Bleomycin-induced Alkaline-labile Damage and Direct Strand Breakage of PM2 DNA

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ABSTRACT

Bleomycin-induced breakage of an isolated covalently closed circular DNA from bacteriophage PM2 was assayed fluorometrically after agarose gel electrophoresis and staining with ethidium bromide. When bleomycin-damaged DNA was assayed under neutral conditions, there was a decrease in the amount of Form I DNA and a simultaneous increase in both Forms II and III of DNA. However, when the damage was assayed under non-denaturing alkaline conditions, there was a greater decrease in the amount of Form I DNA and a corresponding increase in both Forms II and III DNA compared with neutral conditions. Approximately one alkaline-labile site was formed for every single-strand break introduced. The rate of formation of Form III DNA was found to be approximately twice as fast when measured under alkaline conditions compared with neutral conditions. Reaction of bleomycin-treated PM2 DNA with the Escherichia coli apurinic specific endonuclease IV demonstrated that the loss of Form I DNA was very similar to that observed with alkali treatment suggesting that the bleomycin-induced alkaline-labile damage is the result of base removal from the DNA helix. Similar results were obtained when the bleomycin-treated DNA was reacted with E. coli endonuclease III. However, no increase in double-strand breakage was observed with either endonuclease III or IV, which may reflect an inability of the endonuclease to cleave at a site of base loss across from a single-strand break or another lost base. Alkali treatment of purified Form I DNA which was pretreated with bleomycin revealed that some of the alkali-induced double-strand breaks arose from Form I molecules, suggesting that two alkaline-labile sites are formed across from each other. In addition, some Form II molecules produced by bleomycin treatment were converted to Form III molecules by non-denaturing alkaline conditions.

INTRODUCTION

The bleomycins are a group of glycopeptide antibiotics that were originally isolated from Streptomyces verticillus by Umezawa et al. (24). This group of antibiotics has been shown to be effective in the treatment of squamous cell carcinomas, certain lymphomas, and testicular carcinomas (1, 8, 20).

Bleomycin causes fragmentation of DNA in vitro and in vivo (6, 16, 23). The presence of various sulfhydryl compounds greatly enhances bleomycin-induced breakage of DNA (7, 15). Müller et al. (15), assayng free base release by Sephadex chromatography, reported that only thymine was removed from the DNA helix after bleomycin treatment and that the amount of thymine released was related to the base composition of the DNA. In contrast, Haidle et al. (7), using higher concentrations of bleomycin and assaying by paper chromatography, demonstrated the release of all 4 common bases from DNA as well as the removal of uracil from deoxyuridylic acid-containing PBS-1 bacteriophage DNA. In addition to the release of free bases from DNA, Haidle et al. (7) and Kuo et al. (9) demonstrated that cleavage of the sugar-phosphate backbone of DNA by bleomycin yielded primarily 3'-hydroxy groups. These workers also observed no release of P1 from the DNA and that free bases were not released from nucleotides and nucleosides; in addition, they showed bleomycin-induced damage to the deoxyribose moiety.

Since bleomycin has been shown previously to cause single-strand and possibly double-strand breaks (6) and to cause the release of free bases, the question arose as to what proportion of bleomycin-induced DNA damage was in the form of alkaline-labile sites and what proportion was the result of direct strand breakage. Recently, Povirk et al. (17) reported the occurrence of alkaline-labile bonds and double-strand breaks in DNA after bleomycin treatment. Using ColE1 DNA, approximately twice as many single-strand breaks were found under alkaline conditions than under neutral conditions. Ross and Moses (18) reported that reaction of bleomycin with φX 174 RF DNA in the presence of 1 mM EDTA produced alkaline-labile sites with no strand scission. Apurinic and apyrimidinic sites in DNA are known to be alkaline labile (5, 10, 11). Therefore this study was initiated to analyze the relative number of direct strand breaks compared with the amount of alkaline-labile damage induced by bleomycin.

Superhelical covalently closed circular DNA, isolated from the bacteriophage PM2 (2, 3), was used to assay single- and double-strand breaks and alkaline-labile sites (Chart 1). Molecules were distinguished on the basis of the differences in electrophoretic mobilities of the native superhelical molecule (Form I), a nicked relaxed circular molecule (Form II, arising from a single-strand break), and a linear molecule (Form III, arising from a double-strand break). In order to test for alkaline-labile sites induced by bleomycin, identical bleomycin-treated DNA samples were assayed under either neutral conditions or non-denaturing alkaline conditions which effectively hydrolyze alkaline-labile sites but do not denature the DNA.

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the Microelectrode Inc. pH electrode, the pH was 11.6; how-

ever, salt correction factors for this electrode were not
available. The final pH was also determined by increasing
all reaction constituents 30-fold and measuring the bulk
solution with a Beckman Century SS-1 pH meter and 0 to 14
pH electrodes; the final pH was 11.7.

The samples were layered either onto 0.9% agarose tube
gels (22) and electrophoresis performed at 2 ma/gel for 7.5
hr, or onto 1.4% agarose gels and electrophoretically
treated for 6 hr at 100 V. After electrophoresis the gels were
stained with ethidium bromide (0.5 \mu g/ml) in electrophore-
sis buffer (40 mm Tris, 5 mm sodium acetate, and 1 mm
EDTA, pH 7.8) in the dark for a minimum of 4 hr. Quantita-
tive measurements of the amount of DNA that had migrated
in the gel were based on the fluorescence enhancement
after ethidium bromide intercalation. The fluorescence
measurements were made by scanning the gels in an
Amino-Bowman spectrophotofluorometer (American In-
struments Co., Silver Spring, Md.); the incident wavelength
was 510 nm and the emission wavelength was 590 nm. The
relative fluorescence intensity was plotted with a strip chart
recorder, and the areas under the DNA peaks were mea-
 sured with a Dietzgen compensating polar planimeter. With
this method and 0.9% gels, Form I DNA migrated the
fastest, followed by Form III and Form II DNA; with 1.4%
gels, Form III DNA migrated fastest followed by Forms I and
II of DNA. All 3 peaks were completely resolved with both
methods.

To eliminate the possibility that some of the DNA was
denatured under the nondenaturing experimental condi-
tions used, we have performed experiments (not shown) in
which the DNA was denatured by either alkali or heat.
Electrophoresis in 1.4% agarose tube gels of such dena-
tured DNA reveals bands that migrate either more rapidly or
more slowly than the native DNA bands. Such denatured
DNA bands were never observed in our experiments with
the nondenaturing alkaline pH. Additionally, since dena-
tured and neutralized DNA does not intercalate as much
ethidium bromide as does native DNA, one would expect a
decreased total fluorescence if some of the DNA were
denatured. Such a decreased fluorescence was not ob-
served.

Multiple scans of 1 gel have shown that this procedure
and the areas measured are reproducible to \pm 1%. The
correction factor applied to account for the reduced ethid-
ium bromide intercalation in Form I DNA versus Forms II
and III of DNA has been determined by 3 methods (13); the
observed area under the Form I peak was multiplied by the
factor 1.42, thus making the area equivalent to the actual
DNA concentrations in each peak.

**Purification of Forms I and II of PM2 DNA.** A standard
0.5-\mu g/ml bleomycin-DNA reaction was run for 10 min and
stopped with an EDTA-containing layering solution. Control
DNA reactions without bleomycin were also performed.
Bleomycin-treated or control DNA (1.25-\mu g) samples were
each layered on nine 1.4% agarose gels and subjected to
electrophoresis for 6.0 hr at 100 V in the dark. Two of the 9
gels for each reaction mixture were stained with ethidium
bromide (0.5 \mu g/ml) and visualized with shortwave (254 nm)
UV light, and the DNA bands were located. The individual
DNA bands in the remaining unstained and unirradiated
gels that corresponded to the DNA forms previously located
by ethidium bromide staining were cut out under yellow

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**MATERIALS AND METHODS**

**Reagents.** *Escherichia coli* endonuclease IV was the
generous gift of Dr. Siv Ljungquist (Department of Chemis-
try, Karolinska University, Stockholm, Sweden). *E. coli*
endonuclease III was the generous gift of Dr. Stuart Linn
(Department of Biochemistry, University of California at
Berkeley). Endonuclease II was concentrated from Frac-
tion VII of CM-25 Sephadex column as previously described
(4). The bleomycin used in these studies was a gift from
Bristol Laboratories (Syracuse, N. Y.). All other chemicals
were purchased from general suppliers. The PM2 DNA was
prepared by the Strong and Hewitt (21) modification of
Salditt et al. (19).

**Analysis of Bleomycin Damage to PM2 DNA by Agarose
Gel Electrophoresis.** The bleomycin-DNA reaction was
performed at 37° according to the following protocol: 75 \mu l
of PM2 DNA (300 \mu g/ml), 144 \mu l of 2-mercaptoethanol (100
mm), and 180 \mu l of CaCl2 (0.27 mm)-Tris-HCl (50 mm, pH
8.0) were mixed; for the control sample minus bleomycin,
22.2-\mu l aliquots were removed and pipetted into 30 \mu l of
either a neutral pH solution at 23° [20 mm EDTA, 50 mm
Tris-HCl, 10% (w/v) sucrose, and 0.025% (w/v) bromphenol
blue, pH 7.6] or a nondenaturing alkaline solution at 23° [20
mm EDTA, 50 mm Tris-HCl, 10% (w/v) sucrose, and 0.025%
(w/v) bromphenol blue] with the final pH of the reaction
mixture being pH 11.7. To the remaining DNA-2-mercapto-
ethanol-Tris mixture, bleomycin (0.5 \mu g/ml) was added,
and incubated at 37°. Twenty-seven-\mu l aliquots were
removed during the course of the reaction and mixed with 30
\mu l of neutral or nondenaturing alkaline solutions for 2.5 hr.
The EDTA concentration used in these solutions has been
shown to stop the bleomycin-DNA reaction very rapidly
(Haidle, unpublished results).

The pH of the final nondenaturing alkaline reaction solu-
tion was measured in 2 ways. After the pH meter was
standardized with a freshly prepared pH 12.0 solution with
the Microelectrode Inc. pH electrode, the pH was 11.6; how-
ever, salt correction factors for this electrode were not

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**Chart 1.** A schematic representation showing the effects of damage to a
PM2 DNA molecule when assayed under either neutral or nondenaturing
alkaline conditions.
light. These bands were either soaked in electrophoresis buffer (pH 7.8) or soaked in 100 ml of a NaOH solution (pH 11.7) for 4 hr. The gel slices were placed on top of new 1.4% agarose gels and subjected to electrophoresis for 6.0 hr at 100 V in the dark followed by staining with ethidium bromide and visualization of the DNA bands.

RESULTS

Test for Alkaline Lability in Partially Depurinated DNA and Bleomycin-treated DNA. For effective analysis of alkaline-labile damage in the PM2 DNA, it was necessary to determine the conditions at which known alkaline-labile sites could be hydrolyzed, without irreversibly denaturing the DNA. A method, similar to one outlined by Lindahl and Nyberg (11), of acid-heat treatment of the DNA to remove purines selectively was used to generate an alkaline-labile DNA population.

A solution of PM2 DNA (125 µg/ml) containing 0.1 M NaCl-0.01 M sodium citrate, pH 5.0, was heated at 60° for increasing times, and aliquots containing 1.25 µg of DNA were removed for electrophoretic analyses. Chart 2 shows the mass fraction of surviving Form I DNA with increasing time of incubation at 60°. The open circles, representing analyses under neutral conditions (pH 7.6), show a very slow rate for the introduction of single-strand breaks. However, when the pH from aliquots of the same population of acid-heat-treated DNA was raised to 11.7 for 2.5 hr, neutralized, and analyzed on agarose gels, a very significant loss of Form I DNA with a corresponding increase in Form II DNA (not shown) was detected. This demonstrates that the alkaline-labile damage in the form of purine base removal can be assayed effectively with PM2 DNA at pH 11.7 without irreversibly denaturing the DNA. Alkaline treatment beyond 2.5 hr showed no further alkaline hydrolysis, indicating complete hydrolysis of alkaline-labile sites.

For determination of whether 2.5 hr in alkali were sufficient time to hydrolyze all alkaline-labile sites introduced by bleomycin, a standard bleomycin-DNA-2-mercaptoethanol reaction mixture was incubated for 10 min at 37° and stopped with 12 mM EDTA. The final pH was 7.6 for neutral analyses or 11.7 for alkaline analyses. Aliquots containing 1.25 µg of DNA were neutralized at 0, 0.25, 0.5, 1.2, and 3 hr. Only 34% of Form I DNA remained after the 10-min bleomycin treatment and the 3-hr neutral analysis. However, under nondenaturing alkaline conditions, the percentage of Form I DNA dropped quickly during the first hr to 18%; continued incubation up to 3 hr yielded an additional 2% loss of Form I DNA. This indicated that hydrolysis of alkaline-labile sites introduced by bleomycin was nearly complete after 1 hr.

Determination of the Extent of Alkaline-labile Damage Introduced by Bleomycin. Chart 3 shows a composite of a time-course reaction with bleomycin (0.5 µg/ml), 2-mercaptoethanol, and PM2 DNA analyzed under both neutral and non-denaturing alkaline conditions. It is evident that bleomycin (0.5 µg/ml) causes extensive damage to the DNA in a short time. The mass fraction of Form I DNA at the zero time point (before bleomycin was added) was 0.839 in the neutral analysis and 0.814 after alkali treatment. This shows that approximately 2.5% of the starting DNA population contained sites which were hydrolyzable under the non-denaturing alkaline conditions. At the start of the experiment, the mass fraction of Form II DNA comprised 0.161 and 0.186 in the neutral and alkaline analyses, respectively; Form III DNA was present at time zero in amounts not detectable by fluorescence scanning. Under neutral conditions, the mass fraction of Form I DNA (open circles) decreased to less than 0.05 after 20 min of reaction with bleomycin. In contrast, analysis by nondenaturing alkaline conditions in which both breaks and hydrolyzed alkaline-labile sites contribute to the loss of Form I DNA revealed that all detectable superhelical DNA was absent after only 10 min of incubation with bleomycin. The mass fraction of Form II DNA increased fairly rapidly to approximately 0.65 after 10 min and remained fairly constant throughout the remainder of the neutral experiment. However, Form II DNA when analyzed with nondenaturing alkaline conditions in which both breaks and hydrolyzed alkaline-labile sites contribute to the loss of Form I DNA revealed that all detectable superhelical DNA was absent after only 10 min of incubation with bleomycin. 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bleomycin. These curves were normalized by dividing the initial fraction of Form I DNA ($f_0$) into the fraction present at each time point ($f_t$) and taking the negative natural logarithm of the resulting ratio. Analysis of the slopes of these lines reveals that the rate of loss of Form I DNA treated with the nondenaturing alkaline conditions was 2.1 times greater than the loss of the Form I DNA under neutral conditions. This demonstrates that approximately 1 hydrolyzable site was produced for every direct single-strand break. The ratio is in excellent agreement with those obtained over a wide range of bleomycin concentrations and reaction temperatures (not shown). The average rate at which damage was introduced into isolated DNA in the form of single-strand breaks and alkaline-labile sites showed that the reaction proceeds 1.9 times faster when analyzed under alkaline conditions compared with neutral conditions. Therefore, in the bleomycin-DNA fragmentation reaction, for every single-strand break directly introduced by the drug, a second alkaline-labile site is formed.

The rate of appearance of Form III DNA molecules was also studied under the neutral and nondenaturing alkaline conditions. Over a wide range of bleomycin concentrations and reaction temperatures, approximately 1.5 to 2 linear DNA molecules were produced when analyzed under alkaline conditions for every one produced under neutral conditions.

**Analysis of Alkaline-labile Sites by E. coli Endonuclease IV versus Alkali Treatment.** Endonuclease IV isolated from *E. coli* has been shown to introduce a single-strand break in the phosphodiester backbone only at a site of a lost base (12). Endonuclease IV was also shown to be completely active in the presence of EDTA, indicating no metal requirement, and to have a broad salt optimum between 0.2 and 0.3 M NaCl. We wished to test the effect of this apurinic endonuclease on bleomycin-treated DNA to determine whether alkaline-labile sites introduced by bleomycin were the result of missing bases. Aliquots of a reaction mixture containing 0.27 mM CaCl$_2$, bleomycin (0.5 µg/ml), DNA (50 µg/ml), 25 mM 2-mercaptoethanol, and incubated at 37°C for increasing times were subjected to 3 analyses: the bleomycin-treated DNA was (a) mixed with a neutral (pH 7.6) 20 mM EDTA-containing solution for 2.5 hr; (b) mixed with a 20 mM EDTA-containing solution with the final pH adjusted to pH 11.7 for 2.5 hr; (c) mixed with a solution containing 20 mM EDTA, 0.4 M NaCl, and 30 units of endonuclease IV for 2.5 hr at pH 7.6. Control experiments demonstrated that 30 units of endonuclease IV were able to convert a DNA population containing 85% Form I with an average of 3 apurinic sites/molecule (11) into Form II DNA within 2 hr. Chart 5 A shows the loss of Form I DNA after increasing reaction with bleomycin and the subsequent treatments described above. The initial DNA population contained approximately 0.8 of the mass fraction of Form I DNA which continuously decreased to approximately 0.15 after 20 min. However, when the bleomycin-treated DNA was assayed by either alkali or endonuclease IV treatment, the surviving Form I DNA fraction decreased much faster than in the neutral assay and the mass fraction of Form I DNA was the same with either the alkali or endonuclease IV treatment. This demonstrates that bleomycin is introducing alkaline-labile sites in isolated DNA, some of which are the result of missing bases. Earlier in this paper we reported that approximately 1.5 to 2 times as many double-strand breaks were measured after alkali treatment as compared with neutral conditions. Chart 5, A and B, shows the increase in Forms II and III, respectively. B shows that incubation with endonuclease IV does not result in any increase in the number of double-strand breaks compared with the neutral assay conditions while double-strand breaks increased significantly under nondenaturing alkaline conditions.

For determination of whether different types of base damage were being produced, experiments similar to those in which endonuclease IV was used were performed with endonuclease III (4). Endonuclease III is specific for DNA damaged by UV light, osmium tetroxide, acid, or X-rays. The specificity for endonuclease III attack on DNA appears to be a site of a lost base or the 5,6-dihydroxydihydrothymine type (4). The endonuclease III was tested on acid-heat-treated DNA and completely converted Form I DNA into
Form II DNA. As in the case of the reaction of endonuclease IV with bleomycin-treated DNA, reaction of bleomycin-damaged DNA with endonuclease III revealed the loss of Form I DNA to be very similar to that of alkali treatment, but no increase in Form III DNA above that observed under neutral analysis conditions was seen (not shown).

The failure of either endonuclease III or IV to produce an increase in the total mass fraction of Form III DNA may reflect an inability of these endonucleases to bind to and cleave at a site of base loss across from a single-strand break or another lost base. Another possible explanation for these results is that the increase in Form III DNA after alkali treatment was the result of strand separation of widely spaced single-strand breaks on complementary strands. This would be possible since the alkali treatment is near denaturing conditions and widely spaced breaks could result in double-strand break formation.

**Determination of the Source of Alkaline-Induced Double-Strand Breakage.** The observed increase in the frequency of Form III DNA molecules after treatment in non-denaturing alkaline conditions could have arisen from 2 alkaline-labile sites in close proximity on opposite strands, and/or a Form II DNA molecule with an alkaline-labile site in close proximity to a single-strand break on the opposite strand. In order to resolve the question of whether Form III DNA arose from Form I or II of DNA, Forms I and II of DNA which had been treated with bleomycin were purified from gels after electrophoresis. In order to determine that no breakage had occurred in the purification procedure, Forms I and II of DNA that were reacted with all components except bleomycin were also purified. Gel slices that contained the separated DNA forms were incubated at neutral or alkaline conditions and again subjected to electrophoresis (see "Materials and Methods"). Form I DNA that was not reacted with bleomycin was recovered as 100% pure Form II DNA. However, after alkali treatment purified bleomycin-treated Form II DNA was again subjected to electrophoresis, and Form III DNA was observed in the gel pattern. The presence of Form III DNA strongly suggests that some Form II molecules contain an alkaline-labile site across from a single-strand break. Similar results could be obtained if Form II molecules contained 2 alkaline-labile sites in close juxtaposition on complementary strands with a single-strand break in a distant part of the same molecule.

Bleomycin-treated Form I DNA, after alkali treatment and electrophoresis, revealed the presence of all 3 DNA forms. The appearance of Form II DNA molecules would be expected to arise from the alkaline hydrolysis of a depurinated (or depyrimidinated) site (Figs. 3 and 5). The appearance of Form III DNA after alkali treatment of Form I DNA reveals that bleomycin does induce a type of DNA damage which is converted to a double-strand break upon treatment with alkali.

**DISCUSSION**

From this study it is evident that the interaction of bleomycin with isolated DNA causes a wide variety of damage to the DNA structure. This damage includes single- and double-strand breakage, alkaline-labile damage, and various combinations of single-strand breaks and alkaline-labile damage within a small region of complementary DNA. Previously, we proposed a model for the interaction of bleomycin with DNA as revealed through the breakage reactions (13, 14). It was postulated that bleomycin could exist either as a monomer or as a dimer composed of 2 monomers held together by weak hydrogen bands. Evidence in support of this dimer model came from the observation that both the clinical mixture of bleomycins and the purified A<sub>2</sub> derivative promote single- and double-strand breaks. Within the clinical mixture of bleomycin, there may exist at least 1 derivative which has 2 reactive sites within the structure of the molecule. One site could be responsible for the single-strand breakage of the phosphodiester backbone of one of the strands on the 3' side of the phosphate and the other reactive group could remove a base from the opposite DNA strand. It is possible that this derivative may exist as either a monomer or a dimer. Such a dimer has been constructed with the use of CPK space-filling models. The steric properties of this constructed dimer would allow for its binding in the major groove of the DNA and thus could cause both single-strand breaks and alkaline-labile damage (base removal) on each DNA strand. The binding of the monomer or dimer bleomycin molecules would not necessarily mandate or preclude single-strand breakage or base removal by the putative reactive sites on the molecule. This monomer-dimer model would be capable of producing the following combinations of structural damage to isolated DNA: either 1 single-strand break or 1 base removed; or a single-strand break on 1 strand with a base removed on possibly the same or opposite strand; or this dimer could also induce direct double-strand breakage or cause a base to be removed on each strand.

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