BLEOMYCIN-INDUCED BREAKAGE OF CLOSED-CIRCULAR DNA

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SUMMARY: The amount of breakage induced by bleomycin in closed-circular DNA from bacteriophage PM2 was investigated under various reaction conditions. Analysis of the extent of breakage, by alkaline band centrifugation and agarose gel electrophoresis, yielded quite different results from those previously reported for the breakage of closed-circular SV40 DNA by bleomycin. Compared to these earlier results, we found (a) the reaction required the addition of 2-mercaptoethanol; and (b) the breakage reaction was further stimulated by the addition of 2-mercaptoethanol in the absence of EDTA.

The antitumor effectiveness of bleomycin, a glycopeptide antibiotic discovered by Umezawa et al. (1), has generally been considered to be related to its ability to induce intracellular breakage of DNA (2-4). It also causes fragmentation of isolated DNA in vitro (2, 5).

The methods previously used to analyze the DNA-breakage reaction (5) are not readily usable for more detailed studies of the kinetics of the reaction, since several breaks in each DNA molecule are required in order to produce measurable changes in the sedimentation profile. A much more sensitive and accurate method for the detection of bleomycin-induced breakage has been described by Umezawa et al. (6). This procedure, using closed-circular SV40 DNA, relies on the difference in sedimentation behavior between a DNA molecule (Form I), in which both strands are continuous and therefore do not separate after alkaline denaturation, and a molecule (Form II) with one or more single-strand breaks in which the strands do separate, and sediment much more slowly, under alkaline conditions (7). Since SV40 DNA has a molecular weight of 3.6×10^6 daltons (8), or about 6000 base pairs, this method can

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detect the breakage of a single phosphodiester bond out of 12,000, a great increase in sensitivity over methods using linear DNA.

We have developed a method based on the use of circular DNA from the bacteriophage PM2 (9). This DNA can be obtained in considerably larger quantities than animal virus DNAs, such as SV40. In addition, since PM2 DNA has a molecular weight of 6 x 10^6 daltons (10), a further increased sensitivity in the detection of bleomycin-induced breakage should be possible.

MATERIALS AND METHODS

Chemicals: The bleomycin used in these studies was a generous gift from Bristol Laboratories (Syracuse, New York). Optical grade cesium chloride was manufactured by Metallgesellschaft AG (Frankfurt, Germany), ethidium bromide was purchased from Calbiochem (San Diego, California); and Tris (base), "Ultra pure", from Schwarz/Mann (Orangeburg, New York). All other chemicals were from general suppliers. The PM2 DNA used was a generous gift from Drs. J. E. Strong and R. R. Hewitt, and was prepared by their modification (11) of the procedures of Salditt et al. (12).

Analysis of Bleomycin-Induced DNA Breakage by Alkaline Band Centrifugation: Bleomycin was allowed to react with the purified closed-circular DNA, adjusted to a concentration of 0.2 mg/ml, under a variety of experimental conditions, as shown in "Results". The reactions were terminated at the desired times by the addition of 1/4 volume of 1.5 M NaOH-3.5 M NaCl. The percentage of DNA which remained as unbroken, closed circles was then determined by band-sedimentation velocity (13), under alkaline conditions, in the Beckman Model E analytical ultracentrifuge. The DNA solution (10-15 µl) was placed in the sample well of a Type II band-forming centerpiece (14); the bulk solution was 2.83 M CsCl-0.3 M NaOH (ρ=1.35 g/ml). Sedimentation of the DNA bands, at 25°C and 23,150 rpm (or 31,410 rpm in some experiments), was monitored by photoelectric scanning of each cell. The amount of DNA in each band was determined by measuring the area of the peaks with a Dietzgen compensating polar planimeter.

Analysis of Bleomycin-Induced DNA Breakage by Agarose Gel Electrophoresis: For all agarose gel electrophoresis experiments, the reaction mixtures contained 1.25 µg of PM2 DNA, 25 mM 2-mercaptoethanol and bleomycin as indicated in the legend to Figure 3 in a total volume of 27 µl of 0.02 M Tris, pH 8.0. The reactions were incubated at 40°C for various times, and were stopped by the addition of 30 µl of 20 mM EDTA containing 10% (w/v) sucrose and 0.025% (w/v) bromphenol blue, pH 7.6. The samples were layered onto 0.9% agarose tube gels; electrophoresis was performed at 2 m amperes per gel for 7.5 hr. After electrophoresis, the gels were stained with a solution containing 0.5 µg/ml of ethidium bromide. Quantitative measurements of the amount of DNA which 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 Aminco-Spectrophotofluorometer (American Instruments Co., Silver Spring, Md.); the incident wavelength was 510 nm and the emission wavelength was 590 nm. The relative fluorescence intensity was plotted using a strip chart recorder, and the areas under the DNA peaks were measured with the planimeter. Using this method, Form I DNA migrated the fastest followed by Form III (double-stranded linear DNA) and Form II; all peaks were completely resolved.

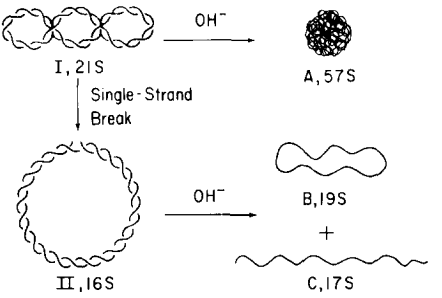

Figure 1: Diagrammatic representation of the different forms of PM2 DNA studied in this work, and the reactions which convert one form to another. Each form is identified by a Roman numeral (for double-stranded DNAs) or a letter (for alkaline-denatured forms) and its sedimentation coefficient, as determined by neutral or alkaline band centrifugation under the conditions described in the text. These S° (experimental) values have not been corrected to standard ($S_{20,w}$) conditions.

RESULTS

Band-Centrifugation Analysis of DNA Breakage: Figure 1, modified from diagrams of Vinograd et al. (7) and Tooze (8) to fit our experimental system, shows schematic diagrams of the different forms of PM2 DNA in these experiments. It also shows, beneath each diagram, the sedimentation coefficient (in Svedberg units) for that form, under the conditions of sedimentation used here; these coefficients have not been converted to standard ($S_{20,w}$) conditions. When the native superhelical DNA molecule (Form I, shown at the upper left in Fig. 1) is denatured in alkali, the strands cannot separate, and the resulting cyclic coil ("A" in Fig. 1) is a very compact, rapidly-sedimenting structure. If a single phosphodiester bond is broken, in either strand of the native DNA molecule, the superhelical turns are lost, and it is converted to the relaxed, open-circular Form II molecule. When this molecule is denatured, the strands can separate, producing one single-stranded, circular molecule ("B" in Fig. 1) and one single-stranded unit-length linear molecule ("C"), both of which sediment much more slowly than the cyclic coil (A).

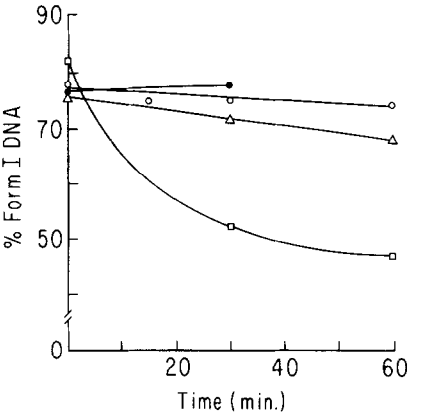

Figure 2: Percent of Form I DNA remaining in the presence and absence of 2-mercaptoethanol and EDTA. All reaction mixtures contained 0.2 mg/ml of PM2 DNA: ● , 2.5 mg/ml of bleomycin, 10 mM EDTA in 0.1 M Na borate, pH 9.1, incubated at 20°C; O , 100 µg/ml of bleomycin in 50 mM Tris-HCl, pH 8.0, incubated at 37°C; ▲ , 1.0 mg/ml of bleomycin in 50 mM Tris-HCl, pH 8.0, incubated at 37°C; □ , 50 µg/ml of bleomycin, 10 mM EDTA, 25 mM 2-mercaptoethanol in 50 mM Tris-HCl, pH 8.0, incubated at 37°C.

The results of a series of experiments in which the buffer conditions, bleomycin concentrations, and times of the reaction mixtures were varied over fairly wide ranges (in the absence of reducing agents) showed little or no fragmentation of DNA; these reactions all contained 10 mM EDTA. Initially, we tried conditions similar to the standard conditions used by Umezawa *et al.* (6). When no DNA breakage was observed in these experiments, we changed to the reported pH optimum of the reaction mixture, 9.1 (6). No reaction was observed at this pH after 30 min., even at very high concentrations (2.5 mg/ml) of bleomycin (Figure 2, closed circles). However, when 2-mercaptoethanol was added (open squares), considerable DNA breakage was observed at significantly lower bleomycin concentrations. A series of experiments in the absence of EDTA was run under increasingly severe conditions of incubation. The EDTA had previously been included in experiments of this type to inhibit any nucleases which might be present. Two such experiments are presented in Figure 2 (open circles, open triangle). It can be

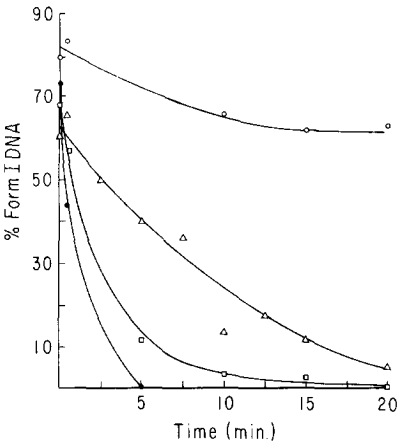

Figure 3: Percent of Form I DNA remaining as a function of increasing bleomycin concentration in the presence of 2-mercaptoethanol. All reaction mixtures contained 46 μg/ml of PM2 DNA, 25 mM 2-mercaptoethanol in 0.2 M Tris-HCl, pH 8.0, and were incubated at 40°C: ○, 0.2 μg/ml of bleomycin; △, 0.5 μg/ml, □, 1.0 μg/ml; ●, 5.0 μg/ml.

seen that there is still very little DNA breakage produced by concentrations of bleomycin up to 1 mg/ml under non-reducing conditions.

Agarose Gel Electrophoresis Analysis of DNA Breakage: In all subsequent experiments, 2-mercaptoethanol has been included and EDTA has been deleted from the reaction mixtures. The reducing agent is required for the reaction, and we have observed (unpublished results) that EDTA is capable of inactivating bleomycin. To avoid the limited sample capacity and the time consuming aspects of the analytical ultracentrifugation analyses, we have adopted the agarose gel electrophoresis system for breakage analyses. This system has the added advantages of greater sensitivity and better resolution of the three forms of DNA.

It can be seen from Figure 3 (open circles) that 0.2 μg/ml of bleomycin has about the same capacity to fragment Form I DNA as was observed using 50 μg/ml of bleomycin in the presence of 10 mM EDTA (Figure 2, open squares). Further, as the concentration of bleomycin is increased (Figure 3), the complete disappearance of Form I DNA occurs at an increasing rate as the

bleomycin concentration is increased. Preliminary results indicate that the first order rate constants for the loss of Form I DNA are directly proportional to the bleomycin concentration. Control experiments (not shown) (a) in the absence of 2-mercaptoethanol and (b) in the absence of bleomycin show no decrease in the amount of Form I DNA. The results are qualitatively similar when the analyses are performed under alkaline conditions.

DISCUSSION

The DNA used in our experiments, from bacteriophage PM2, has both a larger molecular size and a higher superhelix density than does SV40 DNA (15), in addition to the obvious and expected differences in nucleotide sequences. The higher molecular weight would be expected to make the PM2 DNA more susceptible to breakage by bleomycin, rather than less. Preliminary experiments on the reaction of bleomycin with nonsuperhelical PM2 DNA molecules have not revealed any differences in susceptibility as a result of changes in the superhelix density. This nonsuperhelical, closed-circular DNA was prepared by nicking native PM2 DNA molecules with DNase I, which allows the superhelical turns to unwind (Form II, Fig. 1), and resealing the nicks with DNA ligase (16). Finally, although it is impossible to rule out differences in sensitivity as a result of different base sequences, no such specificity has been detected in previous studies on the reaction of bleomycin with different DNAs (17, 18). These considerations make it seem unlikely that properties of the purified DNAs themselves would be responsible for the differences observed. Another factor which might be important is the extent of purification of the DNAs. It is possible that the SV40 DNA contained sufficient reducing agents to allow the reaction to proceed. In any case, the procedure which we have described will allow for a detailed kinetic analysis of the reaction.

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