STIMULATION OF BLEOMYCIN-INDUCED FRAGMENTATION OF DNA BY INTERCALATING AGENTS

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SUMMARY: The effect of three intercalative drugs on the *in vitro* reaction of bleomycin with DNA was determined. In the presence of proflavine, ethidium bromide, or actinomycin D, the bleomycin-induced breakage of native, double-stranded DNA is enhanced. This increased level of fragmentation is seen in the presence or absence of 2-mercaptoethanol, and is dependent on the presence of the native, double-stranded DNA structure.

Bleomycin, an antitumor antibiotic discovered by Umezawa *et al.*(1, 2), causes the breakage of isolated DNA *in vitro* (3, 4), a reaction which is greatly stimulated by reducing agents such as 2-mercaptoethanol. Since this DNA-specific endonuclease-like activity is probably closely related to the antitumor activity of the drug, we have been studying the molecular mechanism of the *in vitro* reaction. Previous studies on this reaction have concentrated on a characterization of the optimum reaction conditions (4), the final molecular species produced by extensive fragmentation of the DNA (5-7), and the susceptibilities of different natural and synthetic polynucleotides (8). The present report represents a different approach to the study of this reaction. The object is to determine the effect of other molecules, which bind to DNA at known sites, on the early stages of the bleomycin-DNA reaction. The first three compounds investigated -- proflavine, ethidium bromide and actinomycin D -- all bind to DNA by the intercalation mechanism originally proposed by Lerman (9) for acridine dyes. Additional evidence

that these three compounds bind to DNA by intercalation has been presented by Sobell (10, 11), Waring (12, 13), and others. It has been shown that the intercalation of each of these three compounds produces similar changes in the structure of closed-circular DNA (13), but they show very little structural similarity except for the common property of intercalation. It was expected that this would enable us to determine the effects on the bleomycin-DNA reaction which were the results of intercalation alone.

MATERIALS AND METHODS:

Radioactively-labelled DNA: [^{3}H]- or [^{14}C]-thymine-labelled DNA was isolated from *Bacillus subtilis* and *Escherichia coli*, using the methods previously described (4). All DNA samples used in the experiments reported here were treated with Pronase at a concentration of 0.1 mg/ml for 60-90 minutes at 37°C, after a 60-minute self-digestion of the Pronase solution (1.0 mg/ml) at 37°C. DNA samples were dialyzed against 0.1 M Tris-HCl, pH 8.0, and their concentration determined by the diphenylamine procedure (14).

Chemicals: The bleomycin used in these experiments, a mixture of 67.4% bleomycin A_2, 30.1% bleomycin B_2, and 2.5% other components (see reference 15 for the primary structure of these components), was a generous gift from Bristol Laboratories (Syracuse, New York). Proflavine (sulfate), ethidium bromide, actinomycin D, and Pronase were obtained from Calbiochem, and used without further purification. Other chemicals were obtained from general suppliers.

Bleomycin-DNA reactions: All reactions were run in a total volume of 100µl of 0.05M Tris-HCl, pH 8.0. The DNA concentration was 2.5 x 10^{-4}M (nucleotide equivalents) in each case. Bleomycin, if present, was at a final concentration of 1.0 mg/ml; 2-mercaptoethanol, if present, was at a final concentration of 25mM. Intercalating agents were included as indicated in the legends to the figures. All reaction mixtures were incubated for one hour at 37°C; at the end of this time, the reactions were stopped by adding 25µl of concentrated alkaline solution (1.5M NaOH, 3.5M NaCl) (16). Since preliminary results showed that some breakdown of the DNA could be caused by the intercalating agents alone if the reaction mixtures were exposed to light, all reactions in which an intercalating agent was present were incubated in total darkness.

After termination of the reaction by addition of the alkaline solution, the reaction mixtures were layered onto linear 5-20% alkaline sucrose gradients, 4.9 ml each. The gradients were centrifuged in a Beckman SW50.1 rotor at 40,000 rpm for 4 hours (unless otherwise indicated) at 5°C; fractions were collected, processed, and counted as previously described (4).

RESULTS:

Reactions with native, double-stranded DNA: Figure 1 shows the effects of proflavine on the reaction of bleomycin with native DNA, both in the presence and absence of 25 mM 2-mercaptoethanol. Similar results were observed with actinomycin D and ethidium bromide. In these experiments, no DNA break-

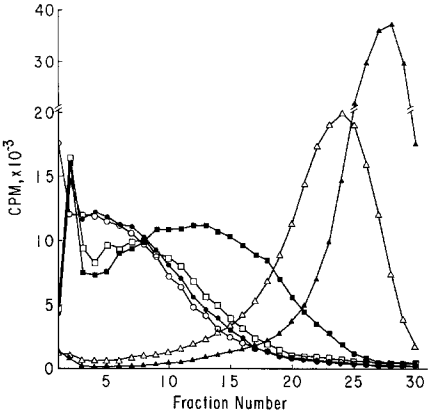

Figure 1: Effect of proflavine on the reaction of bleomycin with native, double-stranded DNA. In this and all following figures, the concentration of DNA in each sample was 2.5 x 10^{-4}M (nucleotide equivalents); bleomycin (if present), 1.0 mg/ml; 2-mercaptoethanol (if present), 25 mM. In all samples in this figure which contained proflavine, the concentration was 2.5 x 10^{-4}M. Alkaline sucrose gradient sedimentation, in all figures, is from right to left. Samples shown here are: ○, DNA only; ●, DNA + 2-mercaptoethanol + proflavine; □, DNA + bleomycin; ■, DNA + bleomycin + proflavine; △, DNA + bleomycin + 2-mercaptoethanol; ▲, DNA + bleomycin + 2-mercaptoethanol + proflavine.

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age appeared to be caused by either proflavine or ethidium bromide, either in the presence or absence of 2-mercaptoethanol (Figure 1). This was also true of actinomycin D, used alone; however, a small amount of breakage was sometimes observed in samples containing both actinomycin D and 2-mercaptoethanol. In the bleomycin reactions without 2-mercaptoethanol, each of the three intercalating agents caused a substantial increase in DNA breakage over that produced by bleomycin alone, which has very little effect under these conditions (4). Finally, the effects of the intercalating agents and 2-mercaptoethanol appear to be additive, since a stimulation of the reaction was also seen, in each case, in the presence of 2-mercaptoethanol.

Effect of different concentrations of intercalating agents: A series of reactions was run with each intercalating agent in which the concentration was varied from 5 x 10^{-5}M to 1 x 10^{-3}M. Since the DNA concentration was held constant at 2.5 x 10^{-4}M (in nucleotides), this corresponded to a range of

input dye-to-nucleotide ratios of 1:5 to 4:1. Figure 2 shows that for ethidium bromide the maximum stimulatory effect on the bleomycin-DNA reactions was observed at a concentration of 2.5 x 10^{-4}M; higher concentrations actually appear to be inhibitory. In similar dose-response experiments with proflavine and actinomycin D (not shown), the results were quite similar to those shown for ethidium bromide in Figure 2. Maximum stimulation of the reaction by proflavine was also observed at 2.5 x 10^{-4}M; however, the higher concentrations of proflavine did not produce as great an inhibition as did the ethidium bromide. The two highest concentrations of actinomycin (5 x 10^{-4}M and 10^{-3}M) stimulated the reaction slightly more than did the next lower concentration (2.5 x 10^{-4}M), which was the optimum concentration for the other two drugs. The input dye-to-nucleotide ratios in all of these samples exceeded the amount which would be required to saturate the reported number of actinomycin binding sites in various DNAs (11). Since additional stimulation of the reaction was observed up to a ratio of 2:1, this may indicate that only a small fraction of the actinomycin added actually binds

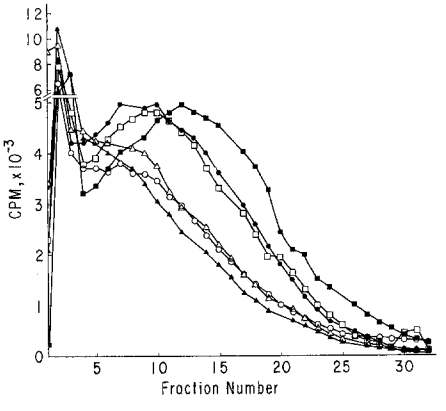

Figure 2: Effect of different concentrations of ethidium bromide on the reaction of bleomycin with native DNA. Samples contained DNA, bleomycin, and the following concentrations of ethidium bromide: ○, None (DNA and bleomycin only); ●, 5 x 10^{-5}M; □, 10^{-4}M; ■, 2.5 x 10^{-4}M; △, 5 x 10^{-4}M; ▲, 10^{-3}M.

to the DNA under these conditions. The concentration dependence of this effect may also explain why it was not observed in our earlier experiments with actinomycin (17), in which input ratios of 1:12 and lower were used.

Reactions with heat-denatured DNA: For these reactions, the DNA solution was heated to 100°C for 5 minutes and quickly cooled in an ice bath, immediately before beginning the reactions. The results of one of these experiments, using ethidium bromide, are shown in Figure 3; similar results were observed with proflavine and actinomycin D. In these experiments, little or no stimulation of the reaction by the intercalating agents was observed, either in the presence or absence of 2-mercaptoethanol, which still had a substantial effect. This is another indication that these intercalating agents and 2-mercaptoethanol probably stimulate the bleomycin reaction by different mechanisms.

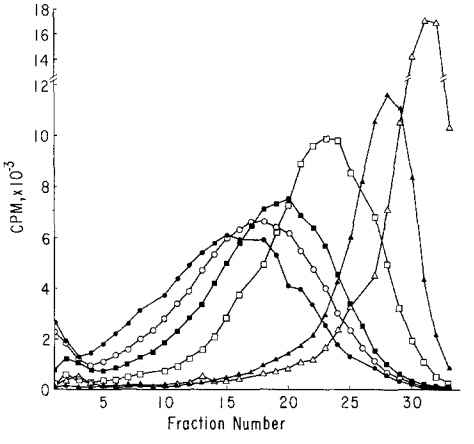

Figure 3: Effect of ethidium bromide on the reaction of bleomycin with heat-denatured DNA. DNA was denatured, by heating to 100°C and quick cooling, and run in reactions as described, except that the gradients were run for five hours at 40,000 rpm. Concentration of ethidium bromide (if present) was 2.5 x 10^{-4}M. ○, DNA only; ●, DNA + 2-mercaptoethanol + ethidium bromide; □, DNA + bleomycin; ■, DNA + bleomycin + ethidium bromide; △, DNA + bleomycin + 2-mercaptoethanol; ▲, DNA + bleomycin + 2-mercaptoethanol + ethidium bromide.

DISCUSSION:

The experiments reported here show that at least three compounds which can intercalate into DNA also increase the extent of bleomycin-induced breakage of the DNA, both in the presence and the absence of 2-mercaptoethanol. It was also observed that (a) this stimulation of the bleomycin reaction does not occur with heat-denatured DNA, and (b) a maximum effect is observed at intermediate concentrations of proflavine or ethidium bromide. It is probable, therefore, that intercalation is the specific property of these compounds which causes the stimulation. The secondary binding reaction of each of these compounds, which has been characterized as binding to the DNA-phosphate groups on the outside of the double helix (18), does not seem to have any stimulatory effect on the reaction (and may even be inhibitory in the case of ethidium bromide), since this is the type of binding which would be favored at higher concentrations (Figure 2) or with denatured DNA (Figure 3).

At least two general hypotheses may be proposed to explain this phenomenon. The first possibility is that the structural changes in the DNA produced by any intercalating agent, *i. e.*, the unwinding and extension of the native double helix (9), could make the DNA more susceptible to attack by the bleomycin. Alternatively, this may be the result of a more specific interaction between the bleomycin and the intercalated drug-DNA complex. Experiments now in progress are designed to test which of these hypotheses is correct. This information, together with model-building studies also in progress, should lead to a better understanding of the molecular interaction of bleomycin with DNA.

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