Biochemistry

 Volume 32, Number 28 July 20, 1993

Accelerated Publications

DNA Photolyase Repairs the *trans-syn* Cyclobutane Thymine Dimer†

Sang-Tae Kim,‡ Khushbeer Malhotra,‡ Colin A. Smith,§ John-Stephen Taylor,§ and Aziz Sancar*,‡

Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599, and Department of Chemistry, Washington University, St. Louis, Missouri 63130

Received April 7, 1993; Revised Manuscript Received May 19, 1993

ABSTRACT: DNA photolyases catalyze the splitting of the cyclobutane ring joining the two dihydropyrimidines of a pyrimidine dimer by a photoinduced electron-transfer reaction. Previous studies concluded that photolyase repairs only the *cis-syn* form of the eight stereoisomers of the cyclobutane pyrimidine dimer (Pyr[ ]Pyr). In this study we found that *Escherichia coli* photolyase binds to the *trans-syn*-I isomer of T[ ]T with about 10^4-fold lower affinity than the *cis-syn* isomer but it repairs it relatively efficiently.

Ultraviolet light (200–300 nm) induces many photoproducts in DNA. Of these, the cyclobutane pyrimidine dimer (Pyr[ ]Pyr) is by far the most abundant [see Wang, (1976) and Cadet and Vigny (1990)]. There are eight potential isomers of Pyr[ ]Pyr; however, because of steric constraints only two of these form in DNA: Pyr[*c,s*]Pyr, which forms in both double- and single-stranded DNA, and Pyr[*t,s*]Pyr, which forms in single-stranded DNA only (Wang, 1976). In single-stranded DNA, T[*c,s*]T[1] and T[*t,s*]T are formed at a 7:1 ratio. Though it has not been rigorously established, the major *trans-syn* product of single-stranded DNA is likely to be the *trans-syn*-I dimer (5′T-5S6S, 3′T-5S6S) (Liu & Yang, 1978), which results from photodimerization of the thymidines in the 5′T-*syn*, 3′T-*anti* glycosyl conformation. This conformation leads to a much more favorable overlap between the 5,6 double bonds of the thymidines in B-form DNA than does the 5′-*anti*, 3′-*syn* conformation which leads to the *trans-syn*-II dimer (Kao *et al.*, 1993). Since single-stranded DNA forms transiently during replication, recombination, and transcription, the T[*t,s*]T isomer is of potential biological significance as a lethal and mutagenic lesion (Banerjee *et al.*, 1990) in most organisms which carry genetic information in double-stranded DNA. In contrast, ssDNA phages are in single-stranded form for most of their life cycle and thus are a more serious target for induction of T[*t,s*]T lesion and its consequences.

Only limited data exist on the repair of T[*t,s*]T. We previously reported (Svoboda *et al.*, 1993) that the *trans-syn*-I isomer was repaired 6 times faster than the T[*c,s*]T by *Escherichia coli* (A)BC excinuclease. However, excision repair by its very nature works only on double-stranded DNA, and it is of only limited use for correcting a lesion that forms only in ssDNA. Photolyase is equally active on single- and double-stranded DNA (Sancar *et al.*, 1985; Payne & Sancar, 1990), and thus it might be the appropriate enzyme for repairing T[*t,s*]T. However, previous studies indicated that photolyase was highly specific for T[*c,s*]T and was incapable of repairing the *trans* isomer. Ben-Hur and Ben-Ishai (1968) reported that partially purified yeast photolyase was capable of repairing T[*c,s*]T but not T[*t,s*]T in heavily irradiated ssDNA when the reaction products were analyzed by paper chromatography. This finding was confirmed by Banerjee *et al.* (1990), who used an 11-nt oligomer containing either a T[*c,s*]T, or a T[*t,s*]T in the center as a substrate for a homogeneous preparation of *E. coli* photolyase. The authors reported that even though both isomers were photoreversible by irradiation with 254 nm (direct photoreversal), only the *cis* isomer could be repaired by treatment with photolyase and black light. Essentially the same results were obtained by us in a very similar experimental system (Svoboda *et al.*, 1993;

† This work was supported by National Institutes of Health Grants GM31082 and CA40463.

‡ University of North Carolina School of Medicine.

§ Washington University.

[1] Abbreviations: T[*c,s*]T and T[*t,s*]T, *cis-syn* and *trans-syn*-I isomers of cyclobutane thymine dimer.

0006-2960/93/0432-7065$04.00/0

Smith & Taylor, 1993). However, these previous studies, after the reaction, measured the substrate and product only and did not address the question of whether the lack of repair was due to lack of binding of photolyase to the *trans* isomer or to its inability to transfer an electron to a T[ ]T with the cyclobutane ring in the *trans* configuration. In the present study we measured both the binding and photolysis of T[*t,s*]T by *E. coli* photolyase. We found that even though the enzyme binds to this isomer very poorly, it repairs it with photochemical efficiency comparable to that of the *cis* isomer.

MATERIALS AND METHODS

Substrates. The 49-bp duplexes containing a thymine dinucleotide, a T[*c,s*]T or a T[*t,s*]T, were prepared as described previously (Smith & Taylor, 1993). The sequence of the damaged strand of the duplex is (thymines involved in dimer formation in bold type)

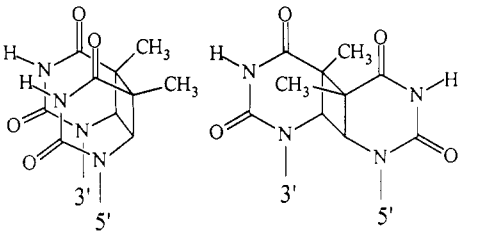
d(AGCTACCATGCCTGCACGAA**TT**-
AAGCAATTCGTAATCATGGTCATAGCT)

This strand was labeled with [γ-^{32}P]ATP (7000 Ci/mmol; ICN) and T4 polynucleotide kinase (New England Biolabs). The labeled strand was annealed with a 5-fold molar excess of the complementary oligomer in 50 mM Tris·HCl, pH 8.0, and 100 mM NaCl by heating at 75 °C for 10 min and cooling to 30 °C over a 1-h period. Double-stranded DNA was purified by electrophoresis through a 12% polyacrylamide gel, followed by electroelution and precipitation. After the DNA was resuspended in 10 mM Tris·HCl, pH 7.4, 10 mM NaCl, and 1 mM EDTA, concentrations were determined by Cerenkov counting of an aliquot.

Enzymes. E. coli DNA photolyase was purified as described previously (Sancar *et al.*, 1984), and the concentration was calculated from the absorption at 580 nm using an extinction coefficient of ϵ_{580} = 5000 M^{-1} cm^{-1} (Payne *et al.*, 1987). The *Mse*I restriction endonuclease which incises between the two thymines of the TTAA sequence was purchased from New England Biolabs.

Binding Assay. The gel retardation assay to measure binding of photolyase to DNA was conducted as described elsewhere (Husain & Sancar, 1987). Briefly, ca. 2000 cpm of labeled DNA was mixed with the indicated amounts of photolyase in a 25-μL reaction buffer containing 50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 5% glycerol and incubated at 5 °C for 30 min. The samples were then loaded onto 5% polyacrylamide gels, and free and bound DNA were separated by electrophoresis. The DNA was located by autoradiography, the bands were cut out, and the radioactivity in bound and free DNA was determined by Cerenkov counting.

Photolysis Assay. A coupled enzyme assay was used to measure photocycloreversion of T[ ]T. The reaction mixture (200 μL) contained 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 5% glycerol, and substrate and enzyme at the indicated concentrations. The sample was made anaerobic by flushing with argon, and then the flavin was reduced to the $FADH^-$ (active) form by exposing the sample to 200 camera flashes filtered through a Schott RG630 (long-pass 630 nm) filter (Payne & Sancar, 1990). The samples were then exposed to photoreactivating light of 366 nm using a Quantacount monochromator/actinometer as the light source. The fluence rate was measured by the ferrioxalate method. To measure repair, following photoreactivation the DNA was extracted with phenol/chloroform, precipitated with ethanol, and digested with *Mse*I as described by the supplier.



cis-syn *trans-syn*-I

FIGURE 1: *cis-syn* and *trans-syn*-I isomers of cyclobutane dithymine used in this study.

*Mse*I incises the phosphodiester bond between the thymine dinucleotide making up the dimer, and unless the cyclobutane ring is cleaved, *Mse*I is unable to hydrolyze this bond; even if it did, the two halves of the labeled strand would remain together because of the cyclobutane ring. Following digestion the DNA was separated on a 12% polyacrylamide gel. The full-length and incised fragments were located by autoradiography and quantified by Cerenkov counting. To obtain the quantum yield, repair rate was measured under several fluence rates, and using $k_{cat} \sim k_p I$ (where k_p = photolytic constant and I = fluence rate) the value of k_p was obtained from the slope of a k_{cat} versus I plot. From this value the quantum yield was calculated using $\phi = (5.2 \times 10^9) k_p/\lambda\epsilon$ [see Payne and Sancar (1990)].

RESULTS AND DISCUSSION

Binding of T[c,s]T and T[t,s]T to Photolyase. As is apparent from Figure 1, these two isomers of the cyclobutane pyrimidine dimer have very dissimilar structures, and the *trans* isomer is not expected to fit into an enzyme active site optimal for the *cis* isomer. However, the two dihydropyrimidines joined by the cyclobutane ring contribute only a small fraction of photolyase binding free energy, the rest being provided by the sugar–phosphate backbone of neighboring nucleotides (Husain *et al.*, 1987; Kim & Sancar, 1991). Therefore, *a priori*, high-affinity binding of photolyase to T[*t,s*]T could not be eliminated, and this question needed to be addressed experimentally.

Figure 2 shows the binding of photolyase to 49-bp duplexes containing one of the isomers of T[ ]T or a normal dinucleotide in the middle of the substrate. In agreement with previous findings photolyase binds with about 10^5-fold higher affinity to the T[*c,s*]T substrate compared to an undamaged duplex (Husain & Sancar, 1987). In contrast, in this electrophoretic mobility shift assay the T[*t,s*]T isomer was retarded only at micromolar concentrations of photolyase in a slowly migrating nonspecific complex identical to that obtained with nonsubstrate DNA. Thus, within the resolution of our assay the affinity of photolyase for T[*t,s*]T is identical or very close to its nonspecific affinity for DNA. Furthermore, this 10^4–10^5-fold lower affinity is sufficient to explain the failures in earlier attempts to detect photorepair of T[*t,s*]T because these assays did not have the sensitivity to detect 0.01% repair rate compared to T[*c,s*]T. Thus, it was still conceivable that photolyase would repair this isomer provided that a high concentration of the enzyme was used to ensure binding.

Repair of T[t,s]T by Photolyase. We conducted photoreactivation experiments under conditions where variable amounts of T[*t,s*]T were bound "nonspecifically" (Figure 3A) and a single dose of photreactivating light was delivered or under conditions where all the DNA was bound (Figure 3B) and the photoreactivating light dose was the variable. As apparent from these figures, a fraction of T[*t,s*]T is repaired,

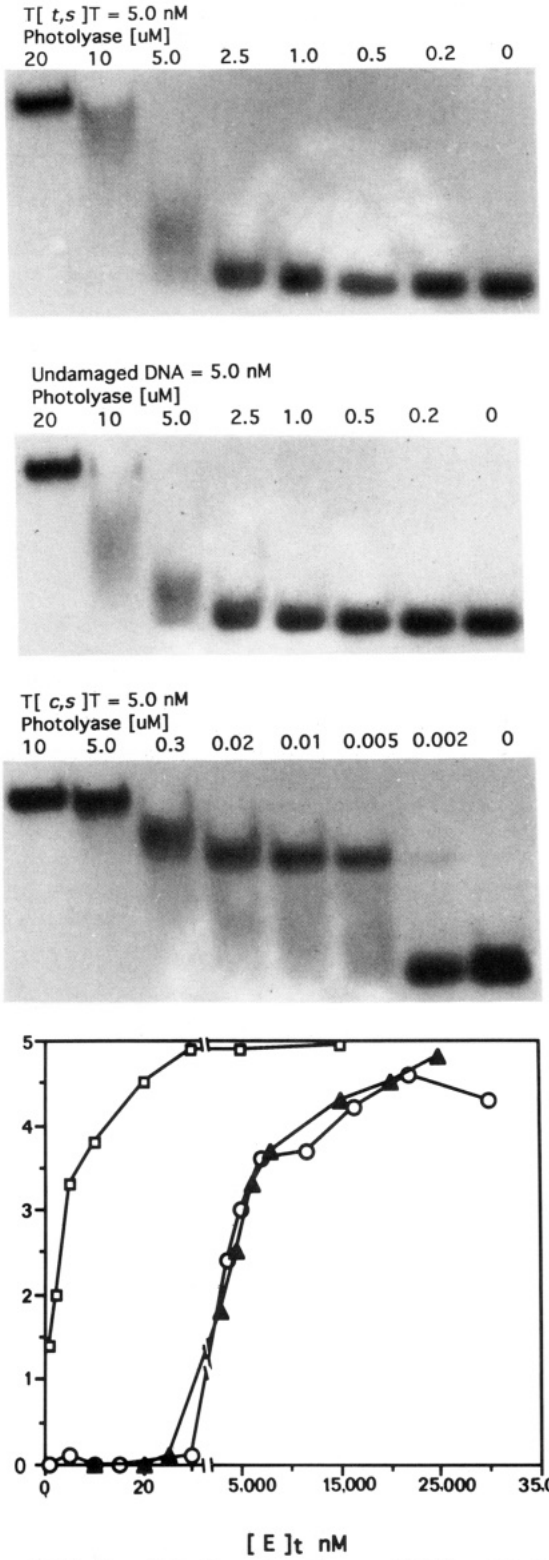


FIGURE 2: Binding of photolyase to undamaged DNA and to DNA with T[*c,s*]T or T[*t,s*]T lesions. (A, top three panels) Autoradiographs of gel retardation assays. Reaction mixtures (25 μL) containing 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 5 nM 5′-end-labeled DNA substrate (5 nM) were mixed with photolyase at the indicated concentrations and incubated at 5 °C for 30 min. Then the samples were subjected to electrophoresis on nondenaturing 5% polyacrylamide gel. Note the different retardation levels of the specific and nonspecific complexes. (B, bottom panel) Binding curves for the three substrates. The binding curves were generated by analyzing the retarded bands corresponding to the photolyase–DNA complexes, which migrate slower than the "free" DNA bands on the native polyacrylamide gels. Symbols: circles, undamaged DNA; triangles, T[*t,s*]T substrate; squares, T[*c,s*]T substrate.

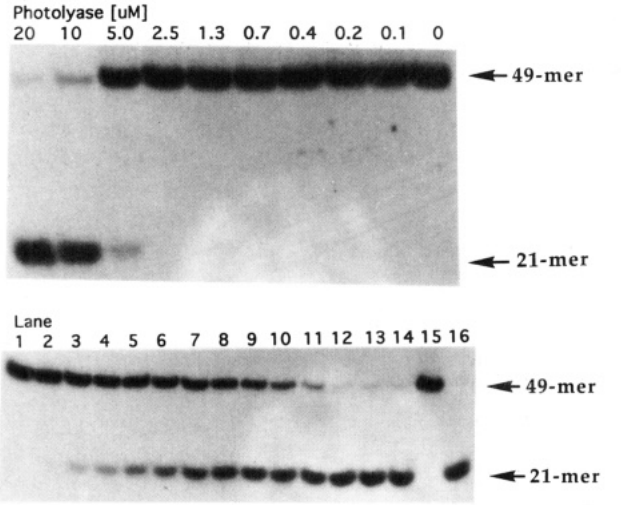


FIGURE 3: Repair of T[*t,s*]T by photolyase. (A, top panel) The 49-mer (T[*t,s*]T, 5 nM) was mixed with the indicated concentrations of photolyase and exposed to photoreactivating light (12 kJ/m^2). (B, bottom panel) The substrate (5 nM) was mixed with 20 μM photolyase and exposed to the indicated fluences of photoreactivating light. The light fluences were as follows (kJ/m^2): lane 1, none; lane 2, photoreactivation of Enz–FADH^0–MTHF to Enz–FADH$^-$–MTHF with camera flashes filtered through a 550-nm cutoff filter; lanes 3 and 16, 1.2 kJ/m^2, lane 16 contained 49-mer T[*c,s*]T; lane 4, 2.4 kJ/m^2; lane 5, 3.6 kJ/m^2; lane 6, 4.8 kJ/m^2; lane 7, 6 kJ/m^2; lane 8, 7.2 kJ/m^2; lane 9, 8.4 kJ/m^2; lane 10, 9.6 kJ/m^2; lane 11, 10.8 kJ/m^2; lane 12, 12 kJ/m^2; lane 13, 13.2 kJ/m^2; lanes 14 and 15, 14.4 kJ/m^2, lane 15 had no photolyase. After photoreactivation the DNA was extracted with phenol/chloroform and treated with *Mse*I restriction endonuclease which digests only the repaired DNA to generate the 21-mer. The positions of *Mse*I-resistant full-length substrate (49-mer) and the *Mse*I-generated 21-mer product from repaired DNA are indicated.

proportional to the amount of DNA bound and to the photoreactivating light dose delivered. Similar results were obtained when ssDNA substrates were used in these assays. We conclude that photolyase does repair T[*t,s*]T and that earlier attempts failed to detect this activity because lower concentrations of photolyase were used.

Photochemistry of T[t,s]T Repair. Model studies with photosensitized splitting of T[t,s]T have yielded conflicting results. Ben-Hur and Rosenthal (1970) reported that anthraquinone-sensitized photosplitting of both isomers, which is initiated by electron abstraction from the dimer, occurred with similar efficiencies. In contrast, Pac *et al.* (1982) found that in redox photosensitized splitting by phenanthrene/*p*-dicyanobenzene pair T[*c,s*]T was split much more efficiently than T[*t,s*]T of a 1,3-dimethylthymine dimer. These authors suggested that the high efficiency of splitting of T[*c,s*]T was due to the more strained cyclobutane ring in this isomer because of the *cis* relationship of the methyl groups and the dihydropyrimidine rings (Pac *et al.*, 1982). Although in DNA different constraints exist on the *cis* and *trans* isomers compared to those in free bases, we considered the possibility that the low quantum yield of splitting of T[*t,s*]T contributed to its low efficiency of repair.

Quantum yields are obtained by conducting kinetic experiments under either enzyme saturating or substrate excess conditions. However, because of low specific binding of photolyase to T[*t,s*]T, it is not possible to conduct photolysis experiments when all substrate is in the form of the ES complex (Payne & Sancar, 1990; Kim & Sancar, 1991). Similarly, because of very inefficient repair when enzyme is limiting, it is not practical to measure repair rate under substrate excess conditions (Eker *et al.*, 1986). Instead, we conducted

photolysis experiments under conditions where all the substrate was bound, mostly nonspecifically. Under these conditions we found that the repair rate (k_{cat}) was proportional to the fluence rate (I) over the range of 5–40 ergs mm^{-2} s^{-1}. From the slope of k_{cat} *vs* I a first-order rate constant of 3.3×10^{-6} $mm^2 \cdot erg^{-1}$ was obtained, and using the approximation of $k_{cat} \sim k_p I$ a lower limit of the quantum yield of $\phi > 0.003$ was calculated. This contrasts with the value of $\phi = 0.6$ found for T[*c,s*]T (Payne & Sancar, 1990). Since the vast majority of the T[*t,s*]T substrate under our experimental conditions is bound nonspecifically, we think this is a minimum estimate and that the real value could be much higher in the absence of nonspecific binding.

In conclusion, photolyase does repair T[*t,s*]T. The lower rate of repair compared to T[*c,s*]T was mainly due to the lower affinity of T[*t,s*]T for photolyase. The lower affinity can be attributed to the altered stereochemistry about the cyclobutane ring and its disruptive effect on DNA structure as indicated by NMR, melting temperature (Taylor *et al.*, 1990), and bending (Wang & Taylor, 1993) studies. Furthermore, we consider that this repair has a biological significance because thymine dimers in ssDNA are not substrates for any other repair enzyme which would maintain the integrity of the DNA while eliminating the lesion.

REFERENCES

Banerjee, S. K., Borden, A., Christinsen, R. B., LeClerc, J. E., & Lawrence, C. W. (1990) *J. Bacteriol. 172*, 2105–2112.

Ben-Hur, E., & Ben-Ishai, R. (1968) *Biochim. Biophys. Acta 166*, 9–15.

Ben-Hur, E., & Rosenthal, I. (1970) *Photochem. Photobiol. 37*, 3164–3167.

Cadet, J., & Vigny, P. (1990) in *Bioorganic Photochemistry* (Morrison, H., Ed.) Vol. 1, pp 1–272, John Wiley & Sons, New York.

Eker, A. P. M., Hessels, J. K. C., & Dekker, R. H. (1986) *Photochem. Photobiol. 44*, 197–205.

Husain, I., & Sancar, A. (1987) *Nucleic Acids Res. 15*, 1109–1120.

Husain, I., Sancar, G. B., Holbrook, S. R., & Sancar, A. (1987) *J. Biol. Chem. 262*, 13188–13197.

Kao, J., Nadji, S., & Taylor, J.-S. (1993) *Chem. Res. Toxicol.* (in press).

Kim, S.-T., & Sancar, A. (1991) *Biochemistry 30*, 8623–8630.

Liu, F.-T., & Yang, N. C. (1978) *Biochemistry 17*, 4865–4876.

Pac, C., Kubo, J., Majima, T., & Sakurai, H. (1982) *Photochem. Photobiol. 36*, 273–282.

Payne, G., & Sancar, A. (1990) *Biochemistry 29*, 7715–7727.

Payne, G., Heelis, P. F., Rohrs, B. R., & Sancar, A. (1987) *Biochemistry 26*, 7121–7127.

Sancar, A., Smith, F. W., & Sancar, G. B. (1984) *J. Biol. Chem. 259*, 6028–6032.

Sancar, G. B., Smith, F. W., & Sancar, A. (1985) *Biochemistry 24*, 1849–1855.

Smith, C. A., & Taylor, J.-S. (1993) *J. Biol. Chem. 268*, 11143–11151.

Svoboda, D. L., Smith, C. A., Taylor, J.-S., & Sancar, A. (1993) *J. Biol. Chem. 268*, 10694–10700.

Taylor, J.-S., Garrett, D. S., Brockie, I. R., Svoboda, D. L., & Telser, J. (1990) *Biochemistry 29*, 8858–8866.

Wang, C.-I., & Taylor, J.-S. (1993) *Chem. Res. Toxicol.* (in press).

Wang, S. Y. (1976) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., Ed.) Vol. 1, pp 295–356, Academic Press, New York.