Degradation of Structurally Modified DNAs by Bleomycin Group Antibiotics

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ABSTRACT: Bleomycin-mediated DNA strand scission has been shown to be diminished at certain sequences in proximity to 5-methylcytidines. We have investigated the molecular basis of this observed diminution using selective bleomycin (BLM) modifications at the C-terminus. Of the four different bleomycin congeners investigated, only bleomycin A_2 and bleomycin BAPP were substantially affected by cytidine methylation. We have also examined the effect of other DNA modifications on bleomycin-mediated strand scission. Methylation at the N^6 position of adenosine resulted in diminution of DNA cleavage by all four bleomycin congeners. The presence of bulky 5-(glucosyloxy)methyl groups in the major groove of T4 DNA had little effect on the efficiency of DNA strand scission mediated by bleomycin A_2 or B_2, suggesting the absence of important steric interactions between Fe(II)·BLM and DNA in the major groove. In contrast, DNA cleavage mediated by bleomycin congeners was very sensitive to a major DNA conformational change, the B → Z transition. Salt and $MgCl_2$ titrations of the DNA copolymers poly(dG-dC)·poly(dG-dC) and poly(dG-^{Me}dC)·poly(dG-^{Me}dC) demonstrated that bleomycin A_2 and B_2 did not cleave Z-DNA efficiently. In addition, circular dichroism titrations of these copolymers revealed that both bleomycin congeners increased the cation concentration necessary to induce the B → Z transition, implying that bleomycin preferentially binds to and stabilizes B-form DNA. These results are consistent with a model in which cytidine methylation at appropriate sequences of DNA is sufficient to induce subtle conformational changes that render the helix unreceptive to cleavage by some bleomycin congeners.

The bleomycins (BLMs)[1] are a family of antitumor antibiotics that are thought to elicit their chemotherapeutic effects by degrading cellular DNA [for reviews, see Hecht (1979) and Sugiura et al. (1985)]. Bleomycin binds to DNA and mediates cleavage in a reaction that is dependent on metal ions and molecular oxygen (Ishida & Takahashi, 1975; Sausville et al., 1978a,b; Ehrenfeld et al., 1985). The bithiazole moiety and C-terminal substituent of bleomycin participate in DNA binding, while the N-terminus is responsible for metal ion chelation and oxygen activation (Chien et al., 1977; Povirk et al., 1979; Kross et al., 1982a; Umezawa et al., 1984; Kilkuskie et al., 1985). DNA strand scission by the bleomycins occurs at a limited number of sites, predominantly at 5′-GT-3′ and 5′-GC-3′ sequences (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Mirabelli et al., 1982; Kross et al., 1982b).

We have recently shown that bleomycin-mediated DNA strand scission was diminished substantially at a number of sites in proximity to cytidines that had been methylated at the 5-position in the major groove (Hertzberg et al., 1985). Two mechanisms for bleomycin selectivity on the basis of DNA methylation seem possible. There may be an unfavorable steric interaction between the methyl group on cytidine and part of the bleomycin molecule, which could lower the affinity of bleomycin for the methylated sequence. This would imply that part of the bleomycin molecule is positioned in the major groove of DNA. Alternatively, the diminished bleomycin strand scission could be due to local conformational changes of the DNA in proximity to the methylated cytidines. Methylation of cytidine residues in DNA is known to facilitate a major DNA conformational change, the B → Z transition (Behe & Felsenfeld, 1981).

In order to study the molecular basis of this observed diminution of BLM-mediated DNA strand scission, we have investigated the effects of selective bleomycin modifications on the efficiency of DNA strand scission. Reported herein are parameters of BLM structure necessary for discrimination of BLM-mediated cleavage in response to cytidine methylation. Also reported are the effects of DNA modification on susceptibility to DNA strand scission by bleomycin. Total cleavage of T4 DNA, which contains bulky 5-(glucosyloxy)-methyl groups in the major groove (Revel & Luria, 1970), was little changed from cleavage of T4 DNA lacking these groups, suggesting that major groove contacts were negligible in the bleomycin–DNA complex. Interestingly, methylation of the N^6 position in adenosine, a major groove modification thought to induce relatively minor changes in DNA conformation (Cheng et al., 1985), also resulted in diminution of DNA strand scission by bleomycins. In addition, we demonstrate that there is a significant reduction of DNA strand scission mediated by bleomycin when poly(dG-dC)·poly(dG-dC) and poly(dG-^{Me}dC)·poly(dG-^{Me}dC) undergo the B → Z conformational transition. These data are consistent with a model in which the methylation of a small number of cytidines, within appropriate sequences of DNA, causes subtle conformational changes that render the helix unreceptive to cleavage by certain bleomycin congeners.

Experimental Procedures

Materials

Bleomycin BAPP (Tanaka, 1977) and blenoxane were obtained from the National Cancer Institute. The latter was fractionated to provide pure bleomycin demethyl-A_2 (Fujii et al., 1973a), bleomycin A_2, and bleomycin B_2 as described (Fujii et al., 1973b; Chen et al., 1977; Oppenheimer et al., 1979). DNA polymerase I (Klenow fragment) and bacterial alkaline phosphatase (unit definition: 1 unit hydrolyzes 1 nmol of ATP in 30 min at 37 °C) were purchased from Bethesda Research Labs; DNA polymerase I (Kornberg polymerase), DNase, and nuclease P1 were purchased from Boehringer Mannheim. Supercoiled pBR322 plasmid DNA was isolated from *Escherichia coli* strain Ja221 according to the procedure of

[1] Abbreviations: BLM, bleomycin; bp, base pair(s); DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; TBA, 2-thiobarbituric acid; DNase, deoxyribonuclease.

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Clewell (1972) or purchased from Bethesda Research Labs. Calf thymus DNA (Sigma Chemicals) was sonicated, deproteinized with phenol, and dialyzed extensively against 10 mM Tris, pH 7.5, containing 50 mM NaCl. All restriction methylases [unit definition: 1 unit is the amount required to protect 1 μg of substrate DNA for 1 h at 37 (*Hha*I and *Hpa*II methylases) or 65 °C (*Taq*I methylase) against cleavage by the respective restriction endonuclease], restriction endonucleases (unit definition: 1 unit is the amount required to produce complete digestion of 1 μg of substrate DNA in 60 min), and polynucleotide kinase (unit definition: 1 unit catalyzes the transfer of P_i from ATP to the 5′-OH terminus of a polynucleotide, producing 1 nmol of acid-insoluble ^{32}P in 30 min at 37 °C) were purchased from New England Biolabs. Solutions of Fe(II) and dithiothreitol (DTT) were prepared immediately before use. Poly(dG-dC)·poly(dG-dC) and poly(dG-^{Me}dC)·poly(dG-^{Me}dC) were obtained from Pharmacia Molecular Biologicals. T4 DNA was obtained from Sigma Chemicals. [α-^{32}P]dATP and [γ-^{32}P]ATP were purchased from New England Nuclear.

Methods

Preparation of DNA Restriction Fragments. Supercoiled pBR322 plasmid DNA was digested with *Bam*HI and labeled at the 3′-end with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I (Sanger & Coulson, 1975). Enzymatic digestion with *Hin*cII and *Eco*RI yielded two singly end labeled DNA fragments, 381 and 280 base pairs in length, that were isolated from a 5% polyacrylamide gel as described (Maxam & Gilbert, 1980). In a separate preparation, pBR322 DNA was cleaved with *Sty*I, 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Maxam & Gilbert, 1980), digested with *Bgl*I, and isolated from a polyacrylamide gel to yield a singly end labeled DNA fragment, 207 base pairs in length.

In Vitro Methylation of DNA Fragments. End-labeled DNA fragments (0.1–1 μg) were methylated at 37 °C for 2 h in 100 μL of 50 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, 5 mM 2-mercaptoethanol, and 300 μM *S*-adenosylmethionine with 48 units of *Hha*I methylase and/or 10 units of *Hpa*II methylase. The reaction was terminated by ethanol precipitation. In order to verify that the methylase reaction was complete, these DNA fragments were subjected to *Hha*I and/or *Hpa*II digestion for 1 h using at least 10 enzyme units per microgram of DNA. Similarly, end-labeled DNA fragments were methylated on adenosine by using 11 units of *Taq*I methylase at 65 °C for 2 h in a buffer containing 100 mM Tris-HCl, pH 7.8, 10 mM EDTA, and 300 μM *S*-adenosylmethionine. The reaction was terminated by ethanol precipitation, and the pellet was resuspended in water. An aliquot was cleaved with *Taq*I to determine the success of the methylation. In all cases, methylated DNA fragments were fully protected from restriction enzyme cleavage as judged by analysis on polyacrylamide gels, while the nonmethylated substrates were fully cleaved.

DNA Cleavage by Fe(II)·BLM. The standard reaction mixture (20-μL total volume) contained 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1000–2000 cpm of ^{32}P-end-labeled DNA made up to 50 μM (bp) with sonicated calf thymus DNA, 1 mM DTT, and 0.1–5 μM Fe(II)·BLM. After incubation at ambient temperature for 60 min, the DNA was isolated by ethanol precipitation, washed with cold 80% ethanol, dried, and suspended in 4 μL of a 100 mM Tris–borate/50% formamide loading buffer, pH 8.3. Some reaction mixtures were treated with 1 M hydrazine hydrate for 30 min at 0 °C before ethanol precipitation. The sample was heat denatured at 90 °C for 2 min, chilled on ice, loaded onto a 0.4 mm thick, 40 cm long, 8% polyacrylamide, 1:20 cross-linked/50% urea gel, and electrophoresed at 1500–1850 V for 2–4 h. Autoradiography was carried out with an intensifying screen at −70 °C on Kodak X-Omat AR film.

Analysis of DNA Cleavage Gels. The autoradiograms from the gels were scanned with an LKB 2202 laser densitometer interfaced to a Hewlett-Packard 3390 integrator. The background absorbance due to the film density was substracted from the scans. The data are reported as histograms in the form of fractional cleavage, $f = A_i/A_t$, where A_i is the area of a peak corresponding to cleavage efficiency at band i and A_t is the sum of the areas of all of the cleavage band peaks within the gel lane.

Preparation of Cytidine-Containing T4 DNA. The nucleoside content of wild-type T4 DNA was determined by nuclease P1/bacterial alkaline phosphatase digestion, followed by HPLC analysis, as described by Gehrke et al. (1984). Very little unmodified cytidine was detected by this analysis. There were two nucleosides that had chromatographic and spectral (Lehman & Pratt, 1960; Lichtenstein & Cohen, 1960) behavior consistent with their representation as the α and β anomers of [(glucosyloxy)methyl]cytidine. Assuming that all of the cytidine derivatives have the same HPLC response factor, 94% of the cytidines in T4 DNA were glucosyloxymethylated. This DNA was nick-translated for 16 h at 16 °C in a reaction mixture (0.5 mL) containing 30 μg of T4 DNA, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 80 units of DNA polymerase I from *E. coli* [units as defined by Richardson et al. (1964)], 6.4 milliunits of DNase [units as defined by Kunitz (1950)], and 200 μM each of dATP, dCTP, dGTP, and TTP (Rigby et al., 1977). The mixture was phenol-extracted twice, ether-extracted 3 times, ethanol precipitated, washed with cold 80% ethanol, and dried. Nucleoside analysis indicated that 89% of the cytidine moieties in the nick-translated product were unmodified.

Bleomycin Cleavage of Modified and Unmodified T4 DNA. Each reaction mixture (250 μL) was carried out in 10 mM Tris-HCl, pH 7.5, containing 178 μM DNA, 100 μM Fe(II)·BLM A_2 or B_2, 0.5 mM DTT, and 50 mM NaCl. After 30 min at ambient temperature, a 50-μL aliquot was removed for analysis with 2-thiobarbituric acid (Burger et al., 1980), and the remaining material was ethanol precipitated. The ethanol supernatant was concentrated in vacuo and analyzed for free base content by HPLC, as described (Hertzberg & Dervan, 1984).

Bleomycin Cleavage of DNA Copolymers. Each reaction mixture (100-μL total volume) contained 200 μM (bp) DNA copolymer in 10 mM Tris-HCl (pH 7.5) and varying amounts of NaCl and $MgCl_2$ (as noted). The reaction mixture was heated at 60 °C for 10 min and then allowed to cool. Fe(II)·BLM A_2 or Fe(II)·BLM B_2 was added (100 μM) followed by 0.5 mM DTT. The reaction was incubated at 25 °C for 30 min and assayed for thiobarbituric acid reactive products as described (Burger et al., 1980). Similar reaction mixtures, lacking DTT (400-μL total volume) were analyzed for free base and base propenal release as described (Hertzberg & Dervan, 1984).

Circular Dichroism Titrations of DNA Copolymers. Poly(dG-dC)·poly(dG-dC) or poly(dG-^{Me}dC)·poly(dG-^{Me}dC) was dissolved in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl at a concentration of 50 μM (bp), and the CD spectrum was recorded. Aliquots of NaCl or $MgCl_2$ were added, and the solutions were heated at 60 °C for 10 min and allowed to cool to ambient temperature in order to allow the DNA to reach conformational equilibrium (Behe & Felsenfeld, 1981;

Pohl & Jovin, 1972). The molar ellipticity at 293 nm was measured at increasing NaCl or $MgCl_2$ concentrations in order to determine the midpoint for the B → Z transition. The titrations were repeated in the presence of 25 μM Fe(III)·BLM, 25 μM bleomycin, or 25 μM Fe(III).

Results

Effect of Cytidine Methylation on DNA Cleavage Mediated by Bleomycin Congeners. Four different congeners of bleomycin that differ only at the C-terminus were analyzed with respect to their sequence specificity of DNA strand scission and the sensitivity of this cleavage to cytidine methylation. Two ^{32}P-end-labeled DNA fragments from plasmid pBR322 were methylated either with *Hha*I methylase or with both *Hha*I and *Hpa*II methylases in order to introduce methyl groups on cytidines at specific sequences. *Hha*I recognizes and methylates the internal cytidines of the sequence GCGC, while *Hpa*II recognizes and methylates the internal cytidines of the sequence CCGG. These methylated, end-labeled DNA fragments, in addition to the corresponding nonmethylated counterparts, were subjected to cleavage by Fe(II)·BLM A_2, Fe(II)·BLM B_2, Fe(II)·BLM demethyl A_2, and Fe(II)·BLM BAPP. The resultant ^{32}P-labeled DNA fragments were analyzed by denaturing polyacrylamide gel electrophoresis and densitometry to determine the relative efficiencies of DNA cleavage at specific sequences.

The sequence specificities of DNA cleavage by these four bleomycin congeners were similar but not identical (Figures 1–5). In most cases, each congener mediated strand scission at the same DNA sequences, but the relative band intensities were somewhat different for individual bleomycins. This is consistent with other studies that have shown that the C-terminal substituent contributes only slightly to the sequence recognition by bleomycin (Takeshita et al., 1981; Kross et al., 1982b). Interestingly, when some of the BLM congeners were used to effect cleavage of a single DNA fragment having different extents of cytidine methylation, the relative efficiency of DNA cleavage in proximity to the methylated cytidines was diminished. For example, Fe(II)·BLM A_2 mediated cleavage of a 280 bp DNA fragment from pBR322 was substantially lower within a 14 base pair sequence surrounding CCGG sequences methylated by *Hpa*II (Figure 1, lanes 1–3; Figure 2a, sites 26, 30, 35, 39) as was previously reported (Hertzberg et al., 1985). Most of the diminished cleavage occurred between or adjacent to methylated cytidines, rather than directly at the methylated base. In comparison, cleavage of this DNA fragment by bleomycin demethyl A_2, which differs from bleomycin A_2 only by the absence of one methyl group on the C substituent, was only slightly diminished by cytidine methylation (Figure 2c). In addition, the DNA cleavage efficiency by bleomycin B_2 at most sites in proximity to the methylated sequences was only slightly diminished (Figure 1a, lanes 4–6; Figure 2b), in contrast to the result with bleomycin A_2. The fourth bleomycin congener tested, bleomycin BAPP, displayed a marked diminution of cleavage within this 14 bp sequence for those DNA fragments that had been methylated by *Hpa*II (Figure 2d). The histograms shown in Figures 2 and 5 illustrate cleavage intensities within the sequences surrounding the methylation region. Histograms of the entire DNA sequences resolvable on the gel are presented as supplementary material (see paragraph at end of paper regarding supplementary material).

The effect of methylation on DNA cleavage efficiency by bleomycin congeners was examined by using another DNA fragment from pBR322 (Figure 3). Bleomycin A_2 cleavage at a methylated cytidine (site 118) was significantly diminished, while cleavage at all other sites was similar for methylated and unmethylated DNA substrates. As increasing concentrations of bleomycin A_2 produced larger numbers of cleavage events, strand scission at the methylated cytidine remained diminished. Cleavage of this DNA fragment by bleomycin B_2 was much less affected by the presence of cytidine methyl groups at all concentrations tested (Figure 3a, lanes 14–21). There was no difference in cleavage efficiency at the other *Hha*I methylation site in this DNA fragment for any of the bleomycin congeners tested. For the other site (bases 144–147), BLM-mediated cleavage occurred only at the outer, nonmethylated cytidine of the *Hha*I GCGC recognition sequence.

Several analyses of the DNA cleavage efficiency by the bleomycin congeners were carried out to determine the variation of strand scission at the methylated site and the extent

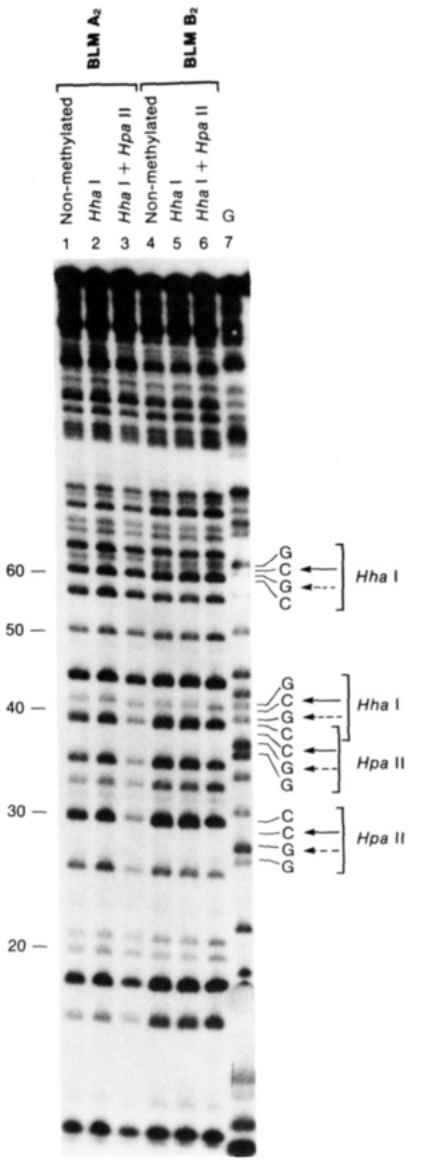

FIGURE 1: Polyacrylamide gel analysis of the cleavage of a fragment of pBR322 DNA with BLM A_2 and BLM B_2. A 3′-^{32}P-end-labeled 280 bp DNA fragment that had been methylated with *Hha*I (lanes 2 and 5) or *Hha*I and *Hpa*II (lanes 3 and 6), along with the nonmethylated DNA (lanes 1 and 4), was allowed to react with Fe(II)·BLM + 1 mM DTT. Lanes 1–3, 1.0 μM Fe(II)·BLM A_2; lanes 4–6, 1.0 μM Fe(II)·BLM B_2; lane 7, G lane (Maxam & Gilbert, 1980). Numerals in the margin of the autoradiogram refer to oligonucleotide size. Solid arrows in the right margin refer to cytidines that are methylated by the indicated enzymes; dotted arrows refer to guanosines that are base paired to methylated cytidines.

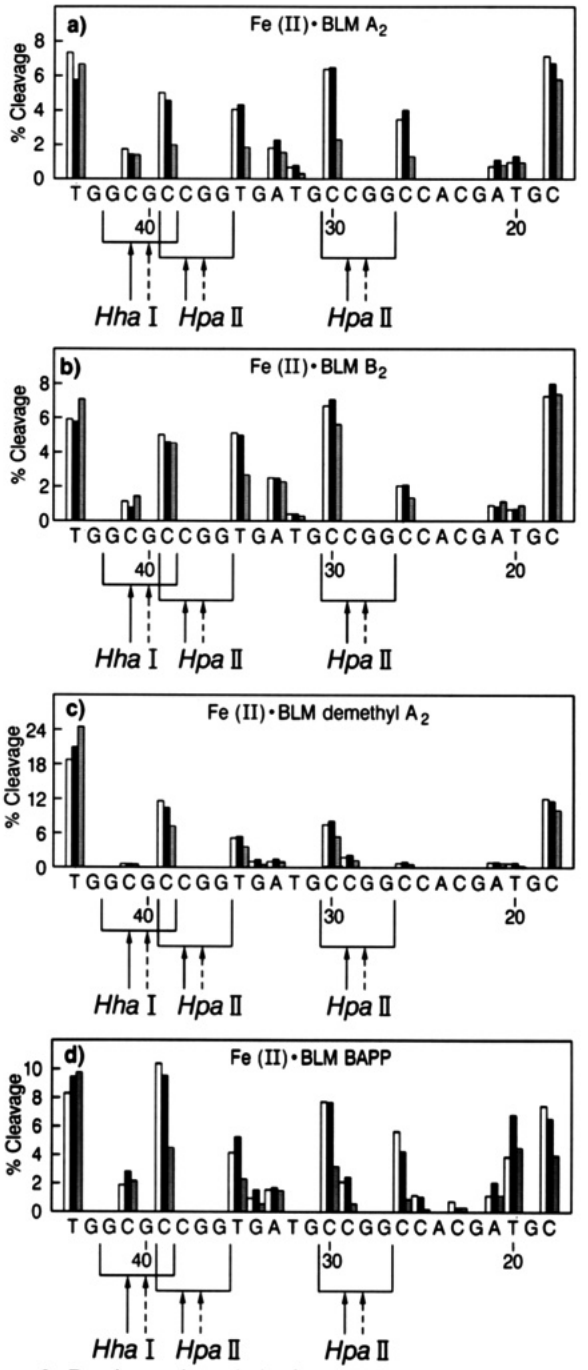

FIGURE 2: Densitometric analysis of DNA cleavage by four congeners of BLM. The methylated and unmethylated 280 bp DNA fragments were cleaved and analyzed as indicated in the legend to Figure 1; only the 27 bp from the methylation region is illustrated. Bars indicate nonmethylated DNA (open bars) or methylation by either *Hha*I methylase (solid bars) or *Hha*I and *Hpa*II methylases (hatched bars). Panel a, densitometric analysis of lanes 1–3 from Figure 1; panel b, the same sites from lanes 4–6; panel c, 0.5 μM Fe(II)·BLM demethyl A_2 + 1 mM DTT; panel d, 0.5 μM Fe(II)·BLM BAPP + 1 mM DTT. Numerals and arrows refer to the same sites as in Figure 1.

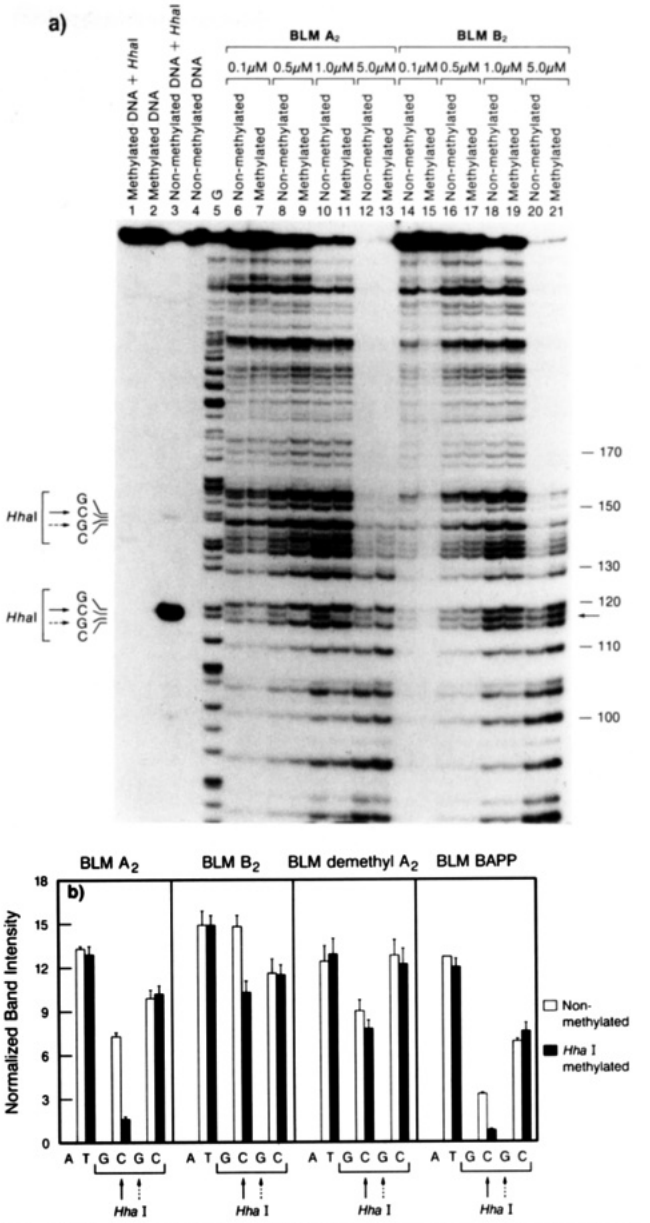

FIGURE 3: Polyacrylamide gel analysis of the cleavage of a 381 bp fragment of pBR322 DNA with BLM A_2 and BLM B_2. In panel a, the 3′-^{32}P-end-labeled DNA fragment was methylated with *Hha*I, and the methylated (lanes 1, 2, 7, 9, 11, 13, 15, 17, 19, 21) and unmethylated (lanes 3–6, 8, 10, 12, 14, 16, 18, 20) DNA fragments were digested with varying concentrations of Fe(II)·BLM A_2 (lanes 6–13) or Fe(II)·BLM B_2 (lanes 14–21) + 1 mM DTT. Lanes 6, 7, 14, and 15, 0.1 μM Fe(II)·BLM; lanes 8, 9, 16, and 17, 0.5 μM Fe(II)·BLM; lanes 10, 11, 18, and 19, 1.0 μM Fe(II)·BLM; lanes 12, 13, 20, and 21, 5.0 μM Fe(II)·BLM. Lanes 1 and 3 were treated with *Hha*I endonuclease; lanes 2 and 4 were untreated; lane 5 is a G lane (Maxam & Gilbert, 1980). Arrows and numerals in the margin of the autoradiogram are as described in Figure 1. In panel b is shown a statistical analysis of the diminution of cleavage at sites 116 (C), 118 (methylated cytidine), and 120 (T) of this DNA fragment. The autoradiogram shown in panel a and several other autoradiograms were analyzed by densitometry as described under Experimental Procedures. BLM A_2 results are the average of nine determinations; BLM B_2 results are the average of four determinations; BLM demethyl A_2 and BLM BAPP results are the average of two determinations for each. Values represent the mean ± SEM, in arbitrary units.

of experimental error due to the densitometric analysis (Figure 3b). Diminution of cleavage efficiency at the methylated cytidine (site 118) was significant for bleomycin A_2 (78%) and bleomycin BAPP (76%). In contrast, cleavage of this DNA fragment by bleomycin B_2 (30% diminution) and bleomycin demethyl A_2 (13% diminution) was much less affected by the presence of cytidine methyl groups. As these replicate determinations were performed at several bleomycin concentrations and utilized different methylated DNA preparations, the statistical analysis indicates that the observed effects are reproducible. All of the DNA preparations were analyzed for complete methylation as judged by resistance to cleavage by the appropriate restriction endonuclease (for example, Figure 3a, lanes 1–4).

Effect of Adenosine Methylation on DNA Cleavage by Bleomycin Congeners. The restriction methylase *Taq*I in-

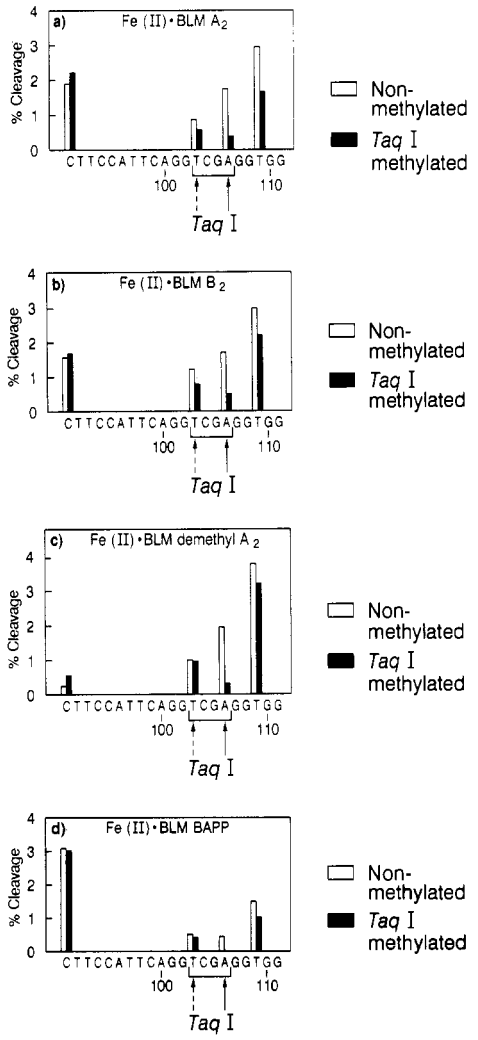

FIGURE 4: Analysis of the cleavage of a 207 bp fragment of pBR322 DNA with each of four BLM congeners following methylation with *Taq*I. The *Taq*I-methylated and unmethylated 5′-end-labeled DNA fragments were treated with 2 μM Fe(II)·BLM + 1 mM DTT and then analyzed on a polyacrylamide gel (Figure 3 of the supplementary material). The autoradiogram was subjected to densitometric analysis; a 21 bp fragment in the methylation region is shown. Panel a, Fe(II)·BLM A_2; panel b, Fe(II)·BLM B_2; panel c, Fe(II)·BLM demethyl A_2; panel d, Fe(II)·BLM BAPP.

troduces methyl groups on N^6 of adenosine within the recognition sequence TCGA. A DNA fragment of pBR322 was methylated with *Taq*I methylase in order to determine whether this major groove modification would have any effect on the efficiency of strand scission by bleomycin congeners. We chose a DNA fragment that contained the sequence $^{5'}$-GTCGA-$^{3'}$ in order to investigate two different bleomycin recognition sites ($^{5'}$-GT-$^{3'}$ and $^{5'}$-GA-$^{3'}$). Bleomycin-mediated strand scission at the GT sequence would show the effect of adenosine methylation on the strand opposite to the cleaved nucleotide, while bleomycin-mediated strand scission at the GA sequence would show the effect of adenosine methylation on the same strand. The end-labeled, *Taq*I-methylated DNA fragment, in addition to the corresponding nonmethylated DNA fragment, was subjected to cleavage by each of the bleomycin congeners in the presence of Fe(II) (supplementary material, Figure 3). In each case, DNA strand scission was diminished in the vicinity of the methylated adenosine residues relative to the cleavage of the nonmethylated substrate. The base sequences at which this diminution occurred are illustrated in histograms (Figure 4). Bleomycin-mediated strand scission at the GA site (106) was clearly diminished when the adenosine was methylated. Bleomycin-mediated strand scission at the GT site (103) was also diminished by methylation, but the extent of diminution was less pronounced. At this cleavage site, the adenosine on the opposite strand (base paired to thymidine 103) was methylated. Interestingly, there was another strong cleavage site adjacent to the methylation region (thymidine 109) at which cleavage was diminished for those DNA substrates that were methylated by *Taq*I. This effect was especially apparent with Fe(II)·BLM A_2 (Figure 4a). Thymidine-109 is three nucleotides away from the nearest methylated adenosine. Thus, in contrast to the results observed with cytidine methylation, the methylation of adenosine residues resulted in diminished strand scission for all of the tested bleomycin congeners.

Table I: BLM-Mediated Production of Thiobarbituric Acid (TBA) Reactive Material and Thymine Concomitant with T4 DNA Degradation[a]

	substrate			
	wild-type T4 DNA[b]		nick-translated T4 DNA[c]	
metallo-BLM	TBA-reactive material (nmol)	thymine (nmol)	TBA-reactive material (nmol)	thymine (nmol)
Fe(II)·BLM A_2	1.65 ± 0.12	1.86 ± 0.12	1.91 ± 0.12	1.50 ± 0.21
Fe(II)·BLM B_2	1.50 ± 0.10	1.70 ± 0.15	1.81 ± 0.16	1.72 ± 0.12

[a] The reactions were carried out and analyzed as described under Experimental Procedures; product yields were derived from two independent analyses; the data represent the mean ± SD. [b] The extent of cytidine glucosylation was determined by nucleoside analysis (Experimental Procedures); 94% of the cytidines were found to be glucosyloxymethylated. [c] Nucleoside analysis indicated that 11% of the cytidines were glucosyloxymethylated.

Effect of Major Groove Blocking Groups on Bleomycin-Mediated Cleavage of DNA. The methylation of cytidine by *Hha*I and *Hpa*II methylases occurs at the C-5 position in the major groove of DNA. Similarly, the methylation of adenosine by *Taq*I methylase occurs at the N^6 position in the major groove. The diminution of bleomycin-mediated strand scission in the presence of these major groove modifications could simply be due to an unfavorable steric interaction between the methyl group on the nucleotide base and a part of the bleomycin molecule. This model predicts that part of the bleomycin molecule must lie in the major groove when the drug is bound in the orientation that leads to DNA strand scission. If this were true, then DNA substrates containing groups that block the major groove should not be able to bind bleomycin as efficiently and would be expected to be partially or totally resistant to cleavage.

We tested this prediction using T4 bacteriophage DNA, which has most of its cytidine residues modified with bulky (glucosyloxy)methyl groups (Revel & Luria, 1970) in the major groove. The corresponding "nonglucosylated" T4 DNA was prepared by nick-translation of the natural T4 DNA to replace the modified cytidines with cytidine. The amount of glucosyloxymethylated cytidine contained in each of these DNA fragments was determined by enzymatic degradation of the DNA followed by HPLC analysis of the resultant nucleosides (Gehrke et al., 1984). In the natural T4 DNA, 94% of the cytidines were glucosylated, while only 11% of the cytidines in the nick-translated product were glucosylated (Table I). These two DNA substrates were subjected to cleavage by Fe(II)·BLM A_2 and Fe(II)·BLM B_2; DNA cleavage was assayed indirectly with 2-thiobarbituric acid (TBA), which reacts efficiently with the base propenals that

Table II: BLM-Mediated Production of Nucleotide Base and Base Propenals Concomitant with Copolymer Degradation[a]

substrate	nmol of product			
	cytosine	5-methylcytosine	C-propenal[b]	5-MeC-propenal[c]
poly(dG-dC)·poly(dG-dC)	7.63 ± 0.83		8.96 ± 0.58	
poly(dG-^{Me}dC)·poly(dG-^{Me}dC)		6.79 ± 0.05		5.47 ± 0.54

[a] The reactions were carried out and analyzed as described under Experimental Procedures; the NaCl concentration was 0.1 M; product yields were derived from two independent analyses; the data represent the mean ± SD. [b] *trans*-3-(Cytidin-1′-yl)propenal. [c] *trans*-3-(5′-Methylcytidin-1′-yl)propenal.

are produced by bleomycin in amounts equal to DNA scission events (Burger et al., 1982). Also measured by HPLC analysis was the release of thymine, which accompanies the formation of alkali-labile product (Burger et al., 1982; Wu et al., 1983; Sugiyama et al., 1985a). The amount of TBA-reactive material produced by either bleomycin A_2 or bleomycin B_2 was only slightly diminished for wild-type DNA compared to nick-translated DNA. The amount of free thymine released by these bleomycins from both DNA substrates was also similar, although a slightly greater proportion of thymine, as compared with TBA-reactive product, was formed from wild-type T4 DNA (Table I). Using the same global assay to measure the effect of DNA methylation on bleomycin strand scission, we found the cleavage of methylated poly(dG-dC)·poly(dG-dC) to be diminished by 39 ± 2.9% in comparison to that of the unmethylated copolymer (Table II and Figure 6a). Thus, while the methylation of every cytidine in this DNA substrate diminished total cleavage mediated by bleomycin, the glucosylation of 94% of the cytidines of T4 DNA had little effect on the efficiency of DNA strand scission by bleomycin A_2 or bleomycin B_2. In addition, cleavage of an end-labeled DNA fragment from glucosyloxymethylated T4 DNA by bleomycin A_2 revealed the expected sequence specificity, one nucleotide to the 3′ side of guanine (data not shown). These results imply the absence of important steric interactions between Fe(II)·BLM and DNA in the major groove.

The reaction of Fe(II)·BLM with DNA leads to two different types of degradation. Direct DNA cleavage results in the stoichiometric production of base propenals, while the formation of an alkali-labile lesions is accompanied by the release of free nucleotide bases (Giloni et al., 1981; Burger et al., 1982; Wu et al., 1983, 1985a,b; Sugiyama et al., 1985a). Since the chemistry that leads to alkali-labile sites on DNA would presumably not be measured by sequencing gels or the TBA assay, the formation of free nucleotide bases from the alternating copolymers poly(dG-dC)·poly(dG-dC) and poly(dG-^{Me}dC)·poly(dG-^{Me}dC) was measured by HPLC analysis (Table II). The methylation of poly(dG-dC)·poly(dG-dC) resulted in a diminution of both base propenal and free base product formation mediated by Fe(II)·BLM A_2. A greater proportion of free base, as compared with base propenal, was formed from the methylated copolymer. This effect has also been observed for DNA oligonucleotides (Long and Hecht, unpublished results).

These results prompted another examination of the sequence selectivity of DNA degradation by BLM A_2 at methylated sites (Figure 5). In this experiment, unmethylated (lanes 3 and 4) or methylated (lanes 5 and 6) DNAs were first treated with Fe(II)·BLM A_2; half of each sample was subsequently treated with hydrazine (lanes 4 and 6). Hydrazine treatment of the

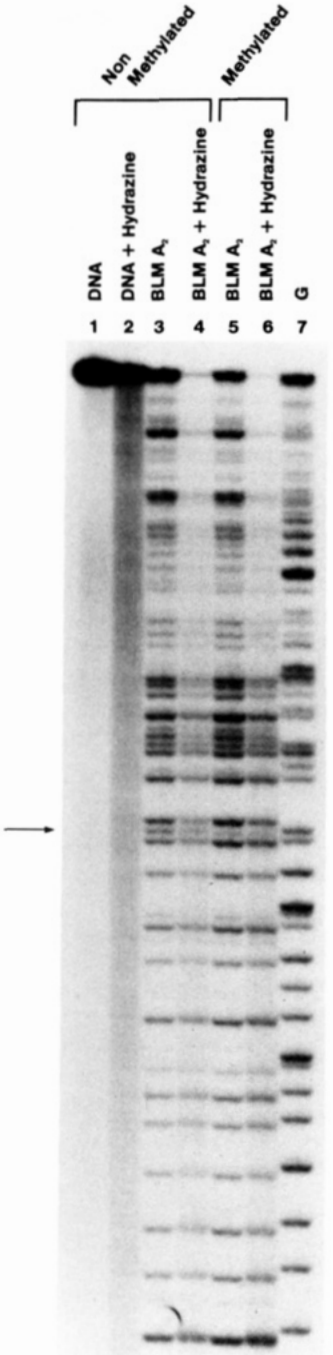

FIGURE 5: Analysis of the cleavage of a 381 bp fragment of pBR322 DNA with BLM A_2 and hydrazine. The 3′-^{32}P-end-labeled DNA fragment was methylated with *Hha*I, and the methylated (lanes 5 and 6) and unmethylated (lanes 1–4) DNA was treated with 1.0 μM Fe(II)·BLM A_2 + 1 mM DTT (lanes 3–6). Lane 1, untreated DNA; lane 2, DNA + hydrazine; lane 3, BLM A_2; lane 4, BLM A_2 + hydrazine; lane 5, BLM A_2; lane 6, BLM A_2 + hydrazine treatment; lane 7, G lane.

alkali-labile lesions mediated by bleomycin effects strand scission by further modification of these lesions (Sugiyama et al., 1988). Lanes 4 and 6 illustrate that successive treatments with bleomycin and hydrazine resulted in the production of a greater number of cleavage events compared to bleomycin alone. The diminution of cleavage at the methylated site was not altered by hydrazine, indicating that the observed diminution was not simply due to alteration of the chemical mechanism of DNA degradation.

Bleomycin-Mediated Cleavage of DNA in Different Conformations. Methylation can affect the conformation of DNA,

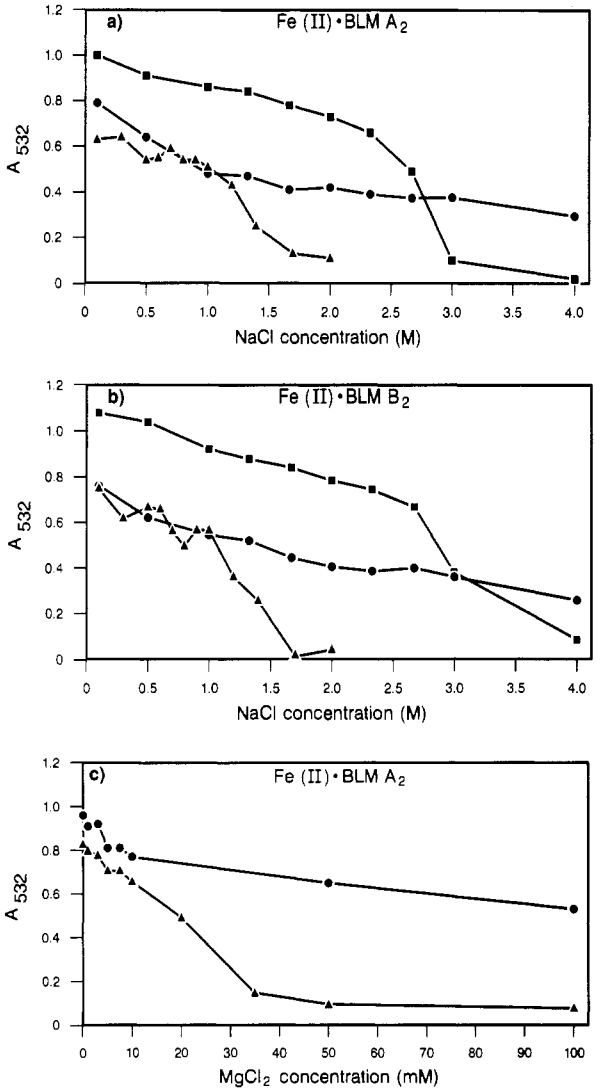

FIGURE 6: Production of thiobarbituric acid reactive material concomitant with Fe(II)·BLM-mediated degradation of calf thymus DNA (●), poly(dG-dC)·poly(dG-dC) (■), or poly(dG-^{Me}dC)·poly(dG-^{Me}dC) (▲). Degradation was carried out by using 100 μM Fe(II)·BLM A_2 + 0.5 mM DTT (panels a and c) or 100 μM Fe(II)·BLM B_2 + 0.5 mM DTT (panel b). The NaCl concentration in panel c was 50 mM.

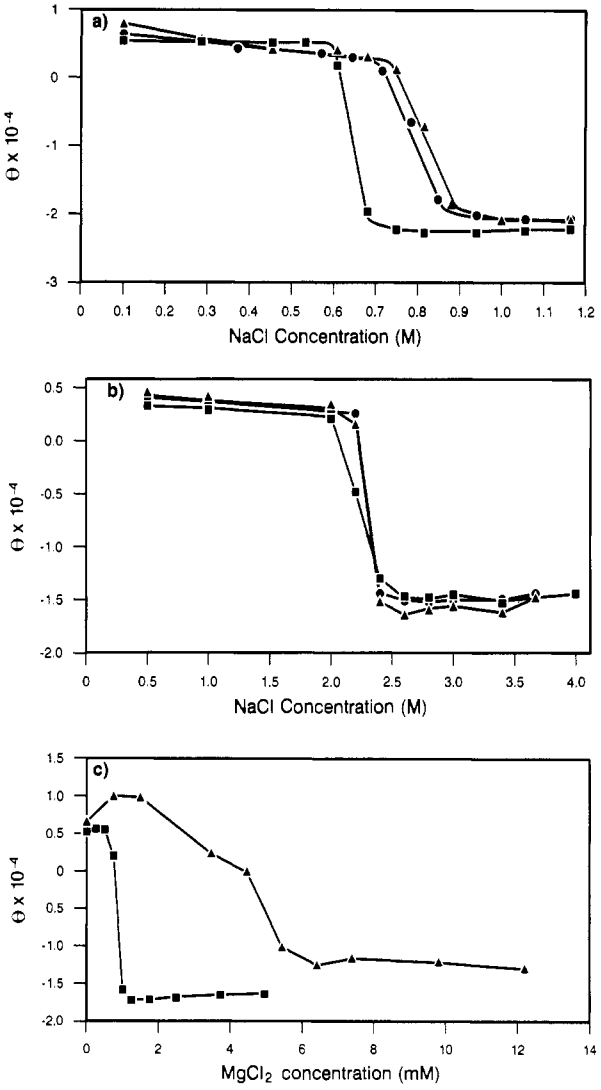

FIGURE 7: Determination of the circular dichroism of poly(dG-^{Me}dC)·poly(dG-^{Me}dC) (panels a and c) and poly(dG-dC)·poly(dG-dC) (panel b) at varying NaCl concentrations (panels a and b) or $MgCl_2$ concentrations (panel c) in the presence of 25 μM Fe(III)·BLM A_2 (▲) or 25 μM Fe(III)·BLM B_2 (●) or in the absence of added Fe(III)·BLM (■). The NaCl concentration in panel c was 50 mM.

and it is possible that alteration of DNA conformation was the cause of the observed diminution of bleomycin-mediated cleavage. This explanation appears most likely for changes in the extent of DNA strand scission that accompany cytidine methylation, since the affected cleavage sites sometimes occurred at bases that were not directly methylated but were near or between methylated CCGG sequences within a 14 bp segment of DNA (Figures 1 and 2; Hertzberg et al., 1985). Cytidine methylation is known to lower the concentration of counterions necessary to induce a B → Z transition in poly(dG-dC)·poly(dG-dC) (Behe & Felsenfeld, 1981). Therefore, in order to assess the effect of DNA conformational changes on BLM-mediated DNA degradation, the efficiency of Fe(II)·BLM A_2 and Fe(II)·BLM B_2 mediated strand scission of the synthetic alternating copolymers poly(dG-dC)·poly(dG-dC) and poly(dG-^{Me}dC)·poly(dG-^{Me}dC) was examined at several different sodium ion concentrations and Mg(II) concentrations (Figure 6). For comparison, DNA of heterogeneous sequence (calf thymus DNA) was also subjected to bleomycin-mediated cleavage at several salt and magnesium concentrations.

The cleavage of calf thymus DNA by both bleomycin congeners decreased slightly as the NaCl concentration was increased. Strand scission of poly(dG-dC)·poly(dG-dC) by bleomycin A_2 also decreased slightly from 0.1 to 2.3 M NaCl and then fell sharply as the NaCl concentration was raised above 2.3 M (Figure 6a). At 3.0 M NaCl, the amount of TBA-reactive products produced by bleomycin A_2 was less than 10% of the amount produced at 0.1 M NaCl. A similar NaCl titration curve was observed with bleomycin B_2 mediated cleavage of poly(dG-dC)·poly(dG-dC), but the decrease was not as sharp and occurred at a slightly higher NaCl concentration (Figure 6b). The decrease in cleavage mediated by these bleomycin congeners occurred near the NaCl concentration at which this DNA copolymer begins to adopt a Z conformation (Behe & Felsenfeld, 1981; Pohl & Jovin, 1972; Figure 7), suggesting that these bleomycins do not cleave Z-DNA efficiently.

Bleomycin A_2 mediated cleavage of the methylated copolymer, poly(dG-^{Me}dC)·poly(dG-^{Me}dC), yielded less TBA-reactive products than did cleavage of the nonmethylated polymer at all of the salt concentrations tested (Figure 6a). Since TBA-reactive product formation is stoichiometric with strand scission (Burger et al., 1982), this result is consistent with the diminution of cleavage observed on sequencing gels. As the salt concentration was increased, a sharp decrease in bleomycin-mediated strand scission was observed at concen-

trations above 1.0 M NaCl. Both bleomycin congeners produced similar amounts of products from this copolymer, and the NaCl titration curves were similar (Figure 6a,b). The release of 5-methylcytosine from poly(dG-^{Me}dC)·poly(dG-^{Me}dC) mediated by bleomycin A_2 and bleomycin B_2 also decreased sharply at NaCl concentrations above 1.0 M. Also, because the presumed product of this reaction, *trans*-3-(5′-methylcytidin-1′-yl)propenal, had not been characterized previously as a product of Fe(II)·BLM-mediated oligodeoxynucleotide degradation, we verified that this compound had formed by HPLC comparison with an authentic, synthetic sample (data not shown).

For the $MgCl_2$ titration, the cleavage of calf thymus DNA by bleomycin A_2 decreased gradually as the $MgCl_2$ concentration was increased (Figure 6c). Although strand scission of poly(dG-^{Me}dC)·poly(dG-^{Me}dC) decreased more sharply between 10 and 35 mM Mg(II) than did strand scission of calf thymus DNA, the expected sharp decrease near the midpoint of the B → Z transition [0.6 mM Mg(II), 50 mM NaCl; Behe & Felsenfeld, 1981] did not occur. It seems plausible that the B → Z transition was perturbed by the presence of bound BLM. Similarly, for the NaCl titration the sharp decrease in DNA strand scission observed with poly(dG-^{Me}dC)·poly(dG-^{Me}dC) did not occur precisely at the NaCl concentration previously shown to induce Z-DNA in this copolymer (Behe & Felsenfeld, 1981) but occurred at a slightly higher NaCl concentration, also consistent with the suggestion that the transition was perturbed by the bound BLM.

Circular Dichroism of DNA Copolymers in the Presence of Bleomycins. The B → Z transition of poly(dG-dC)·poly(dG-dC) and poly(dG-^{Me}dC)·poly(dG-^{Me}dC) can be monitored by the inversion of the circular dichroic spectra (Pohl & Jovin, 1972). The midpoint of the B → Z transition for these DNA copolymers was determined in the presence and in the absence of Fe(III)·BLM A_2 and Fe(III)·BLM B_2 by raising the NaCl or $MgCl_2$ concentration while observing the circular dichroism at 293 nm (Figure 7). The ferric complexes of bleomycin were used in order to preclude DNA strand scission during the course of the titration, and the bleomycin to DNA copolymer ratio (1 bleomycin to 2 bp) was the same as that used for the cleavage titrations above. For poly(dG-^{Me}dC)·poly(dG-^{Me}dC), the B → Z transition midpoint occurred at 0.65 M NaCl in the absence of bleomycin, and at 0.8 M in the presence of either Fe(III)·BLM A_2 or Fe(III)·BLM B_2. Bleomycin or Fe(III) alone had little effect on B → Z transition midpoint (data not shown). For poly(dG-dC)·poly(dG-dC), the transition midpoint occurred at 2.25 M NaCl in the absence of bleomycin, and at 2.3 M in the presence of either Fe(III)·BLM congener. For poly(dG-^{Me}dC)·poly(dG-^{Me}dC) the B → Z transition occurred at 0.87 mM $MgCl_2$ in the absence of bleomycin, and at approximately 3.5 mM in the presence of Fe(III)·BLM A_2. Thus, for each titration Fe(III)·BLM raised the cation concentration necessary to induce Z-DNA in these DNA copolymers, suggesting that the drug preferentially binds to B-DNA.

Discussion

The molecular basis for the recognition of specific sites on DNA by bleomycin is not well understood. The roles of the bithiazole moiety and C-terminal substituent in the interaction of bleomycin with DNA have been studied by the use of bithiazole derivatives (Chien et al., 1977; Povirk et al., 1979; Lin & Grollman, 1981; Fisher et al., 1985; Kilkuskie et al., 1985). Certain structurally modified DNA molecules have provided mechanistically useful information as well (Hertzberg et al., 1985; Sugiyama et al., 1985b, 1986). Also carried out have been NMR and unwinding experiments (Lin & Grollman, 1981; Sakai et al., 1981, 1982, 1983; Booth et al., 1983; Fisher et al., 1985; Henichart et al., 1985); in the aggregate, these studies suggest that the planar, aromatic bithiazole moiety of bleomycin may (partially) intercalate into DNA, with the cationic group at the C-terminus binding to a DNA phosphate group and thereby stabilizing the interaction.

Experimental evidence strongly suggests that the chemistry which leads to DNA strand scission originates with abstraction of the deoxyribose C-4′ hydrogen (Burger et al., 1980; Giloni et al., 1981; Wu et al., 1983, 1985a,b; Murugesan et al., 1985), implying that the metal binding domain of bleomycin has access to the minor groove. In addition, agents that bind to DNA in the minor groove are known to perturb the sequence specificity of DNA strand scission (Sugiura & Suzuki, 1982). In contrast, other agents that bind to guanine in the major groove do not alter the DNA cleavage specificity of bleomycin (Suzuki et al., 1983). These results suggest that bleomycin interacts with DNA only in the minor groove.

The observation that cytidine methylation diminished DNA strand scission mediated by bleomycin can be explained by either of two hypotheses. It is possible that a localized unfavorable steric interaction between the cytidine methyl group and part of the bleomycin molecule decreased the affinity of the drug for the methylated sequence. This decreased binding affinity would then be reflected in a diminished frequency of DNA strand scission at that site. Alternatively, it is possible that the presence of methyl groups effect conformational changes in DNA within the vicinity of the methylated sequence. These conformational alterations may introduce unfavorable interactions with the bleomycin molecule and thereby lower the binding affinity of the drug for that particular methylated site and the adjacent sequences that have undergone the conformational change.

The cytidine methyl group is situated in the major groove of B-DNA. Most of the experiments directed toward understanding the nature of bleomycin–DNA interactions support a partial intercalation model, with most or all of the drug oriented in the minor groove (Kross et al., 1982a,b; Booth et al., 1983; Fisher et al., 1985; Henichart et al., 1985). Because the intercalation by bleomycin must almost certainly involve the bithiazole moiety (Chien et al., 1977; Povirk et al., 1979; Kross et al., 1982a; Kilkuskie et al., 1985), and the ability of the metal chelated N-terminus of BLM to effect transformations at C-4′ of deoxyribose (Burger et al., 1980; Giloni et al., 1981; Wu et al., 1983, 1985a,b; Murugesan et al., 1985; Sugiyama et al., 1985a,b, 1986) argues for its localization in the minor groove, the only portion of bleomycin that appears to have the possibility of direct interaction in the major groove is the C substituent attached to the bithiazole. Therefore, to test whether methylation-induced diminution of bleomycin strand scission was caused by direct steric interaction of BLM with the methyl group of 5-methylcytidine in the major groove, the effect of modification of the C substituent was investigated. The sequence specificities of DNA cleavage induced by the four bleomycin congeners tested—bleomycin A_2, bleomycin B_2, bleomycin demethyl A_2, and bleomycin BAPP—were very similar but not identical. Methylation of specific cytidine residues by restriction methylases substantially diminished strand scission mediated by only two of the congeners, bleomycin A_2 and bleomycin BAPP (Figures 1–3). That the diminution of strand scission occurred at some sites that were not directly methylated suggests strongly that unfavorable steric interactions between the cytidine methyl group and bleomycin are not likely to account for the diminution of strand

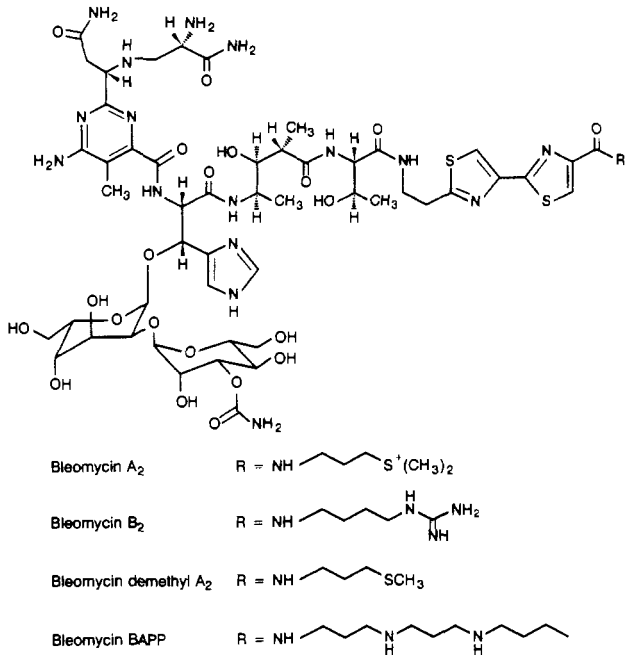

FIGURE 8: Chemical structures of bleomycin congeners.

scission observed. However, since neither the size of the bleomycin/DNA binding site nor the stoichiometry of BLM binding at these sites is known, we cannot rule out the possibilities that direct steric interaction with a single BLM molecule may extend as far as three base pairs from the cleavage site or that more than one BLM may bind at a single DNA site in a cooperative fashion.

The effect of cytidine methylation on BLM-mediated DNA strand scission was much smaller for two other BLM congeners, which also differed in structure solely within the C substituent. It has been noted previously (Kross et al., 1982a) that the number and location of positive charges within the alkyl substituents in series of structurally related bithiazole derivatives had a dramatic effect on their DNA affinity. Interestingly, those BLM congeners whose DNA cleavage was insensitive to the presence of 5-methylcytidine (BLM B_2 and BLM demethyl A_2) differed from the other tested congeners (BLM A_2 and BLM BAPP) in the number and location of positive charges within the C substituent (Figure 8). It seems possible that bleomycin B_2 and bleomycin demethyl A_2 bind to DNA in a different orientation than bleomycin A_2 and therefore are less susceptible to DNA conformational changes occasioned by the introduction of a methyl group on cytidine. Alternatively, there may be a common orientation that is more readily altered for some of the BLM congeners on the basis of C substituent–DNA interactions. It is of interest that Sakai et al. (1983) reported that bleomycin demethyl A_2 displayed no intercalation of the aromatic bithiazole into poly(dA-T)·poly(dA-T). The present results indicate that if the binding geometry of these bleomycin congeners is different, this difference is not a major contributor to the DNA sequence recognition since the cleavage patterns mediated by all of the bleomycins are similar.

The effect of a different major groove modification on bleomycin-mediated cleavage was also investigated by N^6 methylation of adenosine. Gel electrophoresis studies have shown that adenosine methylation introduces a detectable but extremely small structural perturbation in DNA (Cheng et al., 1985). We tested the possible recognition of this structural change by congeners of bleomycin. A clear diminution of strand scission was observed at the methylated adenosine for each of the congeners tested (Figure 4). This effect has also been observed for blenoxane (a mixture of several bleomycins) by comparing the cleavage of genomic α-DNA to a similar sequence cloned in *E. coli* (Murray & Martin, 1985). Our results demonstrated that in addition to the affected $G^{Me}A$ site (Figure 4, site 106), there was a small but significant reduction in the cleavage of two other sequences adjacent to the methylated adenosine. One of these nucleotides (thymidine-103) is located on the strand opposite to a methylated adenosine. The diminution of cleavage observed at sites 103 and 106 could be explained by direct steric interaction between the methyl groups in the major groove and bleomycin. However, the strong cleavage obtained at the thymidine-109, three nucleotides distant from the nearest ^{Me}A, was also diminished for those DNA substrates that were methylated. Given the differences in the location of the methyl groups relative to the three sites of DNA strand scission (vide supra), it seems unlikely that the same direct methyl group–bleomycin steric interaction could account for diminution for strand scission at all three sites. The alternative explanation for diminished strand scission, i.e., that a slight structural perturbation in this region was induced by adenosine methylation and selectively recognized by each bleomycin, seems more plausible. Presumably, this structural perturbation would be different from that induced by cytidine methylation since the different bleomycin congeners were all affected similarly by adenosine methylation, in contrast to the situation observed for cytidine methylation.

Additional data pertinent to the issue of steric interaction between bleomycin and DNA in the major groove was derived from experiments using phage T4 DNA (Table I). The major groove of T4 DNA is substantially blocked by bulky glucose residues. This DNA has been used to study the groove specificity of two minor groove binders, daunomycin (Yen et al., 1983) and neocarzinostatin (Dasgupta & Goldberg, 1985). In both cases, the presence of glucose residues was found to have a negligible effect on the binding affinities, consistent with other studies indicating minor groove specificities for daunomycin and neocarzinostatin. Similarly, for bleomycin A_2 and bleomycin B_2, we observed that the presence of glucose residues in the major groove had very little effect on the efficiency of DNA strand scission (Table I). Although we did not directly measure DNA binding, the similarity in the type and amount of cleavage products derived from both DNA substrates suggests that the interaction between bleomycin and glucosylated DNA is not very different from that between bleomycin and unmodified DNA. This is consistent with other results which suggest that bleomycin binds in the minor groove.

The reaction of bleomycin with the alternating copolymers poly(dG-dC)·poly(dG-dC) and poly(dG-^{Me}dC)·poly(dG-^{Me}dC) was performed at different salt concentrations to determine the susceptibility of DNA in different conformations to bleomycin cleavage. A sharp reduction in the amount of TBA-reactive products formed by bleomycin was observed near the salt concentrations at which these copolymers have been reported to exist in Z conformation (Pohl & Jovin, 1972; Behe & Felsenfeld, 1981) (Figure 6). This reduction in cleavage was not due solely to decreasing electrostatic interactions between bleomycin and DNA, since the cleavage of calf thymus DNA was affected much less by salt. The copolymers were resistant to bleomycin-mediated cleavage when they existed in the Z conformation, presumably because the affinity of the drug for Z-DNA is very low. Several other DNA binding agents, primarily intercalators, have also been demonstrated to bind more tightly to B-DNA than to Z-DNA

(Van de Sande & Jovin, 1982; Mirau & Kearns, 1983). The circular dichroism titration showed that the binding of Fe(III)·BLM to poly(dG-^{Me}dC)·poly(dG-^{Me}dC) increased the NaCl concentration necessary to induce Z-DNA (Figure 7) and significantly raised the $MgCl_2$ concentration needed to induce the Z form. This is consistent with Fe(III)·BLM binding to and stabilizing B-DNA until a high enough cation concentration was able to effect the dissociation of the drug concomitant with the transition to the Z conformation. In addition, these results indicate that bleomycin can bind to methylated DNA, despite the observation on polyacrylamide gels that less cleavage occurred near methylated sites.

Both bleomycin A_2 and bleomycin B_2 behaved similarly with these copolymers. Neither congener was efficient at mediating cleavage of Z-DNA, and both congeners inhibited the B → Z transition to the same degree. Therefore, with respect to this major DNA conformational change, it appears that bleomycin A_2 and bleomycin B_2 possess very similar selectivities. However, there were differences between bleomycin A_2 and bleomycin B_2 when isolated cytidines were methylated within a nonmethylated flanking sequence (Figures 1–3). Therefore, it is likely that a few isolated 5-methylcytidines are not sufficient to induce Z conformation within these flanking sequences (Nickol & Felsenfeld, 1983). It is more reasonable to suggest that conformational changes induced within these linear DNA fragments are subtle, and it is interesting that a relatively small molecule can recognize these differences. It has been observed that methylated pyrimidines stabilize an "alternating B" conformation of DNA in solutions at moderate NaCl concentration (Wu & Behe, 1985; Chen et al., 1983). Wu and Behe (1985) have proposed that the methylation of CpG sequences may shift a region of DNA toward a more alternating structure and that regulatory proteins can recognize such subtle conformations. On the basis of the results presented here, it seems likely that certain bleomycin congeners are also capable of discriminating between B-DNA and alternating B structures. In addition, the fact that several CCGG and GCGC sequences within certain genetic regions in tumor cells have been found to be undermethylated (Riggs & Jones, 1983; Kuo et al., 1984) is consistent with the possibility that diminished cleavage of methylated regions of DNA may provide a source of selectivity for BLM as a therapeutic agent. Within the bleomycin/DNA complex, the positioning of the C-4′ hydrogen of the deoxyribose relative to the putative iron-bound activated oxygen (Hecht, 1986) could be different with DNAs of slightly altered conformations. Our results do not establish whether the diminution of cleavage of methylated sequences on DNA is due to the low affinity of bleomycin for these sites or to an improper orientation between the reactive center of bound activated bleomycin and the deoxyribose C-4′ hydrogen.

ACKNOWLEDGMENTS

We thank David Killian, University of Virginia, for an authentic sample of 3-(5′-methylcytidin-1′-yl)propenal and Eric Long for providing data on bleomycin degradation of a methylated oligodeoxynucleotide.

SUPPLEMENTARY MATERIAL AVAILABLE

Figure 1 showing densitometric analysis of 72 bp from the 280 bp DNA fragment described in Figure 1 of this paper, Figure 2 showing densitometric analysis of 117 bp from the 381 bp DNA fragment described in Figure 3 of this paper, Figure 3 showing analysis of the cleavage of a 207 bp fragment of pBR322 DNA with each of four BLM congeners following methylation with *Taq*I, and Figure 4 showing densitometric analysis of 77 bp from the 207 bp DNA fragment illustrated in Figure 3 of the supplementary material (15 pages). Ordering information is given on any current masthead page.

Registry No. Bleomycin BAPP, 69177-41-9; blenoxane, 9041-93-4; bleomycin demethyl A_2, 41089-03-6; bleomycin A_2, 11116-31-7; bleomycin B_2, 9060-10-0; poly(dG-dC), 36786-90-0; poly(dG-^{Me}dC), 51853-63-5; deoxycytidine, 951-77-9.

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