INHIBITION OF LIGASE REACTION BY BLEOMYCIN

MICHIKO MIYAKI and TETSUO ONO

Department of Biochemistry, Cancer Institute,
Japanese Foundation for Cancer Research,
1-37-1, Kami-Ikebukuro, Toshima-ku, Tokyo, Japan

HAMAO UMEZAWA

Institute of Microbial Chemistry,
Shinagawa-ku, Tokyo, Japan

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ATP-dependent DNA ligase prepared from ascites hepatoma of rat was markedly inhibited by bleomycin at the low concentration. The inhibition was observed in the absence of a thiol compound. However, the degree of inhibition was increased by addition of mercaptoethanol to the reaction mixture. The principal mechanism of inhibition was found to involve the strand scission of DNA which was not repaired by the ligase. The inhibition at rather low concentration of bleomycin suggested that this antibiotic might affect the joining of DNA by binding to DNA strand or by interaction with the enzyme.

DNA-ligase[1~4] appears to take part in DNA replication, recombination, and repair, and its activity rises in virus-infected cells[5] and regenerating rat liver[6]. We found that this enzyme was inhibited by bleomycin at a concentration much lower than other inhibitors such as acridine orange, ethidium bromide, actinomycin D, and sarkomycin. Such a fact suggests that bleomycin differs in the mechanism of inhibition from other chemicals. This paper describes the mode of inhibition of DNA ligase by bleomycin A_2 (copper free) *in vitro*.

Materials and Methods

Bleomycin A_2 (copper free) was the product of Nippon Kayaku Co. *E. coli* C_{600}, *E. coli* C_{600} (thymine$^-$), and phage λ_{CI} were obtained from Dr. J. TOMIZAWA. Pancreatic DNase (Worthington), ^{3}H-thymidine and acridine orange (Daiichi Pure Chemicals), actinomycin D (Merck), cycloheximide (Upjohn), ethidium bromide (Boots Pure Drug), and hydroxyapatite (Sigma) were commercial products.

The ligase was partially purified as follows: Rat ascites hepatoma, AH-130, cells were disrupted by sonication at 20 kC, 250 W for 1~2 minutes in a solution containing 0.35 M sucrose, 35 mM $KHCO_3$, 4 mM $MgCl_2$ and 10 mM mercaptoethanol, and the sonicate was centrifuged at $100,000 \times g$ for 2 hours. To the supernatant was added ethylene glycol to 30 % concentration, and the solution was dialyzed against 0.02 M potassium phosphate - 1 mM EDTA - 10 mM mercaptoethanol - 30 % ethylene glycol (pH 6.5), and applied to a phosphocellulose column. The ligase was eluted with a linear gradient of potassium phosphate buffer (pH 6.5) from 0.02 M to 0.5 M containing 1 mM EDTA, 10 mM mercaptoethanol, and 30 % ethyleneglycol. The ATP-dependent DNA ligase fraction eluted at 0.2 M~0.25 M

phosphate was dialyzed for 3 hours against three changes of 0.02 M potassium phosphate - 1 mM EDTA - 30 % ethylene glycol (pH 6.5). The partially purified enzyme was free from endonuclease as judged by alkaline sucrose gradient centrifugation analysis of DNA incubated with this fraction.

^{3}H-λ-DNA was prepared as follows: *E. coli* C_{600} (thymine$^-$) was infected with phage λcI and shaken in λ-broth (containing 10 mg/ml of Casamino acid (Difco), 2.5 mg/ml of NaCl, and 0.5 μg/ml of thyamine, pH 7.0) supplemented with ^{3}H-thymidine (10 μg/ml) at 37°C for 2.5 hours. The phage collected by centrifugation was purified by banding in CsCl (R. I. = 1.3820) centrifugation at 70,000 × *g* for 19 hours. ^{3}H-λ-DNA was extracted by phenol saturated with 10 mM Tris-HCl - 1 mM EDTA (pH 8.0) and the aqueous phase was dialysed against 10 mM Tris-HCl - 1 mM EDTA (pH 8.0). Unlabeled DNA was prepared in the same manner as the labeled one using *E. coli* C_{600}. Unlabeled DNA (100 μg/ml) was cross-linked by incubation with 10 μM N-methyl-bis(2-chloroethyl)amine-HCl at 25°C in 25 mM Tris-HCl (pH 7.2). A mixture of cross-linked λ-DNA (70 μg/ml) and ^{3}H-λ-DNA (7.5 μg/ml) was incubated at 50°C for 30 minutes in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 0.5 M NaCl, and cooled to 0°C (hydrogen-bonded mixed dimer).

Ligase activity was assayed according to the method described by ZIMMERMAN *et al.*[7] Hydrogen-bonded mixed dimer (7.7 μg/ml) was incubated at 30°C with the ligase fraction (50 μg protein/ml) in a buffer (pH 8.0) containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM mercaptoethanol and 0.5 mM ATP. Each 0.2 ml of the reaction mixture was mixed with 0.25 ml of a solution containing 0.1 M Na_3PO_4 (pH 12.6), 5 mM EDTA and 50 μg calf thymus DNA, and incubated at 60°C for 10 minutes, then neutralized by the addition of 0.10 M sodium phosphate buffer (pH 6.8) (2 ml) containing 3 % formaldehyde. To this solution was added hydroxyapatite (16 mg) which was then collected on a glass filter, washed by 0.1 M sodium phosphate - 3 % formaldehyde (pH 6.8), dried, and the radioactivity adsorbed by hydroxyapatite was measured in toluene scintillator.

The sizes of damaged and repaired DNAs were analyzed by alkaline sucrose gradient centrifugation. ^{3}H-λ-DNA (100 μg/ml) was digested at 20°C with pancreatic DNase (0.05 unit/ml) in a solution containing 10 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM EDTA (pH 8.0). The DNase-I-nicked DNA (0.3 μg/ml) thus prepared was incubated at 30°C for 60 minutes with ligase fraction (150 μg protein/ml) in 0.3 ml of 50 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$, 10 mM mercaptoethanol, and 0.5 mM ATP. To the reaction mixture were added 0.5 M EDTA (pH 8.0) (30 μl) and 1 N NaOH (30 μl), and the solution was layered on 5~20 % alkaline sucrose gradient containing 0.1 N NaOH, 0.9 M NaCl and 1 mM EDTA. The tube was centrifuged at 25,000 rpm for 16 hours at 20°C in Spinco SW-25.3 rotor. The radioactivity of each 1 ml fraction was measured in toluene-triton scintillator.

The size of DNA treated with bleomycin was determined by sedimentation analysis. A mixture of ^{3}H-λ-DNA (0.7 μg/ml) and cross-linked λ-DNA (7 μg/ml) was incubated with bleomycin at 30°C for 60 minutes in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 0.5 mM ATP in the presence or absence of 10 mM mercaptoethanol. To the reaction mixture were added 0.5 M EDTA (pH 8.0) and 1 N NaOH and the solution was layered on 5~20 % alkaline sucrose gradient which was then centrifuged at 20,000 rpm in Spinco SW-25.3 rotor for 17 hours at 20°C.

Results and Discussion

The effect of chemicals on ATP-dependent DNA ligase obtained from AH-130 is shown in Table 1. The enzyme reaction was inhibited by ethidium bromide and acridine orange which have been described to intercalate to DNA molecule[8]. Actinomycin D is known to bind to guanine residue in DNA[9] and inhibition of the ligase was observed at a rather high concentration (3~70 μg/ml) of this antibiotic at which

concentration DNA polymerase was also inhibited. Bleomycin inhibited the ligase even at a concentration as low as 0.01~1 μg/ml. Sarkomycin reduced the ligase activity at 1 mM, while cycloheximide and 4-hydroxyaminoquinoline 1-oxide exhibited only slight effect at this concentration.

Table 1. Effect of chemicals on ligase reaction

Chemicals	Concentration	Ligase activity (%)
Nil		100
Acridine orange	0.1 mM	36
	1.0 〃	0
Ethidium bromide	0.1 〃	24
Cycloheximide	1.0 〃	77
4-Hydroxyaminoquinoline 1-oxide	0.1 〃	81
	1.0 〃	78
Sarkomycin	1.0 〃	53
Actinomycin D	3.0 μg/ml	60
	30.0 〃	27
	70.0 〃	0
Bleomycin	0.01 〃	74
	0.1 〃	55
	1.0 〃	0

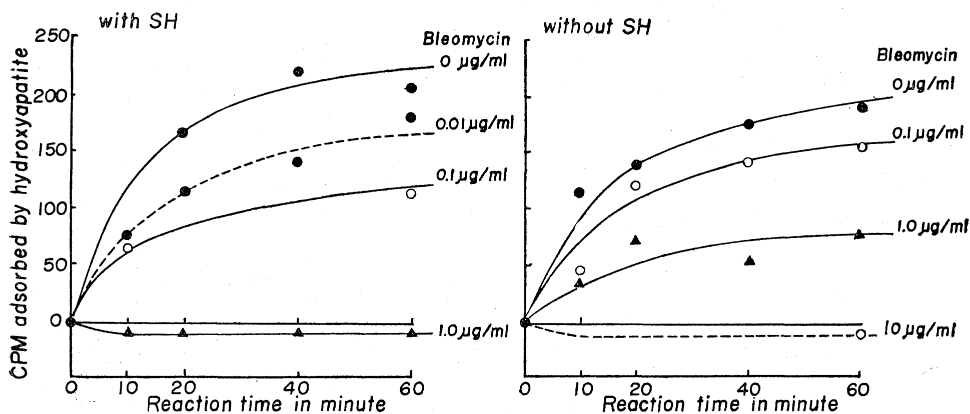
The ligase fraction (50 μg protein/ml) was incubated with chemicals under the conditions described in Methods in the presence of 10 mM mercaptoethanol. Ligase activity indicated is the mean of values at different incubation time of 10, 20, 40 and 60 minutes.

The inhibition of ligase by bleomycin was further examined in the presence or absence of mercaptoethanol. This inhibition was markedly affected by mercaptoethanol as indicated in Fig. 1. The ligase activity decreased to 55 % of the control by 0.1 μg/ml of bleomycin with 10 mM mercaptoethanol, while it was reduced to 80 % without the thiol compound. One μg/ml of the antibiotic with mercaptoethanol exhibited complete inhibition of ligase but 40 % activity remained without mercaptoethanol. High concentration of bleomycin (*e.g.*, 10 μg/ml) inhibited the reaction even in the absence of thiol compound.

Rejoining of the DNA nicked by DNase I was also inhibited by 1 μg/ml of bleomycin in the presence of 10 mM mercaptoethanol (Fig. 2).

^{3}H-λ-DNA was incubated with bleomycin under the assay conditions without the enzyme and change in the size of the strand was analyzed by alkaline sucrose gradient centrifugation (Fig. 3). The strand scission occurred when DNA was incubated with a mixture of 1 μg/ml of bleomycin and 10 mM mercaptoethanol which completely inhibited the ligase. The far smaller amount of scission was observed at 0.1 μg of the antibiotic and 10 mM mercaptoethanol which decreased the ligase activity to 55 %

Fig. 1. Inhibition of joining of hydrogen-bonded cohesive end of λ-DNA by bleomycin.
Hydrogen-bonded mixed dimer (7.7 μg/ml) of ^{3}H-λ-DNA and cross-linked λ-DNA was incubated with the ligase (50 μg protein/ml) and various concentrations of bleomycin in the presence or absence of 10 mM mercaptoethanol under the conditions described in Methods.

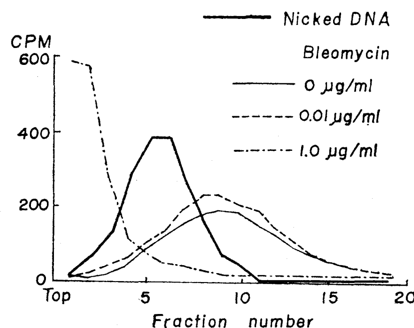


of the control. Change in the size of DNA was hardly observed at 0.01 μg/ml of bleomycin which reduced the ligase activity to 74 %. Incubation of DNA without thiol compound with 10 μg/ml of bleomycin, which strongly inhibited the ligase, produced scission. The antibiotic at 1 μg/ml did not damage DNA without thiol compound, while it reduced the activity to 40 %.

Neither DNA degraded by bleomycin nor that by means presented in Fig. 4 could be repaired by ligase (Fig. 4 A, B, C, E, F) under the conditions employed for the rejoining of DNA nicked by DNase-I (D).

Fig. 2. Inhibition of joining of DNase-I-nicked DNA by bleomycin.

^{3}H-λ-DNA (0.3 μg/ml) nicked by DNase-I was incubated with ligase (150 μg protein/ml) in the presence of bleomycin, and the product was centrifuged in 5~20 % alkaline sucrose gradient at 25,000 rpm for 16 hours at 20°C.



The present results suggested the following mechanism for the inhibition of ligase by bleomycin. Bleomycin at higher concentration (*e.g.*, above 1 μg/ml with 10 mM mercaptoethanol, or 10 μg/ml without mercaptoethanol) produced strand scission of DNA which could not be repaired by the ligase. This antibiotic at lower concentration (*e.g.*, 0.01 μg/ml with 10 mM mercaptoethanol, or 0.1 μg/ml and 1 μg/ml without mercaptoethanol) was presumed also to inhibit the joining of hydrogen-bonded cohesive end of λ-DNA by binding to DNA strand or to ligase.

To confirm the latter mechanism, we are planning the experiments using the method of OLIVERA and LEHMAN[2] or WEISS and RICHARDSON[3] to assay the ligase, in which nicking of DNA does not interfere the assay.

Bleomycin[10,11] is said to inhibit cell multiplication[12] at a low concentration, have lethal effect on mammalian cells[13], and to decrease the incorporation of ^{3}H-thymidine into DNA[14]. It lowered the Tm of DNA[15] and produced single-strand scission in the presence of thiol compound[16], while it did not inhibit the DNA polymerase[17].

Fig. 3. Scission of DNA by bleomycin.

^{3}H-λ-DNA (0.7 μg/ml) was incubated with bleomycin and cross-linked DNA (7 μg/ml) in the presence or absence of 10 mM mercaptoethanol under the conditions described in Methods, and the broken DNA was centrifuged in 5~20 % alkaline sucrose gradient at 20,000 rpm for 17 hours at 20°C.

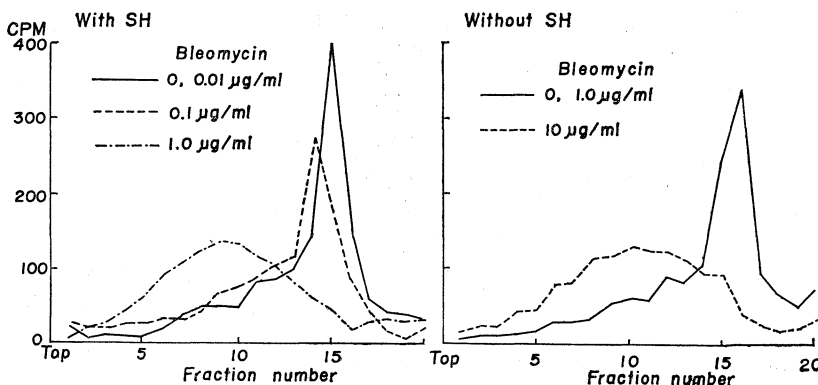
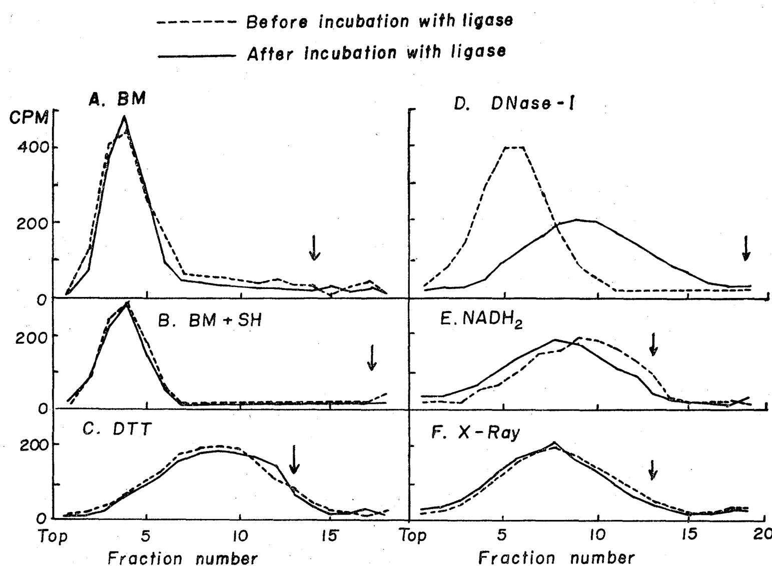


Fig. 4. Repair of damaged DNAs by ligase.

^{3}H-λ-DNA (1 μg/ml) was treated with DNase-I (D), bleomycin (10 μg/ml) (A), bleomycin and mercaptoethanol (1 μg/ml and 10 mM respectively) (B), dithiothreitol (10 mM) (C), $NADH_2$ (10 mM) (E), or X-ray (100 rad) (F), and dialyzed against 0.1 M Tris-HCl-1 mM EDTA (pH 7.0). The damaged DNA (0.3 μg/ml) was incubated with the ligase fraction (150 μg protein/ml) and then sedimented in alkaline sucrose gradient at 20,000 rpm for 17 hours (A), 22,000 rpm for 20 hours (B), 20,000 rpm for 16 hours (C, E, F) or 25,000 rpm for 16 hours (D). The arrow indicates the peak of intact λ-DNA.



If the primary action of bleomycin is the scission of DNA *in vivo*, the repair system including enzymes other than ligase will be required, because the damaged DNA is not rejoined by ligase alone. Such a system, the presence of which has been suggested[18], may be different depending on the kind of cells. On the other hand, if the antibiotic at rather a low concentration inhibits the ligase *in vivo*, it may be supposed that the inhibition of the ligation process[19] by bleomycin results in inhibition of DNA synthesis and cell multiplication.

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