5,6-Saturated thymine lesions in DNA: production by ultraviolet light or hydrogen peroxide

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ABSTRACT

Thymine analogs with saturated 5-6 bonds are important types of DNA damage that are recognized by the DNA N-glycosylase activity of *E. coli* endonuclease III. Seeking agents which could preferentially form 5,6-hydrated thymine residues in duplex DNA both *in vivo* and *in vitro*, we exposed purified duplex DNA to 325- or 313-nm light; however, after such exposure pyrimidine dimers greatly predominated over 5,6-hydrated thymine. Hydrogen peroxide, on the other hand, formed significant numbers of endonuclease III-sensitive sites *in vitro* which were not apurinic/apyrimidinic lesions and thus were likely to be 5,6-hydrated thymines.

INTRODUCTION

DNA is inherently somewhat chemically unstable, even in the absence of exogenous damaging agents (1). In addition, aerobic cells, which obtain energy by the reduction of oxygen, produce high-potential intermediates (2) which react with DNA (3,4). The fact that oxygen radicals profoundly affect cells is witnessed by the multilevel defenses which respiring organisms have erected and by the pronounced sensitivity to oxygen of cells lacking adequate protection against free radicals (2).

Oxidizing agents, ultraviolet (UV) light and X-rays produce certain types of DNA damage in common (5,6). Osmium tetroxide has been useful in preparing polynucleotide substrates with one such type of damage (6), 5,6-saturated thymine residues, since the reagent seems to be relatively specific in producing these residues (7). However, partial or total denaturation of the DNA is required during treatment with this agent. Therefore, to study duplex DNA in whole cells or viruses, we have sought other agents to form 5,6-saturated thymine residues in DNA. Previous reports had indicated a greater relative efficiency of formation of such damage by near UV light (>300-nm) compared to far (<300-nm) UV light (8). Contrary to expectation, we found, however, that pyrimidine dimers predominate over thymine glycols as photoproducts at both

short and long UV wavelengths. Conversely, hydrogen peroxide provided a ready means of introducing 5,6-saturated thymine into DNA with relatively few accompanying strand breaks, apurinic/apyrimidinic (AP) sites, or other apparent types of damage.

MATERIALS AND METHODS

Materials. Hydrogen peroxide (30% solution) was reagent grade from Mallinckrodt, [methyl-³H]thymidine was purchased from Schwarz-Mann, Sarkosyl NL-30 was from Geigy Chemical Co., Triton X-100 was from Sigma, nitrocellulose filters (type B-6) were from Schleicher and Schuell, and bovine catalase (25,000 units/mg) was from Worthington.

E. coli endonuclease III was Fraction VII prepared according to Gates and Linn (6) and was assayed as described. T₄ UV endonuclease was Fraction IV prepared according to Friedberg and King (9) and was stored as described by Demple and Linn (7). Endonuclease IV was Fraction V of Ljungquist (10), was assayed as noted therein and was generously supplied by Dr. Tomas Lindahl, Imperial Cancer Research Fund, Mill Hill, London.

Phage PM2 [³H]DNA (7000-13,000 cpm/nmol) was prepared according to Kuhnlein et al. (11). DNA concentrations are expressed as nucleotide-residues.

DNA nicking assays. The enzymatic or non-enzymatic nicking of form I PM2 DNA was assayed as described by Kuhnlein et al (11) using nitrocellulose filters after incubation of the treated DNA at pH 12.3 for 2 min at 25°C. Reactions contained 67 mM Tris-HCl, pH 8.2, 25 mM KCl, 4 mM EDTA and 0.005% Triton X-100 in 50 µl, and were incubated 30 min at 37°C.

Irradiation of DNA. The source of 254-nm light was a Westinghouse germicidal lamp (G15T8). Monochromatic light of 325-nm wavelength was provided by a Liconix He:Cd laser with 15 mW output and a 1 mm beam, on loan from the San Francisco Laser Center. The beam was expanded to 1-2 cm using a quartz lens of 50 cm focal length, and deflected through 90° to the sample using a front surface mirror (Edmund Scientific). For irradiation with a 313-nm maximum intensity, light from a xenon short arc lamp (mounted in a Hitachi Perkin Elmer MPF 2A Spectrofluorimeter) was passed through several layers of soft glass to eliminate wavelengths below 300 nm and deflected to the sample with a first surface mirror.

Incident radiation fluxes at 254 or 313 nm were determined by potassium ferrioxalate actinometry according to Jagger (12). The 325-nm dose rates were determined from the known output power of the laser and from the area of the

incident beam, or by direct measurement with a photometer. All samples were irradiated in a thin layer (<2 mm) on a watch glass kept on ice.

Hydrogen Peroxide Treatment. Reactions of H_2O_2 with PM2 [3H]DNA (7000 cpm/nmol nucleotide in 10 mM Tris-HCl, pH 7.5) were incubated at 37°C for the indicated time. Unless otherwise stated, reactions were stopped by the addition of 30- μ l aliquots to tubes containing 120 units of bovine catalase. After 10 min at room temperature, the samples were heated 2 min at 70°C and chilled for assay of endonuclease-sensitive sites.

RESULTS

Irradiation of DNA with Near UV Light

Far UV light (<300 nm) causes the formation of a broad spectrum of base damage in DNA, including pyrimidine dimers (13) and pyrimidine photohydrates (14). Though the latter are formed with considerably lower efficiency than pyrimidine dimers (perhaps 1-2%) by 254 nm light (6,8), they are nonetheless generated in significant amounts. However, terrestrial organisms are not normally exposed to light of such short wavelengths because solar radiation which penetrates the atmosphere in significant yield is in the near-UV range (300-400 nm). Therefore, it is important to examine alterations of DNA structure which appear in response to wavelengths near 300 nm.

Because of their stability and the ease with which they can be detected, pyrimidine dimers have long been known to be a major type of damage resulting from near UV light (15). Using an indirect, degradative assay for 5,6-dihydroxydihydrothymine (thymine glycol), Hariharan and Cerutti (8) measured the ratio of that adduct to pyrimidine dimers formed in vivo by UV doses of various wavelengths. They reported that the efficiency of formation of both products was severely reduced as the wavelength of the radiation increased to 313 nm, but that the ratio of thymine glycol to pyrimidine dimers seemed to increase dramatically, from 0.06 at 280 nm to 0.73 at 313 nm (8).

We have established that our preparations of E. coli endonuclease III contain a DNA glycosylase activity which specifically removes 5,6-saturated thymines (including thymine glycol) from duplex DNA (7). However, a study of the UV action spectrum for the formation of sites sensitive to the enzyme indicated a maximum around 280 nm, with no detectable endonuclease sites formed by radiation of 300 nm or greater (16). As the intensity of light on the samples was not determined exactly in that early experiment, it was necessary to pursue the matter further using monochromatic radiation of known intensity.

Phage PM2 superhelical DNA circles were irradiated in a thin film at 0°C, then examined for sites sensitive either to endonuclease III (i.e., thymine glycol residues) or to phage T4 UV endonuclease (taken to be a measure of pyrimidine dimers). These tests are highly sensitive, being capable of detecting 1-2 base lesions per 10^5 base-pairs. Indeed, sites sensitive to the T4 endonuclease were formed with moderate efficiency and in significant amounts by monochromatic 325-nm light from a He:Cd laser (Fig. 1A). Concomitantly, however, there was no evidence for the induction of endonuclease III-sensitive sites, even with doses up to 7×10^5 J/m². If 0.1 endonuclease III-sensitive site per PM2 genome were the limit of detection, thymine glycol residues would be formed by the 325 nm light in amounts less than one-ninth the number of pyrimidine dimers.

PM2 DNA exposed to light of approximately 313-nm wavelength from a xenon short arc lamp (Fig. 1B) was also tested. Again, even with doses of up to 9300 J/m², no significant endonuclease III-sensitive sites were formed. In contrast, T4 UV endonuclease incised at a relatively low but significant number of sites in the DNA exposed to the largest doses of 313-nm light. For comparison, the production of sites sensitive to both endonucleases by 254-nm

Figure 1. Formation by UV light of sites in duplex DNA sensitive to T4 UV endonuclease and *E. coli* endonuclease III. PM2 [³H]DNA (0.32 mM) was irradiated as indicated in Materials and Methods. Assays contained 1 nmol DNA and either 66 units of T4 UV endonuclease (circles) or 5-10 units of endonuclease III (squares). Panel A: 550 J/m²/sec 325-nm light from a He:Cd laser. Panel B: 2 J/m²/sec 254-nm light (filled symbols) or 15.5 J/m²/sec 313-nm light (open symbols), from a germicidal lamp or a xenon short arc lamp, respectively.

radiation is also shown in Figure 1B. Clearly, the amounts of enzyme used were sufficient to affect cleavages at many more sites than were observed in the substrates treated with near UV light.

Because endonuclease III cleaves DNA at 5,6-hydrated thymine residues formed by osmium tetroxide (7) and probably by UV or gamma rays (6), it appears that, for DNA irradiated in vitro, the production of 5,6-saturated thymine-type lesions does not markedly approach that of pyrimidine dimers as the wavelength of the radiation increases beyond 300 nm. These observations are at variance with those of Hariharan and Cerutti (8), but it is conceivable that the degradative assay employed by Hariharan and Cerutti (8) also measures some product other than thymine glycol. Alternatively, intracellular conditions, or specifically, the nuclear environment in HeLa cells, may have promoted a greatly increased efficiency of formation of thymine-glycol-type damage, perhaps through the generation of hydroxyl radicals. In this regard, for example, the DNA damaging agent hydrogen peroxide is produced in significant amounts by irradiation with near UV light of solutions of tryptophan or tryptophan-containing proteins (17). However, when E. coli systems (intact cells, or phages λ , T4 and T7) were irradiated with the 325-nm light in vivo, pyrimidine dimers appeared to be the major cause of biological inactivation. This conclusion was based upon the effectiveness of photoreactivation, or of the E. coli uvr^+ or T4 $denV^+$ gene products in promoting the survival of cells or phages irradiated with 325-nm light (B. Demple, James Halbrook and S. Linn, unpublished). However, it is important to note that at 365 nm other photoproducts are quite important in these systems (see review by Webb, reference 18), and it is likely that these photoproducts are not without effect after 325-nm irradiation.

Treatment of DNA with Hydrogen Peroxide

Hydrogen peroxide breaks down to produce oxygen radicals which in turn have been shown to cause DNA damage by way of altered bases (17), particularly pyrimidines (19). A preliminary study has indicated that hydrogen peroxide produces thymidine glycol from thymidine (20). We thus tested whether duplex DNA treated with hydrogen peroxide might contain thymine glycol residues (Table I). After reaction followed by dilution and dialysis, the DNA was tested for cleavage by alkali, by endonuclease III, and by the similarly purified, AP site-specific endonuclease IV of E. coli (11). In no case were a significant number of sites formed by peroxide which could subsequently be cut by alkali treatment or by the AP-site specific endonuclease IV. Endonuclease III, however, effected cleavage at a significant number of sites, the concen-

Table I. Nicking of PM2 DNA treated with 0.1M hydrogen peroxide

<u>Treatment</u>	<u>Incubation at 37°</u>	
	-	+
	<u>nicks per PM2 DNA molecule</u>	
None	0.11	0.44
Endonuclease IV	0.11	0.44
Alkali	0.10	0.48
Endonuclease III	0.20	1.16

Reactions with PM2 [³H]DNA (0.25 ml) were incubated at 37°C for 60 min where indicated, then diluted 4-fold into 1 M NaCl-10mM Tris-HCl, pH 7.5, at 0°C and dialyzed versus 10 mM Tris-HCl, pH 7.5. Endonuclease reactions contained 2 nmol PM2 DNA and 6 units of endonuclease III or endonuclease IV. Alkaline hydrolysis was performed by incubation for 3 hr at 25°C in 0.15 M potassium phosphate, pH 12.3, then neutralization.

tration of which increased with the length of hydrogen peroxide treatment. Although the reagent also gave rise to strand breaks (as seen by the increase in the "Blank" level of nicks per DNA molecule during treatment), the major portion of damage evidently consisted of modified bases. (Strand breaks due to the hydrogen peroxide-mediated formation of hydroxide radicals have been previously noted (3,17).) Judging from the specificity of endonuclease III (7), the modified bases would appear to be 5,6-saturated thymine residues, perhaps specifically 5,6-dihydroxydihydrothymine. The sensitivity of the endonuclease assay employed here allows the measurement of lesions in substrates at levels for which base excision could not be directly detected. A 100-fold or greater increase in the extent of DNA oxidation would be necessary to permit an unambiguous determination of base release by the thymine glycol glycosylase function of endonuclease III (7). However, the resistance of endonuclease III-sensitive sites to endonuclease IV precludes the action of endonuclease III simply as having been at base-free sites.

Not surprisingly, the rate and extent of derivitization of the DNA is dependent upon the concentration of hydrogen peroxide used (Figure 2). As the initial rates of derivitization were of interest in these experiments, the peroxide was destroyed rapidly by the addition of catalase. (When dilution and dialysis were used instead for this purpose (Table 1), a greater extent of

Figure 2. Formation of endonuclease III-sensitive sites in double-stranded DNA by hydrogen peroxide. PM2 [³H]DNA (0.34 mM) was treated with hydrogen peroxide as indicated (see Materials and Methods). Reactions contained 3 nmol PM2 DNA and 6 units of endonuclease IV or 12 units of endonuclease III. A small number of nicks formed by the endonuclease IV were subtracted to obtain the number of non AP sites sensitive to endonuclease III. Triangles, degree of nicking by 0.6 M hydrogen peroxide alone. H₂O₂ concentrations forming endonuclease III-sensitive sites: open circles, 0.1 M; filled circles, 0.2 M; open squares, 0.3 M; filled square, 0.6 M.

lesion formation occurred.) The apparent initial rate of formation of sites sensitive to endonuclease III is in direct proportion to the initial hydrogen peroxide concentration (Figure 2, inset). Although it is unlikely that the peroxide itself reacts directly with DNA, the concentration of free radicals formed by H₂O₂ decay, which apparently mediate base damage (21), should reflect the initial peroxide concentration (22).

DISCUSSION

DNA damage of the thymine glycol type can be formed by ultraviolet light, but only short (<300-nm) wavelengths of the radiation seem to produce such lesions with measurable efficiency, and then in small numbers relative to pyrimidine dimers. In fact, as shown by an indirect analysis (18, 23), wavelengths less than 305 nm are responsible for virtually all of the biological consequences of solar radiation mainly through pyrimidine dimer formation. In contrast, hydrogen peroxide does seem to form in significant amounts sites in DNA which are cleaved by endonuclease III. These enzyme-sensitive sites are damaged bases, not AP sites. From the known specificity of endonuclease

III (7) and the observations of Schellenberg (20), the lesions are likely to be 5,6-hydrated thymines. Although the peroxide reactions carried out in these experiments were not especially efficient, the rates can be increased by the addition of metal catalysts (e.g., Fe^{+++} or Cu^{++}), which are known to promote the rate but not the product distribution of H_2O_2 breakdown (22), or by treating single-stranded DNA. For example, upon the addition of 100 μM CuSO_4 to 0.1M H_2O_2 , the rate of inactivation by hydrogen peroxide of λ phage DNA packaged in vitro is increased more than 10^4 -fold (unpublished). Also noteworthy is the observation that peroxide produces base damage at a six-fold greater rate than it does strand breaks (see Figure 2). This is a clear advantage of H_2O_2 over γ -rays in producing endonuclease III-sensitive sites for experimental work (4,6).

Hydrogen peroxide is clearly lethal to E. coli and to eukaryotic cells (24). Certain E. coli mutants deficient in DNA repair are particularly sensitive to mutagenesis (24) or killing (24,25; B. Demple, J. Halbrook and S. Linn, unpublished) by peroxide, indicating the importance of DNA damage in biological responses to H_2O_2 . The exact molecular species responsible for these responses in vivo have not been identified, although the hydroxyl radical ($\text{HO}^\bullet$) appears to be the main agent responsible for the formation of strand breaks, as well as alkali-labile and other lesions in DNA during γ - or X-irradiation in vitro (4,21,24). Since hydrogen peroxide decomposes to form a variety of reactive species (22), experiments with specific scavengers (2) are necessary to define further which radicals form sites sensitive to endonuclease III.

Intact cells can be readily treated with hydrogen peroxide, and a probe, E. coli endonuclease III, is available for thymine glycols produced by this agent. It is thus possible to examine the effect of 5,6-saturated thymines on cell survival and mutagenesis and to increase our understanding of the physiological responses to the ubiquitous oxygen radicals (2).

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