Effect of Apurinic/Apyrimidinic Endonucleases and Polyamines on DNA Treated with Bleomycin and Neocarzinostatin: Specific Formation and Cleavage of Closely Opposed Lesions in Complementary Strands†

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ABSTRACT: Bleomycin and neocarzinostatin induce modified apurinic/apyrimidinic (AP) sites by oxidation of the sugar moiety in DNA. In order to quantitatively assess the susceptibility of these lesions to repair endonucleases, drug-treated ^{3}H-labeled colE1 DNA was mixed with ^{14}C-labeled heat-depurinated DNA, and endonuclease-susceptible sites in the mixture were titrated with various AP endonucleases or with polyamines. Single- and double-strand breaks were quantitated by determining the fractions of supercoiled, nicked circular, and linear molecules. Exonuclease III and endonucleases III and IV of *Escherichia coli*, as well as putrescine, produced a nearly 2-fold increase in single-strand breaks in bleomycin-treated DNA, indicating cleavage of drug-induced AP sites. The bleomycin–induced AP sites were comparable to heat-induced sites in their sensitivity to *E. coli* endonucleases III and IV but were cleaved by exonuclease III only at high concentrations. Bleomycin-induced AP sites were much more sensitive to cleavage by putrescine than heat-induced sites. Treatment with putrescine or very high concentrations of endonuclease III also increased the number of double-strand breaks in bleomycin-treated DNA, suggesting a minor class of lesion consisting of an AP site accompanied by a closely opposed break in the complementary strand. These complex lesions were resistant to cleavage by endonuclease IV. However, when colE1 DNA was treated with neocarzinostatin, subsequent treatment with putrescine, endonuclease IV, or very high concentrations of endonuclease III produced a dramatic increase in double-strand breaks but no detectable increase in single-strand breaks. These results suggest that virtually all neocarzinostatin-induced AP sites are accompanied by a closely opposed strand break. The presence of a closely opposed strand break apparently renders AP sites resistant to cleavage by at least some AP endonucleases, although the degree of resistance to specific enzymes may depend on the precise structure and/or exact position of both the AP site and the accompanying break. The fraction of such closely opposed lesions induced by both bleomycin and neocarzinostatin is significant, even at very low levels of DNA damage, and the resistance of these lesions to repair enzymes suggests possible involvement in both the cytotoxic and mutagenic effects of these drugs. Several characteristics of the putative AP site/strand-break lesions induced by neocarzinostatin suggest that they may correspond to certain AP-like lesions which were previously detected on DNA sequencing gels as endonuclease IV susceptible sites and which have been strongly implicated in neocarzinostatin-induced mutagenesis.

Bleomycin and neocarzinostatin (NCS)[1] are antitumor antibiotics which specifically oxidize DNA sugars, producing both strand breaks and apurinic/apyrimidinic (AP) sites [see reviews by Povirk (1983) and by Goldberg et al. (1985)]. In the case of bleomycin, the structure of the AP site has recently been shown to involve oxidation of C-4′, to form a ring-opened structure with an aldehyde at C-1′ and (unlike heat-induced AP sites) a ketone at C-4′ (Sugiyama et al., 1985; Rabow et al., 1986). The structure(s) of NCS-induced AP sites has (have) not been determined, although there is evidence that they differ chemically from heat-induced sites, and thus probably also contain an oxidized deoxyribose (Povirk & Goldberg, 1985).

AP endonucleases are enzymes which catalyze the single-strand cleavage of the phosphodiester backbone at AP sites in DNA, the first step in repair of these lesions [reviewed by Lindahl (1982)]. Type I enzymes cleave at the 3′ side of the AP site [probably by catalyzing β-elimination from the C-1′ aldehyde (Bailly & Verly, 1987)], leaving a 5′-phosphate terminus and an AP sugar attached to the 3′ terminus. Type II enzymes cleave at the 5′ side of the AP site, leaving a 3′-hydroxy terminus and a 5′-phosphoryl AP sugar attached to the 5′ terminus. *Escherichia coli* possess two major type II AP endonucleases, endonuclease IV and exonuclease III. Both these enzymes can also remove phosphates and fragmented sugars from the 3′ end of double-stranded DNA, producing a suitable template-primer for repair synthesis by DNA polymerase I (Niwa & Moses, 1981: Demple et al., 1986), and exonuclease III has an additional 3′ → 5′ exonuclease activity. The third major AP endonuclease of *E. coli* is endonuclease III, a type I enzyme, with an associated glycosylase activity which removes certain oxidized or fragmented bases from DNA. Somewhat similar to endonuclease III are the pyrimidine dimer glycosylase/AP endonucleases (UV-endonucleases) of *Micrococcus luteus* and phage T4. These type I enzymes cleave one pyrimidine of the dimer from its

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[1] Abbreviations: AP, apurinic/apyrimidinic; DSB, double-strand break; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NCS, neocarzinostatin; Tris, tris(hydroxymethyl)aminomethane.

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sugar and then cleave the DNA backbone at the resulting AP site.

There is evidence that various AP endonucleases differ substantially in their ability to recognize and cleave AP sites containing oxidized sugars. X-Irradiated DNA contains alkali-labile sites, probably AP sites with an oxidized C-2′, which are substrates for yeast endonuclease E but not *E. coli* endonuclease III (Wallace, 1983). AP sites in bleomycin-treated DNA are reportely susceptible to rat liver AP endonuclease (Schyns et al., 1978) and *E. coli* endonucleases III and IV (Lloyd et al., 1978), but not exonuclease III (Niwa & Moses, 1981). Recently, we reported that at least one form of NCS-induced AP site was susceptible to endonuclease IV but not to endonuclease III or exonuclease III (Povirk & Goldberg, 1985). While these results would suggest that the bleomycin- and NCS-induced AP sites have different structures, caution must be exercised in comparing the available data, which come from several studies in which different enzyme preparations, different drug and enzyme treatment conditions, and different DNA cleavage assays wee employed. Because of these uncertainties, and because of the failure of host cell deficiencies in AP endonucleases to have any effect on survival and mutagenesis of bleomycin-damaged λ phage (Povirk & Houlgrave, 1988), we undertook a quantitative comparison of the susceptibilities of AP sites induced by heat, bleomycin, and NCS to cleavage by four AP endonucleases and two primary amines. The results suggest that, although both bleomycin and NCS induce endonuclease-resistant AP sites, endonuclease resistance may in most cases be due to the presence of a closely opposed break in the complementary strand, rather than to the chemical structure of the AP site itself.

Materials and Methods

Materials. Purified bleomycin A2, obtained from Dr. W. T. Bradner, was dissolved in distilled water at a concentration of 2 mM (A_{290} = 28). Fe(III)·bleomycin was formed by adding an equal volume of 2 mM ferric ammonium sulfate (12-hydrate) in 0.5 mM HCl. The complex was stored at −20 °C and was stable in this form, although a slight loss in activity was noted upon repeated freezing and thawing. Use of Fe(III)·bleomycin rather than metal-free bleomycin improved the reproducibility of the strand breakage assays. NCS chromophore, prepared as described previously (Povirk et al., 1981), was a gift of Dr. I. H. Goldberg.

Radiolabeled colE1 was prepared from strain JC411 (obtained from Dr. W. D. Rupp), grown in K medium containing 4 μg/mL thymine and either 0.4 μCi/mL [2-^{14}C]thymine or 0.5 μCi/mL [*methyl*-^{3}H]thymidine. Supercoiled DNA was prepared by the alkaline lysis method (Maniatis et al., 1982), banded twice in CsCl/ethidium bromide gradients, and dialyzed against 50 mM Hepes–KOH/0.1 mM EDTA, pH 8.

To obtain depurinated DNA, ^{14}C-labeled colE1 was ethanol-precipitated, dissolved in 0.1 M KCl/10 mM sodium citrate, pH 4, and heated at 60 °C for 3 min (Schaaper & Loeb, 1981). The sample was neutralized by addition of Hepes–KOH, pH 8, to a concentration of 50 mM. This DNA contained 1.0–1.3 AP sites per molecule.

Highly purified endonuclease III, isolated from the overproducing strain λN99/pRPC79*nth*$^{+}$, was a gift of Dr. Richard P. Cunningham. Highly purified endonuclease IV was a gift of Dr. Bruce Demple. One unit of endonuclease IV is defined as the amount of enzyme required to release 1 pmol of 3′-phosphoglycolaldehyde per minute from a synthetic DNA substrate [see Demple et al. (1986)]. Exonuclease III was from New England Biolabs. *M. luteus* UV-endonuclease was from Applied Genetics.

Drug–DNA Reactions. Bleomycin reactions contained 100 μg/mL supercoiled [^{3}H]DNA, 25 mM 2-mercaptoethanol, 40 mM Hepes–KOH, pH 8, 0.1 mM EDTA, and 0–0.4 μM Fe(III)·bleomycin. Fe(III)·bleomycin was diluted just before use in distilled water. The reaction mixture was prepared on ice and then incubated at 37 °C for 1 h. The EDTA concentration was then increased to 50 mM and the incubation continued for 30 min to assure complete chelation of Fe(III). EDTA had little effect on bleomycin activity at 0.1 mM but effectively stopped the DNA cleavage reaction at 50 mM.

NCS chromophore reactions contained 100 μg/mL supercoiled [^{3}H]DNA, 5 mM glutathione, 40 mM Hepes–KOH, pH 8, 0.1 mM EDTA, and 0–0.05 μM chromophore. Chromophore was diluted to 10 times the final concentration in 20 mM sodium citrate (pH 4)/20% methanol, just before addition. The mixture was prepared on ice and incubated for 20 min at 22 °C in the dark.

Enzyme Treatments. Drug-damaged DNA was diluted to a concentration of 30 μg/mL, with addition of appropriate reagents to yield the indicated buffers. A trace (~2 μg/mL) of depurinated [^{14}C]DNA was added. The mixture was treated for 1 h at 37 °C with various AP endonucleases, or with polyamines. Endonuclease III, endonuclease IV, and polyamine treatments were performed in 0.2 M NaCl/50 mM Hepes–KOH/0.1 mM EDTA/0.1 mM dithiothreitol, pH 8. *M. luteus* UV-endonuclease treatments were performed in 50 mM NaCl/50 mM Hepes/KOH/1 mM EDTA/0.1 mM dithiothreitol, pH 8. In both cases, bleomycin-treated samples contained an additional 15 mM EDTA. Exonuclease III treatments were performed in 50 mM Hepes–KOH/5 mM $CaCl_2$/1 mM EDTA/0.1 mM dithiothreitol, pH 8. Use of calcium as a cofactor allows assay of the AP endonuclease actiivty with minimal interference from the exonuclease activity (Rogers & Weiss, 1980). Bleomycin-treated samples were ethanol-precipitated prior to treatment with exonuclease III in order to remove EDTA and bleomycin. All enzymes were diluted on ice in the appropriate buffer just before use, except that exonuclease III was diluted in the absence of $CaCl_2$.

Gel Electrophoresis. Samples, usually 20 μL, containing ~2500 cpm of ^{3}H and ~400 cpm of ^{14}C, were run on horizontal 1.6% agarose gels containing 16 mM Hepes–KOH, 16 mM sodium acetate, 0.8 mM EDTA, and 0.5 μg/mL ethidium bromide, pH 7.5. Electrophoresis was for 2 h at 12 V/cm. Under these conditions, linear colE1 molecules ran fastest, followed by supercoiled and nicked circular. This arrangement was essential for quantitating very small fractions of linear DNA. Hepes buffer was used because AP sites, particularly those induced by NCS, were found to be unstable in Tris buffers. Constant exchange between the anode and cathode reservoirs was required to maintain buffer pH.

Bands representing the three topological forms were visualized under long-wave ultraviolet light and excised from the gel. The gel slices were melted in an autoclave for 10 min in 0.8 mL of water, and radioactivity was counted after addition of 12 mL of Scintiverse E (Fisher).

Strand Break Calculations. The number of double-strand breaks per molecule (n_2) was calculated from the fraction of radioactivity found in linear molecules. Assuming that the distribution of double-strand breaks among individual molecules follows a Poisson distribution, the fraction of total molecules having exactly one break (f_1) (i.e., full-length linear molecules) is

$$f_1 = n_2 e^{-n_2}$$

The fraction of molecules having no double-strand breaks (f_0)

(i.e., supercoiled and nicked circular molecules) is

$$f_0 = e^{-n_2}$$

Thus

$$\frac{f_1}{f_1 + f_0} = \frac{n_2 e^{-n_2}}{n_2 e^{-n_2} + e^{-n_2}} = \frac{n_2}{1 + n_2}$$

or

$$n_2 = \frac{1}{(f_1 + f_0)/f_1 - 1} = \frac{1}{(R_S + R_N + R_L)/R_L - 1}$$

where R_S, R_N, and R_L are the radioactivity associated with supercoiled, nicked circular, and linear DNA, respectively. It may be noted that $f_1/(f_1 + f_0)$ is the proportion of linear molecules expressed as a fraction of total *full-length* molecules, rather than of total molecules. Shorter molecules with two or more double-strand breaks will migrate faster than the linear form and will not be included in the total radioactivity counted.

The number of total peaks per molecule (n_{tot}) (single-strand breaks plus double-strand breaks) was calculated from the fraction of unbroken (supercoiled) molecules (f_s) according to the formula $n_{tot} = -\ln f_s = -\ln (R_S/R_T)$ where R_T is the total radioactivity. Due to the fact that radioactivity in molecules with more than one double-strand break is not counted, R_T must be calculated from the radioactivity in full-length molecules and the known number of double-strand breaks, i.e.

$$(R_S + R_N + R_L)/R_T = f_0 + f_1$$

$$R_T = (R_S + R_N + R_L)/(f_0 + f_1)$$

where

$$f_1 + f_0 = n_2 e^{-n_2} + e^{-n_2}$$

In the experiments presented here, the fraction of molecules with multiple double-strand breaks was small (<5%), so that the factor $f_0 + f_1$ was very close to 1 and never less than 0.95.

Results

Cleavage of Bleomycin-Induced Lesions. Bleomycin can be activated by several pathways, all of which lead to production of a specific iron chelate complex termed "activated bleomycin", which is active in DNA degradation (Burger et al., 1981). In preliminary experiments, it was found that the most reproducible results were obtained when performed Fe(III)·bleomycin was added to DNA, followed by 2-mercaptoethanol. There is strong evidence that the effect of thiol in bleomycin reactions is to reduce Fe(III)·bleomycin to Fe(II)·bleomycin, which is then converted to activated bleomycin (Povirk, 1979, 1983; Burger et al., 1981). The exact mechanism of bleomycin activation in cells is unknown. However, glutathione proved to be inefficient in bleomycin activation in vitro, and since the nature of the thiol cofactor would not be expected to have qualitative effects on the nature of bleomycin-induced DNA damage, 2-mercaptoethanol was chosen as a cofactor in bleomycin reactions.

ColE1 [^{3}H]DNA incubated in the absence of bleomycin contained 0.1–0.2 total breaks and <0.01 double-strand break per molecule. Treatment of DNA with 0.2–0.3 μM bleomycin resulted in an additional 0.5–0.7 total breaks and 0.06–0.08 double-strand breaks per molecule. Subsequent treatment with AP endonucleases, including endonuclease III, endonuclease IV, and exonuclease III of *E. coli* (Figure 1) and the UV-endonuclease of *M. luteus* (not shown), produced a further increase in total strand breaks. In agreement with previous studies (Lloyd et al., 1978), the number of endonuclease-susceptible sites was approximately equal to the number of direct strand breaks induced by bleomycin. Titration of a mixture of depurinated [^{14}C]DNA and bleomycin-treated [^{3}H]DNA revealed that heat-induced and bleomycin-induced AP sites were indistinguishable in their sensitivity to the type I endonucleases (endonuclease III and UV-endonuclease). However, bleomycin-induced AP sites were slightly less sensitive to endonuclease IV, and profoundly (about 30-fold) less sensitive to exonuclease III, than heat-induced sites, probably because of the difference in chemical structure. Cleavage of heat-induced AP sites by endonuclease IV appeared to be

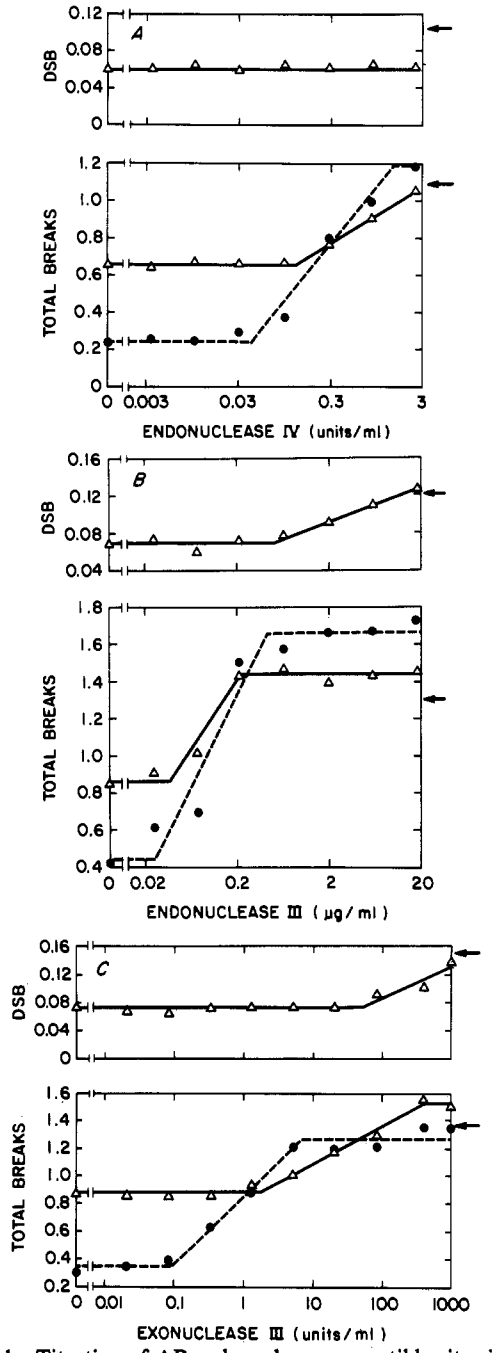

FIGURE 1: Titration of AP endonuclease susceptible sites in bleomycin-treated DNA (△) or in depurinated DNA (●). Bleomycin concentrations were 0.2–0.3 μM. DSB = double-strand breaks. Breakage seen in untreated DNA (0.1–0.2 total break and <0.01 double-strand break) has not been subtracted. All enzymes induced <0.05 total break in untreated DNA. Double-strand breakage in depurinated DNA (not shown) was always <0.02 per molecule and was not increased by treatment with enzymes or polyamines. Arrows show the level of cleavage produced in bleomycin-treated DNA by 100 mM putrescine.

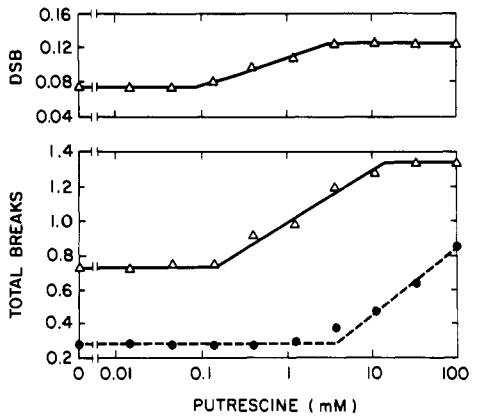

FIGURE 2: Cleavage by putrescine of putative AP sites in bleomycin-treated DNA (△) or in depurinated DNA (●). DSB = double-strand breaks. For further details, see Figure 1.

significantly inhibited by the presence of bleomycin-treated DNA (compare Figure 1A and Figure 3A), perhaps due to the large number of blocked 3′ ends which are formed by bleomycin and which are also believed to be substrates for endonuclease IV (Demple et al., 1986).

Concentrations of endonuclease III at which the increase in total breaks was fully expressed did not increase the number of double-strand breaks in bleomycin-treated DNA in all. However, very high concentrations of endonuclease III or exonuclease III (Figure 1) did increase double-strand breakage by approximately 70–95%. When even higher concentrations of these enzymes were employed, apparent cleavage of undamaged DNA and anomalous DNA migration on the gel (perhaps due to enzyme binding) resulted; thus, it was not possible to determine whether the increase in double-strand breaks had reached a plateau. The increase in double-strand breakage induced by exonuclease III required enzyme concentrations approximately 200 times greater than those needed to cleave heat-induced AP sites. Thus, it is possible that, even in the presence of Ca^{2+}, sufficient exonuclease activity was present to digest DNA between proximal single-strand breaks in opposite strands, resulting in apparent double-strand breakage. However, the endonuclease III induced increase in double-strand breaks suggests that bleomycin produced a class of complex lesion consisting of an AP site accompanied by a closely opposed strand break (or another AP site) in the opposite strand. The presence of the closely opposed lesion might distort DNA so as to render these sites either partially or totally resistant to endonuclease III, endonuclease IV, and exonuclease III, all of which require a double-stranded substrate (Lindahl, 1982). This possibility was previously noted by Lloyd et al. (1978).

To obtain more information on this question, chemical cleavage of bleomycin-induced AP sites by polyamines (Lindahl & Andersson, 1972) was attempted. Bleomycin-induced AP sites were highly sensitive to cleavage by putrescine (Figure 2), much more so than heat-induced sites. Complete cleavage was effected by 20 mM putrescine at 37 °C. More importantly, in addition to the increase in total strand breaks, putrescine produced a 1.7-fold increase in double-strand breaks. This result indicates the presence of AP sites or other putrescine-susceptible sites which are accompanied by a closely opposed strand break or another putrescine-susceptible site in the complementary strand. The quantitative similarity in the sensitivity of the closely opposed putrescine-susceptible sites to that of the nonopposed sites is consistent with the proposal that both types of sites have the same chemical structure. Furthermore, both the closely opposed and the nonopposed

Table I: Cleavage and Protection of Bleomycin-Induced AP Sites by Polyamines[a]

	0.20 μM bleomycin		0.28 μM bleomycin	
posttreatment	total breaks[b]	double-strand breaks	total breaks[b]	double-strand breaks
none	0.51	0.08	0.90	0.12
10 mM spermidine[c]	0.55	0.10	0.94	0.14
1 mM putrescine	0.91	0.14	1.56	0.21
10 mM spermidine + 1 mM putrescine	0.66	0.11	1.21	0.12

[a] Bleomycin-damaged DNA was treated with polyamines for 1 h at 37 °C, as indicated. [b] Breakage seen in samples not treated with bleomycin has been subtracted. [c] Complete cleavage of heat-induced AP sites was effected by 10 mM spermidine, with half-maximal cleavage at 1 mM.

sites were markedly less sensitive to cleavage by spermidine than heat-induced sites and were partially protected from putrescine-induced cleavage by high concentrations of spermidine (Table I). Thus, all the data are consistent with the existence of a minor component of bleomycin-induced AP sites which has the same chemical structure as the majority of bleomycin-induced AP sites but is partially or completely resistant to endonucleolytic cleavage as a result of the presence of a closely opposed break or AP site in the complementary strand. As has been noted in the case of the bleomycin-induced direct double-strand breaks (Povirk et al., 1977), the occurrence of the closely opposed AP lesions is considerably greater than would be expected from the coincidence of randomly distributed AP sites and strand breaks in bleomycin-treated colE1 DNA.

To determine more precisely the origin of the putrescine-induced double-strand breaks, bleomycin-treated DNA was electrophoresed, and gel slices containing supercoiled and nicked circular molecules were excised, soaked in putrescine at 37 °C, and inserted into and run on a second gel. These experiments revealed that virtually all of the linear molecules generated by putrescine treatment were derived from nicked circular molecules; there was no detectable conversion of supercoiled molecules to linear molecules (data not shown). These results suggest that putrescine-induced double-strand breaks result from lesions consisting of an AP site and a closely opposed strand break and that lesions consisting of two closely opposed AP sites are rarely if ever produced.

Surprisingly, UV-endonuclease did not produce any detectable increase in double-strand breaks in bleomycin-treated DNA even at 50 μg/mL, about 10 times the concentration required for half-maximal cleavage of nonopposed AP sites in both heat-depurinated and bleomycin-treated DNA (not shown). This result suggests that AP sites with a closely opposed strand break are poor substrates for UV-endonuclease, even though this enzyme can cleave AP sites in both single-stranded and double-stranded DNA (Lindahl, 1982).

Cleavage of NCS-Induced Lesions. The effects of AP endonucleases and primary amines on NCS chromophore-treated DNA were considerably different from the effects on bleomycin-treated DNA. AP endonucleases and polyamines had little effect on the number of total breaks in chromophore-treated DNA (Figures 3 and 4). The increase in total breaks was never greater than 15%, and in many experiments, no increase was detectable (the error in estimation of breaks was approximately ±10%). However, when DNA treated with chromophore in the presence of glutathione was subsequently treated with putrescine, spermidine, endonuclease IV, or high concentrations of endonuclease III or exonuclease III, there

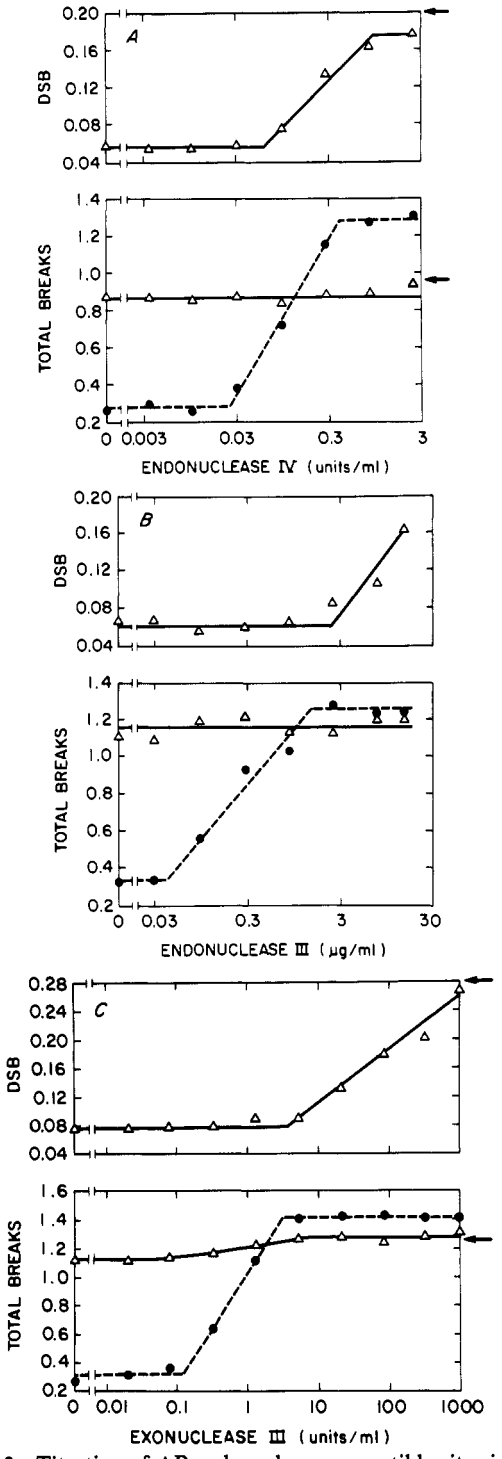

FIGURE 3: Titration of AP endonuclease susceptible sites in NCS chromophore treated DNA (△) or in depurinated DNA (●). Chromophore concentrations were 0.04–0.05 μM. DSB = double-strand breaks. Arrows show the level of cleavage produced in chromophore-treated DNA by 100 mM putrescine [not determined in (B)]. For further details, see Figure 1.

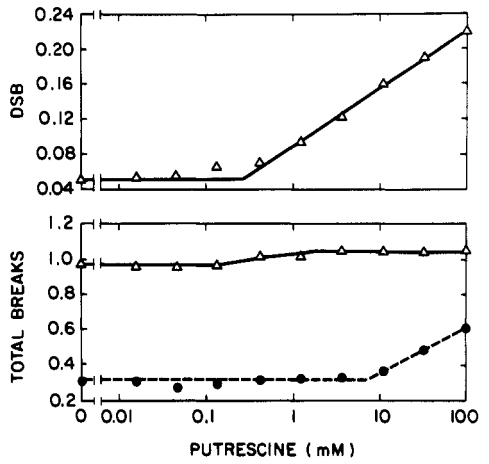

FIGURE 4: Cleavage by putrescine of putative AP sites in NCS chromophore treated DNA (△) or in depurinated DNA (●). DSB = double-strand breaks. For further details, see Figure 1.

Table II: Formation and Cleavage of NCS Chromophore Induced AP Sites[a]

	NCS chromophore cofactor			
	glutathione		2-mercaptoethanol	
posttreatment	total breaks[b]	double-strand breaks	total breaks[b]	double-strand breaks
no addition, 0 °C	0.78	0.04	0.71	<0.01
no addition, 37 °C	0.83	0.05	0.69	0.01
endonuclease IV	0.83	0.24	0.88	0.03
putrescine	0.88	0.27	0.76	0.05

[a] ColE1 DNA was treated with 0.04 μM NCS chromophore in the presence of 5 mM glutathione or 10 mM 2-mercaptoethanol and incubated for 1 h at 37 °C with endonuclease IV (2.8 units/mL) or putrescine (100 mM). The difference concentrations of the sulfhydryl cofactors reflect their differential efficiency in chromophore activation. [b] Breakage seen in samples not treated with NCS has been subtracted.

was a dramatic increase in the number of double-strand breaks. Putrescine (100 mM) was most effective, increasing double-strand breaks by a factor of 4.3 ± 0.6 (n = 8); however, endonuclease IV was nearly as effective as putrescine (Figure 3, Table II). After putrescine treatment, double-strand breaks accounted for approximately one-third of the total breaks in chromophore-treated DNA. Although titration of putrescine-induced cleavage did not reach a plateau (Figure 4), increasing the incubation temperature to 60 °C increased cleavage only slightly (~10%), suggesting that most of the susceptible sites were cleaved by 100 mM putrescine at 37 °C (data not shown).

Since there was little or no increase in total breaks, the increase in double-strand breaks must represent, for the most part, conversion of single-strand breaks to double-strand breaks by endonuclease IV or putrescine, presumably by cleavage of AP sites. Thus, it may be inferred that virtually all chromophore-induced AP sites are accompanied by a closely opposed break in the complementary strand and that these AP site/strand break lesions constitute a substantial fraction of the lesions induced by NCS chromophore. As in the case of bleomycin, linear molecules generated by putrescine treatment of chromophore-damaged DNA were found to be derived almost exclusively from nicked circular, rather than supercoiled, molecules, again suggesting that lesions consisting of two closely opposed AP sites occur rarely, if at all (data not shown). It is not known whether the small number of double-strand breaks seen in DNA treated with chromophore alone arise from true direct double-strand cleavage or from spontaneous cleavage of a small proportion of the AP site/strand break lesions.

The putative closely opposed AP site/strand break lesions induced by NCS chromophore were only slightly resistant to endonuclease IV, requiring about 3-fold more enzyme for half-maximal cleavage than heat-induced AP sites (Figure 3A). Thus, these lesions were more sensitive to cleavage by endonuclease IV than closely opposed AP site/strand break lesions induced by bleomycin, even though nonopposed bleomycin-induced sites were efficiently cleaved. This difference could be due to the fact that the bleomycin-induced strand

break involves loss of a base, while the NCS-induced break does not (Giloni et al., 1981; Kappen & Goldberg, 1983); thus, the NCS-induced sites might more easily adopt a configuration similar to double-stranded DNA. Alternatively, the exact position of the opposed strand break relative to the AP site (which is unknown in either case) may be critical.

Endonuclease III also increased double-strand breakage in chromophore-treated DNA (Figure 3B), but only at high concentration, at least 30-fold higher than that required to cleave heat-induced AP sites (endonuclease III denatured at 70 °C for 10 min had no effect at any concentration). Thus, closely opposed AP lesions induced by NCS chromophore were comparable to those induced by bleomycin in sensitivity to endonuclease III; again, the exact degree of resistance was difficult to determine, since cleavage did not reach a plateau at the highest attainable concentration of endonuclease III. The same was true in the case of UV-endonuclease; only 20% of the putrescine-susceptible chromophore-induced lesions were cleaved by 50 μg/mL UV-endonuclease, while half-maximal cleavage of heat-induced AP sites required only 5 μg/mL (not shown). The chromophore-induced AP lesions appeared to be about 100-fold less sensitive to exonuclease III than heat-induced AP sites (Figure 3C). However, as noted above, the effect of exonuclease III could be an artifact due to residual exonuclease activity rather than AP endonuclease activity.

Quantitatively, the sensitivity of chromophore-induced AP lesions to polymaines was different from that of either heat-induced or bleomycin-induced AP lesions. The chromophore-induced lesions were more sensitive to putrescine than heat-induced AP sites, but slightly less sensitive than bleomycin-induced AP sites (Figure 4). The majority of chromophore-induced lesions were comparable to heat-induced AP sites in sensitivity to spermidine, with half-maximal cleavage requiring 1 mM spermidine; there was a component of sites (20–35%) that were cleaved by putrescine but not by spermidine (not shown), although this component was smaller than was seen with bleomycin.

When 2-mercaptoethanol was used as a cofactor for chromophore activation, double-strand breakage induced by chromophore plus putrescine was 5-fold lower than with glutathione cofactor, even though the levels of the total breakage were comparable (Table II). In agreement with previous results of Boye et al. (1984), double-strand breakage in DNA treated only with chromophore and 2-mercaptoethanol was barely detectable, less than 2% of the total breakage. Thus, efficient production of closely opposed AP site/strand break lesions by NCS chromophore apparently requires some specific interaction between glutathione and chromophore. As has been discussed previously (Hensens et al., 1983; Povirk & Goldberg, 1983), chromophore activation may involve covalent addition of the sulfhydryl cofactor, and different sulfhydryl–chromophore addition products may have different activities. There is very strong evidence that glutathione is the in vivo cofactor of NCS (DeGraff et al., 1985; Kappen et al., 1987). Undoubtedly, the general use of 2-mercaptoethanol rather than glutathione as a cofactor in NCS–DNA reactions accounts for the fact that specific formation of closely opposed AP site/strand break lesions had previously been overlooked.

Discussion

Several previous studies have dealt with putative AP sites in bleomycin- and NCS-treated supercoiled DNA. However, as the unique chemical structure of the bleomycin-induced AP site was in most cases not considered. Our results are in good agreement with those of Lloyd et al. (1978), who reported that the number of endonuclease IV susceptible or endonuclease III susceptible sites in bleomycin-treated PM2 DNA was approximately the same as the number of alkali-labile sites, as judged by the loss of supercoiled molecules. These authors reported that nondenaturing alkali (pH 11.5) also increased the number of double-strand breaks, while endonuclease IV and endonuclease III did not. This effect was attributed to either (i) alkaline cleavage of closely opposed AP lesions which were resistant to AP endonuclease or (ii) local denaturation of short double-stranded regions between nearby breaks in opposite strands. Our results show that the effect of alkali can be mimicked either by putrescine or by sufficiently high concentrations of endonuclease III, strongly suggesting that there are, in fact, closely opposed AP site/strand break lesions in bleomycin-treated DNA which are resistant to endonucleolytic cleavage.

Niwa and Moses (1981) reported that exonuclease III, in citrate buffer, did not cleave bleomycin-induced alkali-labile sites (presumably AP sites) in RF φX174 DNA, under conditions where depurinated DNA was efficiently cleaved. In the absence of added metal cofactors, exonuclease III activity must be attributed to adventitious transition metals in the reaction mixtures. Thus, even though a considerable excess of enzyme was used, the level of AP endonuclease activity may have been insufficient to cleave bleomycin-induced AP sites, due to insufficient metal cofactor. In any case, our results clearly show that, at sufficiently high concentrations, exonuclease III can cleave bleomycin-induced AP sites (Figure 1).

Bose et al. (1980) reported that an AP endonuclease from human lymphocytes increased the number of total strand breaks in NCS-treated colE1 by a factor of approximately 1.6, as judged by loss of supercoiled molecules (linear molecules and double-strand breaks were not quantitated). Although we sometimes detected a slight endonuclease-dependent increase in total strand breaks, it was much smaller than that reported by Bose et al. At present, we have no satisfactory explanation for this discrepancy between the two studies. It is possible but unlikely that lesions are formed whcih are sensitive to the human AP endonuclease, but not to any of the microbial AP endonucleases or to polyamines.

More recently, Povirk and Goldberg (1985) analyzed NCS chromophore-treated DNA on sequencing gels and noted the occurrence of alkali-dependent breaks at certain cytosine residues, particularly those in the trinucleotide sequence A-G-*C*. It now appears highly likely that these lesions correspond to the closely opposed AP site/strand break lesions described in the present report. In both cases, the lesions were formed with much greater frequency with glutathione cofactor than with 2-mercaptoethanol. Furthermore, the putative AP lesions detected on sequencing gels were about 5-fold less sensitive to endonuclease IV than heat-induced AP sites and at least 10-fold less sensitive to endonuclease III and exonuclease III (Povirk & Goldberg, 1985). These susceptibilities are in good agreement with those of the closely opposed AP site/strand break lesions detected in supercoiled DNA (Figure 3). In the earlier study, it was noted that the 5′ sugar–phosphate bond of the NCS-induced AP site was more labile than that of heat-induced sites, suggesting a difference in chemical structure. It was proposed that this difference accounted for the resistance of the lesion to endonucleolytic cleavage. However, the presence of a closely opposed strand break, as revealed by the present study, might also contribute to endonuclease resistance.

A strong correlation has been found between the sequence specificity of certain NCS-induced mutations (particularly G·C

→ A·T transitions) and that of the endonuclease-resistant AP sites at cytosine residues, suggesting that these lesions are highly mutagenic (Povirk & Goldberg, 1985, 1986). The hypothesis that these AP lesions are always accompanied by a closely opposed strand break suggests a novel mechanism of mutagenesis; that is, repair of the strand break may require synthesis of a repair patch on an AP site containing template. Thus, mutagenesis (i.e., the change in DNA sequence) could occur before DNA replication. This is an important consideration, since the NCS-induced AP lesions can be cleaved either by endonuclease IV or by polyamines and may have a short lifetime in the cell. It should also be noted that the results with supercoiled DNA suggest that closely opposed AP site/strand break lesions account for about 20–25% of the total strand breaks induced by NCS, whereas previous base release data (Povirk & Goldberg, 1985) indicate that the number of AP sites at cytosine residues is less than one-tenth the total number of strand breaks. Thus, it is likely that AP sites (accompanied by closely opposed strand breaks) are formed at adenine and thymine residues as well but were not detected as alkali-dependent strand breaks on sequencing gels due to the prominence of direct strand breaks. Thus, these lesions could also be responsible for NCS-induced mutations at A·T base pairs, which are at least as frequent as those at G·C base pairs. Endonuclease IV susceptible sites have in fact been detected at a few thymine residues where direct strand breakage is less prominent (Kappen & Goldberg, 1983).

Closely opposed AP site/strand break lesions induced by NCS are highly sensitive to polyamines, and it is likely that in bacteria, a significant proportion are converted to double-strand breaks before they can be repaired. Thus, these lesions probably play a critical role in cell killing as well as mutagenesis. In eukaryotes, similar cleavage reactions could be promoted by lysine residues of DNA-bound histones. Cleavage of heat-induced AP sites by at least some amines involves formation of a covalent Schiff's base linkage between the amine and aldehyde of of the AP site (Coombs & Livingston, 1968); furthermore, covalent linkage of histones to depurinated DNA has been demonstrated in vitro (Mirzabekov et al., 1978). If a similar conjugate were formed upon cleavage of the NCS-induced AP sites by histone amine groups, the result would be a double-strand break with a histone covalently linked to one of the 3′ ends. Such a structure might be even more difficult to repair than a simple double-strand break. These reactions could explain the rather high proportions of double-strand breaks (Shiloh et al., 1983; Kuo et al., 1984) and protein-linked strand breaks (Samy et al., 1986) which have been detected in DNA of mammalian cells treated with NCS.

Evidence for involvement of closely opposed AP lesions in bleomycin-induced mutagenesis and cytotoxicity is less convincing, although, theoretically, similar processing might be expected. Bleomycin-induced mutations in λ phage (Povirk 1987) do occur primarily at expected positions of AP sites (G-*C*, G-*T*, and A-*T* sequences). However, the majority of bleomycin-induced sites are not accompanied by a closely opposed break, and the particular sequence specificity of closely opposed AP lesions is not known. If these lesions are in fact involved in bleomycin-induced mutagenesis, their resistance to AP endonucleases could explain why mutation frequencies of bleomycin-damaged phage are not affected by host cell deficiencies in these enzymes (Povirk & Houlgrave, 1988).

As has been previously noted in the case of double-strand breaks (Povirk et al., 1977), the occurrence of closely opposed AP site/strand break lesions in bleomycin-treated DNA is much greater than would be expected from coincidence of randomly distributed strand breaks and AP sites. The ratio of double-strand breaks to single-strand breaks in bleomycin-treated DNA is nearly constant over a wide range of bleomycin concentrations and reaction times (Povirk et al., 1977; Lloyd et al., 1978), and this has been taken as evidence that the double-strand break is produced in a single bleomycin–DNA interaction (Povirk, 1983). Yet, the chemistry of activated bleomycin gives no suggestion of bifunctionality (Burger et al., 1981). Alternatively, an apparent excess of closely opposed lesions would also be expected if bleomycin had markedly higher affinity for DNA sites with preexisting damage in the opposite strand. Recently, Keller and Oppenheimer (1987) have obtained fairly strong evidence for this latter mechanism. Using synthetic double-stranded oligonucleotide substrates, they showed that the presence of a one-base gap with 3′- and 5′-phosphate termini (a structure very similar to a bleomycin-induced strand break) dramatically increased the frequency of bleomycin-induced cleavage in the complementary strand at a site directly opposite the gap. Enhanced cleavage was not seen if either terminus was an uncharged hydroxyl, suggesting that the increase in local negative charge at the break site is essential for enhanced cleavage. Thus, generation of closely opposed AP site/strand break lesions is probably attributable to similar selective formation of AP sites by bleomycin opposite existing bleomycin-induced strand breaks; the failure of the drug to produce lesions consisting of two closely opposed AP sites (see Results) is expected, since formation of the first AP site would not increase the local negative charge.

With NCS chromophore, however, the specific production of closely opposed lesions is even more striking. In chromophore-treated DNA, the majority of AP sites are accompanied by a closely opposed strand break, even when there is, on average, less than 1 break per 12 000 bases (i.e., per colE1 molecule). Thus, formation of the two closely opposed lesions is highly concerted and probably occurs in a single chromophore–DNA interaction. Such a mechanism would imply that the chromophore is a bifunctional agent, a view consistent with recent proposals for a biradical form of activated chromophore (Kappen & Goldberg, 1985; Myers, 1987). Experiments to determine the exact position of the closely opposed strand break, relative to the AP site, are in progress. However, it may be noted that NCS-induced alkali-dependent breaks (presumably AP sites) at C residues were only detected at sites where an A or T residue was located two bases upstream (Povirk & Goldberg, 1985); no such requirement was seen at either of the two downstream bases. Since NCS-induced direct strand breaks occur almost exclusively at T and A residues (Goldberg et al., 1985), we speculate that the required T or A residue, opposite the base two positions upstream from the AP site, is probably the site of the strand break. The two DNA sugar moieties that would be attacked in formation of such a lesion, although formally separated by two nucleotide positions, actually lie directly facing each other in the minor groove. Simultaneous attack on these two sugars by the proposed biradical form of activated NCS chromophore (Myers, 1987) provides a simple model for formation of these lesions. If this model is correct, mutagenesis occurring during repair synthesis, as postulated above, would require an exonuclease (e.g., exonuclease III) to remove two nucleotides upstream from the strand break, in order to expose the AP site in the template.

Initially, a major goal of the present study was to determine whether bleomycin- and NCS-induced AP sites have the same chemical structure, on the basis of a quantitative analysis of

their susceptibilities to various forms of chemical and enzymatic cleavage. However, even though differences in susceptibility have been found, particularly with endonuclease IV, these could be due to differences in DNA secondary structure resulting from closely opposed breaks in the complementary strand, rather than to chemical differences in the AP site itself. Even chemical cleavage can be influenced by DNA secondary structure, as evidenced by the much greater alkaline lability of heat-induced AP sites in single-stranded DNA, as compared to double-stranded DNA (LaFleur et al., 1980). Thus, due to complications arising from the presence of closely opposed lesions, it is still uncertain whether the structures of bleomycin- and NCS-induced AP sites are the same; this question will probably only be resolved by direct chemical determination of the structure of the NCS-induced AP site.

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