Photoalkylated DNA and ultraviolet-irradiated DNA are incised at cytosines by endonuclease III

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ABSTRACT

Photoalkylation, the ultraviolet irradiation of DNA with isopropanol and di-tert-butylperoxide, causes a variety of base alterations. These include 8-(2-hydroxy-2-propyl)purines, 8-(2-hydroxy-2-propyl)adenines and thymine dimers. An E. coli endonuclease against photoalkylated DNA was assayed by conversion of superhelical PM2 phage DNA to the nicked form. Enzyme activities were compared between extracts of strain BW9109 (xth), lacking exonuclease III activity, and strain BW434 (xth,nth), deficient in both exonuclease III and endonuclease III. The endonuclease level in the double mutant against substrate photoalkylated DNA was under 20% of the activity in the mutant lacking only exonuclease III. Irradiation of the DNA substrate in the absence of isopropanol did not affect the activity in either strain. Analysis by polyacrylamide gel electrophoresis identified the sites of DNA cleavage by purified E. coli endonuclease III as cytosines, both in DNA irradiated at biologically significant wavelengths and in photoalkylated DNA. Neither 8-(2-hydroxy-2-propyl)purines, pyrimidine dimers, uracils nor 6-4-((pyrimidin-2-one)pyrimidines were substrates for the enzyme.

INTRODUCTION

Photoalkylation is the addition of alcohols to C-8 positions of purines in ultraviolet-irradiated DNA (1). In addition to 8-(2-hydroxy-2-propyl)purines, pyrimidine dimers and uncharacterized adenine adducts are also formed in photoalkylated DNA (2,3). An endonuclease activity that incises photoalkylated DNA was found in crude extracts of M. luteus (4). While the enzyme was neither purified nor characterized, 8-(2-hydroxy-2-propyl)purine moieties were suggested as the sites of endonucleolytic incision (4,5). We found an E. coli endonucleolytic activity which incises photoalkylated superhelical DNA and compared its levels in different strains. The sites of cleavage by the purified enzyme were demonstrated to be DNA cytosine photoproducts, rather than 8-(2-hydroxy-2-propyl)purines, thymine glycols, pyrimidine dimers, uracils or 6-4-(pyrimidin-2-one)pyrimidines. Modified cytosines are formed in the absence of isopropanol and are therefore photoproducts without covalent additions of alcohols. Comparative studies of the mutant strains show the enzyme activity against photoalkylated DNA to be identical to the well-characterized enzyme endonuclease III.
MATERIALS AND METHODS

PM2 Phage DNA Preparation and Irradiation

PM2 phage was grown in Alteromonas espejana and the DNA radiolabeled by addition of [3H]thymidine according to Duker and Teebor (6). The DNA was purified as described (6) and stored in 0.1 M KH₂PO₄, pH 7.0. DNA was photoalkylated in 1.3 M isopropanol and 2% di-tert-butylperoxide for 5 minutes as previously described (2,3). Mixed-wavelength irradiation was with a Bausch and Lomb unit equipped with a 200 W Osram Hg bulb and a Schott WG 320 filter at an incident dose of 6.6 x 10⁻⁵ einstein/cm²/min (2,3). The following damaged moieties were present in the photoalkylated substrate: eight 8-(2-hydroxy-2-propyl)guanines, three 8-(2-hydroxy-2-propyl)adenines and four thymine dimers per molecule (3). Broad spectrum ultraviolet irradiation was performed under identical conditions, except that both isopropanol and di-tert-butylperoxide were absent from the reaction mixture. Following irradiation, the DNA was precipitated with ethanol and the pellet resuspended in buffer A (40 mM KH₂PO₄, pH 7.4, 1 mM EDTA). Unirradiated DNA was heated according to Teebor and Brent, introducing approximately 3.7 apurinic sites per molecule (7). Alkali-labile sites and DNA strand breaks were both determined by binding of labeled single-stranded DNA molecules to nitrocellulose filters (7). Enzyme reactions with approximately 0.05 μg of PM2 DNA were carried out in 100 μl of buffer A at 37°C for 10 minutes. The reactions were stopped by addition of 50 μl of Proteinase K (1 mg/ml) in 10 mM Tris-HCl pH 7.4, 10 mM EDTA, and incubated for an additional 5 minutes.

Bacterial Growth and Preparations of Crude Extracts

E. coli strains HB101, the wild type, BW9109, deficient in exonuclease III (xth⁻), and BW434, deficient in both exonuclease III and endonuclease III (xth⁻,nth⁻) were grown in L Broth. E. coli strain BW531, transformed by a plasmid containing the gene encoding endonuclease III, was grown in L Broth supplemented with ampicillin (8). 100 ml of overnight culture were centrifuged to pellet the bacteria. The cells were resuspended in 5 ml buffer B (50 mM KH₂PO₄, pH 7.0, 10 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol) and sonicated with six one-minute bursts at maximal output. Samples were then centrifuged at 25,000 x g for 20 minutes and the supernatants were assayed for endonuclease activity. Protein concentrations were determined using the Bio-Rad protein assay.

Enzyme Purifications

E. coli strain BW531 was grown overnight in 4 liters of L broth supplemented with ampicillin. The bacteria were pelleted, resuspended in 50 ml buffer B, sonicated and centrifuged as above. Nucleic acids were removed from the supernatant (fraction I) by addition of an equal volume of 5% streptomycin sulfate.
in buffer B followed by centrifugation at 25,000 x g for 20 minutes. The supernatant (fraction III) was applied to a 13 x 2.5 cm phosphocellulose column which had been equilibrated with buffer B. The enzyme was eluted with a linear KH₂PO₄ gradient (0.05 M - 0.3 M) in buffer B. The active fractions (between 110 and 280 mM salt) were pooled (fraction III) and dialyzed against buffer B containing 50 mM NaCl. Fraction III was applied to a 25 ml single-stranded DNA-Sepharose column which had been prepared according to Amrdt-Jovan et al. (9) and equilibrated with buffer B containing 0.05 mM NaCl. The enzyme was eluted with a linear NaCl gradient (0.05 M - 1.0 M) in buffer B and assayed against photoalkylated PM2 DNA. Active fractions (between 225-325 mM salt) were pooled and dialyzed against buffer B before freezing in 50% glycerol (fraction IV).

Uracil-DNA glycosylase was purified from B. subtilis according to Cone et al. (10). Fraction VI was used in the sequencing studies. Endonuclease v (pyrimidine dimer-DNA glycosylase) was purified from T4-infected E. coli according to Friedberg et al. (11). Fraction IV was used in the sequencing studies. Modifications of the purification protocols have been published elsewhere (12,13).

Labeling and Sequencing of Alphoid DNA

The alphoid sequence, which was obtained by EcoRl digestion of a pUC9 plasmid containing the inserted fragment, was labeled at the 3' end and recut as previously described (14). The 92 base pair sequence was photoalkylated or ultraviolet-irradiated for 10 min in buffer C (50 mM Tris-HCl pH 7.4, 1 mM EDTA) and ethanol-predpitated. Six units of endonuclease III (purified fraction IV) was reacted with photoalkylated, ultraviolet-irradiated, or untreated DNA for 30 minutes at 37°C. In 100 μl buffer C; 1 unit of activity introduced 0.01 breaks per molecule of photoalkylated PM2 DNA in 1 minute at 37°C. Reactions of 23 units of uracil-DNA glycosylase with alphoid DNA were performed as described (14); 1 unit of activity released 1 pmol of uracil from 0.75 mmol nucleotide equivalent of PBS-2 DNA in 1 minute at 37°C. (12). Photoalkylated and untreated DNA samples were reacted with 5 units of endonuclease v for 30 minutes according to Gallagher and Duker (15); 1 unit of activity released 1 pmol of thymine from 2.4 μg of ultraviolet-irradiated and photoreversed E. coli DNA in 1 minute at 37°C. (13). Following all enzymic reactions, the samples were ethanol-precipitated. Samples reacted with uracil-DNA glycosylase were resuspended in 25 μl 1 M piperidine and incubated at 37°C for 20 minutes. Unreacted photoalkylated and unirradiated DNA were treated with 1 M piperidine at 37°C for 20 minutes or 90°C for 30 minutes. These were then lyophilized and washed twice by the addition of 10 μl H₂O. All samples were lyophilized and resuspended in gel-loading buffer (90% formamide, 10 mM sodium hydroxide, 1 mM EDTA, 0.05% bromophenol blue, 0.05%
Standard DNA sequencing reactions were performed according to Maxam and Gilbert (16) except that 25 μl of 1 M piperidine was used. Samples were electrophoresed through an 8% sequencing gel. The gel was transferred to filter paper and dried in a gel dryer prior to autoradiography.

RESULTS

Crude extracts of the mutant E. coli strains were first assayed for activities against partially depurinated PM2 DNA. The results are shown in figure 1. The double mutant BW434 showed reduced DNA apurinic/apyrimidinic site endonuclease activity compared to the HB101 wild type or the BW9109 mutant. Endonucleolytic activity of the double mutant against photoalkylated DNA was reduced, as shown in figure 2. Extracts of wild-type HB101 and mutant strain BW9109, with deficient exonuclease III but unaltered endonuclease III, both showed increasing activity against photoalkylated DNA with increasing amounts of protein. Maximum cleavage yielded over 2 breaks per superhelical molecule. By contrast, crude extracts from E. coli strain BW434, deficient in both enzymes, had less activity against the photoalkylated substrate than that observed with the other strains (figure 2). Enzyme activity from extracts of BW434 was maximal at approximately 0.3 breaks.

Figure 1. E. coli Endonuclease Activity Against Partially Depurinated Radiolabeled PM2 Phage DNA. The substrate was incubated for 10 minutes at 37° C. with increasing amounts of cell extracts of strains HB101 (x), BW9109 (o) or BW434 (•). DNA strand breaks were measured by the filter assay. Each point represents the average of two determinations.
Figure 2. E. coli Endonuclease Activity Against Photoalkylated Radiolabeled PM2 Phage DNA. The substrate was incubated for 10 minutes at 37°C with increasing amounts of cell extracts of strains HB101 (x), BW9109 (o) or BW434 (a). DNA strand breaks were measured by the filter assay. Each point represents the average of two determinations.

per DNA molecule. Therefore endonuclease III recognizes and incises a damaged moiety in photoalkylated DNA.

DNA irradiated in the absence of isopropanol and di-tert-butylperoxide also was substrate for extracts of E. coli strain BW9109. Figure 3 shows that endonucleolytic activity increased to greater than 2 breaks per DNA molecule. Enzymic incision of this substrate was diminished in the double mutant strain BW434. Therefore, the presence of an alcohol in the irradiation mixture is not necessary for production of base damage substrate for endonuclease III. It is rather an ultraviolet irradiation product without covalent binding of isopropanol.

The sites of enzymic incision of substrate DNA by endonuclease III were therefore investigated. The end-labeled 92 base pair 3' terminus of the human alphoid sequence was irradiated under photoalkylating conditions and examined for sites of DNA incision with a variety of probes. The results are shown in figure 4. Reaction of photoalkylated DNA with endonuclease III resulted in bands corresponding to all cytosines (lane 8). No bands were observed upon incubation of endonuclease III with unirradiated DNA; therefore these breaks cannot result from nonspecific DNA cleavage. The identical sites of enzymic incision were present
Figure 3. E. coli Endonuclease Activity Against Ultraviolet-Irradiated Radiolabeled PM2 Phage DNA. The substrate was incubated for 10 minutes at 37° C. with increasing amounts of cell extracts of strains BW9109 (○) or BW434 (●). DNA strand breaks were measured by the filter assay. Each point represents the average of two determinations.

When the substrate DNA was irradiated in the absence of isopropanol and di-tert-butylperoxide (lane 9). This confirms that formation of cytosine photoproduct substrate sites for endonuclease III incisions is unrelated to photoalkylation additions to DNA bases. No sites of incision of either photoalkylated or ultraviolet-irradiated DNA were present at any thymines, adenines or guanines.

Further analysis demonstrated that these cytosine photoproducts did not result from deamination to uracil. Photoalkylated DNA was reacted with uracil-DNA glycosylase, followed by hydrolysis by piperidine at 37° C. to cleave any apyrimidinic sites that might arise from release of the damaged base (lane 10). There was no difference between the pattern of bands at pyrimidine sites between this protocol and that obtained by piperidine treatment alone (lane 11). Therefore no uracils are formed upon DNA photoalkylation. Both these lanes showed a band pattern identical to lane 12, which was reacted with piperidine at 90° C. The bands of lanes 10 and 11 are less intense than those of lane 12. It is likely that all the bands in these three lanes are due to formation of 6-4'-(pyrimidin-2'-one)-pyrimidines, which are completely labile in hot alkali. The cytosine photoproducts detected in lanes 8 and 9 do not appear in lanes in which the photoalkylated DNA is treated with piperidine (lanes 10, 11, and 12) and are therefore not alkali-labile. Since the cytosine photoproducts were present at all cytosines and not restricted to
Figure 4. Detection of Cytosine Photoproducts in the Human Alphoid Sequence. Aliquots of 3'-end labeled DNA were lyophilized (lane 1); ultraviolet-irradiated and lyophilized (lane 2); photoalkylated and lyophilized (lane 3); subjected to base-specific chemical cleavages: G (lane 4), G-A (lane 5), C-T (lane 6), C (lane 7); photoalkylated and reacted with endonuclease III (lane 8); ultraviolet-irradiated and reacted with endonuclease III (lane 9); photoalkylated and reacted with uracil-DNA glycosylase followed by incubation in 1 M piperidine at 37° C. (lane 10); photoalkylated and incubated in 1 M piperidine at 50° C. (lane 11); photoalkylated and reacted with endonuclease v (lane 13); unirradiated control DNA reacted with endonuclease III (lane 14). The arrows indicate some of the cleavage loci at cytosine photoproducts by endonuclease III.

Those with adjacent pyrimidines, they are neither pyrimidine dimers nor 6-4'- (pyrimidin-2'-one)pyrimidines. This was confirmed by reaction of the DNA with endonuclease v (lane 13), which did not incise DNA at these products. Some cleavage bands were detected at purine sites in this lane (data not shown). This indicates that purine-containing photoproducts are also produced in photoalkylated DNA.

**DISCUSSION**

It has been previously shown that photoalkylation of DNA produces a variety of damaged moieties. These include 8-(2-hydroxy-2-propyl)guanines, 8-(2-hydroxy-2-
propyadenines and pyrimidine dimers (2,3). An activity in *M. luteus* which cleaved photoalkylated PM2 DNA was reported, but neither purified nor characterized (4). We found that purification of the endonuclease against photoalkylated DNA was complicated by the presence of pyrimidine dimers in the substrate combined with the abundance of pyrimidine dimer-DNA glycosylase activity in *M. luteus*. Therefore *E. coli*, which lacks a pyrimidine dimer-DNA glycosylase, was used as the enzyme source in these studies. These results demonstrate that photoalkylated DNA is incised by an *E. coli* endonuclease. Studies with the mutant strains showed the identical endonuclease deficiency against depurinated DNA demonstrated by Cunningham and Weiss, who used a different assay (17). Mutants deficient in endonuclease III showed reduced incision of both photoalkylated and ultraviolet-irradiated DNA. Therefore this enzyme is responsible for cleavage of such damaged DNAs by *E. coli* extracts.

Endonuclease M is known to be active against non-pyrimidine dimer damage in ultraviolet-irradiated DNA (18,19,20). It contains an endonuclease against DNA apurinic/apyrimidinic sites and glycosylase activities that release thymine glycol and urea from oxidized DNA (21,22,23). In addition, endonuclease III cleaves DNA irradiated by 254 nm light at cytosine photoproduc
ts, apparently by a coupled glycosylase-endonuclease mechanism (24). These experiments demonstrate that cytosine photoproduc
ts are produced by ultraviolet irradiation of higher wavelengths than 254 nm. The sequence studies show that these cytosine photoproduc
ts do not result from deaminations to uracils. The chemical structures of these photoproduc
ts are currently under investigation. However, since such damaged moieties result without isopropanol present during irradiation, they must be photoproduc
ts rather than a covalent additions of alcohol groups to DNA bases. Cytosines have been found to be inert to such additions (25).

No cleavage sites by endonuclease III at adenines, guanines, or thymines were seen in photoalkylated or ultraviolet-irradiated DNA samples. Therefore, 8-(4(2-hydroxy-2-propyl)purines, pyrimidine dimers, thymine glycols and 6-4'-(pyrimidin-2'-one)pyrimidines cannot be the loci of enzymic incision of these substrates. Since thymine glycol-DNA glycosylase is present on the endonuclease III molecule (21,22,23), the enzymic cleavage patterns indicate that thymine glycols are not formed by irradiation of DNA at these conditions. It has been reported that irradiation of cultured human cells by monochromatic 313 nm light yields DNA thymine glycols (26). Further studies are necessary to resolve this discrepancy.

These sequences reveal the presence of damaged adducts in photoalkylated DNA that have not been previously noted. These include 6-4'-[(pyrimidin-2'-one)pyrimidines, detected in the irradiated samples that had been hydrolyzed by
piperidine. In addition to pyrimidine dimers, endonuclease V cleavage sites were present at purine loci in photoalkylated DNA (data not shown). These had been previously detected in DNA irradiated by mixed 250-400 nm light (15). Analyses of damaged adducts in photoalkylated DNA by high performance liquid chromatography have demonstrated two adenine photoproducts in addition to 8-(2-hydroxy-2-propyl)adenines (2,3). Among the _M. luteus_ activities against ultraviolet-irradiated DNA are a pyrimidine dimer-DNA glycosylase (27,28), an endonuclease against purine photoproducts (29) and an endonuclease against uncharacterized non-dimer damaged adducts (30,31,32). An endonuclease has been described that incises gamma-irradiated DNA which is probably identical to the latter activity (32,33,34,35). This enzyme has not been completely characterized but appears to be similar in many ways to _E. coli_ endonuclease III (32). This multiplicity of repair enzymes, along with the variety of damaged adducts in the substrate, is sufficient to explain the endonucleolytic activity detected in _M. luteus_ extracts against photoalkylated DNA substrates (4).

Incision of gamma-irradiated DNA at cytosine loci by a partially purified _M. luteus_ endonuclease has been reported (36). While those cytosine moieties have not been identified, it is possible that they are similar or identical to the photoproducts detected in photoalkylated and ultraviolet-irradiated DNA in these studies. Cytosine glycols are formed by chemical oxidation of DNA. These can then be dehydrated or deaminated, yielding 5-hydroxycytosines, uracil glycols or 5-hydroxyuracils (37). While the stability of these products is unknown, it is possible that one or more of them is substrate for _E. coli_ endonuclease III or the _M. luteus_ endonuclease. Determination of the yields of these modified cytosines at various irradiation conditions and of the chemical natures of the moieties recognized by these enzymes are under investigation.

Irradiation at 254 nm is most commonly used in studies of ultraviolet DNA damage and repair. The carcinogenic effects of actinic radiation result from wavelengths between 280 and 300 nm, since shorter wavelengths are filtered by atmospheric ozone (38). Thus it appears that photoproducts formed at uvB wavelengths are of importance. These may include the cytosine moieties described here as well a diverse family of purine photoproducts (15,29). The distribution and yield of these various products may indicate which, if any, of them is significant in actinic carcinogenesis.

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