Accelerated Publication

Light-Induced Nicking of Deoxyribonucleic Acid by Cobalt(III) Bleomycins†

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ABSTRACT: The anticancer drug bleomycin is a glycopeptide that causes strand scission of DNA both in vivo and in vitro. Cleavage of DNA by bleomycin has been studied extensively in vitro, with the findings that ferrous ion and molecular oxygen must be present and that addition of reducing agents greatly enhances the reaction. To date, only iron has been shown to be an effective metal cofactor for the cleavage of DNA by bleomycin. Here it is reported that two stable cobalt(III) complexes of bleomycin are strikingly effective in causing single-strand breaks (nicks) in supercoiled DNA in the presence of ultraviolet or visible radiation. For example, 366-nm light from an 18-W long-wavelength mercury lamp for 1 h causes 10^{-6} M cobalt(III) bleomycin to completely convert supercoiled ϕX174 DNA (10^{-8} M DNA, 10^{-4} M phosphate) into the nicked circular form. Furthermore, numerous alkali-labile sites are produced on the DNA during this treatment. The observed reactions are not caused by adventitious iron, and they occur only in the presence of cobalt(III) bleomycin and light.

The anticancer drug bleomycin (BLM)[1] is a glycopeptide that causes strand scission of DNA both in vivo (Suzuki et al., 1969) and in vitro (Suzuki et al., 1969, 1970; Haidle, 1971). The in vitro degradation of DNA by BLM has been shown to require molecular oxygen (Sausville et al., 1976) and ferrous ion (Sausville et al., 1976, 1978) as cofactors. In the presence of reducing agents such as 2-mercaptoethanol, the efficiency of DNA breakage is greatly enhanced (Suzuki et al., 1970; Haidle, 1971; Umezawa et al., 1973; Bearden et al., 1977). Thus, the cleavage of DNA by BLM has been proposed to involve an oxygen-requiring oxidation/reduction cycle of iron bleomycin (Lown & Sim, 1977; Sausville et al., 1978), and the cytotoxicity of the drug has been correlated with its ability to degrade DNA (Kohn & Ewig, 1976).

In several studies, divalent metal ions (including cobalt) that compete with iron for the same binding sites on BLM have been found to *suppress* the cleavage activity of BLM on isolated DNA (Suzuki et al., 1970; Umezawa et al., 1973; Takeshita et al., 1976; Lown & Sim, 1977; Sausville et al., 1978). It is nevertheless possible that metallobleomycins other than iron bleomycin may induce cleavage of DNA under conditions that are somewhat different from those used previously. There are some indications that copper(I) bleomycin can do this (Oppenheimer et al., 1981; Freedman et al., 1982; C.-H. Chang and C. F. Meares, unpublished observations).

We show in this report that two cobalt(III) complexes of bleomycin can actively introduce single-strand breaks in ϕX174 supercoiled DNA in the presence of ultraviolet or visible radiation. The degradation reaction caused by these light-activated Co(III) BLM's is strikingly efficient. Single-stranded scission of DNA can be detected with as low as 10^{-8} M Co(III) BLM after treatment with 366-nm radiation from an 18-W mercury lamp for 2 h. That the observed reactions were not caused by adventitious iron but only occurred in the presence of light and Co(III) BLM was carefully examined and verified, as shown below.

Materials and Methods

Materials. BLM, supplied as the clinical mixture Blenoxane, was a generous gift of Bristol Laboratories, Syracuse, NY. ϕX174 RF DNA was purchased from Bethesda Research Laboratories and contained >95% form I. Ethidium bromide and agarose (type I) were purchased from Sigma. Other chemicals were reagent grade. Deionized distilled water was used throughout, and labware was acid washed and thoroughly rinsed to avoid heavy metal contamination.

Preparation of Cobalt(III) Bleomycins. $CoCl_2$ (100 mM, 55 μL) was added to a solution of Blenoxane (5.5 mM, 1 mL), and the pH was adjusted to neutrality with dilute NaOH. The yellowish green reaction mixture, after being allowed to stand at room temperature overnight, was applied to a Sephadex C-25 column (NH_4^+ form, 1 × 50 cm) and eluted with a linear gradient of 0.05–0.5 M ammonium formate, pH 5.7. The major reaction products were separated into four bands that were eluted at 0.14, 0.23, 0.33, and 0.43 M ammonium formate, corresponding to A_2 green, B_2 green, A_2 brown, and B_2 brown, respectively. [Bleomycin A_2 and bleomycin B_2 contain different "terminal amine" residues (Umezawa, 1978).] The green complexes were further purified on a Waters C_{18} μ-Bondapak column with 0.1 M ammonium acetate/acetonitrile (85/15 v/v) as the solvent. The green complexes were identical with those previously described by DeRiemer et al. (1979); the brown Co(III) BLM's will be described elsewhere (Chang

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[1] Abbreviations: BLM, bleomycin; Co(III) BLM, cobalt(III) bleomycin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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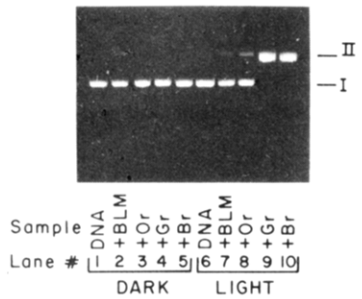

FIGURE 1: Light-induced nicking of ϕX174 RFI DNA by cobalt(III) bleomycins. Reaction mixtures containing (in a total volume of 25 μL) 25 mM Tris–borate, pH 8.1, 190 μM EDTA, and 1 μg of ϕX174 RFI (>95%) DNA were incubated at room temperature either in the dark (lanes 1–5) or under the illumination of a 366-nm UV lamp for 1 h with the following additions (final concentration 1 μM): lanes 1 and 6, none; 2 and 7, blenoxane; 3 and 8, orange Co(III) BLM A_2; 4 and 9, green Co(III) BLM A_2; 5 and 10, brown Co(III) BLM A_2. After the addition (10 μL) of a dye/EDTA mixture containing 48% (w/v) sucrose, 120 mM EDTA, pH 7, and 0.02% (w/v) bromophenol blue, electrophoresis was carried out at 50 mA for 4 h.

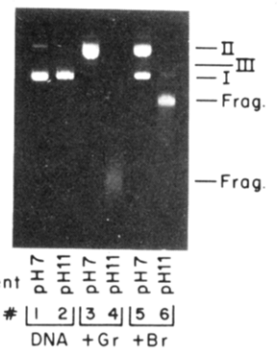

FIGURE 2: Light-induced damage of ϕX174 RFI DNA by cobalt(III) bleomycins includes production of many alkali-labile sites. The reaction conditions were the same as in Figure 1, with the following additions (final concentration 1 μM): lanes 1 and 2, none; lanes 3 and 4, green Co(III) BLM A_2; lanes 5 and 6, brown Co(III) BLM A_2. All six samples were irradiated for 1 h. Three samples (lanes 2, 4, and 6) were then hydrolyzed at pH 11.5 for 2 h in the dark following the addition of 10 μL of a sucrose/EDTA/dye mixture (pH 13) and 1 μL of 1.67 M NaOH/25 mM EDTA. The other three samples (lanes 1, 3, and 5) were not exposed to high pH; 10 μL of a *neutral* sucrose/EDTA/dye mixture was added. Electrophoresis was at 50 mA for 3 h. The diffuse band in lane 4 moved with the dye front.

et al., unpublished observations). The orange Co(III) BLM's were prepared by heating the reaction mixture as described previously (DeRiemer et al., 1979). Purified Co(III) BLM's were dissolved in 10 μM EDTA (pH 7) at known concentrations and stored at −20 °C before use. The complexes were protected from exposure to light.

DNA Nicking Assay. The reaction conditions for the light-induced strand scission of ϕX174 RF DNA by three chromatographically purified Co(III) BLM A_2's are described in the legend to Figure 1. For the light reaction the DNA solutions were irradiated at a distance of 10 cm from an 18-W long-wavelength UV 366-nm lamp (Model UVL-56, Ultraviolet Products, San Gabriel, CA). Electrophoresis of DNA was performed at room temperature in an agarose gel (1% w/v) in horizontal slabs immersed in the electrophoresis buffer. The electrophoresis buffer consisted of 83 mM Tris–borate, pH 8.1, 2.5 mM EDTA, and 1 μg/mL ethidium bromide. After electrophoresis, the gel was placed on top of a long-wavelength UV light source and photographed on Polaroid type 55 positive/negative film through a 560-nm cutoff filter. When desired, the negative films of gels were scanned with a Gilford linear transport apparatus to determine the relative band intensities.

Results

Strand Scission of Superhelical DNA as a Result of Light Action on Cobalt(III) Bleomycins. The light-induced activity of Co(III) BLM's in degrading DNA is demonstrated in Figure 1. The cleavage of DNA was followed by monitoring the conversion of covalently closed circular (form I) ϕX174 DNA to nicked circular (form II) and linear duplex (form III) DNA. A single-strand break changes form I DNA to form II DNA, while a double-strand break, produced either in a direct manner or as a result of two close-spaced single-strand breaks in complementary strands, changes either form I or form II DNA into form III DNA. As shown in Figure 1, the untreated DNA sample (lane 1) showed a major band corresponding to the superhelical form I DNA and a weak band corresponding to the nicked circular form II DNA. For the DNA solutions incubated in the dark in the presence of BLM (lane 2) or Co(III) BLM's (lanes 3–5), the band intensities of form I relative to form II DNA showed very little change from that observed for the untreated specimen, indicating the inability of these reagents to cleave DNA under the conditions examined. That the inherent DNA cleavage activity of the green and brown Co(III) BLM's can be induced by light is shown by the presence of a predominant concentration of form II DNA in the irradiated DNA solutions containing these two complexes (lanes 9 and 10). A small DNA cleavage activity inducible by light was also observed with the orange complex (lane 8). No form III DNA was observed in these reactions. In the absence of Co(III) BLM's, the action of 366-nm light alone did not cause any detectable strand breakage of DNA either in the BLM-omitted or in the BLM-added reaction mixture (lanes 6 and 7).

Under similar conditions [2 μM green Co(III) BLM B_2, 10-min irradiation], cleavage of DNA was also inducible by 290-, 370-, or 450-nm light from a 150-W xenon lamp in a fluorescence spectrophotometer; the shortest wavelength was the most effective.

Single-Strand Breaks and Alkali-Labile Sites. The result of DNA treatment with the light-activated Co(III) BLM's is shown in Figure 2 to consist not only of single-strand breaks but also of numerous alkali-labile sites. Alkali-labile sites (which presumably result from loss of nitrogenous bases on DNA) are converted into single-strand breaks by exposure to high pH (Bayley et al., 1961; Lloyd et al., 1978). When DNA samples were first treated with Co(III) BLM's and light and then incubated for 2 h at pH 11.5 in the dark, the DNA was found to be degraded entirely into smaller fragments (Figure 2, lanes 4 and 6), whereas those samples that had not been exposed to high pH (lanes 3 and 5) showed forms I and II. The diffuse band in Figure 2, lane 4, moved with the bromophenol blue tracking dye, indicating that these DNA fragments had lengths on the order of a few hundred bases (intact ϕX174 DNA contains 5386 base pairs).

Comparison of DNA Breakage by Iron and Cobalt Bleomycins. It is important to establish unequivocally that this novel DNA-nicking activity results exclusively from the effect of light on Co(III) BLM's, rather than from any contaminating species (in particular, iron bleomycin). As shown in Figure 3, the degradation of DNA in the presence of iron bleomycin (freshly prepared from ferrous ammonium sulfate and Blenoxane) led to rapid formation of form III DNA in addition to form II DNA, whereas Co(III) BLM's did not produce significant amounts of form III DNA. Moreover, the DNA cleavage reaction induced by iron bleomycin proceeded with about the same efficiency in the dark as in the light;[2] by

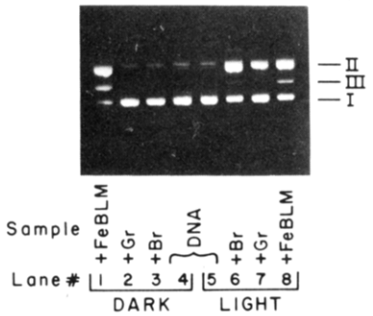

FIGURE 3: Cleavage of ϕX174 RFI DNA: iron bleomycin vs. cobalt bleomycins. The reaction conditions were the same as in Figure 1 except that the solutions were irradiated (lanes 5–8) or incubated in the dark (lanes 1–4) for 2 h with the following additions (final concentration 0.1 μM): lanes 1 and 8, iron bleomycin [freshly prepared from $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and Blenoxane]; lanes 2 and 7, green Co(III) BLM A_2; lanes 3 and 6, brown Co(III) BLM A_2; lanes 4 and 5, none.

comparison, the presence of light is indispensable for the DNA cleavage action of Co(III) BLM's. It is important to note that light and Co(III) BLM's cause degradation of almost as much form I DNA as iron bleomycin under the same conditions.

Additional lines of evidence also rule out the possibility that the observed reactions might be caused by adventitious iron: (1) reducing agents such as 1 mM dithiothreitol showed no effect on the amount of DNA strand breaks induced by light and Co(III) BLM's; (2) when the DNA cleavage reactions were studied under conditions providing the in situ formation of Co(III) BLM's from Co(II) and BLM,[3] the presence of up to 1% iron in the Co(II) solution had no detectable effect on the extent of DNA breakage.

Discussion

Many cobalt complexes undergo photochemical reactions, leading to replacement of a coordinated ligand or to reduction of the Co(III) (Adamson, 1968). The wavelength of the radiation used is important; stimulation of charge-transfer or ligand transitions with near-UV light usually leads to photoreduction of cobalt in complexes such as cobalamins (Pratt, 1972) and cobalt(III) acidopentaammines (Adamson, 1968). The Co(III) BLM's we studied are likely to be similar to cobalamins and cobalt(III) pentaammines in the nature of the ligating atoms around cobalt (Meares et al., 1982; Chang and Meares, unpublished observations), suggesting that photoreduction of cobalt(III) may be a preliminary step in the DNA cleavage reaction. Further study is necessary to clarify the reaction mechanism.

Studies from many laboratories have established that cobalt bleomycin does not enter normal cells efficiently but accumulates selectively in the nuclei of certain types of cancer cells (Nouel et al., 1972; Kono & Kojima, 1972; Poulose et al., 1975; Kahn et al., 1977; Kono et al., 1977) and binds to nuclear DNA (Kono, 1977). Cobalt-57 bleomycin has been used extensively as a radiopharmaceutical to detect cancers in human patients (Poulose et al., 1975; Nouel, 1976; Kahn et al., 1977). In view of our observations, it may be possible to modify these procedures in order to use Co(III) BLM's to selectively destroy tumors in the presence of normal tissue.

[2] At higher concentrations of iron bleomycin ($\sim$10 μM) the light did stimulate DNA cleavage somewhat. A similar observation on light-enhanced DNA cleavage by iron bleomycin has recently been reported (Sugiura et al., 1982).

[3] Reaction mixtures contained (in a total volume of 25 μL) 25 mM Tris–borate, pH 8.1, 190 μM EDTA, 1 μg of ϕX174 RFI DNA, 1 μM BLM, and 400 μM Co(II). Co(II) was added last. In the absence of BLM, 400 μM Co(II) did not induce DNA strand breakage either in the dark or under the light.

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