Enzymatic Analysis of Isomeric Trithymidylates Containing Ultraviolet Light-induced Cyclobutane Pyrimidine Dimers

I. NUCLEASE P1-MEDIATED HYDROLYSIS OF THE INTRADIMER PHOSPHODIESTER LINKAGE

(Received for publication, August 23, 1988)

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Our recent findings suggest that enzymatic hydrolysis of the intradimer phosphodiester bond may constitute the initial step in the repair of UV light-induced cyclobutane pyrimidine dimers in human cells. To examine the susceptibility of this phosphodiester linkage to enzyme-mediated hydrolysis, the trinucleotide d-TpTpT was UV-irradiated and the two isomeric compounds containing a cis-syn-cyclobutane dimer were isolated by high performance liquid chromatography and treated with calf spleen phosphodiesterase. Snake venom phosphodiesterase hydrolyzed only the 5'-isomer (d-TpTpT) but was totally inactive toward the 3'-isomer (d-TpTpT). In contrast, calf spleen phosphodiesterase only operated on the 3'-isomer by cleaving the 5'-internucleotide bond. Kinetic analysis revealed that (i) the activity of snake venom phosphodiesterase was unaffected by a dimer 5' to a phosphodiester linkage, (ii) the action of calf spleen phosphodiesterase was partially inhibited by a dimer 3' to a phosphodiester bond, and (iii) Escherichia coli phr B-encoded DNA photolyase reacted twice as fast with d-TpT as with d-TpTpT as with d-TpTpT. Mung bean nuclease, nuclease S1, and nuclease P1 all cleaved the 5'-internucleotide linkage, but not the intradimer phosphodiester bond, in d-TpTpT. Both phosphate groups in d-TpTpT were refractory to mung bean nuclease or nuclease S1. Incubation of d-TpTpT with nuclease P1, however, generated the novel compound d'TpT containing a severed intradimer phosphodiester linkage. Accordingly, nuclease P1 represents the first purified enzyme known to hydrolyze an intradimer phosphodiester linkage.

Exposure of cellular DNA to UV radiation results in the formation of a number of different lesions (1), which are potentially carcinogenic, mutagenic, and cytotoxic (2). The major photoproduct induced is the cyclobutane dimer formed between adjacent intranucleotid pyrimidines (3). Extensive studies into the metabolic fate of pyrimidine dimers in UV-treated human cells have established that these photoproducts are acted on by the so-called nucleotide mode of excision repair. In this complex multienzymatic process, the dimer-containing site is restored to normalcy by a series of reactions leading to the release of the dimer as part of an oligonucleotide, followed by filling in of the resulting gap with a nucleotide sequence complementary to the opposite intact strand and ligation of newly synthesized and preexisting material (4). However, the nature of individual steps, particularly the critical reaction(s) facilitating dimer removal from human DNA, remains ill defined. Compelling evidence that the initiating event(s) occurring in human cells may in fact differ from those known to arise in either Micrococcus luteus or bacteriophage T4 has been obtained recently in a number of laboratories. Indeed, unlike M. luteus and phage T4, human cells may not employ a pyrimidine dimer-DNA glycosylase to initiate excision repair of UV-induced dimers, as judged by the inability to detect photoliberation of free thymine in dimer-containing excision fragments isolated from post-UV induced human fibroblasts (5, 6). That the repair-initiating event may also differ from that arising in Escherichia coli derives from our recent observation that free thymidine and thymidine monophosphate can be detected after exposure of these same isolated excision fragments to a dimer photoreversing fluence of UV light, implying that the photoactivated thymidine and thymidine monophosphate moieties remain attached to excision fragments only by the cyclobutane ring of the pyrimidine dimers (6, 7). Furthermore, we have demonstrated that pyrimidine dimer-containing sites in DNA are subject to modification (i.e. their intradimer phosphodiester bond may be cleaved) during post-UV incubation of excision-repair-defective xeroderma pigmentosum complementation group A or D cells (7, 8). These cumulative findings prompted us to postulate that enzymatic hydrolysis of the phosphodiester linkage between dimer-forming pyrimidines may constitute an early step in the nucleotide excision-repair pathway operative on these photoproducts in human cells (7).

To elucidate the molecular structure of modified pyrimidine dimer sites in genomic DNA of xeroderma pigmentosum group A or D cells and excised dimer-containing oligonucleotides in normal human cells, a convenient assay capable of distinguishing normal dimer sites from modified ones is required. The current method of choice for direct measurement of pyrimidine dimers in cellular DNA involves chromatographic analysis of radioactive high molecular weight DNA hydrolyzed by acid treatment (9). Since this hydrolysis results in cleavage
of N-glycosyl bonds to liberate free bases and acid-stable photoproducts, the procedure precludes discrimination between thymine dimers with an intact or a cleaved phosphodiester bond. We therefore set out to establish a deoxyribonuclease treatment protocol that digested UV-irradiated DNA to individual mononucleotides and dimeric photoproducts without severing intradimer phosphodiester linkages. As the first of two papers in this series, we delineate here the molecular structure of the two isomeric trithymylate compounds containing unmodified č-syn-cyclobutane pyrimidine dimers, and provide evidence for their differential suitability to serve as a substrate for snake venom exonuclease, calf spleen phosphodiesterase, nuclease S1, mung bean nuclease, or nuclease P1. The latter enzyme was found to be the only deoxyribonuclease capable of hydrolyzing the phosphodiester bond between the two pyrimidines forming a cyclobutane dimer, supporting our recent finding that intradimer phosphodiester bonds can be enzymatically incised (7). Since it was also observed that snake venom phosphodiesterase-mediated hydrolysis of UV-irradiated [3H]thymine-labeled poly(dA):poly(dT) released thymine dimers as part of a trinucleoside disphosphate without attacking the intradimer phosphodiester bond, our results suggest that a nucleoside digestion-HPLC procedure may be employed to assay UV-irradiated DNA for cyclobutane pyrimidine dimer sites with an intact or a severed intradimer phosphodiester linkage. Moreover, this and the accompanying article illustrate the utility of dimer-containing compounds in generating well-defined modifications, such as pyrimidine dimers, as tools for examining the substrate requirements of various DNA-processing enzymes. A preliminary report of our findings has been presented elsewhere (10).

**EXPERIMENTAL PROCEDURES**

**Enzymes—** *E. coli* photolyase, the product of the photolyase gene (11), was a generous gift of Dr. A. Sancar (University of North Carolina, Durham, NC). The enzyme preparation (fraction VII from Sancar et al. (12)) was supplied in a 50 mM Tris-HCl, pH 7.4, buffer containing calf spleen phosphodiesterase, nuclease S1, mung bean nuclease, or nuclease P1. The enzyme preparation (fraction VI1 from Sancar et al.) was supplied in a 50 mM Tris-HCl, pH 7.4, buffer containing calf alkaline phosphatase, staphylococcal nuclease, bovine pancreatic phosphodiesterase, mung bean nuclease, nuclease S1, and bacterial alkaline phosphatase were obtained from Sigma. Calf spleen phosphodiesterase, calf alkaline phosphatase, staphylococcal nuclease, bovine pancreatic DNase I, and nuclease P1 from *Penicillium citrinum* were all purchased from Boehringer Mannheim Canada (Dorval, PQ).

**HPLC and Other Instrumentation—** HPLC instrumentation consisted of a Waters M45 dual piston pump (Waters Associates, Milford, MA), a custom-designed injector with a 2-ml loop capacity, and a Beckman model 160 fixed wavelength absorbance detector (Beckman Instruments, Tokyo, ON). The separation of UV-irradiated (dT)3 was performed on a Whatman C8 Partisol-10 ODS-2 column (250 x 4.6 mm inner diameter; Whatman), employing a gradient elution in which the initial elution buffer, denoted buffer A (50 mM ammonium acetate, pH 7), was applied for 15 min, followed by a linear gradient for 55 min up to a final elution mixture of 40% methanol, 60% buffer A. The flow rate was held throughout at 0.6 ml/min. We occasionally employed a second HPLC system consisting of a Waters 840 control station equipped with two Waters 510 dual piston pumps, a 6-kml universal injector, and a Waters 490 Programmable Multiwavelength detector. We have also separated the radioactive content as determined in a Beckman model LS 5801 liquid scintillation spectrophotometer.

UV absorption measurements were performed on a Perkin-Elmer Lambda 4B UV-visible spectrophotometer (Perkin-Elmer Ltd., Richmond, BC).

**Photochemical Reaction of (dT)3 and Isolation of Photoproducts—** A 1-ml sample of (dT)3 (thymidylid-(3'-5')-thymidylic acid (Sigma)), dissolved in water at a concentration of 0.1 mg/ml (A280 = 23), was placed in a quartz cuvette held at 4 °C and exposed to 290-nm UV light (half-band width, 6.5 nm) emitted by a 2.5-kilowatt HgXe arc lamp in a Schoeffel housing (Schoeffel Instruments Corp., Westwood, NJ) equipped with a predispersion prism. The fluence rate was monitored with a UVX Radiometer (Ultra-Violet Products Inc., San Gabriel, CA). After photoreversal, the ethanol was evaporated and the samples were subjected to HPLC analysis.

**Photochemical Reversal of Cyclobutane Pyrimidine Dimers—** Purified dimer-containing compounds were dissolved in 1 ml of 5% aqueous ethanol and irradiated with a 35-mm diameter Petri dish covered with a 5.5 kJm-2 UV light delivered by two 15-watt (low pressure mercury vapor) germicidal lamps (model GE 15T8, General Electric, Toronto, ON) emitting 97% of their energy at 254-nm wavelength at a fluence rate of ~5.4 watt m-2. During irradiation, the UV fluence rate was monitored with a UVX Radiometer (Ultra-Violet Products Inc., San Gabriel, CA). After photoreversal, the ethanol was evaporated and the samples were subjected to HPLC analysis. The radioactive content was determined in a Beckman model LS 5801 liquid scintillation spectrophotometer.

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**Reaction of E. coli DNA Photolyase with UV-modified Oligonucleotides—** The 80-μl reaction mixture contained 50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1 mg/ml bovine serum albumin, dissolved in water, and stored at -20 °C. The concentration of each product was estimated by assuming that its t<sub>500</sub> was equal to that of thymidines at neutral pH.

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above. The samples were then assayed by HPLC for the production of thymidine 3'-monophosphate, using the sodium phosphate-methanol gradient system. Results, calculated from the integrated peak areas, were expressed as percent total nucleotides hydrolyzed, assuming that the area of the UV-modified products and thymidine monophosphate were equivalent.

**Reaction of Nuclear P1 or Mung Bean Nuclease with UV-modified (dT)₃**—A sample of each dimer-containing compound (680 pmol of d-T₃p>TpT or 740 pmol of d-TpTp>TpT) was treated for 3 h with 1000 units of nuclease S1 or 160 units of mung bean nuclease in a reaction mixture containing 10 mM sodium acetate, pH 5.3, 1 mM ZnCl₂, and 50 mM NaCl (total volume, 90 µl). Ten µl of 0.5 M Tris-HCl, pH 7.5, buffer containing 0.1 µmol of MgCl₂ and 0.4 units of bacterial alkaline phosphatase were then added, and the reaction mixtures were incubated for 1 h. After precipitating the proteins, the supernatants were evaporated and the hydrolysates were examined by HPLC.

**Isolation of Nuclease P1 with UV-modified (dT)₃**—Dimer-containing thymidine diesters were treated for 3 h in a standard 90 µl reaction mixture containing 10 mM sodium acetate, pH 5.3, 1 mM ZnSO₄, 15 units of nuclease P1, and d-TpTp>TpT (740 pmol) or d-T₃p>TpT (680 pmol). Five µl of 1 M Tris-HCl, pH 7.5, 0.2 M MgCl₂ buffer, and 0.4 units of bacterial alkaline phosphatase were then added, and the incubation was continued for an additional 30 min. The supernatants were fractionated into 2.5-µl drops and UV-irradiated with 2 kJ/m².

**Product Analysis on DEAE-Cellulose Chromatography**—Anion exchange chromatography on DEAE-cellulose (Whatman DE23) was used to separate the products from nuclease P1 reaction. A 7.5-ml column (Pharmacia C 10/10; Pharmacia, Dorval, PQ) was equilibrated with 10 mM ammonium acetate, pH 7, and the samples, supplemented with unlabeled thymidine as a UV marker, were dialyzed 10-fold with the equilibration buffer and applied to the column at 30 ml/min. The column was washed with 15 ml of the same buffer. Separation of compounds was achieved over 2 h using a linear gradient of 10–500 mM ammonium acetate, pH 7, at a flow rate of 30 ml/h. In total, 150 0.5-ml fractions were collected and their radioactivity measured.

**Digestion of UV-irradiated [³H]Thymine-labeled Poly(dA):Poly(dT)** with Snake Venom Phosphodiesterase and Alkaline Phosphatase—Radioactive copolymer poly(dA):poly(dT) (525 µm nucleotides, 169 cpm/pmole thymine) was prepared according to an earlier procedure (15). For UV irradiation, 50-µl aliquots of radiolabeled copolymer were fractionated into 2.5-µl drops and UV-irradiated with 2 kJ/m² as described above. [³H]Thymine-labeled copolymer (1.5-nmol nucleotides) was treated with 200 units of DNase I and 150 units of staphylococcal nuclease for 1 h in a 50-µl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, and 2 mM CaCl₂. After incubation, the samples were heated for 5 min at 100 °C and cooled quickly on ice to separate the DNA strands. To these mixtures were added 20 mM Tris-HCl; pH 7.5; 0.5 M NaCl; and 0.4 units of calf alkaline phosphatase, and the samples were incubated overnight. Fresh phosphatase and venom phosphodiesterase (20 and 8 x 10⁴ units, respectively) were then added and the incubation was continued for 3 h, after which the proteins were removed by precipitation as outlined above. The supernatants, containing hydrolyzed material, were recovered and taken to dryness. For analysis of the digestion products, 60 µl of buffer containing 0.7 nmol of d-T₃p>TpT, 0.7 nmol of d-TpTp>TpT, and 0.5 nmol of unlabeled thymidine were added to separate aliquots of the digested copolymers and then examined by reverse-phase HPLC using the phosphate-methanol gradient system.

**Preparation of [³H]Thymine-labeled d-T₃p>TpT—Radiolabeled d-T₃p>TpT** was prepared by calf spleen phosphodiesterase digestion of heavily UV-irradiated [³H]thymine-labeled poly(dA):poly(dT). Ten-nmol nucleotides of UV-treated (~10 kJ/m²; 290-nm light) polynucleotide were digested for 1 h with 300 units of staphylococcal nuclease in 10 mM Tris-HCl, pH 8.8, 1 mM CaCl₂ (total volume, 100 µl). After incubation, the samples were subjected to 95 °C and cooled quickly on ice to separate the DNA strands. To these mixtures were added 0.5 M Tris-HCl, pH 7.5, containing 0.2 units of calf spleen phosphodiesterase and 0.4 units of bacterial alkaline phosphatase were then added to the remaining volume of the reaction mixtures. The samples were then incubated overnight, whereupon the proteins were precipitated with 3 volumes of ice-cold ethanol. The supernatants, containing hydrolyzed material, were dried in a Speed Vac centrifuge, and tritiated d-T₃p>TpT was isolated by reverse-phase HPLC, desalted using a Waters Sep-Pak C₁₈ cartridge, and stored in water at −20 °C.

**RESULTS**

**Isolation and Characterization of UV-modified (dT)₃**

Dimerized pyrimidines, due to saturation of their 5,4-double bonds, exhibit little absorbance at 254 nm (1, 3). Since UV-induced formation of a cyclobutane ring between adjacent thymines can occur at either end of d-TpTpT, the unmodified thymine residue can be used to detect UV-modified compounds at 254 nm. Accordingly, the reverse-phase HPLC elution profile in Fig. 1 reveals that UV (290 nm) irradiation of (dT)₃ yielded two major 254-nm-absorbing products (denoted compounds 1 and 2) which are well separated from the parental oligonucleotide as well as several other minor UV-modified compounds. The two major product substrates displayed a UV absorption maximum at 266.7 nm, a value close to that of the untreated (dT)₃ (265.7 nm) obtained for the unreacted (dT)₃. In contrast, the UV absorption minimum of both photoproducts (240.5 nm) was significantly different from that of the parent compound (234.5 nm), but consistent with the presence of a cyclobutane dimer in each UV-modified oligonucleotide (1, 3). Evidence that compounds 1 and 2 contained a cyclobutane thymine dimer was also obtained from both photochemical reversal (data not given) and photoenzymatic reversal (see below), which regenerated unmodified (dT)₃. Moreover, neither UV-induced thymidylate exhibited any appreciable absorbance at wavelengths greater than 300 nm, indicating the absence of a 6',4'-(pyrimidin-2'-one)-pyrimidine photoproduct (so-called (6-4) photoproduct) (14). Combined with data from physicochemical characterization of two dimer-containing thymidylates with retention properties on reverse-phase HPLC similar to those of the compounds under study here (15), we infer that compound 1 has the structure d-T₃p>TpT and compound 2 the isomeric configuration d-TpTp>TpT. Knowing their exact chemical structure, both dimer-containing oligonucleotides were used as model substrates to
investigate (i) how a cyclobutane dimer affects the activity of various DNA-processing enzymes, and (ii) whether intracyclobutane dimer phosphodiester bonds can be enzymatically incised.

**UV-modified Oligonucleotides As Substrates for E. coli DNA Photolyase**

It has been reported that E. coli DNA photolyase is able to photoreverse cyclobutane dimers present in UV-damaged (dT)_3 (phosphorylated at the 5'-end) (16). As shown in Fig. 2, dimers in unphosphorylated trinucleotides were monomerized at a linear rate for the first 30 min, corresponding to ~90% reaction for d-T<p>TpT and ~45% for d-TpT<p>T.

It would therefore appear that although the enzyme can act some 2-fold faster on the 5'-isomer than on the 3'-isomer, either UV-modified oligomer can serve as a substrate. The 2-fold difference in substrate preference did not arise from any contaminating inhibitor, since a control experiment in which a mixture of the two UV-damaged oligomers was subjected for 1 h to photoenzymatic reversal resulted in complete conversion of d-T<p>TpT to (dT)_3, whereas only about 60% of the dimers in d-TpT<p>T were photoreversed.

**UV-modified Oligomers as Substrates for Snake Venom and Calf Spleen Phosphodiesterases**

The time course of the reaction of snake venom phosphodiesterase or calf spleen phosphodiesterase with the UV-modified compounds, in comparison to (dT)_3, is presented in Fig. 3. Snake venom phosphodiesterase is an exonuclease that catalyzes the stepwise 3'→5' removal of 5'-mononucleotides from polynucleotides (17). As is evident in Fig. 3A, the 5'-isomer is readily hydrolyzed to generate thymidine 5'-monophosphate (detected as thymidine at 254 nm following treatment with bacterial alkaline phosphatase). When the UV absorbance was measured at 230 nm, a second peak with a retention time of ~10 min was readily visible on the HPLC chromatogram; this peak was found to have the same chromatographic and spectral properties as authentic d-TpT<p>T (data not shown). Unlike d-T<p>TpT, d-TpT<p>T proved to be totally refractory to exonucleolytic attack by snake venom phosphodiesterase, even when the reaction was carried out with higher enzyme concentrations (e.g. 10⁻² units) over longer periods (e.g. 24 h). The suitability of the UV-modified oligomers to serve as substrates for calf spleen phosphodiesterase was found to be the opposite of that obtained for snake venom phosphodiesterase (Fig. 3B). Calf spleen phosphodiesterase is a 5'→3' exonuclease releasing 3'-mononucleotides in a processive fashion (18). This enzyme was totally inactive against d-T<p>TpT but was able to degrade d-TpT<p>T to thymidine 3'-monophosphate and d-T<p>T (albeit at a considerably slower rate than for the parental trinucleotide).

Together, these exonucleolytic digestion studies provide further support for the conclusion that compounds 1 and 2 are isomeric trithymidylates possessing a cis-syn-cyclobutane dimer at the 5' and 3' terminus, respectively. Moreover, neither snake venom phosphodiesterase nor calf spleen phosphodiesterase cleaves the intradimer phosphodiester bond in d-T<p>TpT, d-TpT<p>T, or d-T<p>T, a fundamental requirement for either enzyme to be incorporated into an assay designed to measure modified cyclobutane dimer-containing sites.

**Formation of d-TpT<p>T by Snake Venom Phosphodiesterase-mediated Digestion of UV-irradiated Poly(dA):poly(dT)**

To investigate the response of snake venom phosphodiesterase on longer oligonucleotide chains containing a cyclobutane dimer, [3H]thymine-labeled poly(dA):poly(dT) was exposed to 2 kJm⁻² of 254-nm radiation and treated with snake venom phosphodiesterase in concert with DNase I, staphylococcal nuclease, and calf alkaline phosphatase. The digestion products were then resolved by HPLC using d-T<p>TpT and d-TpT<p>T as authentic markers. Fig. 4A portrays the sep-
Reaction of Nucleases with UV-modified Trinucleotides

FIG. 4. Reverse-phase HPLC separation of products from enzymatically digested $[^{3}H]$thymine-labeled poly(dA):poly(dT). Unirradiated copolymer (A) and irradiated (2 kJm$^{-2}$, 254-nm UV light) copolymer (B) were each digested with the snake venom exonuclease-containing mixture after which 10-$\mu$l samples, containing about 0.3 nmol of nucleosides each, were subjected to HPLC analysis as described under "Experimental Procedures." Elution positions for d-T$p$T, d-T$p$TpT, d-TpT$p$T, and thymidine (dT) are indicated by arrows.

FIG. 5. Reaction of mung bean nuclease, nuclease S1, and nuclease P1 with UV-modified trithymidylates. Purified d-T<p$>TpT$ and d-TpT<p$>T$ were treated with each endonuclease, in concert with bacterial alkaline phosphatase, and the reaction products were analyzed on reverse-phase HPLC using the acetate-methanol gradient system. A, untreated d-T$p$T$p$T and d-TpT<p$>T; B, treatment of d-TpT<p$>T with any of the three endonucleases; C, treatment of d-T<p$>TpT$ with mung bean nuclease or nuclease S1; D, treatment of d-T<p$>TpT$ with nuclease P1. Retention times of d-T<p$>TpT$, d-TpT<p$>T$, and thymidine (dT) are indicated by arrows. A$_{254}$ absorbance at 254 nm.

thymine dimers within nuclease-resistant trinucleotides, indicating that the specific inhibition of snake venom phosphodiesterase induced by the cyclobutane dimer is limited to only the intradimer and the 5'-flanking phosphodiester bonds.

UV-modified Oligonucleotides as Substrates for Single-strand Specific Endonucleases

Mung bean nuclease, nuclease S1, and nuclease P1 degrade single-stranded nucleic acids to 5'-mononucleotides by combined endo- and exonuclease activities (20). Purified d-T<p$>TpT$ and d-TpT<p$>T$ were each treated with saturating amounts of each endonuclease in concert with bacterial alkaline phosphatase, and the reaction products were analyzed by reverse-phase HPLC. As illustrated in Fig. 5A, untreated d-T<p$>TpT$ and d-TpT<p$>T had retention times of ~37 and ~39 min, respectively. Treatment of d-TpT<p$>T with any of the three endonucleases, together with phosphatase, yielded thymidine (retention time, ~46 min) and d-T<p$>T
phatase the intriguing result presented in Fig. produce thymidine exclusively. This compound was characterized by a longer retention time phatase yielded a novel compound as illustrated in Fig. 5A, an intradimer phosphodiester bond catalyzed by nuclease P1. In contrast, the retention on ion exchange chromatography depends upon the net charge of the compound, and that no thymidine was released, these results indicated that nuclease P1 had catalyzed the formation of a 5'-terminal phosphate group without loss of a nucleotide from the UV-modified trithymidylate.

Photochemical Reversal of Cyclobutane Dimers—The experimental evidence suggested that the novel molecule produced by nuclease P1 and that the resulting monophosphate group was then removed by alkaline phosphatase. This would render the molecule more hydrophobic and explain its increased retention time on reverse-phase HPLC. To test whether nuclease P1 produced a terminal phosphate group, the strategy outlined in Fig. 6 (steps a and b) was adopted. [3H]Thymine-labeled d-T<\(\text{p}^{\text{i}}\)T was treated with nuclease P1, alone or in combination with bacterial alkaline phosphatase, and the reaction products were analyzed by DEAE-cellulose chromatography. As shown in Fig. 7A, d-T<\(\text{p}^{\text{i}}\)T eluted from the ion exchange column at about 0.23 M ammonium acetate. Treatment of d-T<\(\text{p}^{\text{i}}\)T with nuclease P1 alone yielded a compound that eluted at about 0.28 M ammonium acetate (Fig. 7B). When the nuclease P1-treated product was digested with phosphatase, we observed a new compound characterized by shorter retention than untreated 5'-isomer (Fig. 5C). Given that the retention on ion exchange chromatography depends upon the net charge of the compound, and that no thymidine was released, these results indicated that nuclease P1 had catalyzed the formation of a 5'-terminal phosphate group without loss of a nucleotide from the UV-modified trithymidylate.

Characterization of the Reaction of Nuclease P1 with d-T<\(\text{p}^{\text{i}}\)T

Analysis by DEAE-Cellulose Chromatography—To explain the intriguing result presented in Fig. 5D, we entertained the hypothesis that only the 5'-phosphodiester bond was cleaved (Fig. 5B). As mentioned before, the latter compound was not visible on the HPLC chromatogram. However, using uniformly [\(\text{H}\)]thymine-labeled d-T<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T we could demonstrate the production of d-T<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T (data not shown). After incubation of d-T<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T with mung bean nuclease or nuclease S1, the dimer-containing oligonucleotide was left unchanged, suggesting that neither enzyme was able to hydrolyze either the 3'- or the 5'-phosphodiester linkage in this UV-modified trithymidylate (Fig. 5C). Surprisingly, treatment of d-T<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T with nuclease P1 and bacterial alkaline phosphatase yielded a novel compound as illustrated in Fig. 5D. This compound was characterized by a longer retention time (~52 min) on the reverse-phase column. Control experiments revealed that alkaline phosphatase alone did not modify the retention time of the 5'-isomer. Multiple experiments with nuclease P1 indicated that the unique compound was reproducibly formed in a time-and enzyme-dependent manner.

![Fig. 6. Experimental strategy adopted to test for scission of an intradimer phosphodiester bond catalyzed by nuclease P1.](image)

- **Fig. 6.** Experimental strategy adopted to test for scission of an intradimer phosphodiester bond catalyzed by nuclease P1. a, incision at the dimer site is postulated to occur by nuclease P1 (NP1)-mediated hydrolysis of the 5'-phosphodiester linkage between dimer-forming thymines, leaving 3'-OH and 5'-P termini; b, the resulting 5'-phosphate group is removed by bacterial alkaline phosphatase (BAP) to yield a modified trithymidylate (dT<\(\text{p}^{\text{i}}\)T) in which the 5'-thymidine moiety stays attached to the remainder of the oligonucleotide solely by a cyclobutane ring; c, photochemical reversal (PCR) of dT<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T should generate free thymidine and d-T<\(\text{p}^{\text{i}}\)T; d, treatment of dT<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T with snake venom phosphodiesterase (SVP) plus bacterial alkaline phosphatase should give rise to thymidine and a cyclobutane thymidine dimer (dT<>dT); and finally, e, photochemical reversal of the resulting dT<\(\text{p}^{\text{i}}\)T should produce thymidine exclusively.

![Fig. 7. Analysis of reaction products of nuclease P1 with d-T<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T by DEAE-cellulose chromatography.](image)

- **Fig. 7.** Analysis of reaction products of nuclease P1 with d-T<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T by DEAE-cellulose chromatography. [\(\text{H}\)]Thymine-labeled d-T<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T (1.36 nmol, ~10,000 dpm) was treated for 3 h under standard reaction conditions with 15 units of nuclease P1, and the reaction products were analyzed by ion exchange chromatography. A, no enzymatic treatment; B, treatment with nuclease P1 only; C, successive treatment with nuclease P1 and alkaline phosphatase. Elution positions of d-T<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T, dT<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T, dT<\(\text{p}^{\text{i}}\)T<\(\text{p}^{\text{i}}\)T and of the UV marker thymidine (dT) are indicated by arrows.

\[ ^{5} \text{M. Liuzzi and M. C. Paterson, manuscript in preparation.} \]
were analyzed by reverse-phase HPLC using the acetate-methanol prediction, the modified oligonucleotide was isolated and the data implied that a 5"thymidine residue was phosphatase contained a cyclobutane pyrimidine dimer with by incubating d-T<p>TpT with nuclease P1 and alkaline and d-TpT are indicated by light to photoreverse the dimers, and the resulting products, supplemented with 1 nmol of unlabeled thymine, thymidine and d-TpT, were analyzed by reverse-phase HPLC using the acetic acid-methanol gradient system. The retention times of d-T<p>T, dT<->d,T, dT<->d-TpT and of the UV markers thymine (Thy), thymidine (dT) and d-TpT are indicated by arrows.

by incubating d-T<p>TpT with nuclease P1 and alkaline phosphatase contained a cyclobutane pyrimidine dimer with a cleaved intradimer phosphodiester bond. This interpretation of the data implied that a 5'-thymidine residue was attached to d-TpT solely by a cyclobutane ring. To test this prediction, the modified oligonucleotide was isolated and irradiated with 5.5 kJ m⁻² of 254-nm UV light to photoreverse the dimers, and the resulting products, supplemented with 1 nmol of unlabeled thymine, thymidine and d-TpT, were analyzed by reverse-phase HPLC using the acetic acid-methanol gradient system. The retention times of d-T<p>T, dT<->d,T, dT<->d-TpT and of the UV markers thymine (Thy), thymidine (dT) and d-TpT are indicated by arrows.

FIG. 8. Photochemical reversal of compounds containing a nicked intradimer phosphodiester bond. Purified [³²P]thymine-labeled dT<p>T or [³²P]thymine-labeled dT<->dT<->d<T (~8,000 dpm) (B) was exposed to 5.5 kJ m⁻² of 254-nm UV light to photoreverse the dimers, and the resulting products, supplemented with 1 nmol of unlabeled thymine, thymidine and d-TpT, were analyzed by reverse-phase HPLC using the acetic acid-methanol gradient system. The retention times of d-T<p>T, dT<->d,T, dT<->d-TpT and of the UV markers thymine (Thy), thymidine (dT) and d-TpT are indicated by arrows.

Further evidence for cleavage of the intradimer phosphodiester linkage was obtained as outlined in Fig. 6 (steps d and e). The nuclease P1- and phosphatase-modified 5'-isomer, then the ratio of the radioactivities associated with d-TpT plus d-T<->d<T<->d to that associated with thymidine alone, should equal 2:1. From the data in Fig. 8A, we estimated this ratio to be 1.9:1.

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DISCUSSION

Results presented above demonstrate that exposure of (dT)₃ to 290-nm radiation leads to the formation of two major products, compound 1 and 2, characterized by distinct retention properties on reverse-phase HPLC. Based on (i) photoenzymatic and photochemical-induced reversal as diagnostic probes for cyclobutane thymine dimers and (ii) direction-specific exonucleases (snake venom and calf spleen phosphodiesterase) to localize the photoproducts induced, we also establish that compound 1 has the structure d-T<->d-TpT and compound 2 the isomeric configuration d-TpT<->dT. The structural assignment of both compounds described here using an enzymatic approach is thus fully consistent with data from an independent study by Rycyna et al. (15) in which NMR was used to characterize the two isomers.

Knowing the precise structure of the two dimer-containing isomers, it is instructive to analyze the kinetic data on the activities of the DNA-processing enzymes employed here to toward parental and UV-altered trithymidylates, in order to glean new information concerning substrate requirements of the enzymes. E. coli DNA photolyase binds specifically to cyclobutane dimers in a light-independent step and, upon illumination with 300-600 nm radiation, catalyzes the reversal of the cyclobutane ring (11). Jorns et al. (16) have recently examined the reaction of E. coli DNA photolyase with UV-irradiated oligonucleotides and found a decrease in binding affinity with chain length. These authors, however, were unable to define the position of the cyclobutane dimer within these molecules and hence could only speculate on the modulating influence of neighboring phosphate or base moieties. Our findings clearly show that the enzyme can repair cyclobutane dimers at unphosphorylated termini of oligonucleotides. However, d-T<->d-TpT was operated on about twice as rapidly as d-T<->d-TpT, implying that a 3'-phosphate group adjacent to the dimerized thymine residues may enhance enzyme affinity and in turn accelerate the photoenzymatic reversal reaction. These findings are fully consistent with the mechanism of damage recognition proposed by Sancar and co-workers (22, 23), who argued that the only essential structural feature for substrate recognition is the pyrimidine dimer site, and that while contact is made with the phosphate backbone, predominantly 3' to the dimer, this interaction simply serves to increase enzyme affinity. Experiments in which E. coli photolyase was employed to photoreverse the dimers in dT<->d-TpT or dT<->d-pTpT were unsuccessful, but nonetheless provided further information regarding substrate recognition by the enzyme. We and others have demonstrated previously that photolyases from a variety of organisms preserve full activity toward pyrimidine dimer-DNA glycosylase-modified cyclobutane dimers situated either within an intact, methoxyamine-treated polynucleotide or even at the 5'-end of short oligonucleotides (13, 24, 25). In addition, we have observed that E. coli photolyase can act on long oligonucleotides to photoreverse cyclobutane dimers in which the intradimer phosphodiester bond is broken (7, 26).

The results presented here, on the other hand, mirror a previous observation indicating that the enzyme was unable to photoreverse cyclobutane dimers in dimer-containing excision fragments (estimated average size, ~3.7 nucleotides (6)) isolated from post-UV-irradiated normal human cells.³

³ N. E. Gentner, unpublished results.
Recently, we have also been able to demonstrate that d-TpTpT is not a substrate for the E. coli photoreactivating enzyme. Based on our findings, it appears, therefore, that the enzyme is capable of photoconverting cyclobutane dimers with a cleaved intradimer phosphodiester linkage only in long oligonucleotide chains. The failure to react with modified dimers in short molecules may be due to increased distortion produced by an altered dimer resulting in inefficient binding of photolyase to the substrate. In contrast, modified pyrimidine dimer sites situated in a long single-stranded or double-stranded polynucleotide, may be maintained in a fully photoreactivable configuration, thereby leading to the generation of single-strand breaks (7, 26).

The two exonucleases were each capable of digesting only one isomer, but there was a difference in their reactivity toward the susceptible isomer compared with the parent trithymidylate. Snake venom phosphodiesterase released thymidine 5'-monophosphate from d-TpTpT and (dT)₃ at the same rate. Thus, a modified 5'-base has no discernible effect on the enzyme, and inhibition of its 3'→5' exonuclease activity is confined to the intradimer phosphodiester bond. Calf spleen phosphodiesterase, on the other hand, hydrolyzed d-TpTpT considerably slower than undamaged (dT)₃. Accordingly, UV-induced modification of the nucleoside 3' to the target phosphodiester bond, while not preventing hydrolysis totally, certainly serves to reduce the reaction rate appreciably. In fact, under similar experimental conditions, (dT)₃ was acted on almost 20 times more quickly than the 3'-isomer. We have observed similar substrate restrictions on molecules containing a phosphodiester bond 5' to an apurinic site. Snake venom phosphodiesterase is known to have an associated single strand-specific endonuclease activity (27). The outcome of incubation with the UV-modified trithymidylates shows that the sterically obstructed intradimer phosphodiester linkage is refractory to both the exo- and endonucleolytic functions of the enzyme. Interestingly, the inability of the snake venom phosphodiesterase to act on the remaining phosphodiester linkage in d-TpTpT suggests that modification (possibly loss of aromaticity) of the base 3' to an internucleotide phosphate group is inhibitory to the endonuclease activity. Further evidence for this stems from the observation that snake venom phosphodiesterase cannot cleave the phosphodiester linkage 5' to apurinic sites (28). Digestion of UV-irradiated poly(dA):poly(dT) to mononucleotides and dimer-containing trinucleotides by snake venom phosphodiesterase implies that hydrolysis of phosphodiester linkages further upstream from the cyclobutane dimer is not impeded by the damaged bases.

Nuclease S1, mung bean nuclease, and nuclease P1 gave the same results with respect to the 3'-isomer (d-TpTpT), i.e., each hydrolyzed only the 5'-phosphodiester linkage in d-TpTpT. Treatment of the 5'-isomer (d-TpTpT) with saturating amounts of nuclease S1 or mung bean nuclease had no discernible effect on the dimer-containing oligonucleotide, with both phosphodiester bonds appearing refractory to the action of either enzyme. This finding suggests that the base immediately 5' to the 3'-phosphodiester group may be involved in the mechanism of substrate recognition by both endonucleases, since NMR studies of d-TpTpT indicate that cyclobutane dimers introduce only a minor conformational distortion to the 3'-phosphate group itself (15). The reaction of d-TpTpT with nuclease P1 yielded an intriguing result. Repeated experiments performed with unlabeled dimer-containing oligonucleotide indicated that upon treatment of d-TpTpT with nuclease P1 and bacterial alkaline phosphatase, a novel product with a longer retention time on reverse-phase HPLC was formed. As retention on a reverse-phase column is inversely dependent upon the polarity of the compounds being separated, d-TpTpT must have been altered by the two enzymes in a manner that increased its affinity for the chromatographic support, i.e., by rendering it less polar. This result may be achieved by nuclease P1-mediated cleavage of the 5'-phosphodiester linkage only and removal of the resultant phosphate group by alkaline phosphatase. Until now, enzymatic hydrolysis of phosphodiester linkages between cross-linked adjacent bases has not been observed to occur in vitro. Franklin et al. (14) have reported recently that purified dinucleoside monophosphates containing a (6-4) photoproduction are resistant to digestion by various deoxyribonucleases. Likewise, recent studies by Kumar and co-workers (29) have indicated that the internucleotide phosphodiester bond of a new type of dimeric adenine photoproduction is refractory to the action of nuclease S1, snake venom phosphodiesterase, or calf spleen exonuclease. Together, these findings clearly suggest that the intradimer phosphodiester bond is protected from enzymatic attack by an intrastand cross-link. In sharp contrast, our results unequivocally demonstrate that nuclease P1 from P. citrinum is able to cleave the phosphodiester linkage between dimer-forming pyrimidines, albeit at a much slower rate than normal phosphodiester bonds. To our knowledge, this is the first report of a purified enzyme activity that catalyzes such a reaction on a cyclobutane pyrimidine dimer site. At present, it is not known why the enzyme cleaves the intradimer phosphodiester linkage in d-TpTpT but not in d-TpTpT, and why the 5'- but not the 3'-phosphodiester linkage is cleaved in d-TpTpT. Nor do we know for certain whether normal and intradimer phosphodiester bonds are cleaved by the same enzyme, although analysis by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis of the purified nuclease P1 preparation employed in these studies did not reveal any contaminant. Conditions are currently being sought in which the activity responsible for the cleavage of normal phosphodiester bonds may be suppressed without affecting the other putative activity in order to address these questions.

As noted earlier, the studies reported here were also undertaken to address our specific need for a sensitive method to distinguish a cyclobutane pyrimidine dimer with a severed as opposed to an intact internal phosphodiester linkage. As documented in Fig. 4, this goal was realized by establishing that treatment of UV-irradiated poly(dA):poly(dT) with snake venom phosphodiesterase (combined with staphylococcal nuclease, DNase I, and calf alkaline phosphatase) (i) spares the intradimer phosphodiester bond, (ii) yields only one dimer-containing oligonucleotide (i.e., d-TpTpT), and (iii) leads to complete recovery of all thymine dimers. The quantitative excision of cyclobutane dimers as trinucleotides indicates that both endonucleases, staphylococcal nuclease and DNase I, do not cleave the phosphodiester bond immediately 5' to a dimer in a polynucleotide chain. The function of these endonucleases is merely to shorten the lengthy polynucleotides, thereby facilitating more rapid digestion by the processive snake venom exonuclease to generate mononucleotides and dimer-containing nucleases-resistant oligomers. In our experience, calf spleen phosphodiesterase cannot substitute for snake venom phosphodiesterase in the enzymatic digestion mixture; not only were the dimers not excised quantitatively from the UV-treated copolymer by the former exonuclease, but a diverse mixture of products, including d-

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1 M. Liuzzi and M. C. Paterson, manuscript in preparation.
2 M. Weinfield, M. Liuzzi, and M. C. Paterson, manuscript in preparation.
T$\text{cp}\text{T}$, d-T$\text{cp}\text{TpT}$, and d-T$\text{Tp}\text{cp}$T, was recovered. Un-\noubtedly, the approach used for UV-irradiated poly(dA): poly(dT) can be extended to UV-treated DNA, since similar nuclease-resistant sequences of different base composition have been identified in enzymatic hydrolysates of UV-irradiated DNA (30-32).

Using (i) the enzymatic digestion procedures for dimer analysis described here and (ii) the various UV-modified thymidine molecules as markers, we have demonstrated that cyclobutane pyrimidine dimers with a cleaved internal phosphodiester linkage are excised from DNA as trinucleotides with a ruptured intradimer phosphodiester bond at the 3' terminus (i.e. d-T$\text{TpT}<\text{dT}$) (33). Moreover, the enzyme digestion-HPLC assay has also been successfully employed to detect a novel human activity that hydrolyzes intradimer phosphodiester linkages. The enzyme, termed intracytobutane pyrimidine dimer-DNA phosphodiesterase, is postulated to either initiate the excision-repair process operative on cyclobutane dimers and/or to relieve conformational stress introduced by the cyclobutane ring, thereby presumably increasing the fidelity of de novo synthesis, and transcription on a UV-damaged template.6

Acknowledgment—We wish to thank S. M. Hunt for excellent secretarial assistance.

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