DECREASE OF MELTING TEMPERATURE AND SINGLE STRAND SCISSION OF DNA BY BLEOMYCIN IN THE PRESENCE OF 2-MERCAPTOETHANOL

KAZUO NAGAI*, HIDEO SUZUKI, NOBUO TANAKA and HAMAO UMEZAWA

Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo, Japan

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Decrease of melting temperature (Tm) of *Escherichia coli* 15T⁻ DNA, with little breakage of the DNA strand, was observed in the presence of bleomycin A_2 and 2-mercaptoethanol. DNA digested more drastically with pancreatic deoxyribonuclease (E. C. 3.1.4.5) revealed no shift of Tm. When the DNA, treated with bleomycin and 2-mercaptoethanol, was dialyzed after heat denaturation, marked strand scission occurred. Tm of poly (dG)·poly (dC) decreased by 9°C with a change of transition width at 40 μg/ml of bleomycin, although that of poly d(AT)·poly d(TA) showed no change. Single strand scission of synthetic deoxyribopolymers was observed to occur slightly in the presence of bleomycin and markedly when dialyzed after the incubation. Strand scission without dialysis was produced more significantly on poly (dG)·poly (dC) than poly d(AT)·poly d(TA) in the presence of bleomycin.

The basic glycopeptide antibiotic bleomycin exhibits antibacterial effects and also inhibits EHRLICH carcinoma.[1] Therapeutic activity of this antibiotic against human squamous cell carcinoma was shown by ICHIKAWA *et al.*[2]

As to the action of bleomycin, the followings have been reported *in vivo*: marked inhibition of DNA synthesis with lesser inhibition of protein synthesis[3], single strand scission in DNA of *E. coli* and HeLa cells[4]. *In vitro*, a decrease of Tm of DNA[5] and single strand scission of DNA[4] in the presence of a sulfhydryl compound was demonstrated. It was also suggested that a reaction must occur between DNA and 2-mercaptoethanol and finally the antibiotic[5].

The effects of bleomycin on the synthetic deoxyribopolymers poly (dG)·poly (dC) and poly d(AT)·poly d(TA) are described in this paper, together with a comparsion of the effects of bleomycin and deoxyribonuclease on DNA.

Materials and Methods

Materials: Bleomycin A_2 (lot CM-3 or F-4, copper free) was prepared by Nihon Kayaku Co., Tokyo and supplied by Dr. TAKITA, Institute of Microbial Chemistry, Tokyo and phleomycin (lot A-9331-648) was kindly given by Bristol Laboratories, Syracuse, New York. Pancreatic deoxyribonuclease (E. C. 3.1.4.5) was a commercial product of Calbiochem, Los Angeles, California. [2-^{14}C] thymine (24 mc/mM) was purchased from Daiichi Pure Chemicals Co., Tokyo.

* Laboratory of Microbiology, Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo.

Preparation of [^{14}C]thymine-labeled DNA: *E. coli* 15T$^-$ was cultivated for five generations in Tris-minimal medium[6] supplemented with 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 % casamino acids, 0.2 % glucose and 1 mg/liter of thymine. [^{14}C]thymine was added to the medium to a final concentration of 25 μc/liter at the time of inoculation with the bacteria. DNA was prepared according to the method of MARMUR[7].

Preparation of synthetic deoxyribopolymers: The deoxyribopolymers were synthesized as described by SCHACHMAN *et al.*[8] for poly d(AT)·poly d(TA) and RADDING, JOSSE and KORNEERG[9] for poly (dG)·poly (dC) using the fraction VII of DNA polymerase (E. C. 2.7.7.7) purified following the procedure of RICHARDSON *et al.*[10] After incubation, the reaction mixture was deproteinized with chloroform-isoamyl alcohol (24:1, by vol.) until no further precipitate appeared. The polymers precipitated with cold ethanol were dissolved in 15 mM sodium chloride—1.5 mM sodium citrate. The solution was dialyzed against 500 vol. of 0.2 M sodium chloride—15 mM sodium citrate, and then twice against 1,000 vol. of water.

Determination of Tm: The Tm of DNA and deoxyribopolymers was determined as described in the previous report[5]. Absorbances at 260 mμ of the reaction mixtures before heat denaturation were 0.40 for DNA and 0.20 for deoxyribopolymers in the absence of bleomycin.

Sucrose density gradient centrifugation analysis: The incubation mixture was layered on the top of 4.8 ml of an alkaline (pH 12.5, 0.02 M potassium phosphate) or neutral (pH 7.5, 0.02 M potassium phosphate) sucrose density gradient solution (5~20 %). Centrifugation was carried out in a SW 50 L rotor of a BECKMAN model L2-65B centrifuge at 50,000 r.p.m. for 150 miniutes for DNA and poly (dG)·poly (dC) or 180 minutes for poly d(AT)·poly d(TA) at 20°C. The DNA was precipitated by the addition of cold 5 % trichloracetic acid using 0.5 mg of bovine serum albumin as a carrier, and counted with an Aloka windowless gas-flow counter. After addition of 1 ml of water to each fraction absorbance at 260 mμ was measured (Shimazu spectrophotometer QV-50).

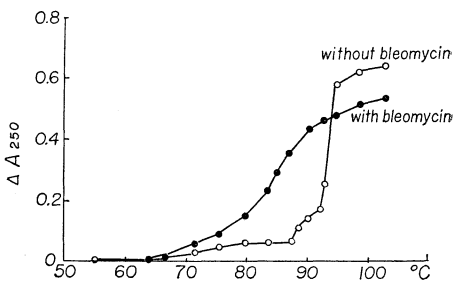
Results

Effect of Bleomycin A_2 on Tm of poly (dG)·poly (dC)

The Tm of poly (dG)·poly (dC) was 93°C in the reaction medium used, and was decreased by bleomycin to 84°C with a change of transition width as shown in Fig. 1. These results are similar to those observed with salmon sperm DNA[5]. In the case of poly d(AT)·poly d(TA), the Tm did not show any shift with bleomycin whereas it increased by 15°C in the presence of 40 μg/ml of phleomycin as reported by FALASCHI and KORNBERG[11].

Fig. 1. Effect of bleomycin on thermal denaturation of poly(dG)·poly(dC).

The reaction mixture contained poly (dG)·poly (dC) at a final concentration of 0.20 absorbance at 260 mμ in 50 mM Tris-HCl (pH 7.6) and 1 mM 2-mercaptoethanol with or without 40 μg/ml of bleomycin. Temperature was raised after 120 minutes of incubation at 37°C.



Effect of Bleomycin A_2 on Molecular Size of DNA

As reported in a previous paper[5], the Tm of *E. coli* B DNA decreased by 12°C in the presence of 40 μg/ml bleomycin and 1 mM 2-mercaptoethanol. A similar Tm decrease was also observed in the case of *E. coli* 15T$^-$ DNA. The change of molecular size was investigated by sucrose density gradient

Fig. 2. Sedimentation profile of DNA after heat denaturation.

20 μg/ml of *E. coli* 15 T⁻ DNA was incubated in 50 mM Tris-HCl (pH 7.6) and 1 mM 2-mercaptoethanol in the presence or absence of 40 μg/ml of bleomycin for 120 minutes at 37℃. The reaction mixture was layered after heat denaturation on a 5 to 20% linear neutral (pH 7.5, 0.02 M potassium phosphate) sucrose density-gradient. Centrifugation was carried out at 50,000 r.p.m. for 150 minutes at 20℃. Fraction 1 represents the bottom fraction of the centrifuge tube.

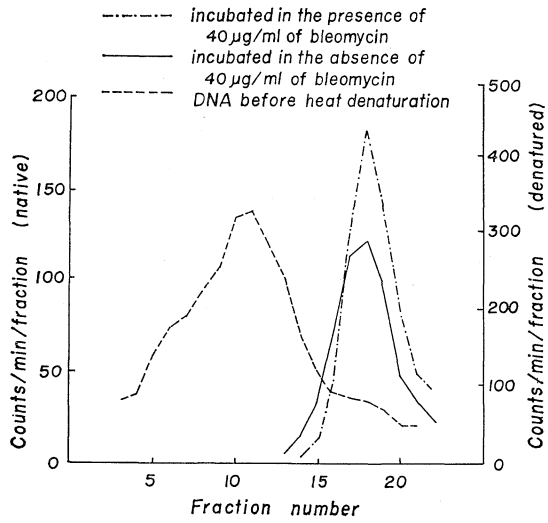


Fig. 3. Effect of dialyzing on heat denatured DNA.

Samples were treated similar to those in Fig. 2 except that they were dialyzd against the reaction medium for 120 minutes at 4℃ before the sedimentation analysis.

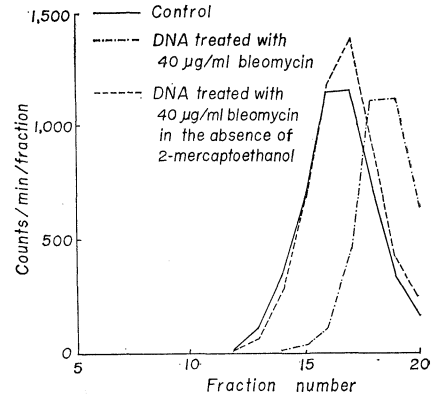
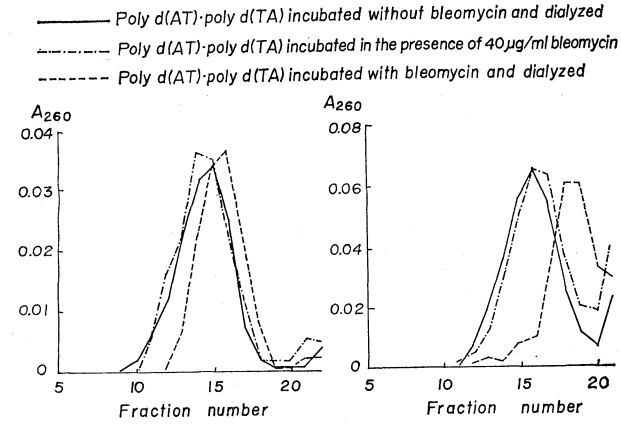


Fig. 4. Sedimentation analysis of poly d(AT)·poly d(TA).

Poly d(AT)·poly d(TA) was incubated at 37℃ for 120 minutes in 50 mM Tris-HCl (pH 7.6) and 1 mM 2-mercaptoethanol with or without bleomycin. The final concentration of poly d(AT)·poly d(TA) was 2.0 absorbance at 260 mμ. 0.1 ml of the reaction mixture, before or after dialysis against 10 vol. of the reaction medium, was layered on a 5 to 20% linear neutral (a) or alkaline (b) sucrose density-gradient. Centrifugtion was carried out at 50,000 r.p.m. for 180 minutes at 20℃.



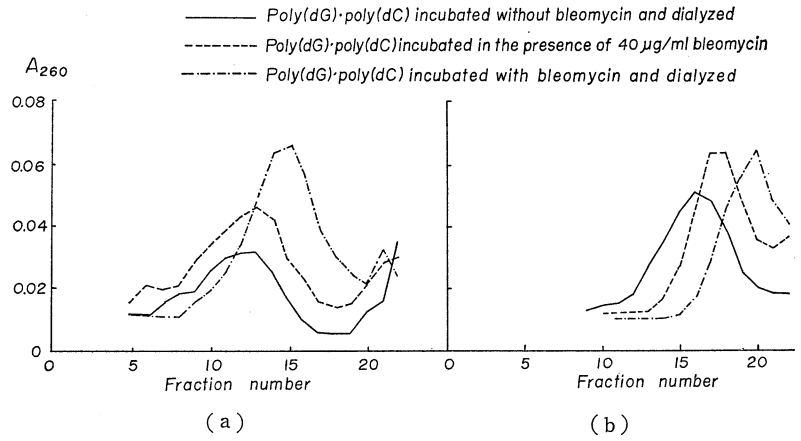
centrifugation after the determination of Tm. In the absence of 2-mercaptoethanol, there was no difference in the molecular size between the control and bleomycin-treated DNA. However, as shown in Fig. 2, using the conditions producing a Tm decrease, that is with 2-mercaptoethanol, the amount of rapidly-sedimenting DNA decreased only slightly, indicating little breakage of DNA caused by bleomycin.

In order to determine whether the small breakage of DNA caused the Tm decrease, the Tm of *E. coli* B DNA was determined after a more drastic digestion using pancreatic deoxyribonuclease, which was reported by STUDIER[12] to produce single strand scission on native DNA. There was no change of the Tm before or after the digestion.

When the DNA was dialyzed against the reaction medium after heat denaturation, as shown in Fig. 3, DNA which had been treated with bleomycin in the presence of 2-mercaptoethanol revealed a peak of a smaller molecular size, indicating

Fig. 5. Sedimentation analysis of poly(dG)·poly(dC).

Poly (dG)·poly (dC) was incubated at 37℃ for 120 minutes in 50 mM Tris-HCl (pH 7.6) and 1 mM 2-mercaptoethanol with or without bleomycin. The final concentration of poly (dG)·poly (dC) was 1.0 absorbance at 260 mμ. 0.2 ml of the reaction mixture, before or after dialysis against 10 vol. of the reaction medium, was layered on a 5 to 20% linear neutral (a) or alkaline (b) sucrose density-gradient. Centrifugation was carried out at 50,000 r.p.m. for 150 minutes at 20℃.



scission of DNA strands.

Single Strand Scission of Deoxyribopolymers

Although bleomycin does not cause a decrease of the Tm of poly d(AT)·poly d(TA), strand scission was shown by sucrose density gradient analysis. Poly d(AT)·poly d(TA) was incubated with bleomycin and 2-mercaptoethanol both with and without subsequent dialysis and subjected to neutral or alkaline sucrose density gradient centrifugation. After dialysis of the bleomycin-treated polymer, a shift of the peak to a smaller molecular size than the control was clearly shown in the case of the alkaline sucrose density gradient analysis (Fig. 4). The results indicate that bleomycin reacts also with poly d(AT)·poly d(TA) causing single strand scission.

In the case of poly (dG)·poly (dC), single strand scission was also observed to occur markedly after dialysis of the reaction mixture. Moreover, it was shown that more significant strand scission of poly (dG)·poly (dC) had been produced by bleomycin without dialysis in comparison with poly d(AT)·poly d(TA).

Discussion

Decrease of the Tm of DNA seemed to be due to decrease of hydrogen bonds supporting the double stranded structure. It has been reported that when DNA is digested with deoxyribonuclease K1 or K2, the Tm of the resulting small molecular products decreases[13]. As described above, even though scission of strands was induced by pancreatic deoxyribonuclease, the Tm did not decrease under the conditions employed. However, in the case of bleomycin treatment, scission of DNA and decrease of the Tm were always observed. Decrease of the Tm caused by bleomycin, therefore, cannot be ascribed solely to scission of DNA strands. It is concluded from the present results that the bleomycin molecule probably is inserted between the double strands, separating each strand. If this is the case, this kind of effect is thought to be dependent not only on the structure of the bleomycin molecule but also on the structure of the DNA. As described above, the Tm of poly (dG)·poly (dC) decreases with bleomycin but that of poly d(AT)·poly d(TA) does not change. This suggests that bleomycin is inserted between

dG and dC but not between dA and dT. The single strand scission of poly (dG)·poly (dC) without dialysis was more marked than that of poly d(AT)·poly d(TA). From this observation, poly d(AT)·poly d(TA) seems to be more stable to the action of bleomycin than poly (dG)·poly (dC). This point is interesting in relation to the sensitivities of synthetic deoxyribopolymers to nucleases. Poly (dG)·poly (dC) has been reported[9] to be much less sensitive than poly d(AT)·poly d(TA) or DNA to three different nucleases from *E. coli* including DNA endonuclease, DNA diesterase and the exonuclease that persists in the purified DNA polymerase fractions.

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