Stability of DNA thymine hydrates

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ABSTRACT

Pyrimidine hydrates are products of ultraviolet irradiation of DNA. We have already demonstrated the formation of both *cis*-thymine hydrate and *trans*-thymine hydrate (6-hydroxy-5,6-dihydrothymine) in irradiated poly(dA-dT):poly(dA-dT). These are released from DNA as free bases by bacterial or human glycosylases. Thymine hydrate stabilities were studied in irradiated DNA substrates using purified *E. coli* endonuclease III as a reagent for their removal. After irradiation, substrate poly(dA-dT):poly(dA-dT), radiolabeled in thymine, was incubated at 50, 60, 70 or 80°C, cooled, and then reacted with the enzyme under standard conditions. Thymine hydrates were assayed by enzymic release of labeled material into the ethanol-soluble fraction. Their identities were confirmed by high performance liquid chromatography. The decay of thymine hydrates in heated DNA followed first-order kinetics with a $k = 2.8 \times 10^{-5}$/sec at 80°C. These hydrates were also detected in lesser quantities in the unirradiated, control substrate. Extrapolation from an Arrhenius plot yields an estimated half-life of 33.3 hours at 37°C for DNA thymine hydrates. Such stability, together with their formation in unirradiated DNA, suggest thymine hydrates to be formed under physiological conditions and to be sufficiently stable in DNA to be potentially genotoxic. This necessitates their constant removal from DNA by the excision-repair system.

INTRODUCTION

Ultraviolet (UV) irradiation of cellular DNA results in a variety of pyrimidine photoproducts, including cyclobutane pyrimidine dimers and 6–4′-(pyrimidin-2′-one)pyrimidines (1,2). Such irradiation of cells causes cell death, mutation and neoplastic transformation (3). DNA pyrimidine hydrates are chemically minor products of UV radiation (1,4). These are removed from DNA as free bases by the glycosylic activity of *E. coli* endonuclease III (5,6). This type of enzyme, sometimes termed a 'redoxyendonuclease', is widely conserved and present in a great variety of species (7–9). It excises a wide variety of modified pyrimidines formed by UV, gamma-, or X-irradiation or chemical oxidation, followed by cleavage at the resultant DNA apyrimidinic site by catalysis of β-elimination (8,9).

Pyrimidine hydrates were originally detected in irradiated solutions of free pyrimidine bases, nucleosides and nucleotides (4). Unequivocal demonstration of their formation in DNA has been relatively recent, and was achieved by a combination of enzyme probes and use of high performance liquid chromatography (HPLC) for definitive characterization of the products (5). We have demonstrated the formation, in UV-irradiated poly(dA-dT):poly(dA-dT), of both *cis*-thymine hydrate and *trans*-thymine hydrate (6-hydroxy-5,6-dihydrothymine); both are excised from DNA by bacterial and human enzymes (6). Previous studies of pyrimidine hydrates have shown them to have differing stabilities. In neutral aqueous solutions, uracil hydrate (6-hydroxy-5,6-dihydrouracil) is stable, but cytosine hydrate (6-hydroxy-5,6-dihydrocytosine) rapidly reverts to cytosine (4,5). The *cis* and *trans* isomers of thymine hydrate each have a half-life of 24 hours in neutral solution (10). The stabilities of pyrimidine hydrates in DNA differ from those of free bases. Cytosine hydrate formed in UV-irradiated poly(dG-dC):poly(dG-dC) is relatively stable, with a half-life of 25 hours at 37°C, unlike cytosine hydrate in aqueous solution (11). By contrast, uracil hydrate in UV-irradiated poly(dA-dU):poly(dA-dU) is somewhat less stable than the free base (11). To determine the stability of thymine hydrates in DNA, we used purified *E. coli* endonuclease III as a reagent for their removal from UV-irradiated poly(dA-dT):poly(dA-dT) (6). Substrates were heated at different temperatures prior to enzymic reactions. This led to decreased photoproduct recoveries from DNA; the higher the temperature, the lower the yields. Thymine hydrates were also detected in lesser quantities in the unirradiated control substrate. The half-life of thymine hydrates in DNA was extrapolated to be 33.3 hours at 37°C, similar to those of free bases in aqueous solution (10). These results indicate that DNA thymine hydrates might be formed under physiological conditions in DNA and are sufficiently stable for potential genotoxicity. This necessitates their constant removal from DNA by the excision-repair system.

MATERIALS AND METHODS

The substrate, double-stranded alternating copolymer poly(dA-dT):poly(dA-dT), was radiolabeled in thymine with (5–^{3}H)TTP (NEN, 20.0 Ci/mmol) by nick translation as previously described

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(6); it was dissolved in 40 mM KH_2PO_4, pH 7.4, 1 mM EDTA. Specific activity was approximately 2.5×10^6 cpm/μg. The substrate was irradiated in the cold by broad-spectrum UV light, using a Bausch and Lomb unit equipped with a 200 W Osram Hg bulb and a Schott WG 320 filter at an incident dose of 6.6×10^{-5} einstein/cm^2/min, as previously described (6,12,13). The dose rate of the 270 nm component was 12 J/m2/sec. Following irradiation, DNA was heated in a water bath in an eppendorf tube and cooled at 37°C for 10 minutes. Small oligonucleotides were removed from the substrate by a Sephadex G-50 (Pharmacia) spin-column (14). Endonuclease III was purified from overproducing *E. coli* strain lambda-N99 containing the plasmid pHIT1 by the method of Asahara et al. (15). After an enzymic reaction for 30 minutes at 37°C, the yield of DNA thymine hydrates was assayed by measurement of release of labeled photoproduct from the irradiated substrate into the ethanol-soluble fraction as previously described (6). The substrate, in the 100μl reaction mixture, was precipitated in a dry ice-ethanol bath, after addition of 100μl calf thymus DNA (1 mg/ml), 30μl of 3.0 M sodium acetate, 60μl of 50 mM $(Co[NH_3]6)Cl_3$ and 660μl of ethanol. Following centrifugation, the radioactive content of the supernatant was determined and analysis of released bases performed both by high performance liquid chromatography (HPLC) and by thin layer chromatography (TLC) as previously described (6).

S1 nuclease digestion was performed to determine the extent of substrate denaturation according to Chao and Duker (16). In this assay, DEAE-cellulose paper, which binds highly negatively charged compounds, is washed free of oligonucleotides while retaining larger segments of DNA (17). Following irradiation, the buffer was changed to 30 mM sodium acetate, pH 4.5, 50 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol, by a Sephadex G-50 spin-column (14). S1 nuclease (Amersham; 0.5 unit/60 ng DNA) was added and the reaction performed at 37°C. The reaction was stopped by 15 mM EDTA and each entire mixture spotted on a 2.5 cm square on marked Whatman DE81 DEAE-cellulose paper. The paper strip was washed and dried; the squares were cut from the strip and counted in POPOP (16). Single-stranded poly(dA), radiolabeled in adenine, was synthesized by terminal transferase (Pharmacia) and used as the positive control (18).

RESULTS

The possible denaturation of the substrate irradiated poly(dA-dT):poly(dA-dT) during heating was examined by S1 nuclease digestion. The results are shown in figure 1. After 12 minutes of digestion, over 70% of the control unirradiated DNA remained bound to the paper and this pattern did not change even after 2 hours of heating at 80°C. Similarly, although UV irradiation caused some denaturation of DNA, 2 hours of subsequent heating did not increase the damage any further. The percentage of radioactivity bound to the paper decreased slightly after further heating, as in the case of unirradiated DNA. By constrast, single-stranded poly(dA) without any treatment, was completely and rapidly digested by S1 nuclease (data not shown). Figure 1, therefore demonstrates that heating did not cause significant substrate denaturation beyond that due to irradiation.

Materials released from irradiated and heated substrate by endonuclease III were analyzed by HPLC. The freshly prepared substrate was irradiated for 60 minutes, heated at 70°C for 5 hours and incubated with 1.0μg of endonuclease III for 30 minutes. Figure 2, a typical HPLC profile of enzyme-released radiolabeled materials, showed two distinct peaks of thymine photoproducts in addition to a minor peak identified as thymine. About 70% of the recovered radioactivity was present in the two major peaks. Analysis of the enzyme released photoproducts by TLC also showed them to be separable from marker thymine (data not shown). These photoproducts were determined to be a mixture of *cis*-thymine hydrate and *trans*-thymine hydrate by their cochromatography in HPLC with appropriate markers, synthesized by both chemical and photochemical means as described (6). Interestingly, control supernatants from unirradiated, but heated DNAs also had two peaks in the positions of thymine hydrates, with no thymine release. These latter peaks, with 71% of the released radioactivity, indicate possible

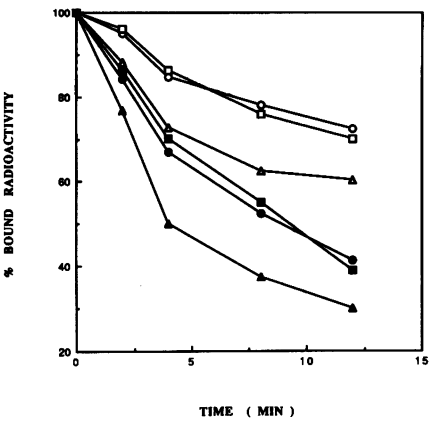

Figure 1. S1 Nuclease Digestion of Irradiated and Heated Poly(dA-dT):poly(dA-dT). Irradiated (filled symbols) or unirradiated (open symbols) poly(dA-dT):poly(dA-dT) was heated at 80°C for 0 (●,○), 2 (■,□), and 5 (▲,△) hours and digested by S1 nuclease. The extent of digestion was assayed by binding of radioactivity to DEAE-cellulose paper as described. Each point represents the average of three independent determinations.

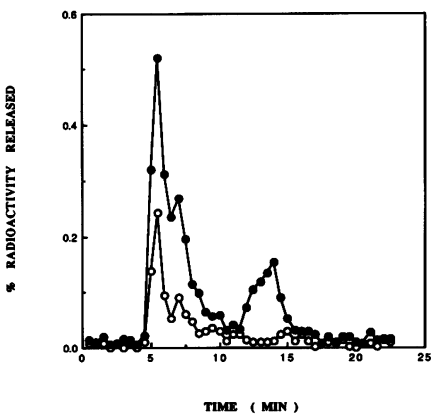

Figure 2. Separation of Enzyme-Released Thymine Hydrates from Thymine by HPLC. Poly(dA-dT):poly(dA-dT), radiolabeled in thymine, was irradiated with broad-spectrum UV light for 1 hour, heated at 70°C for 5 hours, cooled, and incubated with 1.0μg of endonuclease III for 30 minutes at 37°C. After the reaction, the substrate was precipitated. The ethanol-soluble fraction containing enzyme-released thymine hydrates was dried under vacuum, dissolved in water, filtered, and analyzed by HPLC as described. The eluant was 50 mM ammonium formate (1 ml/minute). The 0.5 ml fractions were counted in 20% Biosolve (Beckman). Enzyme-released radioactive material from irradiated (●) or (○) unirradiated substrates.

endogenous formation of thymine hydrates in unirradiated DNAs. Radioactivity release from this control substrate was not affected by heating before the enzymic reaction (data not shown).

The stabilities of DNA thymine hydrates were then examined by reaction with endonuclease III under conditions of enzyme excess. Irradiated substrate was heated, cooled and reacted with 1.0μg of enzyme for 30 minutes at 37°C. Thymine hydrates were assayed by enzymic release of labeled material into the ethanol-soluble fraction. However, some non-enzymic release of labeled materials were detected and these were found to be small oligonucleotides by analysis with HPLC (data not shown). To reduce this background and to maintain parity with the conditions of S1 digestion, the substrates were passed through Sephadex G-50 before enzymic reactions. This resulted in total elimination of non-enzymic label release. Figure 3 demonstrates that the decreased releases of thymine hydrates after heating of DNA at 80°C followed first-order kinetics. With no substrate heating, 0.64% of substrate radioactivity, equivalent to 0.29 pmoles of thymine hydrates (100%), were released; after 5 hours, 0.17 pmoles were recovered. Curve fitting was by the method of least squares. The half-life of thymine hydrates at 80°C was estimated to be 6.9 hours by extrapolation, yielding a k of $2.8\times10^{-5}sec^{-1}$.

Stabilities of thymine hydrates at different temperatures were determined. Substrate DNAs were heated between 50 and 80°C at 10°C increments; data similar to those in figure 3 were obtained. Rate constants for decay of thymine hydrates were determined at each temperature from estimated half-lives. The results are summarized as an Arrhenius plot (figure 4). Curve fitting and extrapolation yield an estimated rate constant of $0.6\times10^{-5}sec^{-1}$ at 37°C. The estimated half-life of thymine hydrates at this temperature is therefore 33.3 hours. The activation energy for this reaction was determined to be 8 kcal/mol from the slope of this line.

DISCUSSION

The DNA glycosylase activity of endonuclease III is known to release 5,6-saturated thymines and their derivatives, such as thymine glycol (5,6-dihydroxy-5,6-dihydrothymine), 5-hydroxy-5-methylhydantoin, methyltartronylurea and urea, as free bases from oxidized DNA (19–23). It has recently been established that this enzyme and its human counterpart remove both cytosine hydrate and uracil hydrate from UV-irradiated DNAs (5,24). Subsequently, both *cis*-thymine hydrate and *trans*-thymine hydrate were shown to be also excised by these enzymes (6). Both these hydrates were synthesized by both organic chemical and photochemical methods. These markers were demonstrated to coelute with the material released from UV-irradiated poly(dA-dT):poly(dA-dT) in three independent systems: HPLC, TLC and paper chromatography (6,25). No thymine glycol was released from this substrate by either preparation (6,25).

Since poly(dA-dT):poly(dA-dT) has an alternating purine-pyrimidine sequence, thymine hydrates are produced by UV irradiation to the exclusion of major photoproducts formed at adjacent pyrimidines. In addition, there is no possiblity of interference with repair glycosylase activity by the presence of cyclobutane pyrimidine dimers (26). Because of the low yields of DNA thymine hydrates, high doses of broad-spectrum UV light were required. This contrasts with the relatively abundant formation of cytosine hydrates in poly(dG-dC):poly(dG-dC) and uracil hydrates in poly(dA-dU):poly(dA-dU) by monochramatic 280 nm light (24,27). The quantum yield of thymine photohydration in aqueous solution is three orders of magnitude lower than that of uracil photohydration (10); the yield of cytosine photohydrate is intermediate (4,28). This is paralleled by the photoreactivity of these bases in DNA; studies of synthetic polydeoxyribonucleotides indicate photohydration of uracil to be greater than cytosine, which in turn far exceeds that of thymine (5,6,11,24,27).

Therefore, the lower photochemical yield of thymine hydrates results in reduced rates of its enzymic excision from poly(dA-dT):poly(dA-dT). However, the possibility exists that, although such photohydrates were produced in higher quantities, those were removed with lesser efficiency. Endonuclease III strongly prefers native DNA as substrate (21,23,29). Although DNA thymine glycol is a substrate for endonuclease III, there was no excision of that base from extensively oxidized and denatured poly(dA-dT):poly(dA-dT) (23). While this substrate is initially double-stranded, high fluences of irradiation and heating could

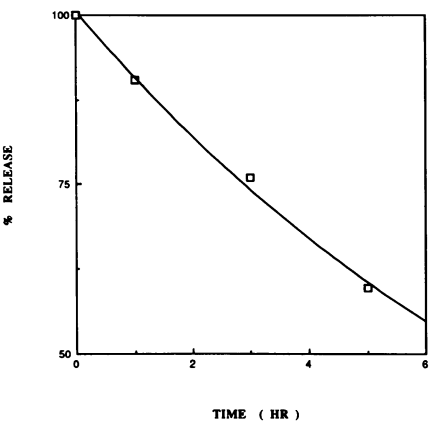

Figure 3. Heat-induced Decay of Thymine Hydrates Formed in Poly(dA-dT):poly(dA-dT). UV-irradiated poly(dA-dT):poly(dA-dT), radiolabeled in thymine, was heated at 80°C for indicated times, cooled, passed through Sephadex G-50, and reacted with endonuclease III as described. Reaction mixtures were precipitated and ethanol-soluble radioactivities determined as described. Each point represents the average of two independent determinations.

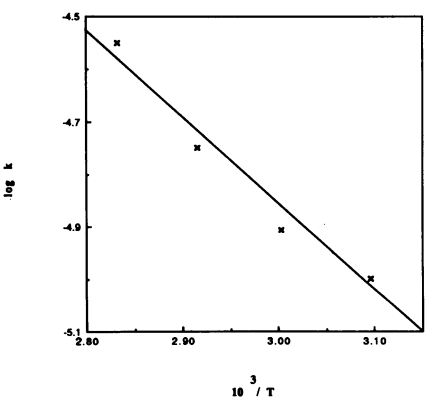

Figure 4. Arrhenius Plot of Heat-induced Decay of Thymine Hydrates Formed in Poly(dA-dT):poly(dA-dT). Rate Constants (sec^{-1}) for decay of thymine hydrates in UV-irradiated poly(dA-dT):poly(dA-dT) as functions of temperature were determined as described.

alter substrate conformation and result in reduced enzyme binding. This might be reflected in low turnover of this enzyme against DNA thymine hydrates (6). Analysis using digestion by S1 nuclease, which degrades single-stranded DNA, was therefore initially used to establish the substrate conformation. Since the DNA molecules larger than oligonucleotides are retained by the DE81 paper, the fraction of radioactivity bound to the paper gives an estimate of the prevalence of undigested double-stranded substrate. The results (figure 1) demonstrate that, although UV irradiation caused some denaturation, this was not increased by heating for 2 hours at 80°C. After 5 hours of heating, the percent bound radioactivity decreased slightly and this was also true for control, unirradiated DNA. Therefore, heating of the irradiated substrate did not yield significant denaturation.

HPLC analysis of released materials showed them to be *trans*-thymine hydrate and *cis*-thymine hydrate, as previously reported (6,25). Both *trans*-thymine hydrate and *cis*-thymine hydrate were chemically and photochemically synthesised. These coeluted with the enzyme released labeled materials. Heating the irradiated substrate prior to enzymic reaction did not alter the distribution of released materials (figure 2), indicating that DNA thymine hydrates do not decay, yielding ring-opened derivatives. It appears that these hydrates are instead reversed to thymines. This contrasts with thymine glycol in DNA, which decays to yield a plethora of derivatives (30). However, enzymic excision of thymine hydrates decreased with heating with release dependent on temperature (figure 3). Thymine hydrate half-lives of 6.9 hours at 80°C increased to 19.2 hours at 50°C. Thus, DNA thymine hydrates are less stable at higher temperatures. Since it is difficult to directly determine the slow rate of reversal of thymine hydrates at 37°C, this rate was estimated from an Arrhenius plot (figure 4). This plot, covering a range of submelting temperatures, results in a straight line; the existence of a threshold temperature for this reaction is therefore unlikely. The half-life of DNA thymine hydrates is estmated to be 33.3 hours at 37°C. This is similar to the half-life of 24 hours of free thymine hydrates at this temperature in neutral solution (10).

The enzymic excision of thymine hydrates from unirradiated DNA is of significance (figure 2). Heating of the substrate did not effect the release, because this occurred, to the same extent, also without heating. Although the UV fluences required to induce thymine hydrates in any significant yields are far in excess of the range of cell survival, the ubiquitous presence of background levels of thymine hydrates in unirradiated poly(dA-dT):poly(dA-dT) indicate the possible biological relevance of these products. Because of the intracellular aquatic environment, thymine hydrates might constantly be formed in DNA under physiological conditions. An estimate might therefore be made of the quantity of thymine hydrates present in a human cell, assuming the amount of thymine hydrates in this substrate to be representative of that in human DNA. 0.15% of substrate radioactivity, equivalent to 0.07 pmoles of thymine hydrates, was released in 30 minutes from 30 ng of unirradiated substrate. The quantities of thymine hydrates present in cellular DNA might be conceivably greater than the estimate from the refrigerated substrates used here. However, extrapolation from this data to the human genome (with 28.5% of its bases as thymine) indicate a possiblity of 1.4×10^3 hydrated thymines present in the human genome. This background indicates hydration of thymines, and possibly other pyrimidines, to be another class of spontaneously occuring DNA damages in addition to oxidative and hydrolytic types. These include depurinations, depyrimidinations, base deaminations, ring openings, and base oxidations (8,31–34).

Therefore, although thymines hydrates are produced by high UV fluences, there exists a background level of these compounds in unirradiated DNA. This renders thymine hydrates, and possible other pyrimidine hydrates, unique among the DNA photoproducts of UV irradiation; significant levels of neither cyclobutane pyrimidine dimers nor 6–4′-(pyrimidin-2′-one)pyrimidines have been reported in unirradiated DNA. Thymine hydrates in DNA have half-lives of 33.3 hours, similar to those in neutral solutions. Left unrepaired, these hydrates could cause biological consequences, including interference with transcription, inhibition of replication, or mutation. Thus, it appears that endonuclease III and its mammalian counterparts in the excision-repair pathway are necessary for the repair of hydrated pyrimidines.

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