(i.e., ionic strength) is as yet unclear.

Registry No. d(CGTTATAATGCG)·d(CGCATTATAACG), 89144-59-2.

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Cobalt–Bleomycins and Deoxyribonucleic Acid: Sequence-Dependent Interactions, Action Spectrum for Nicking, and Indifference to Oxygen†

C.-H. Chang and C. F. Meares*

ABSTRACT: Light-induced strand scission of DNA by cobalt–bleomycins is more likely to occur at certain base sequences than others. By use of ^{32}P-end-labeled DNA restriction fragments as the substrates for cleavage, products have been analyzed on high-resolution polyacrylamide gels and compared to those produced by iron–bleomycin. The results indicate that the sites of damage to DNA are similar in both cases: pyrimidine residues located at the 3′ side of a guanine are preferentially attacked. Consistent with the observed nicking specificity, interactions between cobalt–bleomycin and guanine residues in the trinucleotide sequence GGT are revealed in a dimethyl sulfate methylation experiment. The action spectrum for the light-induced DNA cleavage reaction correlates with the absorption spectrum of cobalt–bleomycin in the wavelength range between 330 and 450 nm. In contrast to iron–bleomycin, the extent of DNA degradation by light-activated cobalt–bleomycins appears to be indifferent to the concentration of dissolved oxygen in the reaction medium, and little or no base propenal is produced. Bases (e.g., thymine) are released by both agents. Fluorescence quenching experiments show that apparent binding constants of cobalt–bleomycin complexes with DNA are in the 10^7 M^{-1} range in 25 mM tris(hydroxymethyl)aminomethane, pH 8, 1 mM NaCl, and 1 mM ethylenediaminetetraacetic acid at 25 °C.

Bleomycin (BLM)[1] is the name of a group of glycopeptide antibiotics used clinically in the treatment of cancer (Umezawa et al., 1966; Blum et al., 1973; Crooke & Bradner, 1976); the drug is thought to act in vivo by chemically degrading cellular DNA (Suzuki et al., 1969; Terasima et al., 1970).

Bleomycin forms complexes with transition metal ions, and its biological activity appears to require metal coordination (Sausville et al., 1976). Metallobleomycins thus formed are capable of inducing strand scission of DNA in vitro. For example, iron–bleomycin actively degrades isolated DNA in the presence of molecular oxygen and a variety of reducing agents (Sausville et al., 1976, 1978a,b; Lown & Sim, 1977). Cleavage of DNA by copper–bleomycin (Murugesan et al., 1982; Freedman et al., 1982; C.-H. Chang and C. F. Meares, unpublished data), and by manganese– and nickel–bleomycins as well (C.-H. Chang and C. F., Meares, unpublished data), may also occur under appropriate conditions.

Recently, we have found that cobalt(III)–bleomycins can cause strand scission of DNA in the presence of light (Chang & Meares, 1982). There are several different cobalt(III)–bleomycins, including an orange complex in which all six ligands to cobalt are supplied by bleomycin, a green complex

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[1] Abbreviations: BLM, bleomycin; Co(III)–BLM, a complex of cobalt(III) with bleomycin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; bp, base pair.

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in which hydroperoxide (OOH^-) acts as a ligand, a brown aquo complex with water as a ligand, a brown formate complex, and so on (Chang et al., 1983). Here we describe experiments which characterize several basic features of DNA nicking by cobalt(III)–bleomycin, including the strength of the association of Co(III)–BLMs with DNA, the preference for certain DNA base sequences, the dependence of the light-induced nicking reaction on wavelength, the lack of dependence on oxygen, and some aspects of the chemistry of cleavage. Where appropriate, comparisons are made to iron–bleomycin.

Materials and Methods

Blenoxane (bleomycin sulfate) was a gift from Bristol Laboratories. Cobalt(III)–bleomycins were prepared and purified as described previously (Chang & Meares, 1982; Chang et al., 1983; DeRiemer et al., 1979). ϕX174 RF DNA (>95% form I), [*methyl*-^{3}H]thymine-labeled ϕX174 RF DNA, T4 polynucleotide kinase, and restriction enzymes *Alu*I and *Hinc*II were purchased from Bethesda Research Laboratories. Calf thymus DNA (type I) and bacterial alkaline phosphatase were from Sigma, [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) was from Amersham, and phenazine methosulfate was from Aldrich. Acrylamide (Sigma) was recrystallized before use. Other chemicals were the purest commercially available reagents. Oxygen and argon were obtained from Liquid Carbonic. In some experiments, argon was scrubbed with a powdered zinc suspension in 1 mM phenazine methosulfate solution (Burger et al., 1979). Enzymatic reactions were performed according to the procedures provided by suppliers. Distilled, deionized water was used throughout.

Preparation of Radiolabeled DNA Fragments. Wild-type bacteriophage T7 DNA, prepared by procedures similar to those of Studier (1969), was digested with restriction endonuclease *Alu*I to completion. The resulting fragments, after treatment with bacterial alkaline phosphatase, were separated by electrophoresis on a 5% polyacrylamide, 40 cm long, 3 mm thick, preparative gel. Electrophoresis was carried out for 24 h at 280 V in 0.089 M Tris–borate and 1 mM EDTA, pH 8.1. The 836 base pair (bp) fragment, which contained the early promoter region, was isolated (Hsieh & Wang, 1976; Siebenlist, 1979), labeled at the 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase, and digested with restriction endonuclease *Hinc*II. The two end-labeled fragments, 68 and 121 base pairs in length, were then isolated by electrophoresis on an 8% polyacrylamide gel. Recovery and purification of DNA fragments from the polyacrylamide gel were performed according to Maxam & Gilbert (1980). The nucleotide sequences of the 68- and 121-bp fragments (Figure 1) were determined by the method of Maxam & Gilbert (1980) and verified by comparison with the sequence of Dunn & Studier (1981). Autoradiography was carried out at −70 °C on Kodak XAR-5 film with an intensifying screen.

DNA Reactions. The reaction conditions for DNA cleavage by light-activated cobalt–bleomycins were similar to those described previously (Chang & Meares, 1982). For inhibition studies, free radical scavengers were added to the reaction mixture at the following concentrations: dimethyl sulfoxide, 2.8–0.28 M; isopropyl alcohol, 2.6 M; methanol, 4.6 M; ascorbic acid, 20 mM; histidine, 5 mM; xanthine, 0.5 mM.

The dimethyl sulfate methylation reactions were carried out in the dark in the absence and presence of cobalt–bleomycin, following the procedure for G-specific reaction described by Maxam & Gilbert (1980). For DNA nicking under anoxic conditions, reaction mixtures in rubber septum stoppered vials were purged with argon for 10 min prior to irradiation. Further experimental details are given in the figure legends.

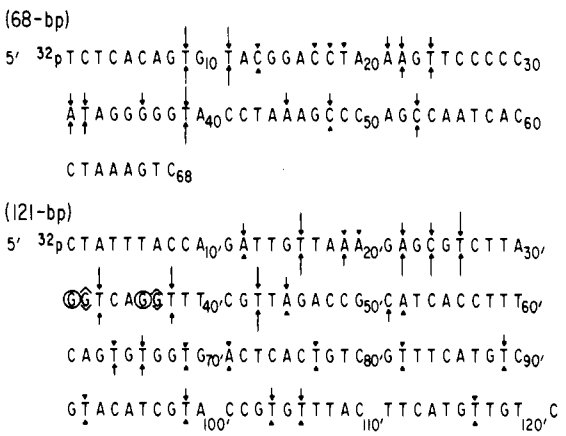

FIGURE 1: Nucleotide sequence of the 68- and 121-bp fragments. Only the strand labeled with ^{32}P is shown; the primed and unprimed numbers distinguish these two fragments. The 5′ termini of the 68- and 121-bp fragments correspond to positions 1 and 836, respectively, in the map of Dunn & Studier (1981). The observed sites of cleavage by cobalt–bleomycins and light are indicated by arrows (↓); the sites of cleavage by iron–bleomycin (Blenoxane plus dithiothreitol) are also shown (↑). Arrow length reflects relative intensity of the fragment band on the autoradiograms in Figures 2A and 3A. Guanine residues at which enhancement of dimethyl sulfate methylation occurs are circled, and those showing relative protection from methylation are bracketed.

Action Spectrum. The solutions to be irradiated were placed in 3-mm fluorescence cells and exposed to light of the following wavelengths: 330, 350, 370, 390, 410, 430, 450, 488, 514, 568, and 633 nm. The irradiatioin time was controlled so that (optically thin) samples irradiated at each chosen wavelength would receive the same total number of photons (2.3×10^9 photons/cm^2). The light source was 150-W xenon lamp in a Perkin-Elmer MPF-44B fluorescence spectrophotometer. Light intensities were measured by means of a Scientech 202 disc calorimeter. Appropriate optical cutoff filters were used to eliminate contamination of the irradiating light by higher orders from the monochromator.

Fluorescence Quenching Experiments. Samples containing a fixed amount of cobalt–bleomycin (1–4 μM) and different concentrations of added DNA were prepared in separate vials and kept in the dark prior to measurements. Conditions were chosen so that each set of experiments covered as wide a range of percent bound Co(III)–BLM as practical. A minimum concentration of 1 μM Co(III)–BLM was needed for adequate fluorescence intensity. Fluorescence intensities were measured at 365 nm on Perkin-Elmer fluorometers (Model MPF-44B or MPF-2A), with excitation at 310 nm. The binding parameters were obtained from the fluorescence data by a method similar to Chien et al. (1977). The fraction (f) of Co(III)–BLM bound at a given fluorescence reading (F) is given by $f = (F_0 - F)/(F_0 - F_\infty)$, where F_0 and F_∞ are the fluorescence intensities of the free and bound Co(III)–BLM, respectively. From the values of f and the known input concentration of Co(III)–BLM, the concentrations of free and bound Co(III)–BLM, B_f and B_b, respectively, were calculated. A least-squares fit of the plot of $1/B_f$ vs. $[PO_4]_0/B_b$, where $[PO_4]_0$ is the total molar DNA–phosphate concentration, yielded the apparent binding constant (K_{app}) from the y intercept and the apparent number of bases per binding site ($2N$) from the x intercept (Kasai et al., 1978). Typical plots are shown in Figure 7, and the results are listed in Table I. The largest binding constant in Table I, $K_{app} = 5 \times 10^7$ M^{-1} for aquo-Co(III)–BLMs at ionic strength 0.02, is subject to some uncertainty since it had to be determined from 10^{-6} M Co(III)–BLM solutions (rather than 10^{-7} M); the true value may

be somewhat higher than 5×10^7 M^{-1}. The binding constants reported by Kakinuma & Orii (1982) for the green and aquo complexes are about an order of magnitude smaller than ours; this may be due to their use of higher concentrations of Co(III)–BLMs.

Molar absorptivity values of $(1.80 \pm 0.05) \times 10^4$ M^{-1} cm^{-1} at 290 nm were found for both the green and the orange complexes, using an Instrumentation Laboratory 751 atomic absorption/emission spectrometer to measure the content of cobalt in solutions of each pure Co(III)–BLM of known absorbance. A molar absorptivity of 6.4×10^3 M^{-1} cm^{-1} at 260 nm was used to calculate the concentration of DNA nucleotide residues (=DNA phosphate).

Release of Free Bases and Base Propenals. Reaction mixtures containing 40 mM Tris-HCl, pH 8, and 1 mM calf thymus DNA were incubated with 1 mM iron(II)–bleomycin [freshly prepared by using $Fe(NH_4)_2(SO_4)_2$] or cobalt–bleomycin in the absence or presence of 366-nm irradiation for 1 h at room temperature. Aliquots (0.1 mL) were then mixed with 1 mL of 0.6% thiobarbituric acid (Aldrich) and heated at 95 °C for 20 min to assay for the presence of base propenals (Giloni et al., 1981). Aliquots were also analyzed for the presence of free bases by thin-layer chromatography (TLC) on silica gel plates using 10% ammonium acetate/methanol (50:50) or ethyl acetate/isopropyl alcohol/H_2O (74:17:9) as the solvent. When [*methyl*-^{3}H]thymine-labeled DNA was used as the substrate, radioactive species were located on silica gel plates by liquid scintillation counting of sections (10 × 10 mm). Release of radiolabeled thymine was also detected by high-performance liquid chromatography (HPLC) on a C_{18} column (Alltech) using 0.05 M ammonium phosphate (pH 4.5) as the solvent (Kappen et al., 1982). Fractions were mixed with Aquasol (New England Nuclear) for liquid scintillation counting.

Results

Sequence-Dependent Cleavage. The results of experiments demonstrating preferred sites for cleavage of DNA by light-activated cobalt–bleomycins are presented in the autoradiograms shown in Figures 2 and 3. The experimental protocols involved treatment of the singly end-labeled 68- or 121-bp fragment either with cobalt–bleomycin or with Blenoxane plus dithiothreitol, according to the reaction conditions specified in the legends. It is assumed that Blenoxane combines with adventitious metal ions (particularly iron) in the medium (Sausville et al., 1978a). To facilitate the identification of the sites of damage to DNA, four sets of base-specific cleavage products (G, A+G, C, C+T) were prepared by the method of Maxam & Gilbert (1980) and run in parallel lanes as standard markers.

As shown in Figure 2A, the 68-bp fragment was broken by cobalt–bleomycins in the presence of light into shorter oligonucleotides which were revealed as discrete bands on the autoradiogram (lanes 7–9). Under similar experimental conditions, incubation of DNA with cobalt–bleomycins in the dark (lanes 2–4) or without cobalt–bleomycins both in the dark and in the presence of light (lanes 1 and 10, respectively) resulted in no cleavage of the double-stranded fragment. In contrast, inspection of the DNA cleavage products resulting from the iron-mediated bleomycin reactions (lanes 5 and 6) indicates that the additional irradiation did not produce a noticeable change in the extent of DNA degradation (Chang & Meares, 1982). The preferred sites of DNA damage by iron-bleomycin were the same under both light and dark conditions and were quite similar to those produced by cobalt–bleomycins upon light activation.

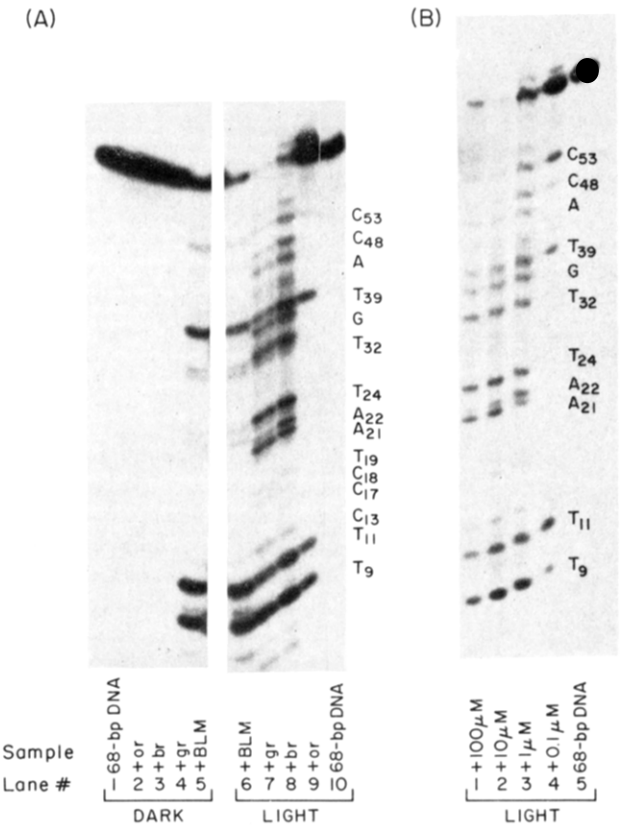

FIGURE 2: Sequence-dependent cleavage of a 68-bp T7 DNA fragment by light-activated cobalt–bleomycins. Labels in the margin indicate nucleotide residues at which cleavage occurs. (A) Reaction mixtures contained (in a total volume of 10 μL) 25 mM Tris–borate, pH 8.1, 190 μM EDTA, and 5′-end-labeled double-stranded restriction fragment, with the following additions (final concentration 10 μM): lanes 1 and 10, none; lanes 2 and 9, orange Co(III)–BLM A_2; lanes 3 and 8, brown Co(III)–BLM A_2; lanes 4 and 7, green Co(III)–BLM A_2; lanes 5 and 6, Blenoxane plus 1 mM dithiothreitol. Samples were incubated in the dark (lanes 1–5) or irradiated with a 366-nm lamp (lanes 6–10) for 1 h. The DNA in each sample was then twice precipitated with ethanol, dried briefly in a vacuum desiccator, and dissolved in 5 μL of a loading buffer containing 80% (v/v) redistilled formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanole, and 0.1% bromophenol blue. The DNA was denatured by heating at 90 °C for 1 min before transferring to the gel well. The sequencing gel (40 × 20 × 0.03 cm) was 8% polyacrylamide containing 8 M urea. (B) Reaction conditions were the same as in (A) except that the following concentrations of green Co(III)–BLM A_2 were added: lane 1, 100 μM; lane 2, 10 μM; lane 3, 1 μM; lane 4, 0.1 μM; lane 5, none. All five samples were irradiated for 1 h. Note that the set of products shows little dependence on the amount of undegraded DNA remaining, except that fewer long fragments are present in the more extensively cleaved preparations (e.g., lane 2 vs. lane 3).

The effect of increasing concentration of cobalt–bleomycins on the light-induced strand scission of DNA is shown in Figure 2B. The same set of site-specific cleavage products was observed for the green Co(III)–BLM over a range of concentrations from 1 to 100 μM (lanes 1–3); at lower concentration (ca. 0.1 μM) there were fewer cleavage products produced (lane 4), but the same major products are evident. The set of products was practically the same, whether there was little (lane 2) or much (lane 3) undegraded DNA remaining in the reaction mixture, except that fewer long fragments remained in the more extensively cleaved preparations.

By use of the 121-bp fragment as the substrate, the sites of damage to DNA by light-activated cobalt–bleomycins were compared to those produced by iron–bleomycin in Figure 3A. As for the 68-bp fragment, similar sites of cleavage are revealed for iron–bleomycin and cobalt–bleomycin by the pro-

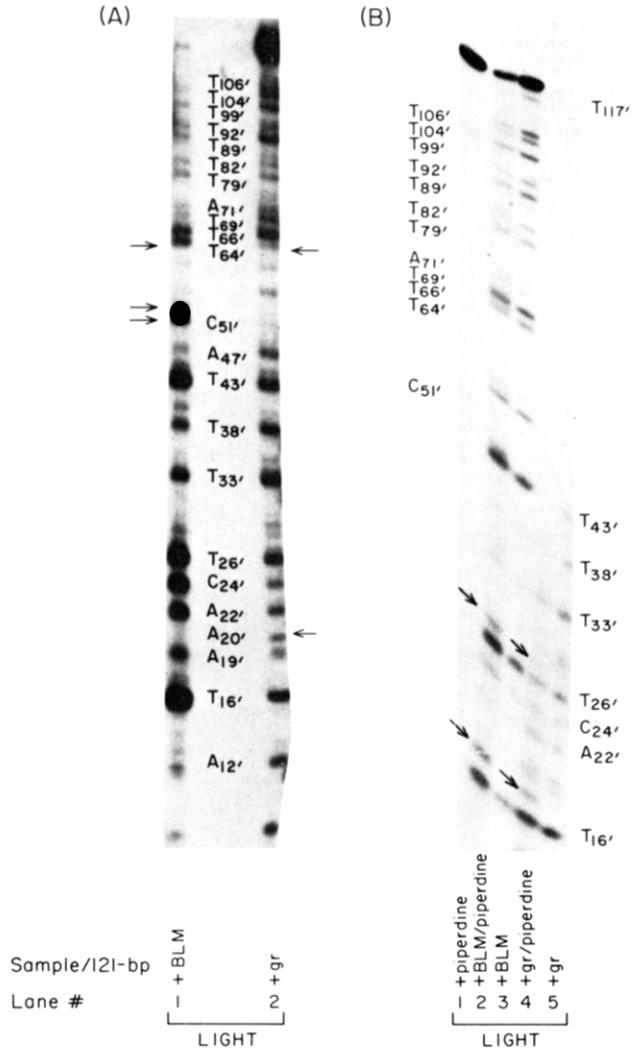

FIGURE 3: (A) Sites of DNA damage by light-activated cobalt–bleomycins as compared to those caused by (iron–) bleomycin, for the 121-bp fragment. Sites where cleavage patterns differ are indicated by arrows in the margin. The 121-bp fragment was reacted with either Blenoxane plus dithiothreitol (lane 1) or green Co(III)–BLM A_2 (1 μM, lane 2) under the conditions described in Figure 2. Irradiation was for 30 min. (B) Upon base hydrolysis of the drug-treated 121-bp restriction fragment, additional cleavage at alkali-labile sites resulted in new bands in the sequencing gel. The new bands observed in lanes 2 and 4 are marked by arrows; their electrophoretic mobilities were similar to the $T_{16'}$ and $T_{26'}$ markers produced by the Maxam–Gilbert procedure. Reaction conditions were the same as in (A), with the following additions: lane 1, none; lanes 2 and 3, Blenoxane plus dithiothreitol; lanes 4 and 5, green Co(III)–BLM A_2. After irradiation for 1 h and ethanol precipitation of DNA as described in Figure 2A, samples 1, 2, and 4 were hydrolyzed in 1 M piperidine for 30 min at 90 °C and lyophilized before dissolving in the loading buffer.

duction of similar distributions of cleaved DNAs. The 121-bp fragment was cleaved by both metallobleomycins at all thymine residues located at the 3′ side of a guanine. Cleavage at the GA and GC sequences, in which the nucleotide residue at the 3′ side of guanine was attacked, was also evident. Minor cleavage products with DNA breaking at some AA, AC, and AT sequences could also be detected. Slight, but reproducible, differences in the site specificity of DNA cleavage by iron–bleomycin and cobalt–bleomycin were observed. Some of these are marked by the arrows in the margin of Figure 3A.

DNA oligonucleotides produced by the action of bleomycin contain glycolic acid residues esterified to the 3′-phosphoryl termini (Giloni et al., 1981). These oligonucleotides migrate slightly faster on gels than the corresponding oligonucleotide 3′-phosphates produced by chemical degradation (Takeshita et al., 1978). Since the DNA cleavage products obtained by the action of light and Co(III)–BLM have the same electrophoretic mobilities as those observed for the corresponding fragments cleaved at the same sites by Blenoxane plus dithiothreitol, it is possible that these cleavage products also contain glycolic acid residues linked to their 3′-phosphoryl termini.

Besides breaking DNA strands, metallobleomycins also produce alkali-labile sites on DNA, which are thought to result from loss of DNA bases (Povirk et al., 1977; Lloyd et al., 1978; Ross & Moses, 1978; Chang & Meares, 1982). It is known that the brief alkaline treatment (0.1 N NaOH, 90 °C, 1 min) used to prepare samples for gel electrophoresis does not cause significant breakage of DNA chains at apurinic sites (D'andrea & Haseltine, 1978). Treatment of the 121-bp DNA restriction fragment with either Blenoxane/dithiothreitol or Co(III)–BLM/light, followed by hydrolysis at 90 °C for 30 min in 1 M piperidine, led to the production of two new DNA fragments (Figure 3B). Each of these new fragments migrated just behind a prominent fragment from the metallobleomycin cleavage reaction ($T_{16'}$ or $T_{26'}$) and each new fragment had approximately the same electrophoretic mobility as the markers ($T_{16'}$ or $T_{26'}$) produced by the Maxam–Gilbert procedure (data not shown). These results suggest that the new fragments have 3′-phosphate termini like the markers, as would be expected if they were produced by alkaline hydrolysis of apyrimidinic sites (Maxam & Gilbert, 1980). The similarity of the results obtained, whether with Blenoxane/dithiothreitol or with Co(III)–BLM/light, is noteworthy.

Points of Interaction between Co(III)–BLM and DNA Residues. The alkylating agent dimethyl sulfate methylates the purine residues in DNA at the N-7 of guanine and, less rapidly, at the N-3 of adenine (Lawley & Brooks, 1962). The methylation method has been successfully applied to the study of DNA–protein binding interactions (Johnsrud, 1978; Ogata & Gilbert, 1978; Siebenlist & Gilbert, 1980). As proteins bound to DNA perturb the methylation reaction at the binding site, points of close contact to certain purine residues can be identified by comparing the extent of methylation at each purine in the absence and presence of a particular DNA-binding molecule. Either protection from, or enhancement of, methylation may be observed for purines in the contact region.

The autoradiograph in Figure 4 shows the effect of bound cobalt–bleomycin on the methylation pattern of the 121-bp fragment. The most obvious effects occur at the two GGT sequences in this fragment. Binding of Co(III)–BLM significantly enhances methylation of $G_{31'}$ relative to methylation of $G_{32'}$, which is adjacent to the cleavage site at $T_{33'}$; the same may be said for residues $G_{36'}$, $G_{37'}$, and $T_{38'}$.

Action Spectrum. The results of varying the wavelength of irradiation on the efficiency of DNA cleavage by cobalt–bleomycins are presented in Figure 5. When the superhelical ϕX174 RFI DNA was used as the substrate, the extent of DNA breakage at each selected wavelength was assayed by agarose gel electrophoresis and quantitated by densitometry as previously described (Chang & Meares, 1982). Under the reaction conditions examined, the action spectrum correlates with the absorption spectrum of cobalt–bleomycins in the wavelength range between 330 and 450 nm, and a small amount of nicked DNA can be detected after irradiation with wavelengths longer than 450 nm. However, with a singly end-labeled linear double-stranded DNA fragment as the substrate, irradiation near the lowest energy ligand-field transition with 614-nm light, from either a 150-W xenon lamp

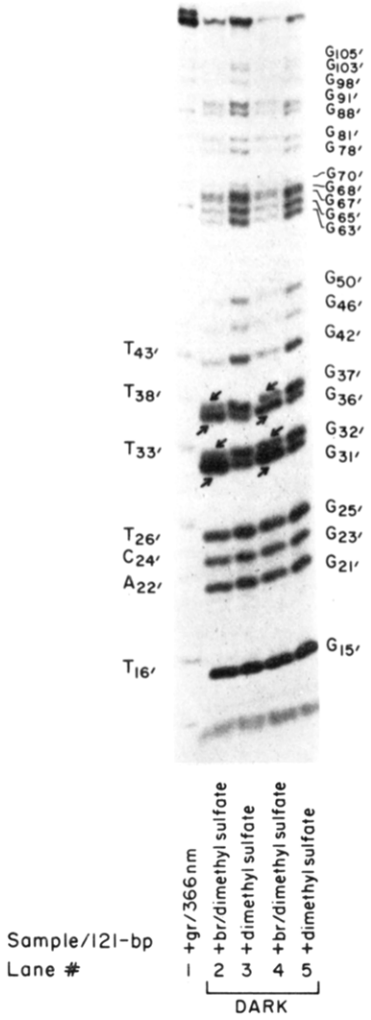

FIGURE 4: Autoradiograph showing effects of bound cobalt–bleomycin on DNA methylation. Arrows point to residues at which susceptibility to methylation is strikingly different in the presence of Co(III)–BLM. The 121-bp fragment was reacted with dimethyl sulfate in the absence (lanes 3 and 5) or presence (lanes 2 and 4) of brown Co(III)–BLM A_2 (final concentration 100 μM). Samples were methylated in the dark for 5 min (lanes 2 and 3) or 10 min (lanes 4 and 5) and then processed as for the "G" reaction (Maxam & Gilbert, 1980). The sites of DNA cleavage produced by green Co(III)–BLM A_2 (1 μM, 1-h irradiation) are shown in lane 1 for comparison. Labels in the margin refer to residues at which cleavage occurs.

(for up to 5 h) or a 0.5-W dye laser source (for 10 min), did not result in any noticeable strand cleavage.

Effect of Oxygen and Free Radical Scavengers. The extent of DNA degradation by light-activated cobalt–bleomycins appears to be indifferent to the concentration of dissolved oxygen in the reaction medium. Figure 6 demonstrates that whereas the iron-mediated bleomycin reaction more actively degrades DNA in the presence of oxygen (lanes 2 and 3 vs. lanes 10 and 11), the light-induced DNA cleavage by cobalt–bleomycins proceeds with about the same efficiency in both oxygen-deprived (lanes 4–6) and oxygen-saturated (lanes 7–9) reaction mixtures. Results similar to those shown in Figure 6 were also observed under anoxic conditions in which argon was further scrubbed with a phenazine methosulfate/zinc suspension. Moreover, for all free radical scavengers examined in this study, little or no inhibitory effect was observed on the light-induced DNA cleavage reaction.

Base Release vs. Base-Propenal Formation. Release of thymine could be detected by HPLC or TLC under conditions for which degradation of DNA by cobalt–bleomycin and light

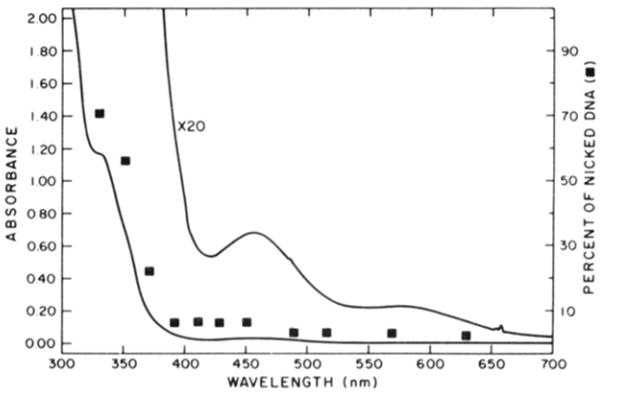

FIGURE 5: Action spectrum for DNA nicking by cobalt–bleomycin, compared to the absorption spectrum of cobalt–bleomycin. Optically thin samples containing 10 μM aquocobalt–bleomycin and 20 μg/mL ϕX174 RFI DNA in 25 mM Tris–borate and 190 μM EDTA, pH 8.1, were irradiated with a total of 2.3×10^{19} photons/cm^2 at each selected wavelength. (■) Percent of nicked molecules as determined by densitometry.

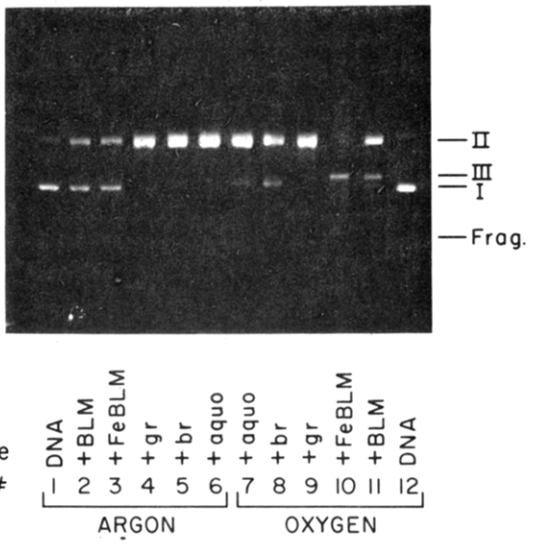

FIGURE 6: Effect of dissolved oxygen on light-induced cleavage of DNA by iron– and cobalt–bleomycins. Reaction mixtures contained (in a total volume of 25 μL) 25 mM Tris–borate, pH 8.1, 190 μM EDTA, and 1 μg of ϕX174 RFI (>95%) DNA with the following additions (final concentration 1 μM): lanes 1 and 12, none; lanes 2 and 11, Blenoxane; lanes 3 and 10, Blenoxane + Fe(II); lanes 4 and 9, green Co(III)–BLM A_2; lanes 5 and 8, brown Co(III)–BLM A_2; lanes 6 and 7, aquo-Co(III)–BLM A_2. Six samples (lanes 1–3 and 10–12) also contained 1 mM dithiothreitol. Samples were equilibrated with argon (lanes 1–6) or oxygen (lanes 7–12) and irradiated with a 366-nm lamp for 1 h. Gel was stained with ethidium after electrophoresis. Note the similarities between lanes 4–6 and lanes 7–9 and the differences between lanes 2–3 and lanes 10–11. Significant quantities of smaller DNA fragments were present only in lanes 10 and 11.

was observed. In the absence of 366-nm irradiation, thymine was not detected by either method. Base propenals were found only in the iron–bleomycin reaction mixtures; there was not a detectable amount of any thiobarbituric acid reactive substance present in the irradiated solutions of cobalt–bleomycin and calf thymus DNA. Consistent with the latter result, no radioactive species other than tritiated thymine was detected by HPLC when [^{3}H]thymine-labeled DNA was used as the substrate.

Binding Parameters of Cobalt–Bleomycins to Calf Thymus DNA. The strength of interaction between three cobalt–bleomycins and calf thymus DNA at different salt concentrations was measured by fluorescence quenching. Least-squares fits of the observed fluorescence data (Figure 7)

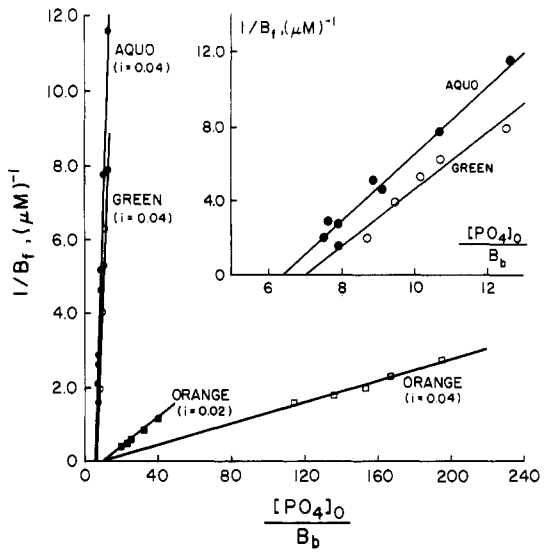

FIGURE 7: Determination of binding parameters of cobalt(III)–bleomycins to calf thymus DNA. The ionic strength of the medium is indicated in parentheses. The insert shows the data for the aquo and green Co(III)–BLM plotted on a different scale. The orange Co(III)–BLM binds 100-fold less strongly to DNA than the other Co(III)–BLMs studied.

Table I: Binding Parameters of Cobalt-Bleomycins to Calf Thymus DNA at 298 K, pH 8.0[a]

Co(III)–BLM species[b]	ionic strength, i	apparent binding constant, K_{app} (M^{-1})	apparent no. of base pairs per binding site, N
aquo[c]	0.02	$(5 \pm 1) \times 10^7$	3 ± 1
A_2 orange	0.02	$(4.7 \pm 0.4) \times 10^5$	5
aquo[c]	0.04	$(11 \pm 1) \times 10^6$	3
A_2 green	0.04	$(13 \pm 3) \times 10^6$	4
A_2 orange	0.04	$(1.5 \pm 0.2) \times 10^5$	5
aquo[c]	0.16	$(1.9 \pm 0.3) \times 10^6$	13

[a] Concentration of DNA is specified as moles of phosphate per liter. [b] The aquo-Co(III)–BLM contains a water molecule (or OH^- ion) coordinated to cobalt; the orange Co(III)–BLM has all six ligands to cobalt supplied by bleomycin; the green Co(III)–BLM contains a hydroperoxide (OOH^-) ion coordinated to cobalt. [c] Mixture of A_2 and B_2.

yielded the apparent association constants (K_{app}) and the number of base pairs per binding site (N) shown in Table I. The aquo and hydroperoxo (green) cobalt–bleomycins display binding constants of approximately the same magnitude (ca. 10^7 M^{-1}) in ionic strength 0.04 buffer, whereas the orange complex has a considerably smaller binding constant (ca. 10^5 M^{-1}) under those conditions. The binding constants decrease as the ionic strength increases, as would be expected if Co(III)–BLM competes with other cations for binding to DNA.

Discussion

DNA Sequence Dependence. From Figures 1, 2A (lanes 6 and 7), and 3A (lanes 1 and 2) it appears that the populations of DNA fragments produced by cleavage with Co(III)–BLM and light are not identical with those produced by Blenoxane. However, closer examination reveals a common preference for causing DNA strand breakage at the 3′ side of guanine residues, particularly if a thymine residue is located there. This has previously been noted for iron–bleomycin (D'andrea & Haseltine, 1978; Takeshita et al., 1978, 1981; Kross et al., 1982; Mirabelli et al., 1982, 1983; Sugiura et al., 1983).

The experiments in Figure 2A (lanes 7–9) imply that changing the sixth ligand of Co(III)–BLM from hydroperoxide (lane 7) to formate (lane 8) has little effect on the sequence specificity for DNA nicking and that orange Co(III)–BLM (lane 9) is much less active than the others (most of the DNA is intact at the top of lane 9). Figure 2B shows that adding Co(III)–BLM beyond the amount required to saturate its binding sites ($K_{app} \simeq 10^7$ M^{-1}) does not lead to significant amounts of cleavage at new positions in the DNA chain. Figure 3B indicates that both metallobleomycins can produce either strand breakage or alkali lability at the same DNA sites, and it suggests that the same types of DNA 3′ termini are produced by both metallobleomycins. Sites of nicking are summarized in Figure 1. The picture which emerges from these experiments is that of a particular set of binding sites on DNA (usually involving guanine) at which cleavage is most likely to occur.

Figure 4 shows that Co(III)–BLM binding to DNA can have different effects on the susceptibility to methylation of two adjacent guanine residues, enhancing the relative reactivity of the residue distal to the bleomycin cleavage site.

Action Spectrum. As Figure 5 shows, light in the near-UV region is very effective at stimulating DNA nicking by cobalt–bleomycin. In the presence or absence of DNA, these irradiation conditions do not cause detectable decomposition of the Co(III)–BLM itself. It is interesting to note that the most effective wavelengths for DNA nicking are those which are likely to excite ligand to metal charge-transfer electronic transitions in the Co(III)–BLM (Adamson, 1968). This is consistent with the possibility that DNA nicking follows photoreduction.

Oxygen. Figure 6 shows that DNA nicking by Co(III)–BLM is not decreased by removal of oxygen from the medium; this is in marked contrast to iron–bleomycin. It suggests that the two metallobleomycins have nicking mechanisms which are quantitatively, and perhaps qualitatively, different. Co(III)–BLM either does not require oxygen at all or (perhaps) uses traces of oxygen with great efficiency.

A further difference is the lack of base-propenal formation associated with DNA nicking by Co(III)–BLM, relative to iron–bleomycin. Hydrogen abstraction from C-4′ of deoxyribose, thought to be an important pathway of DNA cleavage by iron–bleomycin (Takeshita et al., 1978; Giloni et al., 1981; Wu et al., 1983), requires oxygen to lead further to base-propenal formation (Burger et al., 1981, 1982a,b). Thus our data suggest a different chemical mechanism for Co(III)–BLM. However, because both agents produce DNA fragments with similar properties, and both cause the release of bases such as thymine, there appear to be common mechanistic features as well.

Cobalt–Bleomycins. Among the cobalt–bleomycins, the hydroperoxo (green) and aquo and formato (brown) complexes are very similar in binding to DNA and nicking it, while the orange complex is different. The orange complex binds 100× less strongly, shows much less nicking activity, and also is not taken up as well by tumors in experimental animals (DeRiemer et al., 1979; C.-H. Chang et al., unpublished observations). The green and orange Co(III)–BLMs elute very similarly from ion-exchange columns and appear to have the same net electric charge; this suggests that their different properties result from different arrangements of bleomycin side chains around the central cobalt ion. Understanding these differences should provide useful insight into the mechanism of action of bleomycin.

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