DECREASE OF MELTING TEMPERATURE AND SINGLE STRAND SCISSION OF DNA BY BLEOMYCIN IN THE PRESENCE OF HYDROGEN PEROXIDE

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Effect of bleomycin and H_2O_2 on DNA was investigated. Tm of salmon sperm DNA decreased from 76°C to 64°C and that of *Escherichia coli* DNA from 81°C to 65.5°C in the presence of 100 μM of H_2O_2 and 4 μg/ml of bleomycin. The change of Tm was more marked when the concentration of H_2O_2 or bleomycin was increased. The single strand scission of DNA was shown when the DNA was incubated at 37°C with 100 μM of H_2O_2 and 40 μg/ml of bleomycin. It proceeded as the time of incubation was prolonged. The reaction of H_2O_2 at 100 μM on DNA was apparent only in the presence of bleomycin.

A reaction of H_2O_2 and DNA has been known to result in release of bases,[1,2,3] or altered bases,[3,4,5] breakdown of strands and decrease of Tm[3]. This reaction, however, requires a high concentration of H_2O_2 such as 0.1 M and a long period of incubation in the presence of $FeCl_3$. Low concentrations (10^{-2}~10^{-5} M) of H_2O_2 shows no appreciable effect on the viscosity of thymus DNA[6]. BODE[10] has reported that peroxides produced in a reaction mixture containing reducing agent might cause single strand scission of DNA. As reported in previous papers,[7,8,9] bleomycin interacts with DNA which has reacted with a sulfhydryl compound, resulting in decreasing Tm and breaking the strands. In this connection, we investigated whether bleomycin would interact with DNA in the presence of H_2O_2. The results presented in this paper indicate that in the presence of bleomycin a low concentration of H_2O_2 decreases the melting temperature of DNA and causes scission of DNA strands. The molecular size of DNA decreased as the time of incubation was prolonged. However, unlike sulfhydryl compounds, combined effect of H_2O_2 and bleomycin on DNA appears only in their coexistence.

Materials and Methods

Materials: Bleomycin A_2 (lot F-4, copper-free) was prepared by Nihon Kayaku Co., Tokyo and supplied by Dr. TAKITA, Institute of Microbial Chemistry, Tokyo. DNA of *E. coli* B was prepared by the method of MARMUR[11]. Salmon sperm DNA was purchased from Calbiochem, Los Angeles, Calif., U.S.A.

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Determination of Tm: The melting point of DNA was determined in a Gilford recording spectrophotometer as described previously[7]. The reaction mixture containing 20 μg/ml of DNA in 50 mM Tris-HCl (pH 7.6) was kept at 37°C for 120 minutes with H_2O_2 and bleomycin at the concentration indicated. After the incubation, the temperature was raised at the rate of 1°C per 5 minutes.

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 ultracentrifuge at 50,000 or 40,000 rev./min. at 20°C. Absorbance of each fraction at 260 mμ was calculated after addition of 1 ml of water in a quartz cuvette of 1-cm light path (Shimadzu spectrophotometer QV-50).

Results

Effect of H_2O_2 and Bleomycin on the Melting Temperature of DNA

As has been suggested by several authors,[6,12,13] low concentrations of H_2O_2 failed to show any effect on Tm of DNA. However, a marked decrease of Tm was observed in the presence of H_2O_2 and bleomycin. This effect became more apparent as the concentration of either compound was increased. The increase of concentration of H_2O_2 affected it more strongly than that of bleomycin. Similar results were obtained on both DNA's of salmon sperm and *E. coli* B. When salmon sperm DNA was incubated with H_2O_2 and thereafter catalase was added together with bleomycin just before the determination of Tm, then the shift of Tm was only 3.5°C. On the other hand, when H_2O_2 was added after the incubation of DNA and bleomycin and then the Tm was determined, the shift was 9°C. Moreover, the shift was 14°C when bleomycin and H_2O_2 were present throughout the incubation and determination of the melting point.

In an experiment, DNA was incubated with H_2O_2, the reaction mixture was dialyzed, and the Tm of the DNA was determined in the presence or absence of bleomycin.

Table 1. Shift of Tm of DNA in the presence of H_2O_2 and bleomycin

Origin of DNA	Concentration of H_2O_2 (μM)	Concentration of bleomycin				
		0 μg/ml	4 μg/ml		40 μg/ml	
		Tm	Tm	ΔTm	Tm	ΔTm
E. coli B	0	81.0	81.0	0	81.0	0
	10	81.0	77.0	− 4.0	76.0	− 5.0
	100	81.0	65.5	−15.5	63.0	−18.0
Salmon sperm	0	76.0	76.0	0	76.0	0
	10	76.0	73.0	− 3.0	71.5	− 4.5
	100	76.0	64.0	−12.0	62.0	−14.0
		76.0			76.0[a]	0
		76.0			70.0[b]	− 6.0
		76.0			74.5[c]	− 1.5
		76.0			72.5[d]	− 3.5
		76.0			67.0[e]	− 9.0
		76.0			71.0[f]	− 5.0
		76.0			71.0[g]	− 5.0

The reaction mixture was incubated for 120 minutes at 37°C and thereafter the temperature was raised at the rate of 1°C per 5 minutes.

a: DNA was incubated with H_2O_2 (100 μM) and dialyzed against 100 vol. of 50 mM Tris-HCl (pH 7.6) at 4°C for 12 hours. Bleomycin was added before Tm determination.

b: DNA was incubated with bleomycin (40 μg/ml) and dialyzed against 100 vol. of 50 mM Tris-HCl (pH 7.6) at 4°C for 2 hours and against the same volume of the same buffer for another 10 hours. H_2O_2 was introduced before determination of Tm.

c: 1 μg of catalase was added before the incubation.

d: DNA and H_2O_2 were kept at 37°C for 120 minutes and then bleomycin and 1 μg of catalase were added to the reaction mixture.

e: H_2O_2 was added after the incubation of DNA and bleomycin.

f: Tm was determined in the presence of 1 mM of 2-mercaptoethanol in place of H_2O_2.

g: 1 μg of catalase was added to the mixture before the incubation.

Fig. 1. Sedimentation analysis of DNA treated with H_2O_2 and bleomycin.

200 μg/ml of *E. coli* B DNA was incubated at 37℃ in 50 mM Tris-HCl (pH 7.6) containing 100 μM of H_2O_2 with or without bleomycin. 0.1 ml of reaction mixture was layered on 5 to 20% linear neutral (a) or alkaline (b) sucrose density-gradient. Centrifugation was carried out at 40,000 rev./min. for 180 minutes at 20℃.

I: Incubated in the absence of bleomycin for 120 minutes and then dialyzed against 50 mM Tris-HCl (pH 7.6) containing 100 μM of H_2O_2 for 12 hours at 0℃.

II: Incubated in the presence of bleomycin for 120 minutes and dyalyzed as described above.

III: Incubated in the presence of bleomycin for 120 minutes and stored at 0℃ for 12 hours without dialysis.

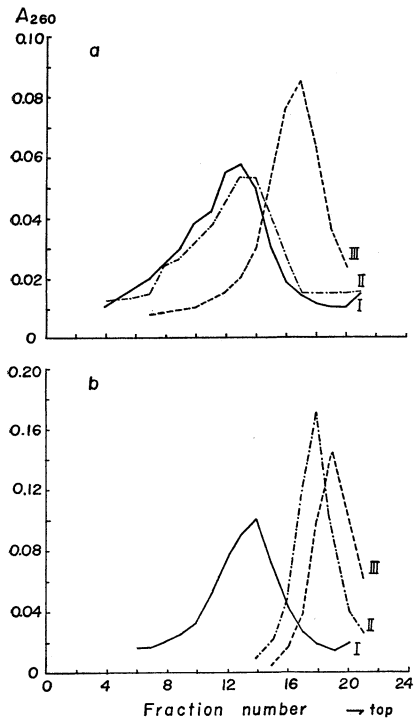
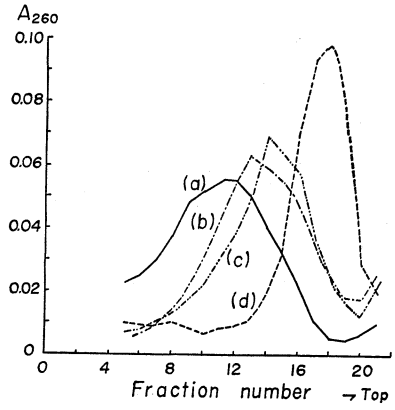


Fig. 2. Decrease of molecular size of DNA after the treatment with H_2O_2 and bleomycin for different periods.

E. coli B DNA was incubated as in Fig. 1. Reaction was stopped with addition of 1 μg of catalase to the 0.5 ml of reaction mixture. 0.1 ml of the reaction mixture was analyzed by alkaline sucrose density-gradient (5~20%) centrifugation which was carried out at 50,000 rev./min, for 150 minutes at 20℃. DNA was incubated in the absence of bleomycin for 6 hours (a) or in the presence of bleomycin for 1.5 hours (b), 3 hours (c) and 6 hours (d).



In this case no change of Tm was observed. In another experiment, DNA was incubated with bleomycin, the reaction mixture was dialyzed, H_2O_2 was added and the Tm was determined, then, Tm of DNA decreased by 6°C. Bleomycin, which has a relatively large molecular weight such as 1,300 and basic functions, is thought to remain in a trace amount after the dialysis and to cause a small decrease of Tm in the presence of H_2O_2. As shown in Table 1, the difference of the effect between 40 μg/ml and 4 μg/ml of bleomycin is very slight.

The results of the experiments are summarized in Table 1. Besides the results of experiments described above, Table 1 includes the result of an experiment testing combined effect of 2-mercaptoethanol and bleomycin on salmon sperm DNA in the presence or the absence of catalase. Catalase shows no influence on the combined effect of the sulfhydryl compound and bleomycin.

Strand Scission of DNA by H_2O_2 and Bleomycin

The molecular size of DNA was analyzed by sucrose density gradient centrifugation after the incubation with H_2O_2 and bleomycin. When the DNA was incubated with H_2O_2 and bleomycin for 120 minutes at 37°C and dialyzed against the buffer containing H_2O_2 at 0°C, no significant change of molecular size was shown by neutral sucrose density gradient centrifugation (Fig. 1-a). However, as presented in Fig. 1-b,

a decrease of molecular size was revealed by alkaline sucrose density gradient analysis. This indicates that single strand breaks occurred during the incubation. As shown in Fig. 1-a, when the reaction mixture was kept at 0°C for 12 hours after the incubation, then the strand breaks were shown even by neutral sucrose density gradient analysis. It was also shown that the number of strand scission increased as the time of incubation was prolonged (Fig. 2). No change in DNA molecular size was observed during 12-hour incubation with either H_2O_2 or bleomycin alone.

Discussion

As shown in previous papers, bleomycin interacts with DNA in the presence of a sulfhydryl compound, resulting in decrease of Tm[7,8] and breakdown of strands[8,9]. The breakdown of strands was more markedly shown when molecular size of DNA was examined after its dialysis. As to the sequence of the reaction it was also shown[7] that the reaction must occur first between DNA and a sulfhydryl compound and finally bleomycin. When the present results are compared with these facts, the reactions of H_2O_2 and sulfhydryl compound with DNA are different in following points:

(1) Decrease of Tm of DNA was observed only in the coexistence of bleomycin and H_2O_2, but in the case of a sulfhydryl compound the decrease of Tm was observed at the same extent when the reaction mixture containing DNA and a sulfhydryl compound was dialyzed after the incubation and thereafter bleomycin was added.

(2) The molecular size of DNA decreased as the time of incubation was prolonged in the presence of bleomycin and H_2O_2. On the other hand, with a sulfhydryl compound, the prolonged incubation did not increase the effect of bleomycin causing breakdown of DNA strands. These indicate that a sulfhydryl compound reacts with DNA during the incubation without bleomycin and this reaction is not reversed even when a sulfhydryl compound is removed by dialysis whereas the reaction between low concentration of H_2O_2 and DNA occurs apparently only in the presence of bleomycin or is reversed when H_2O_2 is removed by dialysis.

The inactivation of pneumococcal transforming activity by ascorbate was suggested to be caused by the peroxide produced during the course of ascorbate autoxidation[14]. Recently single strand scission of DNA has been reported[10,15] to be caused by several reducing agents such as ascorbate, dithiothreitol, 2-mercaptoethanol, reduced diphosphopyridine nucleotide and 4-hydroxyquinoline-N-oxide, and the role of H_2O_2 produced from these reducing agents and oxygen was suggested for the strand scission. However, present results indicate that the strand scission of DNA by a sulfhydryl compound and bleomycin is not caused *via* the reaction of H_2O_2.

According to PHAESE *et al.*[3], the reaction of H_2O_2 with polydeoxynucleotide initiates with the attack of anomeric carbon of deoxyribose by hydroxyl free radical and the reaction in the early steps, that is, before release of the base, are reversible. Though the reaction mechanism among DNA, H_2O_2 and bleomycin remains to be determined, it may be considered that bleomycin might interact with a reaction product of DNA and H_2O_2 which is still in the reversible steps as described above. In this connection, it may be worthy to cite a paper[12] which reported binding of hydrocarbons to DNA in the presence of H_2O_2.

As reported in a previous paper[9], scission of a DNA strand is observed in cells treated with bleomycin. It is not certain, which type of the reactions, that is, those among DNA, H_2O_2 and bleomycin or those among DNA, sulfhydryl compounds and bleomycin woud play a role in cells, causing the strand scission. If the former type is predominant in the cells, it is interesting in connection with the effect of bleomycin against cancer cells[16,17] in which catalase is generally decreased.

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