Excision of cytosine hydrates from Z-DNA

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ABSTRACT

Ultraviolet irradiation of DNA produces cytosine hydrate, released as a free base by *E. coli* endonuclease III. Cytosine hydrate excision was investigated by assaying photoproduct release from cytosine-radiolabeled, irradiated poly(dG-dC):poly(dG-dC). Conformational shifts between B-DNA and Z-DNA were effected by heating the polymer in either nickel chloride or cobaltous chloride, and were determined by circular dichroism. Rates of enzymic cytosine hydrate release did not differ between the different substrate conformations. Irradiation of left-handed poly(dG-dC):poly(dG-dC) resulted in cytosine hydrate formation. Therefore, neither formation nor enzymic excision of ultraviolet-induced cytosine hydrates are substantially affected by these DNA conformational states.

INTRODUCTION

Ultraviolet irradiation of DNA results in formation of a variety of photoproducts, including pyrimidine dimers, 6-4'-(pyrimidin-2'-one)pyrimidines, and cytosine hydrates (5,6-dihydro-6-hydroxycytosines) (1). The latter are excised as free bases by the DNA-glycosylase activity of *E. coli* endonuclease III (2). This type of enzyme, sometimes termed a 'redoxendonuclease', is widely conserved and present in a wide variety of species (3-5). It removes a wide variety of modified pyrimidines formed by ultraviolet, gamma, or X-irradiation or chemical oxidation, followed by DNA breakage at the resultant apyrimidinic site by catalysis of β -elimination (4,5).

Previous studies of enzymic release of cytosine hydrates from ultraviolet-irradiated poly(dG-dC):poly(dG-dC) used substrates in the right-handed B-DNA conformation (2,6,7). However, this alternating purine:pyrimidine sequence can adopt the Z-conformation as a result of reactions with chemical carcinogens, the presence of metal ions, or negative superhelicity (8,9). We therefore examined the possible formation and excision of cytosine hydrates in left-handed poly(dG-dC):poly(dG-dC). Irradiation of the left-handed polymer yielded cytosine hydrate formation. Rates of enzymic photoproduct release did not vary among the different substrate conformations. These results indicate excision-repair of cytosine hydrates to be likely, regardless of the conformational state of the substrate DNA.

MATERIALS AND METHODS

The alternating copolymer poly(dG-dC):poly(dG-dC), radiolabeled with tritium in cytosine to a specific activity of 1.5×10^6 cpm/ μ g, was synthesized as previously described (7). Irradiation was by broad-spectrum ultraviolet light (10). Endonuclease III was purified from overproducing *E. coli* strain lambda-N99 containing the plasmid pHIT1 by the method of Weiss and Duker, with a unit defined as that quantity of enzyme introducing 0.1 breaks per molecule of irradiated PM2 DNA under the conditions described (11). The glycosylase activity for DNA cytosine hydrates was assayed by measurement of the release of labeled photoproduct from irradiated poly(dG-dC):poly(dG-dC) into the ethanol-soluble fraction as previously described (7). The 100 μ l reaction mixture was precipitated by addition of 100 μ l calf thymus DNA, the solution brought to 0.3 M sodium acetate, pH 7, and 450 μ l ethanol added. Following precipitation and centrifugation, the radioactive content of the supernatant was determined by liquid scintillation counting (7).

Poly(dG-dC):poly(dG-dC), in 10 mM HEPES-NaOH, 0.01 mM EDTA, pH 7.6, was incubated in the presence of either nickel chloride or cobaltous chloride according to van de Sande et al. (12). Conformational shifts from B-DNA to Z-DNA were then effected by heating the polydeoxyribonucleotide at 55°C for 15 minutes (12). Conversion of the polymer to Z-DNA was also accomplished by incubation in 60% ethanol (13). Conformational states of substrate alternating copolymers were determined by ultraviolet spectroscopy (12) and by ultraviolet circular dichroism using a Jasco J-600 spectropolarimeter (12-14). Both, assays, ultraviolet spectroscopy and circular dichroism, showed complete conversion of poly(dG-dC):poly(dG-dC) to the Z-conformation. Superhelical bacteriophage PM2 DNA, radiolabeled in thymine, was purified and used as substrate for endonuclease assays by the nitrocellulose filter binding method according to Teebor and Brent (15). Thin layer chromatography was performed on polyethyleneimine-cellulose plates as previously described (16).

RESULTS

The optimum conditions of metal ion concentrations for incubation of endonuclease III with irradiated poly(dG-dC):poly(dG-dC) were first determined. These results are shown in figure 1. Endonuclease III activity was inhibited by the presence of Ni^{+2} or Co^{+2} . At 1 mM concentrations, enzymic release of labeled cytosine photoproduct was inhibited by 30% or 37%, respectively. By contrast, figure 2 demonstrates

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Figure 1. Inhibition of Enzymic Cytosine Hydrate Release from Irradiated Polydeoxyribonucleotide by Nickel Chloride or Cobaltous Chloride. Radiolabeled poly(dG-dC):poly(dG-dC) was irradiated for 60 minutes at a fluence of 16.7 W/m² and incubated with 3.6 units of endonuclease III at 37°C for 30 minutes in the presence of increasing concentrations of NiCl₂ (○) or CoCl₂ (●). Release of radiolabeled material was assayed by ethanol precipitation and recovery of the ethanol-soluble fraction and measurement by liquid scintillation counting. Each point represents the average of three determinations.

Figure 2. Hydrolysis of Poly(dG-dC):poly(dG-dC) by Endonuclease III in Presence of CoCl₂. Irradiated (●) or unirradiated (○) radiolabeled poly(dG-dC):poly(dG-dC) was incubated with 3.6 units of endonuclease III for 30 minutes as described above in the presence of increasing concentrations of CoCl₂. Release of radiolabeled material was assayed by ethanol precipitation and recovery of the ethanol-soluble fraction and measurement by liquid scintillation counting. Each point represents the average of three determinations.

Table 1. Release of Cytosine Hydrate from Irradiated Polydeoxyribonucleotide by Endonuclease III in the Presence of Nickel Chloride or Cobaltous Chloride.

Metal	Conformation at Irradiation	Conformation at Reaction	Slope × 10 ²
Ni ²⁺	B	B	2.18 ± 0.92
Ni ²⁺	B	Z	2.20 ± 0.78
Ni ²⁺	Z	Z	2.05 ± 0.65
Co ²⁺	B	B	1.85 ± 0.88
Co ²⁺	B	Z	1.62 ± 0.52
Co ²⁺	Z	Z	1.64 ± 1.11

Radiolabeled poly(dG-dC):poly(dG-dC) was incubated in 1.0 mM NiCl₂ or 0.5 mM CoCl₂. It was then: (a) irradiated for 30, 60 or 90 minutes and then incubated with 3.6 units of endonuclease III for one hour as described above while in the B-conformation; (b) irradiated for 30, 60 or 90 minutes while in the B-conformation, heated for 15 minutes at 55°C to induce the Z-conformation and then incubated with 3.6 units of endonuclease III for one hour as described above while in the Z-conformation; (c) heated for 15 minutes at 55°C to induce the Z-conformation, irradiated for 30, 60 or 90 minutes and then incubated with 3.6 units of endonuclease III for one hour as described above while in the Z-conformation. Release of radiolabeled material was assayed by ethanol precipitation and recovery of the ethanol-soluble fraction and measurement by liquid scintillation counting. Each point represented the average of three determinations. The results were plotted as percentages of radioactivity released per reaction mixture against times of substrate irradiation. The curves were fitted and the slopes of the lines calculated by the method of least squares.

endonuclease III activity to be stimulated by Co²⁺. Concentrations of CoCl₂ above 1 mM yielded enzymic degradation of both unirradiated and irradiated poly(dG-dC):poly(dG-dC). Release of labeled material increased fivefold at 2 mM Co²⁺. Thin layer chromatographic analysis of the material released from poly(dG-dC):poly(dG-dC) showed its comigration with marker dCMP (data not shown). Under these conditions, superhelical PM2 DNA was not incised by endonuclease III (data not shown). Therefore, endonuclease III digests this polymer in the presence of high concentrations of cobaltous chloride.

These results were used to select the conditions of incubation that would convert right-handed poly(dG-dC):poly(dG-dC) to the left-handed Z-conformation, yet not result in substantial inhibition of the DNA-glycosylase activity of endonuclease III. Incubation in 1.0 mM Ni²⁺ or 0.5 mM Co²⁺, followed by heating, resulted in transition to the Z-form, as shown by circular dichroism (data not shown). Polymers were irradiated in the B or the Z conformations and used as substrates for endonuclease III. The results are shown in table 1. Irradiation of the polymer, followed by induction of the Z-conformation heating in 0.5 mM Ni²⁺, did not affect its suitability as substrate for endonuclease III. Reversing the order by inducing the Z-conformation before irradiation did not change enzymic excision of cytosine hydrate. Therefore, this photoproduct is formed in left-handed DNA and is excised from that substrate.

Similar results, also shown in table 1, were obtained in the presence of 0.5 mM Co²⁺. Poly(dG-dC):poly(dG-dC) was irradiated in either the B or Z conformation and used as substrate for endonuclease III. Irradiation of the polymer, followed by induction of the Z-conformation heating it in 0.5 mM Co²⁺, did not affect cytosine hydrate excision by endonuclease III. Reversing the order by inducing the Z-conformation before irradiation did not alter enzymic photoproduct release. This demonstrates cytosine hydrate to be formed in, and excised from, left-handed DNA.

Since it was possible that formation of cytosine hydrate in Z-DNA was due to the presence of metal cations, poly(dG-

Figure 3. Release of Cytosine Hydrate from Irradiated Polydeoxyribonucleotide by Endonuclease III. Radiolabeled poly(dG-dC):poly(dG-dC) was incubated in 60% ethanol to induce the Z-conformation and irradiated for 60 minutes (●) or irradiated for 60 minutes in the B-conformation in the absence of ethanol (○). The ethanol was removed by dialysis into 40 mM KH_2PO_4 , 1 mM EDTA, pH 7.4, and both substrates reacted with the indicated quantities of endonuclease III (1.2 units/ μl) as described above. Release of radiolabeled material was assayed by ethanol precipitation and recovery of the ethanol-soluble fraction and measurement by liquid scintillation counting. Results were calculated as percentages of radioactivity released of the total present in each reaction mixture. Each point represents the average of three determinations.

dC):poly(dG-dC) was first incubated in 60% ethanol and irradiated; ethanol was removed by dialysis and the resulting substrate incubated with endonuclease III. The results are shown in figure 3. While photoproduct release from DNA irradiated in the Z-conformation was half of that irradiated in the B-conformation, cytosine hydrate was formed under both conditions.

DISCUSSION

Two types of enzymic repair of modified purines do not occur when the DNA substrate is left-handed: enzymic demethylation of O-6-methylguanine and glycosylic excision of 2,6-diamino-4-oxo-5-methylformamidopyrimidine (17–19). However, other enzymes do catalyze reactions with a Z-DNA substrate. These include the restriction endonucleases Hha I and Cfo I, the Hha I DNA methyltransferase, and *E. coli* DNA polymerase I (20,21). The excision of photoproducts from left-handed DNA helices had not been studied. The polydeoxyribonucleotide poly(dG-dC):poly(dG-dC) was examined since it can be easily manipulated to assume the Z-conformation, and, because of the alternating copolymer sequence, cytosine hydrate is produced by ultraviolet irradiation to the exclusion of the major photoproducts formed at adjacent pyrimidines.

Various divalent cations induce transition of this polymer to the Z-conformation. These include Mg^{+2} , Mn^{+2} , Ni^{+2} and

Co^{+2} (12). However, the concentrations of Mg^{+2} and Mn^{+2} necessary to induce this change also inhibited cytosine hydrate excision by endonuclease III (7). Therefore, Ni^{+2} and Co^{+2} were used to induce B-DNA to Z-DNA transitions at concentrations consistent with endonuclease III activity. The facile conversion obtained by heating the alternating copolymer in the presence of these ions allows for examinations of cytosine hydrate release in substrate irradiated in either conformation. While low concentrations of either Ni^{+2} or Co^{+2} do inhibit enzymic cytosine hydrate release, there remains enough glycosylase activity to study substrate conformational effects. One unexpected finding was the degradation of the unirradiated polymer at high concentrations of Co^{+2} ; this was not observed with other cations (7). Since superhelical PM2 DNA was not incised, and released radioactivity was identified as dCMP by thin layer chromatography, it appears that exonucleolytic degradation of poly(dG-dC):poly(dG-dC) by endonuclease III occurs under these conditions. Since only 0.5 mM concentrations of Co^{+2} were used in subsequent experiments, this phenomenon did not affect the results of the glycosylase assays.

Experiments performed in the presence of either Ni^{+2} or Co^{+2} yielded identical results. Comparisons with control DNAs irradiated and reacted with endonuclease III in the B-conformation showed no substantial reduction of enzymic cytosine hydrate excision. These findings were obtained both with DNAs irradiated in the B-conformation and enzyme-reacted in the Z-conformation and with DNAs irradiated in the Z-conformation and enzyme-reacted in the Z-conformation. Therefore, cytosine hydrates are formed in, and excised from, left-handed DNA. There was some reduction in cytosine hydrate formation when poly(dG-dC):poly(dG-dC) was converted to the Z-form by incubation in 60% ethanol. This product is formed by irradiation in the presence of 1.3 M isopropanol (11). It is possible that yields are reduced at higher alcohol concentrations.

The virtually identical yields of cytosine hydrates in both conformations of irradiated poly(dG-dC):poly(dG-dC) is consistent with the findings of identical rates of hydrolytic cytosine deaminations to uracil in these DNA structures (22). Cytosine hydrate and uracil hydrate have been suggested as intermediates in this process (23). The release of cytosine hydrate by endonuclease III was not reduced by heating irradiated DNA at 55°C for 15 minutes. Free cytosine hydrate rapidly reverts to cytosine under such conditions (2,24). This indicates cytosine hydrate to be stable in DNA, probably necessitating its repair. While poly(dG-dC):poly(dG-dC) occurs infrequently in human DNA, the alternating copolymer poly(dG-dT):poly(dA-dC), with the potential for formation of both Z-DNA and cytosine hydrates, is relatively abundant (8). An enzyme very similar to *E. coli* endonuclease III is present in cultured human cells (2,7,25–27). It would appear that excision of photohydrates from such alternating, potentially Z-DNA-forming purine:pyrimidine segments is a function of this type of enzyme. This would prevent somatic mutations resulting either from DNA cytosine hydrate or its derivatives.

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