Interaction of bleomycin with DNA

(sequence analysis/drug-DNA complex)

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ABSTRACT The sequence of oligonucleotides produced by the action of bleomycin and ferrous ion on double- and single-stranded DNA has been determined. In the presence of ferrous ion, bleomycin promotes cleavage at G-T and G-C sequences, while high concentrations of ferrous ion alone result in strand scission that is not base specific. In the presence of bleomycin and ferrous ion, pyrimidine bases located to the 3′ side of guanosine are released preferentially and a low molecular weight product that forms a chromophore with thiobarbituric acid is produced from the deoxyribose moiety. Oligonucleotides produced by the action of bleomycin differ slightly in electrophoretic mobility from those produced by chemical or enzymatic cleavage. A model is proposed to explain the interactions of bleomycin with DNA.

Bleomycin A2 belongs to a family of metal-binding glycopeptide antibiotics (1) that possess antimicrobial (2), antitumor (2), and antiviral (3) properties. These antibiotics bind to and cleave DNA (4) in a reaction that depends on the presence of ferrous ion (5, 6) and molecular oxygen (5-7). During strand scission, nucleoside bases (8, 9), oligonucleotides of various chain lengths (9), and a low molecular weight compound that reacts with thiobarbituric acid (10) are released.

Previously, we reported that the bithiazole and dimethylsulfonium moieties of bleomycin A2 are directly involved in binding to DNA (11). In this paper, we show that bleomycin preferentially cleaves certain base sequences in DNA and present evidence that the product that reacts with thiobarbituric acid derives from the deoxyribose moiety of bases released during the reaction. On the basis, in part, of this information, we propose a model to explain the interactions of bleomycin with DNA.

MATERIALS AND METHODS

Materials. An amber 3 mutant (N-11) of bacteriophage \(\phi X174\) and Escherichia coli HF 4704 were gifts from J. Doniger. Bacterial plasmids pSM1 and pSM15 are described elsewhere (12). Copper-free bleomycin A2 (lot 71L 489) was obtained from Bristol Laboratories, Syracuse, NY; restriction enzymes \(Hae\) III and \(Hin\) II were purchased from Bethesda Research Laboratories, Rockville, MD; pancreatic DNase, from Sigma; calf thymus DNA and bacterial alkaline phosphatase, from Worthington Biochemical; polynucleotide kinase, from P-L Biochemicals; \([\gamma-32P]\)ATP (specific activity, \(\approx 2000\) Ci/mmol), from ICN; \([8,14C]d\)eoxyadenosine (39.8 mCi/mmol), \([8,14C]d\)eoxyguanosine (55.6 mCi/mmol), uniformly labeled \([14C]c\)ytidine (378 mCi/mmol), and \([methyl-14C]\)thymidine (53.5 mCi/mmol), from New England Nuclear (1 Ci = 3.7 \(\times 10^{10}\) becquerels).

Preparation of Restriction DNA Fragments. Bacteriophage \(\phi X174\) was grown in E. coli HF 4704 for 3 hr at 37°C in the presence of chloramphenicol (13). The bacteriophage was purified and DNA was extracted as described by Godson and Vapnek (14) with the following modifications: alkaline sucrose density gradient centrifugation was omitted; instead, DNA was purified by centrifugation at 34,000 rpm for 3 hr in a Beckman SW 41 rotor on cesium chloride density gradients (specific gravity, 1.578) prepared in the presence of ethidium bromide (350 \(\mu\)g/ml). DNA was isolated by extraction with isopropanol followed by precipitation with ethanol, then digested with \(Hae\) III (15). The double-stranded DNA fragment obtained, designated Z6 in the map reported by Sanger et al. (16), was phosphorylated at the 5′ terminus with \([32P]\)ATP and polynucleotide kinase, then digested with \(Hin\) II in a solution containing 6 mM Tris-HCl at pH 7.5, 6 mM MgCl2, and 20 mM NaCl. A portion of the phosphorylated fragment obtained was treated with alkali to obtain single-stranded DNA (16). Single- and double-stranded fragments were purified further by electrophoresis on 4% polyacrylamide gel. Restriction fragments of pSM1 and pSM15 plasmid DNA were prepared as described by Ohtsubo and Ohtsubo (12).

Fragmentation of DNA with Bleomycin. The standard reaction mixture contained 50 mM Tris-HCl at pH 8.5, 10 mM 2-mercaptoethanol, 5′-32P-labeled restriction fragments, calf thymus DNA at 5 \(\mu\)g/ml, and bleomycin A2 at 25 \(\mu\)g/ml in a total volume of 40 \(\mu\)l. After incubation for various periods of time at 37°C, EDTA was added to a final concentration of 10 mM and the solution was rapidly frozen and lyophilized. Each sample was dissolved in 20 \(\mu\)l of 0.1 M NaOH containing 0.2 mM EDTA, to which was added 20 \(\mu\)l of 10 M urea containing 0.05% bromphenol blue/xylene cyanole. The solution was heated at 90°C for 15 sec, and aliquots (5-10 \(\mu\)l) were transferred to 20% polyacrylamide slab gels for sequence analysis.

Sequence Analysis. The nucleotide sequences of restriction fragments and oligonucleotides produced by the action of bleomycin were determined by the method described by Maxam and Gilbert (16). Autoradiography was carried out by exposing polyacrylamide gels to Kodak SB5 film for 5-40 hr at -20°C. The sequence of oligonucleotides was determined by comparison with chemically degraded restriction fragments as described by Maxam and Gilbert (16).

Preparation of 14C-Labeled HeLa Cell DNA. HeLa S2 cells were grown overnight in Eagle’s minimal essential medium containing (A) 0.1 \(\mu\)Ci of \([methyl-14C]\)thymidine, 2.5 \(\mu\)Ci of \([8,14C]d\)eoxyadenosine, and 2.5 \(\mu\)Ci of \([8,14C]d\)eoxyguanosine or (B) 4 \(\mu\)Ci of uniformly labeled \([14C]c\)ytidine. \([14C]D\)NA was isolated from each culture and the purity was determined as described elsewhere (17). \([14C]D\)NA isolated from culture A contained 22% of the total radioactivity in the thymine moiety, 37% as adenine, and 40% as guanine; \([14C]D\)NA isolated from
catalyst B contained 26% of the total radioactivity as thymine, 22% as cytosine, and 52% as deoxyribose.

RESULTS

Fragmentation of DNA Produced by Bleomycin. The φX174 DNA restriction fragment used in this experiment was 139 nucleotides in length. Oligonucleotides, ranging in length from 2 to 60 bases, were identified as reaction products of double-stranded and single-stranded fragments. Longer oligonucleotides were detected, but, because the point of cleavage was not precisely distinguished, these were excluded from the analysis of sequence specificity. Oligonucleotides produced by the action of bleomycin migrate slightly faster on gels than the comparable markers—this difference is most clearly visible when fragments less than 10 nucleotides long are compared (cf. Fig. 2).

The sequence of the part of the restriction fragment used for sequence analysis is shown in Fig. 1. Bases released by treatment with bleomycin and the relative quantity of each oligonucleotide produced are indicated. Analysis of data for the double-stranded fragment reveals that the base located to the 3' side of every guanine residue was released. In contrast, bases located to the 3' side of cytosine, thymine, and adenine were released 6, 12, and 31 percent of the time, respectively.

Under the experimental conditions used, thymine and cytosine were released with a frequency of approximately 0.5; that is, approximately half the total number of pyrimidines were released. The ratio of the frequency of pyrimidines released to that of purines was 3. Seven of the eight oligonucleotides present in largest quantity (i.e., the more intense bands) are those from which a pyrimidine was removed to form a new 3' terminus. Thus, the preferred recognition site for bleomycin in DNA involves G-T or C-C sequences. Sequences with the reverse polarity, T-G and C-G, are not cleaved.

After separation of strands, the experiments and sequence analysis described above were repeated, using the single-stranded fragment. The rate of DNA cleavage by bleomycin was slower when the single-stranded fragment was used; two

nucleotides cleaved in the single-stranded form were not cleaved in the double-stranded material, and four nucleotides cleaved in the double-stranded form were not cleaved in the single-stranded material. There was a qualitative difference in the intensity of some of the bands produced from the two forms; for example, at positions T51 and C44. However, the overall sequence specificity was essentially the same in both forms in that pyrimidines adjacent to guanine or adenine were preferentially released.

The presence of mercaptoethanol in the standard reaction mixture facilitated cleavage of DNA. When mercaptoethanol was replaced by H2O2 or NaBH4, sequence specificity conferred by bleomycin was unchanged.

Effect of Ferrous Ion. The effect of Fe2+ on the restriction fragment was tested in the presence and absence of bleomycin (Fig. 2). Addition of Fe(NH4)2(SO4)2 in ten times the molar concentration of bases produced no detectable cleavage of restriction fragments under the experimental conditions employed. If the concentration of Fe2+ was increased to 1.5 mM, nonspecific fragmentation occurred and the bands corre-

Fig. 2. Autoradiography of a polyacrylamide gel used for sequence analysis of the effects of ferrous ion on cleavage of DNA. The double-stranded fragment of φX174 was incubated with bleomycin as described in the legend to Fig. 1 with the following modifications: 2-mercaptoethanol was omitted from all reaction mixtures and bleomycin was omitted from 1, 3, and 5. Fe(NH4)2(SO4)2 was added as follows: 1, none; 2, none; 3, 0.15 mM; 4, 0.15 mM; and 5, 1.5 mM. A > G, G > A, T > C, and A > C represent standard markers prepared as described by Maxam and Gilbert (16) in which cleavage predominates at the bases indicated. DNase I represents a digest of the restriction fragment. Electrophoresis was for 4 hr.

Fig. 1. Release of bases from double- and single-stranded restriction fragments of φX174 DNA. The standard reaction mixture, containing 32P-labeled restriction fragments, was incubated for 5 min at 37°C, then treated as described in Materials and Methods. The sequences shown represent the part of the double- and single-stranded restriction fragments used for analysis of base specificity. The bases released and the relative intensity of the bands are indicated by asterisks. T10 is T989 in the map of Sanger et al. (15).
spending to oligonucleotides produced were equal in intensity. The electrophoretic mobilities of these oligonucleotides were identical to those of the chemically degraded markers. When bleomycin and 0.15 mM Fe^{2+} were present, cleavage occurred with the same sequence specificity observed in the absence of added Fe^{2+} but in the presence of mercaptoethanol (Fig. 1). Again, oligonucleotides produced under these conditions did not have the same electrophoretic mobilities as the markers nor did they correspond to oligonucleotides from which the 3' phosphate had been removed by treatment with pancreatic DNase I. These differences are most clearly seen when the time of electrophoresis was extended to 8 hr.

**Base Release.** In separate experiments, HeLa cell DNA, labeled with ^14C in thymine, adenine, and guanine or in cytidine and thymidine, was treated with bleomycin and the reaction mixture was subjected to paper chromatography. Identical amounts of thymine were released in both experiments, and the data are combined in Table 1 to illustrate the overall pattern of base release from DNA. As shown, thymine and cytosine were preferentially released; lesser amounts of adenine and guanine were formed. When expressed as a percentage of total bases present in DNA, the relative order of base release from HeLa cell DNA was similar to that observed in comparable sequencing experiments that analyzed the effects of bleomycin on single-stranded restriction fragments prepared from plasmid and φX174 DNA.

**Fragmentation of Deoxyribose.** HeLa cell DNA, labeled in thymidine and cytidine, was prepared as described in the legend to Table 1 and subjected to column chromatography on Sephadex G-10 (Fig. 3). Calf thymus DNA, treated with bleomycin in an analogous manner, was added as carrier. Fractions were tested for radioactivity and for reactivity with thiobarbituric acid. Ten percent of the total radioactivity present was released as thymine or cytosine; 2% (30–40% of the theoretical amount of malondialdehyde that could be formed) was recovered in fractions containing thiobarbituric acid-reactive material.

**Table 1. Preferential release of bases from DNA**

<table>
<thead>
<tr>
<th>DNA</th>
<th>Base attacked or released, %</th>
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<tbody>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td>Plasmid (single-stranded)</td>
<td>31</td>
</tr>
<tr>
<td>φX174 (double-stranded)</td>
<td>47</td>
</tr>
<tr>
<td>φX174 (single-stranded)</td>
<td>41</td>
</tr>
</tbody>
</table>

* Three single-stranded fragments of *E. coli* plasmid DNA (319–420, 416–319, –61–73, of the Ohtsubo's map (12) were digested in a reaction mixture (17) containing 50 mM Tris-HCl at pH 8.5, calf thymus DNA at 50 μg/ml, 10 mM 2-mercaptoethanol, 10 mM ATP, 10 mM MgCl₂, and bleomycin at 250 μg/ml, and sequence analysis was performed. Data for φX174 DNA were taken from Fig. 1. Bases attacked are presented as a percentage of the total number present.

† HeLa cell DNA, labeled as described in Materials and Methods, was incubated for 3 hr with bleomycin as described above for sequence analysis of plasmid DNA and the bases released were determined by paper chromatography in 1-butanol/NH₄OH/H₂O (86:1:13).

‡ Denatured DNA was prepared by heating HeLa cell DNA at 100 µg/ml in 0.02 M Tris-HCl, pH 8.5, at 100°C for 10 min and then rapidly cooling.

**FIG. 3.** Column chromatography of products obtained from digestion of HeLa cell DNA with bleomycin. HeLa cell DNA (0.15 mg), labeled in thymidine and cytidine as described in Materials and Methods, and calf thymus DNA (1 mg) were digested separately under the conditions described in Table 1. The reaction mixtures were combined and subjected to gel filtration on a Sephadex G-10 column equilibrated with 0.1% ammonium carbonate, pH 8.5. The radioactivity in each fraction (1 ml) was measured and the A₅₂₀ was determined after heating for 20 min at 100°C with 0.4% thiobarbituric acid at pH 2. The arrows indicate the peak absorbances of standard markers. O, cpm; †, A₅₂₀.

**DISCUSSION**

The research described here focuses on the binding sites of a complex that involves bleomycin, DNA, O₂, and ferrous ion. The sequencing technique developed by Maxam and Gilbert (16) was fundamental in providing information required to analyze this complex at the molecular level.

Certain assumptions have been made in determining the sequence and end groups of oligonucleotides produced by the action of bleomycin on restriction fragments of DNA. First, radioactive bands detected by autoradiography are assumed to represent integral segments of the fragment, with strand scission and base release having occurred at a specific site to form a new 3' terminus. Furthermore, the affinity of bleomycin for any given base sequence is considered to be uniform throughout the fragment. If these assumptions are correct, pyrimidine bases are released preferentially by the action of bleomycin, thymine being slightly favored over cytosine. Pyrimidines released were almost always located to the 3' side of a purine base which, in most instances, was guanine. Thus, the base sequence most frequently recognized by bleomycin, when it binds to and cleaves DNA, is G-T or G-C; no such specificity is observed when guanine is located on the 3' side of the pyrimidine released. The preferential release of bases deduced by sequence analysis is supported by quantitative measurements of base release from labeled HeLa cell DNA.

A further assumption relates to the small difference in electrophoretic mobility observed between oligonucleotides produced by chemical degradation of restriction fragments (standard markers) and those produced by reaction with bleomycin. We believe that this difference represents an alteration at the 3' phosphate terminus; however, it does not simply reflect hydrolysis of the 3' phosphate ester bond because such a compound (produced by treating the restriction fragment with pancreatic DNase) migrates significantly slower than
The observations above allow us to formulate a model that explains the preferential interactions of bleomycin with DNA (Fig. 4). The model is consistent with most reported observations pertaining to the mechanism of action of this antibiotic. The principal features of the model are intercalation of the bithiazole rings of bleomycin between base pairs in which one chain contains a G-T or G-C sequence. Binding to DNA could be stabilized through electrostatic interactions between the positively charged disulfonium ion and the negatively charged group on the opposite strand. The latter interaction may not be essential for complex formation in view of the apparent biological activity of bleomycin B2, in which the side chain is electronically neutral; however, analogs with negatively charged groups (bleomycinic acid) are inactive (1). We have found that bleomycin B2 acts on DNA with the same specificity as bleomycin A2. Although other bleomycins have not been tested, this result suggests that the terminal amine moiety of bleomycin is not a critical determinant of base specificity and implies that the bithiazole and/or other nearby groups are primarily responsible for recognizing the preferred G-C and G-T sequences.

In Fig. 4, ferrous ion is liganded to some or all of the nitrogen atoms in bleomycin which have been shown in NMR (26) and crystallographic (27) studies to bind copper and other metals. If the same groups were liganded to iron, one coordination position could bind an oxygen atom, O2, H2O, or the carbamoyl moiety of the sugar moiety of bleomycin. Molecular model building studies show that such an oxygen molecule would be positioned near the deoxyribose of the adjacent pyrimidine and, in analogy with metal-catalyzed oxygenations, could facilitate selective cleavage, even by highly reactive OH radicals. Attack by OH radicals at the 4' position of a deoxyribose in DNA might be expected to produce malondialdehyde as a reaction product, release free bases, and leave a 3' terminus containing a carbon fragment esterified to phosphate (28).

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