Effects of Photo-Activated Bleomycin on Deoxyribonuclease I, Exonuclease III and Deoxyribonucleic Acid Polymerase I Reactions

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The activity of bleomycin to break the strand of deoxyribonucleic acid (DNA) in the presence of 2-hydroxy-1-ethanethiol (2-mercaptoethanol) was enhanced by ultraviolet (UV) irradiation. Photo-activated bleomycin stimulated the action of deoxyribonuclease I (DNase I) to degrade DNA and the DNA synthesis by DNA polymerase I with DNase I. On the other hand, although UV-irradiated bleomycin scarcely broke the DNA strand in the presence of 1,2-benzenediol (catechol), it stimulated the action of DNase I to degrade DNA in the presence of catechol. In accordance with the inhibition by catechol, when DNA treated with UV-irradiated bleomycin in the presence of catechol was employed as a primer for the DNA synthesis, the incorporation of precursor into the acid-insoluble fraction by DNA polymerase I with exonuclease III was reduced to about one-half of the incorporation into DNA treated with unirradiated bleomycin. These findings suggest that the ability of bleomycin to bind to double-helical DNA forming regions sensitive to DNase I was increased by an appropriate dose of UV irradiation and that catechol inhibited the activity of the UV-irradiated bleomycin to break the DNA strand rather than to bind to DNA.

Keywords bleomycin; DNase I; exonuclease III; DNA polymerase I; catechol; 2-mercaptoethanol; DNA strand breakage; DNA synthesis; UV irradiation

Introduction

The bleomycins, a family of glycopeptidic antibiotics used clinically in certain cancers, are believed to bind to double-helical deoxyribonucleic acid (DNA) and to degrade it.[1-3] It has been reported that bleomycin is photosensitive; *i.e.*, the ultraviolet (UV) absorption and fluorescence spectra change with UV irradiation.[4-6] These spectrometric changes are due to photorearrangement of the 2,4′-bithiazole rings to 4,4′-thiazole in the bleomycin molecule.[7] Some reports have examined the effects of UV irradiation on the activities of bleomycin for breaking the DNA strand,[6,8] releasing free bases from DNA[5] and decreasing the melting temperature (T_m) of DNA.[6] Recent research has shown that although 1,2-benzenediol (catechol) increased the ability of unirradiated bleomycin to decrease T_m of DNA, it inhibited the activity of bleomycin enhanced by UV irradiation.[9]

It is of interest to examine whether photo-activated bleomycin influences the action of enzymes such as nucleases and DNA polymerase. Studies on the effects of bleomycin on these enzyme reactions have already been reported.[10-12] That is, bleomycin stimulated the degradation of DNA by deoxyribonuclease I (DNase I) and the DNA synthesis by DNA polymerase and inhibited the ligation of the DNA strand by ligase. Moreover, the DNA synthesis which used bleomycin-treated DNA as a primer has been shown to require exonuclease III.[13] In the present study, the effects of UV-irradiated bleomycin on these enzyme reactions, *e.g.*, bovine pancreatic DNase I, *Escherichia coli* (*E. coli*) DNA polymerase I and *E. coli* exonuclease III, were investigated.

Materials and Methods

Materials Bleomycin was obtained from Nippon Kayaku Co. Highly polymerized salmon sperm and calf thymus DNAs, bovine pancreatic DNase I, thymidine triphosphate (TTP), deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP) and bovine serum albumin were obtained from Sigma Co. *E. coli* DNA polymerase I and *E. coli* exonuclease III were from Takara Shuzo Co., and phage PM2 DNA was from Boehringer Mannheim Co. [α-^{32}P]Deoxycytidine triphosphate (dCTP) (3000 Ci/mmol) was obtained from Amersham Laboratories. Scintizol AL-1 for the measurement of the radioactivity of ^{32}P and ethylenediaminetetraacetic acid (EDTA) were from Dojin Chemical Laboratories. Catechol, 2-hydroxy-1-ethanethiol (2-mercaptoethanol) and other reagents were obtained from Wako Pure Chemical Industries Co.

Preparation of UV-Irradiated Bleomycin for Assaying Enzyme Reactions A solution (3 ml) of 48 or 480 μM bleomycin in 5 mM tris-(hydroxymethyl)aminomethane (Tris)–HCl (pH 7.5) in a cuvette was irradiated for the required time with a mercury lamp (15 W) at a distance of 23 cm; *i.e.*, 8 and 90 min at 48 μM or 60 and 300 min at 480 μM. Under these conditions, the irradiation dose was estimated to be 35 erg/mm^2/s by the chemicial densitometer method. The UV absorption spectrum was measured with a Hitachi EPS-3T spectrophotometer.

Effect of UV-Irradiated Bleomycin on the Action of DNase I Reaction mixtures were prepared with the following components: 40 μg/ml salmon sperm DNA, 20 ng/ml bovine pancreatic DNase I, 40 μM UV-irradiated or unirradiated bleomycin and 1 mM catechol or 3 mM 2-mercaptoethanol in 5 mM Tris–HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ and 50 mM KCl. The optical density of each reaction mixture at 260 nm was measured in a cuvette (10 mm light path) with a stopper to obtain the curve of the change with incubation time at 37 °C using a Hitachi EPS-3T spectrophotometer.

Effect of UV-Irradiated Bleomycin on the DNA Polymerase I Reaction The reaction mixtures contained the following components: 1/250 optical density units of phage PM2 DNA, 80 μM UV-irradiated or unirradiated bleomycin and 20 mM 2-mercaptoethanol or 3 mM catechol in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 10 μg/ml bovine serum albumin. The reaction mixtures were incubated for 60 min at 37 °C. Each mixture was used as a solution of DNA to be incorporated with the precursor, [α-^{32}P]dCTP, by the nick translation reaction.[14,15]

The solution for the nick translation reaction contained each DNA mentioned above, 20 mM each of dATP, dGTP and TTP and 3 μCi of [α-^{32}P]dCTP, and was treated with 10 ng/ml bovine pancreatic DNase I for 1 min on ice or with 5 units of *E. coli* exonuclease III for 3 min at 37 °C. Each solution was incubated with 4 units of *E. coli* DNA polymerase I for 40 min at 15 °C in 10 mM 2-mercaptoethanol, 5 μg/ml bovine serum albumin, 5 mM $MgCl_2$ and 50 mM Tris–HCl buffer (pH 7.5). The reaction was carried out in a volume of 10 μl. The respective reaction mixtures are indicated in Tables I and II. An equal volume of a solution containing 2 mg/ml calf thymus DNA, 20 mM EDTA and 0.2% sodium lauryl sulfate (SDS) was then added to the reaction mixture to terminate the enzyme reaction, and 2 volumes of 500 μg/ml calf thymus DNA solution were added.

After the nick translation reaction, each reaction mixture (10 μl) was spotted onto the center of a Whatman GF/C glass-fiber disc of 24 mm in diameter. On the other hand, 10 μl of the reaction mixture was placed into 2.5 ml of ice-cold 10% trichloroacetic acid (TCA), and chilled on ice for 15 min. Then, each precipitate was collected by filtering the solution

through another Whatman GF/C glass-fiber disc. This disc was washed 6 times with 5 ml of ice-cold 10% TCA, followed by 5 ml of 95% ethanol. Both discs were placed in scintilation vials containing 20 ml of scintizol AL-1. The radioactivity of ^{32}P in these vials was measured with an Aloka LSC-700 liquid scintilation counter. The incorporation ratio (%) was determined from the following equation:

$$\text{incorporation ratio (\%)} = \frac{\text{incorporated } ^{32}P \text{ of each reaction mixture}}{\text{incorporated } ^{32}P \text{ of bleomycin-free solution}} \times 100$$

Results

Figure 1 shows the change in optical density at 260 nm based on the breakdown of DNA which was treated with bleomycin in the presence of 2-mercaptoethanol or catechol. Following incubation for 90 min at 37 °C in the presence of 2-mercaptoethanol, the increased values of the optical density of the reaction mixtures which contained bleomycin irradiated by UV for 0, 8 and 90 min were in order of 0.018, 0.071 and 0.021. However, in the presence of catechol, a change in optical density was scarcely caused in comparison with the addition of unirradiated bleomycin; *i.e.*, after incubation for 90 min at 37 °C, the increased values were 0.012, 0.012 and 0.002 with irradiation times of 0, 8 and 90 min, respectively. The optical density did not change in the case of the reaction mixture without bleomycin.

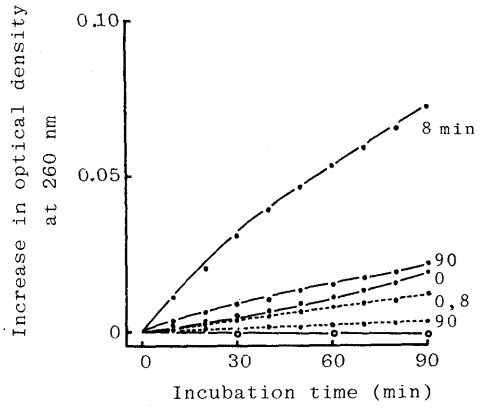

Fig. 1. Effect of UV-Irradiated Bleomycin on the Change in Optical Density of DNA in the Presence of 2-Mercaptoethanol or Catechol

The reaction mixtures contained 40 μg/ml salmon sperm DNA, 40 μM bleomycin irradiated for 0, 8 and 90 min and 3 mM 2-mercaptoethanol or 1 mM catechol in 5 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 50 mM KCl. The optical density at 260 nm was monitored during incubation at 37 °C. —○—, without bleomycin; —●—, with 2-mercaptoethanol; ---●---, with catechol.

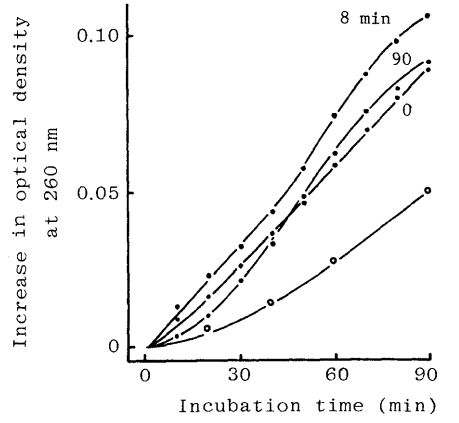

Fig. 2. Effect of UV-Irradiated Bleomycin on the Change in Optical Density of DNA Treated with DNase I in the Presence of 2-Mercaptoethanol

The reaction mixtures contained the same components as indicated in Fig. 1 except for the addition of 20 ng/ml bovine pancreatic DNase I. The optical density was measured as described in Fig. 1. —○—, without bleomycin; —●—, with bleomycin.

Figures 2 and 3 show the effects of UV-irradiated bleomycin on the breakdown of DNA by DNase I. The action of DNase I to increase the optical density of DNA at 260 nm was stimulated by bleomycin in the presence of both 2-mercaptoethanol and catechol. This effect of bleomycin on the action of DNase I was further enhanced by UV irradiation for 8 min. Following incubation for 90 min at 37 °C, the increased values of the optical density were 0.088, 0.106 and 0.092 in the presence of 2-mercaptoethanol and 0.073, 0.097 and 0.059 in the presence of catechol, at irradiation times of 0, 8 and 90 min, respectively. The increased value was 0.049 in the reaction mixture without bleomycin.

Data on the effect of UV-irradiated bleomycin on the incorporation of the precursor, [α-^{32}P]dCTP, into phage PM2 DNA by *E. coli* DNA polymerase I acting in concert with bovine pancreatic DNase I are summarized in Table I. Since the activity of bleomycin to decrease T_m of DNA was most greatly enhanced by UV irradiation for 60 min at 480 μM, as shown previously,[9] it was irradiated for 60 and

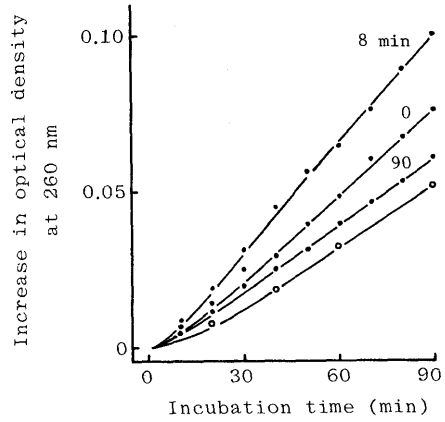

Fig. 3. Effect of UV-Irradiated Bleomycin on the Change in Optical Density of DNA Treated with DNase I in the Presence of Catechol

The reaction mixtures contained the same components as indicated in Figs. 1 and 2, but 1 mM catechol was added instead of 3 mM 2-mercaptoethanol. The optical density was measured as described in Fig. 1. —○—, without bleomycin; —●—, with bleomycin.

TABLE I. Effect of UV-Irradiated Bleomycin on the Incorporation of [α-^{32}P]dCTP into PM2 DNA Using the Nick Translation Reaction with DNase I

Components	Acid-insoluble ^{32}P/ total ^{32}P	Incorporation ratio (%)
Bleomycin-free	0.297	100
Bleomycin + 2-mercaptoethanol	0.203	68.5
Bleomycin (60 min)[a] + 2-mercaptoethanol	0.495	166.7
Bleomycin (300 min)[a] + 2-mercaptoethanol	0.384	129.3

a) Bleomycin was irradiated for 60 or 300 min at 480 μM. Each reaction mixture was prepared and measured for radioactivity of ^{32}P as described in Materials and Methods.

TABLE II. Effect of UV-Irradiated Bleomycin on the Incorporation of [α-^{32}P]dCTP into PM2 DNA Using the Modified Nick Translation Reaction with Exonuclease III

Components	Acid-insoluble ^{32}P/ total ^{32}P	Incorporation ratio (%)
DNA polymerase I with exonuclease III		
Bleomycin-free	0.221	100
Bleomycin + 2-mercaptoethanol	0.414	187.3
Bleomycin (60 min)[a] + 2-mercaptoethanol	0.416	188.2
Bleomycin + catechol	0.491	222.2
Bleomycin (60 min)[a] + catechol	0.270	122.2
DNA polymerase I without nuclease		
Bleomycin-free	0.451	100
Bleomycin + 2-mercaptoethanol	0.391	86.7
Bleomycin (60 min)[a] + 2-mercaptoethanol	0.464	102.9
Bleomycin + catechol	0.440	97.6
Bleomycin (60 min)[a] + catechol	0.362	80.3

a) Bleomycin was irradiated for 60 min at 480 μM. Each reaction mixture was prepared and measured for radioactivity of ^{32}P as described in Materials and Methods.

300 min at 480 μM to assay the incorporation of ^{32}P by the nick translation reaction. In the typical reaction containing DNA polymerase I and DNase I, unirradiated bleomycin depressed about 30% of the incorporation of precursor into the acid-insoluble fraction. On the other hand, the incorporation of ^{32}P was increased to approximately 3- and 2-fold by UV-irradiated bleomycin for 60 and 300 min in comparison with that for unirradiated bleomycin (Table I).

Data on the effect of UV-irradiated bleomycin on the DNA synthesis by DNA polymerase I acting in concert with exonuclease III instead of DNase I are summarized in Table II. The incorporation of ^{32}P into the acid-insoluble fraction was increased about 2-fold by unirradiated bleomycin in the presence of 2-mercaptoethanol or catechol in comparison with that of a solution without bleomycin. The incorporation ratio was not different between UV-irradiated and unirradiated bleomycin in the presence of 2-mercaptoethanol. However, in the presence of catechol, the incorporation of ^{32}P into DNA treated with UV-irradiated bleomycin decreased to approximately one-half of that with unirradiated bleomycin. Under DNase I- or exonuclease III-deficient conditions, the effect of bleomycin on the incorporation of ^{32}P into the acid-insoluble fraction was not different from that without bleomycin.

Discussion

It has been shown that the *in vitro* activity of bleomycin to break the DNA strand could be enhanced by UV irradiation.[6,8] In the present study, the effect of UV-irradiated bleomycin on the enzyme reaction was investigated. The breakdown of DNA caused by bleomycin was examined by a procedure which measured the increase in optical density at 260 nm. In the presence of 2-mercaptoethanol, the breakdown of DNA was greatly increased by bleomycin irradiated for 8 min (Fig. 1). This finding is in harmony with the results indicating that the activities of bleomycin to decrease T_m of DNA and to break the DNA strand were most greatly enhanced by UV irradiation for about 10 min at 48 μM as reported previously.[6] As shown in Fig. 2, UV-irradiated bleomycin stimulated the action of DNase I to degrade DNA. This suggests that the amount of DNase I-sensitive region in the DNA may be increased by treatment with UV-irradiated bleomycin. Further, the reaction of DNA polymerase I with DNase I in which bleomycin-treated DNA was used as a primer was also enhanced about 3-fold by UV-irradiation for 60 min in comparison with treatment by unirradiated bleomycin (Table I). Since the DNase I-deficient DNA polymerase I reaction was scarcely different among the conditions with UV-irradiated and unirradiated bleomycins and without bleomycin, it is possible that enhancement of the DNA polymerase I reaction by UV-irradiated bleomycin may be due to an increase in regions on the DNA susceptible to DNase I.

Catechol has been shown to inhibit the enhanced activity of UV-irradiated bleomycin to decrease T_m of DNA.[9] As demonstrated in Fig. 1, the activity of bleomycin to break the DNA strand was not increased by UV irradiation in the presence of catechol. However, the action of DNase I to degrade DNA was increased by bleomycin irradiated with UV for 8 min, in the presence of catechol (Fig. 3). Since DNase I, an endonuclease, can identify regions of distortion in double-helical DNA, it would seem that DNase I may recognize distorted regions on double-helical DNA bound by the bleomycin molecule rather than sites broken by bleomycin. On the other hand, when DNA treated with UV-irradiated bleomycin in the presence of catechol, followed by exonuclease III, was used as a primer, the incorporation of ^{32}P into the DNA by DNA polymerase I was reduced to about one-half of that employing a primer treated with unirradiated bleomycin (Table II). It has been reported that exonuclease III was required for the DNA synthesis by DNA polymerase I when DNA previously treated with bleomycin was used as a primer,[13] suggesting that catechol may inhibit the formation of sites which are sensitive to exonuclease III. Bleomycin is thought to have bifunctional properties; *i.e.*, the bithiazole moiety intercalates into the base stacking of the double helix [16-18] and radicals such as superoxide or hydroxyl radical produced from the complex between bleomycin and ferrous ions lead to the liberation of free bases and the breakdown of DNA.[19-24] These findings suggest therefore that catechol may exclusively inhibit the activity of photo-activated bleomycin to break the DNA strand rather than to bind to double-helical DNA.

In conclusion, the findings obtained in this study show that the effect of bleomycin on the action of DNase I and DNA polymerase I operating in concert with DNase I was further enhanced by an appropriate dose of UV irradiation and that the photo-activation of bleomycin is due to an increased ability to bind to the double helix by alteration of the bithiazole moiety. Further, catechol may inhibit the function of UV-irradiated bleomycin to break the DNA strand rather than to bind to the double helix. These findings suggest that the formation of nuclease-sensitive regions on DNA by bleomycin may also play an important role in the biological activity, in addition to the activity of bleomycin to break the DNA strand directly.

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