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Copper(I)-Bleomycin: Structurally Unique Complex That Mediates Oxidative DNA Strand Scission†

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Received May 30, 1984

ABSTRACT: Copper(I)-bleomycin [Cu(I)·BLM] was characterized in detail by ^{13}C and ^{1}H NMR. Unequivocal chemical shift assignments for Cu(I)·BLM and Cu(I)·BLM·CO were made by two-dimensional ^{1}H-^{13}C correlated spectroscopy and by utilizing the observation that Cu(I)·BLM was in rapid equilibrium with Cu(I) and metal-free bleomycin, such that individual resonances in the spectra of BLM and Cu(I)·BLM could be correlated. The binding of Cu(I) by bleomycin involves the β-aminoalaninamide and pyrimidinyl moieties, and possibly the imidazole, but not N^{α} of β-hydroxyhistidine. Although no DNA strand scission by Cu(II)·BLM could be demonstrated in the absence of dithiothreitol, in the presence of this reducing agent substantial degradation of [^{3}H]DNA was observed, as was strand scission of cccDNA. DNA degradation by Cu(I)·BLM was shown not to depend on contaminating Fe(II) and not to result in the formation of thymine propenal; the probable reason(s) for the lack of observed DNA degradation in earlier studies employing Cu(II)·BLM and dithiothreitol was (were) also identified. DNA strand scission was also noted under anaerobic conditions when Cu(II)·BLM and iodosobenzene were employed. If it is assumed that the mechanism of DNA degradation in this case is the same as that under aerobic conditions (i.e., with Cu(I)·BLM + O_2 in the presence of dithiothreitol), then Cu·BLM must be capable of functioning as a monooxygenase in its degradation of DNA.

The bleomycins are a structurally related group of antitumor antibiotics capable of inhibiting the growth of transformed cells in vitro and in vivo (Umezawa, 1971). Clinically, the bleomycins are employed for the treatment of squamous cell carcinomas and malignant lymphomas (Carter, 1978; Crooke, 1978; Umezawa, 1979). Interestingly, while the bleomycins are biosynthesized as copper chelates by *Streptomyces verticillus* (Umezawa et al., 1966), and both the Cu(II) and metal-free bleomycins can inhibit the growth of tumor cells in culture (Antholine et al., 1982; Ishizuka et al., 1967; Rao et al., 1980; Umezawa, 1971) and in experimental animals, the therapeutic effect mediated by bleomycin has been attributed to the ability of an Fe(II) complex of bleomycin to degrade DNA in an O_2-dependent transformation.

Umezawa and his co-workers have suggested that the activation of copper(II)-bleomycin (Cu(II)·BLM)[1] in situ involves initial conversion to Cu(I)·BLM; subsequent removal of Cu(I) by specific cellular metal binding proteins would then provide metal-free bleomycin, which could be transformed to the putative "active complex" following chelation of Fe(II)

† This work was supported at the University of Virginia by National Institutes of Health Research Grants CA-27603 and CA-29235 and at the University of California by National Resonance Laboratory Research Resource Center Grants NSF-GP-23633 and NIH-RR-00711.

‡ Fellow of the Economic Development Administration of Puerto Rico. This study was carried out as part of the doctoral program in absentia from Massachusetts Institute of Technology.

§ National Institutes of Health Research Career Development Awardee, 1979-1984.

[1] Abbreviations: BLM, bleomycin; TCA, trichloroacetic acid; 2-D, two dimensional; Me_2SO, dimethyl sulfoxide; CO, carbon monoxide; Me_4Si, tetramethylsilane; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/85/0424-0081$01.50/0

(Takahashi et al., 1977; Umezawa, 1977, 1979). Recently, we described experiments with Cu(I)·BLM which suggested that this metallobleomycin might also mediate O_2-dependent DNA degradation (Oppenheimer et al., 1981), and Chang and Meares have reported that Co(III)·BLM is also capable of effecting DNA strand scission in a transformation that is *hν* dependent (Chang & Meares, 1982). The possibility that some metallobleomycin other than Fe(II)·BLM is actually responsible for the therapeutic effects of bleomycin prompts us to report more fully our findings pertinent to the structure of Cu(I)·BLM and its ability to effect DNA degradation.

Presently, we analyze the ^1H NMR and ^{13}C NMR spectra of Cu(I)·BLM and describe the effect of CO and dithiothreitol on this binary complex, as well as the characteristics of drug–metal interaction. Also described are experimental conditions under which Cu(I)·BLM mediates O_2-dependent DNA degradation. DNA strand scission by Cu(II)·BLM in the presence of the oxygen surrogate iodosobenzene is also demonstrated, and the mechanistic implications of this observation are discussed.

Experimental Procedures

Materials

Electrophoresis-grade agarose was purchased from Bethesda Research Laboratories; glass fiber disks were from Schleicher & Schuell. Chelex-100 resin was purchased from Bio-Rad Laboratories. CuOAc was obtained from Alfa Products and shown to contain about 0.045% Fe by atomic absorption spectroscopy. SV-40 form I DNA was purchased from Bethesda Research Laboratories; cccPM-2 [^{3}H]DNA (21×10^6 cpm/μmol) was prepared as described (Espejo & Canelo, 1968). Blenoxane was obtained from Bristol Laboratories through the courtesy of Dr. William Bradner and was fractionated as described (Chen et al., 1977; Oppenheimer et al., 1979b) to provide bleomycin A_2.

Methods

NMR Spectra. Proton-decoupled, natural abundance ^{13}C NMR spectra were obtained at 20 MHz on a Varian FT-80 NMR spectrometer and at 60.3 MHz. For experiments conducted at 20 MHz the spectral width was 4000 Hz, and typically 80 000 individuals transients were accumulated by using a 45° pulse and 0.5 s between acquisitions. Samples containing Cu(I) were prepared as described previously (Oppenheimer et al., 1981) by using 10-mm tubes with a final blenoxane concentration of 50 mM in 1.5 mL. Dioxane (0.1 M) was used as an internal standard (67.8 ppm from Me_4Si). Higher field natural abundance ^{13}C NMR spectra were obtained on a multinuclei 240-MHz NMR spectrometer equipped with a Nicolet 1180 computer, a 293-B pulse programmer, and a computer-controlled decoupler. The two-dimensional ^1H–^{13}C correlated experiments employed the phase-sensitive pulse sequence of Bax & Morris (1981), thus taking advantage of quadrature detection and a 2-fold increase in digital resolution over conventional sequences. The ^1H 90° pulse was measured directly for each sample by using the decoupler coil for detection. All experiments were conducted with a final blenoxane concentration of 70 mM. For the ^1H–^{13}C correlated experiments the spectral width in the ^{13}C dimension was 103 ppm (6211 Hz at 60.3 MHz) and covered the entire region upfield from the anomeric carbons of the gulose and mannose moieties (from 110 to 1 ppm). Typically, 120 free induction decays were accumulated with 4K data points for each individual spectrum in the ^{13}C dimension, and 256 separate spectral sets were obtained in the second (^1H) dimension. Final data processing gave a digital resolution of 3 Hz in the ^{13}C dimension and 10 Hz in the ^1H dimension. Chemical shifts in both dimensions were referenced to the dimethyl sulfonium resonance (^{13}C = 27.4 ppm; ^1H = 2.95 ppm) and were based on the shifts observed in the corresponding one-dimensional spectra. The copper complexes were prepared in 15% D_2O to provide an internal lock signal and contained 50 mM cacodylate buffer with a final pH of 7.2. Cu(I) was generated in situ from Cu(II) by addition of dithionite under anaerobic conditions. The carbon monoxide complex was formed by bubbling purified CO (Liquid Carbonics) through the solution of Cu(I)·BLM for 10 min.

^1H NMR spectra were obtained at 360 MHz on a Bruker HXS-360 NMR spectrometer equipped with a Nicolet 1180 computer/Fourier transform system, a 293A′ pulse programmer, and a computer-controlled homonuclear decoupling accessory. Samples were prepared as outlined previously (Oppenheimer et al., 1981) by using 5-mm tubes with a final blenoxane or bleomycin A_2 concentration of 5 mM in 0.4 mL. The standard pH electrode correction for deuterium has been employed: pD = meter reading + 0.4 (Glasoe & Long, 1960), and the internal chemical shift standard sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate (TSP) was used.

Bleomycin-Mediated Degradation of [^{3}H]DNA. ^{3}H-Labeled PM-2 DNA (21×10^6 cpm/μmol; radiolabeled in thymine) was employed as a substrate for degradation. Incubation mixtures containing PM-2 [^{3}H]DNA and dithiothreitol in 50 mM sodium cacodylate buffer, pH 7.0, were preincubated at 4 or 37 °C (where indicated in the legends to individual figures, as this procedure was found to improve apparent dependence on metal ions added to initiate the reaction). The solutions were then equilibrated at 37 °C, and bleomycin A_2 was added to the final concentration indicated in the figure legends, followed by Cu(II) [or Fe(II)] as indicated. The reaction mixtures were incubated at 37 °C, and aliquots were removed at predetermined time intervals and applied to glass fiber disks that had been presoaked with thymine. The dried disks were washed with 5% trichloroacetic acid, dried, and used for determination of radioactivity. Zero-time points were determined from duplicate aliquots taken just prior to the addition of bleomycin or metal ion.

Bleomycin-Mediated Strand Scission of cccDNA. Reaction mixtures (80–100 μL total volume) containing 50 mM sodium cacodylate buffer, pH 7.0, and 500 ng of SV-40 form I DNA were treated, as indicated, with 0–25 μM Cu(II)·BLM B_2, 100 μM Na-EDTA, and 10 μM dithiothreitol under aerobic conditions or else with 5–25 μM Cu(II)·BLM B_2 [or Fe(III)·BLM B_2] and 0.5–2.5 mM iodosobenzene under anaerobic conditions. For the aerobic reaction mixtures, the reactions were initiated by the addition of metal ion and incubated at 25 °C for 30 min. For the anaerobic reaction mixtures, the reactions were initiated by the addition of iodosobenzene over a period of 15 min; the reaction mixtures were then incubated at 25 °C for an additional 30 min. The reactions were quenched by the addition of 2 μL of 200 mM Na-EDTA. Half of each reaction mixture was treated with 5 μL of a 40 mM Tris-HCl buffer solution containing 0.15% bromphenol blue and 75% glycerol, and the combined solution was applied to a 1.2% agarose gel containing 1 μg/mL ethidium bromide. Horizontal gel electrophoresis was carried out in 40 mM Tris-HCl buffer, pH 7.8, containing 5 mM Na-EDTA for 7 h at 80 V or 12 h at 45 V. The gels were visualized by UV irradiation.

Results

^{13}C NMR of Cu(I)·BLM. Two-dimensional ^1H–^{13}C correlated NMR spectroscopy was carried out for blenoxane, as well as Cu(I)·BLM and Cu(I)·BLM·CO (see Figure 1 for C

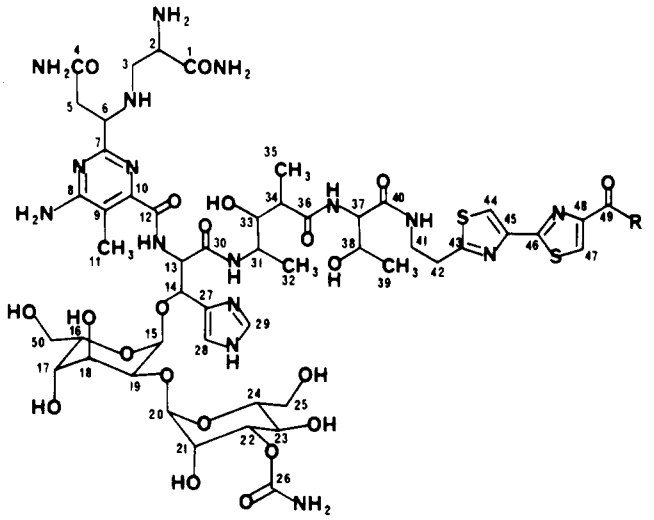

FIGURE 1: Structure of bleomycin with carbon atoms numbered. R = $NH(CH_2)_3S^+(CH_3)_2$, bleomycin A_2; R = $NH(CH_2)_4NHC(NH)NH_2$, bleomycin B_2.

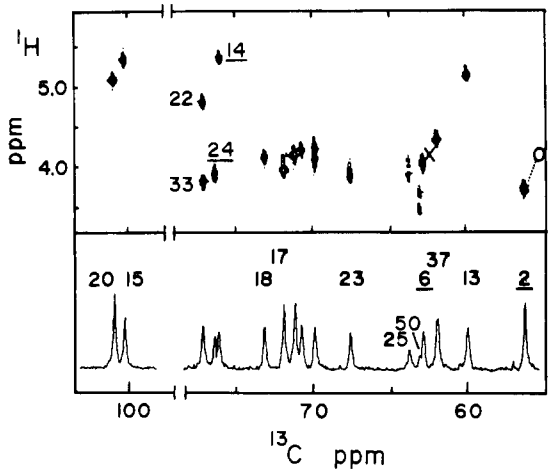

FIGURE 2: Portion of the ^{1}H–^{13}C correlated NMR spectrum for metal-free bleomycin, pD 8.5. The upper portion of the figure shows the contour plot covering 5.5–3.2 ppm in the ^{1}H dimension (vertical axis) and 104–54 ppm in the ^{13}C dimension (horizontal axis). At the bottom of the figure is the projection of the data onto the ^{13}C axis, which results in an ^{1}H-decoupled natural abundance ^{13}C spectrum. The four resonances between those for carbons 17 and 23 (which include carbons 16, 19, 21, and 38) have yet to be assigned definitively. X and O in the contour plots designate the position of the corresponding resonances in the ^{1}H–^{13}C correlated spectrum obtained at pD 7.0. Note that O shifts by ca. 0.4 ppm in the ^{1}H dimension whereas X shifts by less than 0.1 ppm, thus unambiguously assigning X to the propionamide CH (carbon 6) and O to the β-aminoalanine CH (carbon 2).

numbering scheme). The contour plot of the upfield region of metal-free blenoxane at pD 8.5 is shown in Figure 2. Analysis of this plot permitted the assignment of a number of ^{13}C resonances, including those corresponding to the β-CH of hydroxyhistidine, the propionamide CH, and the β-aminoalanine CH. For example, the ^{13}C resonance at 75.7 ppm correlated with the ^{1}H resonance at 5.30 ppm, the latter of which has been assigned unambiguously to the β-CH of hydroxyhistidine by homonuclear spin decoupling experiments (Chen et al., 1977; Oppenheimer et al., 1979a). Likewise, the assignments of the propionamide and β-aminoalaninamide methines could be made for solutions having pH >7, since the latter underwent a larger upfield shift (>0.35 ppm, relative to the propionamide CH) as a result of titration of the primary amine (Oppenheimer et al., 1979a). Analysis of the contour plot (Figure 2) indicated that the ^{13}C resonance at 55.2 ppm

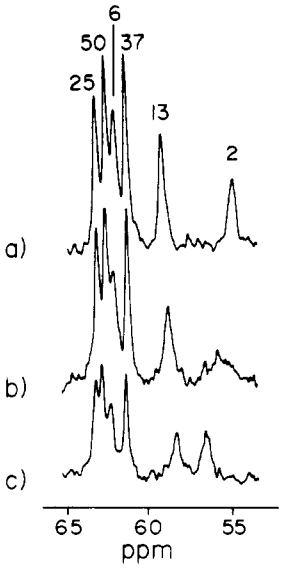

FIGURE 3: Effect of adding Cu(I) on the natural abundance ^{13}C NMR spectrum of bleomycin A_2 at 20 MHz. The ratio of Cu(I):BLM A_2 was 1:4 (a), 2:3 (b), and 9:10 (c). The vertical amplitude in spectrum c is half that of spectrum a or b. Note that the α-histidine (carbon 13) shows a continuous upfield shift with increasing Cu(I) whereas the β-aminoalanine methylene (carbon 2) first broadens and then sharpens with increasing Cu(I). The difference in behavior is attributed to the larger shift for C-2 which results in kinetic broadening. The shift for C-13 is not sufficient to cause drastic line broadening.

experienced the upfield shift in the ^{1}H dimension (to 3.63 ppm), establishing it as the β-aminoalaninamide CH resonance. In contrast, the ^{13}C resonance at 62.4 ppm remained at 3.95 ppm in the ^{1}H dimension and could, therefore, be assigned as the propionamide CH.[2]

Assignments of both ^{13}C and ^{1}H resonances for the Cu(I)·BLM and Cu(I)·BLM·CO complexes were made by a combination of ^{1}H–^{13}C correlated spectroscopy, selective heteronuclear spin decoupling experiments, and, for ^{1}H resonances, homonuclear spin decoupling experiments. The 1:1 Cu(I)·BLM and Cu(I)·BLM·CO complexes gave well-resolved ^{13}C NMR spectra, as shown in Figure 3. When less than stoichiometric amounts of Cu(I) were employed, however, the results were fundamentally different from those obtained with comparable amounts of divalent metal ions such as Zn(II) or Fe(II). The most obvious difference was the rapid exchange of Cu(I) between free and bound forms. This dynamic behavior was manifest as a continuous shift of the ^{13}C resonances with increasing concentrations of Cu(I) until a 1:1 complex was formed (Figure 3).[3] In contrast, two distinct sets of resonances in slow exchange were observed when divalent metals such as Zn(II) were added in less than stoichiometric amounts to solutions of BLM (Cass et al., 1978; Dabrowiak et al., 1978). Computer fitting of the data for both ^{13}C and ^{1}H resonances indicated an exchange rate of about 110 s^{-1}, a rate that was >3000 times faster than observed for Zn(II) under comparable conditions (Lenkinski & Dallas, 1979). The formation of Cu(I) and Cu(I)·CO complexes with bleomycin also led to qualitatively different patterns of ^{13}C chemical shifts compared to those observed for Zn(II) (Dabrowiak et al., 1978). The ^{13}C chemical shift changes observed upon binding

[2] A complete discussion of the ^{13}C spectra of metallobleomycin complexes will be presented elsewhere (N. J. Oppenheimer, unpublished results).

[3] Intermediate to fast exchange is favored at 20 MHz because the rate of exchange is greater than the small difference in the chemical shift between the bound and free forms of the ^{13}C resonances.

Table I: ^{13}C Chemical Shifts for Selected Resonances of Bleomycin, Cu(I)·BLM, and Cu(I)·BLM·CO[a]

resonance	BLM	Cu(I)·BLM	Cu(I)·BLM·CO
β-aminoalaninamide			
CH	55.2	58.3	57.7
CH_2	49.9	53.0	52.9
$CONH_2$	174.2	178.5	178.6
propionamide			
CH	62.4	63.1	63.7
CH_2	42.9	43.7	43.6
$CONH_2$	178.9	177.8	177.2
pyrimidine			
CH_3	13.5	13.9	13.8
C-7	168.0	167.1	167.3
C-8	167.3	165.2	165.3
C-9	114.9	115.7	115.3
C-10	154.9	154.5	155.3
C-12	170.4	170.0	170.0
β-hydroxyhistidine			
H2	139.6	139.7	139.6
H4	120.4	121.0	120.8
C-27	137.5	136.3	137.1
α-H	59.7	59.8	59.6
β-H	75.7	76.0	76.1
C-30	171.7	171.2	171.5
gulose			
1′ (C-15)	100.2	100.2	100.3
mannose			
1′ (C-20)	100.9	101.0	101.1
3′ (C-22)	77.0	77.1	77.0
C-26	160.6	160.5	160.6
valerate			
α-CH	45.3	45.4	45.3
β-CH	77.0	77.1	77.0
γ-CH	50.2	50.3	50.2
α-CH_3	14.6	14.7	14.6
γ-CH_3	17.4	17.5	17.4

[a] Spectra were obtained at 20 MHz at pD 7.1. The samples contained 50 mM bleomycin and 25 mM cacodylate buffer. Assignments for the nonprotonated carbons are those found in Naganawa et al. (1977).

are listed in Table I for selected resonances. Shifts greater than 0.4 ppm were confined to the β-aminoalaninamide, propionamide, pyrimidine, and β-hydroxyhistidine moieties. In addition to these changes, formation of the binary complex between bleomycin and Zn(II) also resulted in substantial shifts for the resonances of the mannose and valerate moieties (Dabrowiak et al., 1978).

^1H NMR of Cu(I)·BLM. (A) Exchangeable Protons. A portion of the ^1H NMR spectra of BLM and Cu(I)·BLM complexes obtained in H_2O are shown in Figure 4, and the chemical shifts of selected resonances are listed in Table II. These spectra revealed the presence of a broad peak at 9.30 ppm in the spectra of Cu(I)·BLM A_2 and Cu(I)·BLM A_2·CO, corresponding to the α-NH of β-hydroxyhistidine. While a small upfield shift of this resonance (9.45 → 9.30 ppm) was observed upon formation of the 1:1 Cu(I)·BLM complex, no further change was noted when conversion of the binary complex to Cu(I)·BLM·CO was effected.

The 4-amino protons of the pyrimidine moiety were extremely sensitive to Cu(I) binding and showed the largest shift of all the exchangeable resonances, 0.512 ppm downfield. The magnitude of this shift was essentially unaltered upon conversion to Cu(I)·BLM·CO and was also comparable to that observed for other metal complexes, e.g., Zn(II)·BLM ($\Delta\nu$ = 0.590 ppm) and Fe(II)·BLM·CO ($\Delta\nu$ = 0.536) (N. J. Oppenheimer, unpublished data). This shift must be a direct consequence of formation of the metal complex since the 4-amino resonance in metal-free bleomycin shows no pH dependence of its chemical shift above pH 4.0; i.e., it is insensitive to protonation of the imidazole and primary amine.

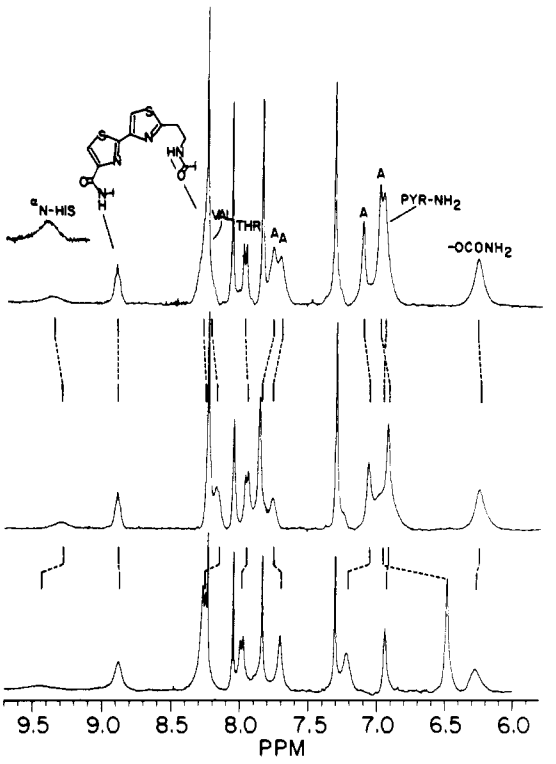

FIGURE 4: Comparison of portions of the ^1H NMR spectra obtained in H_2O at 360 MHz of bleomycin A_2, pH 7.1 (bottom), Cu(I)·BLM A_2, pH 7.1 (middle), and Cu(I)·BLM A_2·CO, pH 7.1 (top). Note the presence of the α-NH of the hydroxyhistidine moiety in the spectra of the copper complexes and the large downfield shift of the 4-amino resonance of the pyrimidine moiety.

Table II: ^1H Chemical Shifts for Bleomycin, Cu(I)·BLM, and Cu(I)·BLM·CO[a]

resonance	BLM	Cu(I)·BLM	Cu(I)·BLM·CO
β-aminoalaninamide			
CH	3.892	3.64[b]	3.69[b]
CH_2	2.966[c]	3.25 ± 0.1,[d] 2.13 ± 0.1[d]	3.30 ± 0.1,[d] 2.15 ± 0.1[d]
propionamide			
CH	3.988	4.16	4.225
CH_2	2.737, 2.662	2.72, 2.68	2.725[c]
pyrimidine			
CH_3	2.044	2.072	2.014
NH_2	6.48	6.96	6.96
β-hydroxyhistidine			
α-NH	9.45	9.30	9.36
N2	7.826	7.862	7.823
H4	7.301	7.302	7.311
α-H	5.084	5.08	5.100
β-H	5.286	5.33	5.254
sugars			
gulose 1′	5.291	5.285	5.247
mannose 1′	5.026	5.027	5.017
mannose 3′	4.692	4.68	4.739
valerate			
α-CH	2.485	2.482	2.525
β-CH	3.738	3.751	3.744
γ-CH	3.897	3.92[b]	3.96[b]
α-CH_3	1.135	1.137	1.138
γ-CH_3	1.150	1.142	1.173

[a] Chemical shifts are in ppm from TSP and were obtained at 360 MHz unless otherwise noted. The samples were prepared either in D_2O or H_2O with 10 mM cacodylate buffer, pD/pH 7.1, and a bleomycin concentration of 5 mM. [b] Chemical shifts were determined at 240 MHz from ^1H–^{13}C correlated experiments. [c] The chemical shift difference of the two methylene protons was less than 0.01 ppm. [d] Chemical shifts were determined at 240 MHz from selective heteronuclear decoupling experiments.

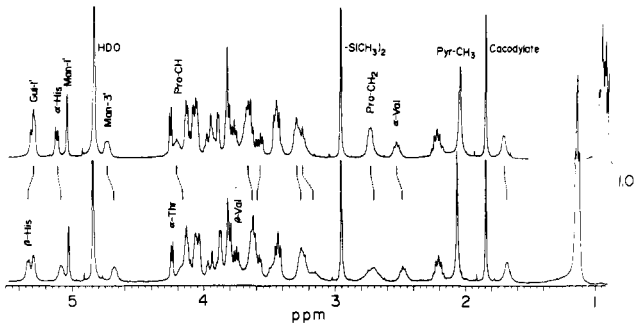

FIGURE 5: Upfield region of the ^1H NMR spectra obtained at 360 MHz for Cu(I)·BLM (bottom) and Cu(I)·BLM·CO (top). His, β-hydroxyhistidine; Gul, gulose; Man, mannose; Pro, propionamide; Thr, threonine; Val, methylvalerate.

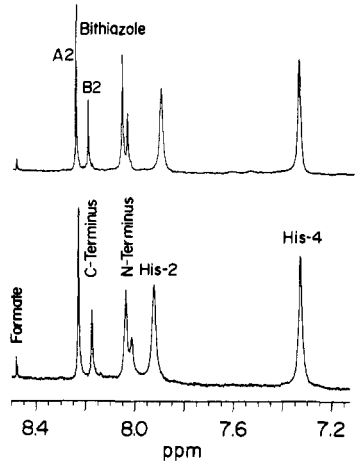

FIGURE 6: Downfield region of the ^1H NMR spectra obtained at 360 MHz for Cu(I)·BLM (bottom) and Cu(I)·BLM·CO (top).

(*B*) *Nonexchangeable Protons.* Formation of the (binary and ternary) Cu(I) complexes of bleomycin had profound effects on both the chemical shifts and line widths of a number of ^1H resonances (Figures 5 and 6; Table II). The resonances of the β-aminoalaninamide moiety were the most sensitive to line broadening; the methylene protons in the binary complex were broadened sufficiently (line width estimated as >150 Hz) so as to be unobservable. Although the line widths remained large in the Cu(I)·BLM·CO ternary complex, one methylene proton of the β-aminoalaninamide moiety was observable as a broad signal in the base line (2.15 ppm, line width estimated as ~100 Hz) under the resonances of the pyrimidinyl methyl group and the central methylene group of the C-substituent.

As noted above, direct measurement of the ^1H chemical shifts of the β-aminoalaninamide methylene protons was precluded both by line width and overlap with other resonances. Nonetheless, these data were accessible from ^{13}C NMR experiments that employed selective ^1H decoupling with a single, unmodulated ^1H frequency. The alteration of the splitting pattern of the ^1H-coupled, ^{13}C resonances could then be correlated with the ^1H decoupling frequency as the latter was changed systematically (Birdsall et al., 1972).[4] From the alteration in the splitting pattern of the ^{13}C resonances with changing decoupling frequency (Figure 7), it was established that complexation of bleomycin with Cu(I) induced a 1.1 ppm nonequivalence of the β-aminoalaninamide methylene protons with one resonance at 2.15 ppm and the other at 3.25 ppm; the latter of these was superimposed on a methylene resonance of the bithiazole moiety. Addition of CO to Cu(I)·BLM did not alter these chemical shifts significantly. It is interesting to note that the nonequivalence of these methylene protons was greater than that observed for the corresponding protons in Zn(II)·BLM (0.87 ppm) (Oppenheimer et al., 1979a) or Fe(II)·BLM·CO (0.28 ppm) (Oppenheimer et al., 1979b). The chemical shift of the methine proton of the β-aminoalaninamide moiety was measured from the ^1H–^{13}C correlated spectrum; at 3.64 ppm, it was significantly further upfield (0.15 ppm) than the corresponding proton in Zn(II)·BLM (Oppenheimer et al., 1979a).

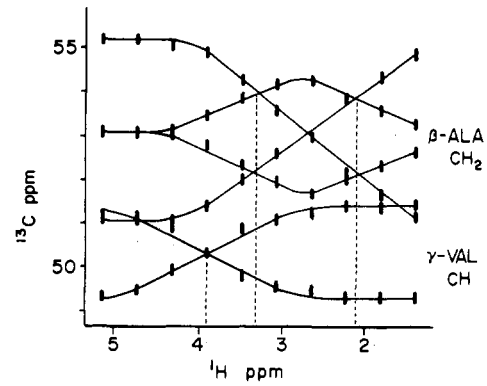

FIGURE 7: Selective off-resonance heteronuclear decoupling experiments conducted at 60.3 MHz for the β-aminoalanine methylene (a triplet centered at 53.0 ppm) and the γ-valerate methine (a doublet centered at 50.3 ppm). Analysis of the alteration in the splitting pattern in the ^{13}C spectrum as a function of the ^1H decoupler frequency gives ^1H chemical shifts for the corresponding protons of 3.90 ± 0.1 ppm for the γ-Val CH (value from ^1H NMR spectrum 3.92 ppm) and 3.25 ± 0.1 and 2.13 ± 0.1 ppm for the β-aminoalanine CH_2.

The proton resonances of the propionamide moiety have been assigned by homonuclear spin decoupling and titration with Cu(I) and confirmed by ^1H–^{13}C correlated NMR spectroscopy. The methylene resonances in the binary complex broadened upon addition of Cu(I) but otherwise showed little change in their chemical shifts. The propionamide CH resonance at 4.17 ppm was quite broad (ca. 25 Hz) and shifted by ~0.1 ppm downfield from the frequency observed in metal-free bleomycin. Upon addition of CO these resonances became much sharper, and the methylene protons became equivalent in chemical shift. From spin decoupling experiments the vicinal coupling constants in the ternary complex were estimated to be about 7.8 Hz, similar to the values for the Zn(II)·BLM complex and the average value for metal-free bleomycin (Oppenheimer et al., 1979a). These values indicate considerable torsional flexibility in the propionamide moiety.

Elsewhere in the molecule the binding of Cu(I) caused only minor changes in the ^1H NMR spectrum. The pyrimidine CH_3 resonance was insensitive to the binding of Cu(I), in contrast to the large downfield shifts observed for this resonance in the Zn(II) (Oppenheimer et al., 1979a) or Fe(II)·CO (Oppenheimer et al., 1979b) complexes. The effects of Cu(I) binding on the resonances of other groups were limited primarily to changes in line widths. For the binary complex the groups for which substantial increases in line width, but little accompanying change in chemical shift, were observed included the mannose moiety, the α and β protons of β-hydroxyhistidine, the α-CH of the valerate moiety, and the proton on the N-terminal thiazole ring. Addition of CO eliminated the effects on all of these groups. A possible explanation for the observed line broadening in the binary complex might involve the formation of transient coordination complexes between the affected groups in bleomycin and Cu(I). Reversal of line broadening upon additional binding of CO might simply reflect blocking of these weak secondary interactions via sta-

[4] It may be noted that the line widths of these ^1H resonances precluded their observation in the 2-D ^1H–^{13}C correlated NMR experiments.

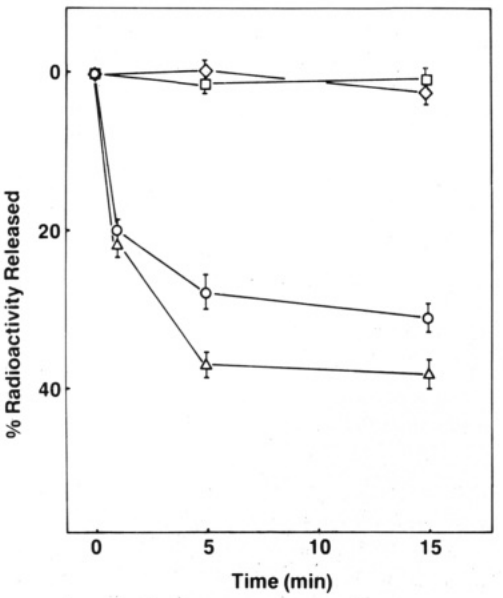

FIGURE 8: Release of radioactivity from PM-2 [^{3}H]DNA by bleomycin A_2 in the presence of Cu(II) and Fe(II). Reaction mixtures (0.35 mL total volume) contained 50 mM sodium cacodylate (pH 7.0), 20 μM dithiothreitol, 3.3 μM ^{3}H-labeled PM-2 DNA, 4.5 μM bleomycin A_2, and 16 μM $Cu(OAc)_2$ (△), 16 μM $Fe(NH_4)_2(SO_4)_2$ (○), or no metal (◇). Dithiothreitol was added to the buffered DNA solution, which was incubated at 4 °C for 4 h prior to addition of bleomycin. The reactions were initiated by the addition of metal ion, where appropriate, and aliquots were taken at predetermined time intervals and analyzed as described under Experimental Procedures. A control experiment (□) included 16 μM Cu(II) and 20 μM dithiothreitol, but no bleomycin.

bilization of the complex between Cu(I) and the primary binding site on bleomycin. Consistent with this suggestion are the published data (Dabrowiak et al., 1978; Pillai et al., 1980) that provide evidence for the close proximity of the mannose, valerate, and β-hydroxyhistidine moieties with the (divalent) metal ions in certain metallobleomycins.

Temperature Effects. Temperature variation (3–55 °C) had little effect on the chemical shifts or coupling constants of Cu(I)·BLM, with the exception of the imidazole H-2 resonance. For the binary complex a minor increase in line width was observed with decreasing temperature, as was a downfield shift of the imidazole H-2 resonance (0.031 ppm, 10 °C). These effects were less pronounced in the ternary complex where some increase in line widths was observed at higher temperature.

Sulfur as a Possible Ligand for Cu(I)·BLM. In order to study the possible participation of a thiol as a ligand in the Cu(I)·BLM complex, an anaerobic solution of 5 mM Cu(I)·BLM was titrated with dithiothreitol, and the effects were monitored by ^{1}H NMR. Addition of dithiothreitol resulted in the immediate formation of a voluminous pale blue precipitate. The effect on the NMR spectrum was a shift of the resonances toward those observed for metal-free bleomycin. At a 1:2 ratio of dithiothreitol and Cu(I)·BLM [i.e., equimolar RSH and Cu(I)], the spectral parameters of the bleomycin resonances became identical with those of metal-free BLM at the same pH. No further change in chemical shifts was noted for these resonances upon further addition of dithiothreitol.

DNA Cleavage by Cu·BLM. Incubation of a buffered solution of [^{3}H]DNA with 20 μM dithiothreitol, 4.5 μM bleomycin A_2, and 16 μM Cu(II) resulted in DNA degradation more substantial than that obtained with the same concentrations of bleomycin and Fe(II).[5] As shown in Figure 8,

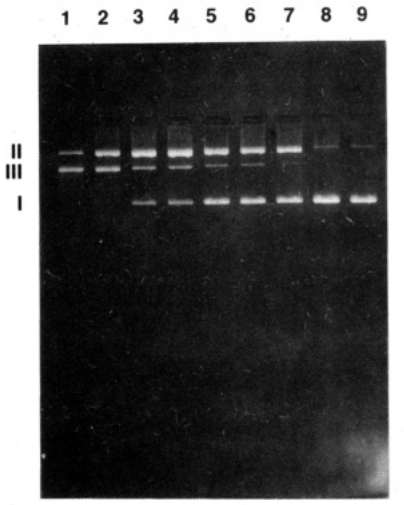

FIGURE 9: Strand scission of SV-40 form I DNA by Cu·BLM. Individual reaction mixtures containing 50 mM sodium cacodylate buffer, pH 7.0, 500 ng of SV-40 DNA, 100 μM Na-EDTA, and 50 μM dithiothreitol were treated with 25 mg of Chelex-100 resin that had been preequilibrated in the same buffer. After this mixture was maintained at 4 °C for 10 min, the resin was removed by centrifugation, and the solution was treated with BLM B_2 (that had been demetalized separately) and the appropriate metal ion. Reaction mixtures contained the following: lane 1, 20 μM Cu(II) BLM·B_2, 100 μM Na-EDTA, and 50 μM dithiothreitol; lane 2, 10 μM Cu(II)·BLM B_2, 100 μM Na-EDTA, and 50 μM dithiothreitol; lane 3, 5 μM Cu(II) BLM·B_2, 100 μM Na-EDTA, and 50 μM dithiothreitol; lane 4, 20 μM BLM B_2, 100 μM Na-EDTA, and 50 μM dithiothreitol; lane 5, 10 μM BLM B_2, 100 μM Na-EDTA, and 50 μM dithiothreitol; lane 6, 5 μM BLM B_2, 100 μM Na-EDTA, and 50 μM dithiothreitol; lane 7, 1 μM BLM B_2, 3 μM $Fe^{II}(NH_4)_2(SO_4)_2$, 100 μM Na-EDTA and 50 μM dithiothreitol; lane 8, 20 μM $Cu^{II}Cl_2$, 100 μM Na-EDTA, and 50 μM dithiothreitol; lane 9, 100 μM Na-EDTA and 50 μM dithiothreitol.

DNA degradation was obtained only in the presence of bleomycin and Cu(II). Another measure of DNA degradation by bleomycin is the ability of the antibiotic to convert supercoiled cccDNA to nicked circular DNA and linear duplex DNA (Haidle et al., 1979; Strong & Crooke, 1979). As shown in Figure 9, when demetalized reaction mixtures containing 500 ng of SV40 form I DNA were treated with Cu·BLM, conversion to form II and form III DNA was observed; the extent of conversion was proportional to the amount of Cu·BLM added. Control experiments carried out in the presence of BLM still resulted in some strand scission. This was presumably due to the presence of adventitious metal ions in the reaction, in spite of the presence of EDTA in the reaction mixtures. However, in each case the extent of strand scission was clearly enhanced by Cu. For example, treatment of SV-40 form I DNA with 10 μM BLM B_2 in the absence of added metal produced a sample (Figure 9, lane 5) containing 51% form I DNA, 41% form II DNA, and 8% form III DNA as judged by densitometry of the gels. In the presence of 10 μM Cu·BLM B_2, all of the SV-40 form I DNA was converted to form II DNA (55%) and form III DNA (45%). The same results were obtained in an experiment that utilized PM-2 DNA.

Strand scission of SV40 form I DNA by a lower (2.5 μM) concentration of Cu(II)·BLM_2 in the presence of dithionite

[5] Precipitation of Cu(0) from anaerobic solutions of Cu(I)·BLM with concomitant generation of blue solutions having the spectral properties of Cu(II)·BLM was observed, and the transformation was facilitated by divalent metal ions such as Zn(II) that have an affinity for BLM. To circumvent these problems, we employed aqueous solutions containing dithiothreitol (Cotton & Wilkinson, 1972).

Table III: DNA Strand Scission by Cu(II)·BLM in the Presence of Sodium Dithionite[a]

additions	form I DNA (%)[b]	form II DNA (%)[b]	form III DNA (%)[b]
complete system [Cu(II), BLM B_2, $Na_2S_2O_4$]	65	29	6
Cu(II)	100	*c*	0
BLM B_2, $Na_2S_2O_4$	95	5	0
none	100	*c*	0
Fe(II), BLM B_2[d]	67	32	1

[a] Reaction mixtures (40 μL total volume) containing 50 mM sodium cacodylate buffer, pH 7.0, 250 ng of SV-40 form I DNA, and 10 μM Na-EDTA were treated sequentially with $Na_2S_2O_4$ (25 μM final concentration), bleomycin B_2 (2.5 μM), and $CuCl_2$ (25 μM) (except for the omissions noted below). The reaction mixtures were incubated at 25 °C for 30 min and then analyzed by gel electrophoresis on a 1.2% horizontal agarose gel, containing 1 μg/mL ethidium bromide. The bands were visualized by UV, and the percent strand scission was determined by densitometry. [b] Relative to untreated control. [c] Trace. [d] Positive control, containing 10 μM $Fe(NH_4)_2SO_4$ in lieu of $CuCl_2$ + $Na_2S_2O_4$.

was also studied. As shown in Table III, the extent of degradation of the DNA by Cu·BLM and Fe·BLM was quite similar; strand scission by Cu·BLM was clearly dependent on the presence of both Cu and BLM B_2.

The lack of activity of Cu(II)·BLM itself in DNA cleavage (unpublished results; Antholine et al., 1982; Asakura et al., 1975; Sausville et al., 1976; Shirakawa et al., 1971; Suzuki et al., 1969; Takahashi et al., 1977; Umezawa, 1974; Umezawa et al., 1976) suggested strongly that the observed release of radioactivity from PM-2 DNA must have been due to Cu(I)·BLM, formed in situ by dithiothreitol (dithionite)-mediated reduction of Cu(II)·BLM. Evidence that the observed DNA cleavage was mediated by Cu(I)·BLM was provided by the findings that identical UV, visible, and ^1H NMR spectra were obtained from samples prepared by admixture of Cu(I) + BLM or Cu(II) + BLM + dithionite (Oppenheimer et al., 1981). Moreover, the activated species formed by admixture of Cu(I)Cl + BLM B_2 + dithiothreitol in the presence of O_2 effected strand scission of SV-40 form I DNA (Table IV).[6]

Also studied was the possible O_2 dependence of DNA cleavage by Cu(I)·BLM, a property that has already been established for Fe(II)·BLM (Sausville et al., 1978b). In the absence of oxygen neither (putative) Cu(I)·BLM nor Fe(II)·BLM effected significant release of radioactivity from [^3H]thymine-labeled PM-2 DNA. Admission of O_2 to the reaction mixtures, however, resulted in rapid DNA degradation; for both Cu(I)·BLM and Fe(II)·BLM, the extent of DNA degradation was very similar (data not shown).

Atomic absorption experiments revealed that the CuOAc employed for this study contained approximately 0.05% mol contaminating Fe(II). As dithiothreitol greatly enhances the extent of DNA degradation by Fe(II)·BLM, presumably via regeneration of Fe(II)·BLM from the Fe(III)·BLM formed as a byproduct of DNA cleavage, the possibility was investigated that the observed degradation of DNA by Cu(I)·BLM was actually due to contaminating Fe(II)·BLM. When 2.5 μM PM-2 [^3H]DNA was preincubated with 2.3 μM BLM A_2, 25 μM Cu(II), and excess dithiothreitol, approximately 20% of the radioactivity was solubilized after 10 min. In comparison, repetition of the experiment in the presence of 12.5 nM Fe(II) [i.e., the amount known to be a contaminant in the Cu(II)] or in the absence of added metal produced <5% degradation. Thus, the amount of Fe(II) contaminating the Cu(II) employed for these studies was insufficient to account for the DNA degradation observed.

[6] It is interesting to note that Me_2SO, a known scavenger of ·OH (Repine et al., 1981), did not suppress the degradation of DNA by Cu(I)·BLM (cf. Table IV). This was consistent with the earlier observation that DNA degradation by Fe(II)·BLM was refractory to Me_2SO (Rodriguez & Hecht, 1982).

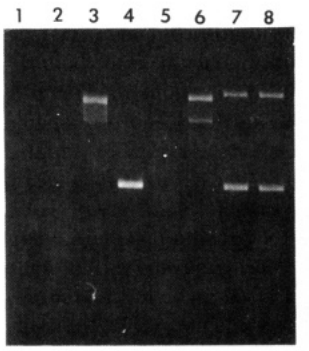

FIGURE 10: SV-40 DNA strand scission by Cu(II)·BLM_2 + C_6H_5IO. Individual reaction mixtures contained the following: lane 1, SV-40 DNA, 25 μM Cu(II)·BLM B_2, and 2.5 mM C_6H_5IO; lane 2, SV-40 DNA, 10 μM Cu(II)·BLM B_2 and 1 mM C_6H_5IO; lane 3, SV-40 DNA, 5 μM Cu(II)·BLM B_2, and 0.5 mM C_6H_5IO; lane 4, SV-40 DNA; lane 5, SV-40 DNA, 1 μM BLM B_2, and 10 μM Fe(II); lane 6, SV-40 DNA, 25 μM Fe(III)·BLM B_2, and 2.5 mM C_6H_5IO; lane 7, SV-40 DNA, 10 μM Fe(III)·BLM B_2, and 1 mM C_6H_5IO; lane 8, SV-40 DNA, 5 μM Fe(III)·BLM B_2, and 0.5 mM C_6H_5IO. Details are given under Experimental Procedures.

DNA cleavage by Fe(II)·BLM in the presence of O_2 has been shown to produce free bases (especially thymine) and base propenals, an observation that prompted analysis of the ability of Cu(I)·BLM to mediate formation of the same species. As shown in Table V, significantly less thymine was formed during degradation of *E. coli* [^3H]thymine-labeled DNA by Cu(I)·BLM than was obtained with Fe(II)·BLM, and Cu(I)·BLM produced no detectable [^3H]thymine propenal, suggesting strongly that the two metallobleomycins do not effect DNA degradation by identical mechanisms. The observation of differences in product formation with Fe(II)·BLM and Cu(I)·BLM also provides additional evidence that the activity observed for the latter is not due to contaminating metal ions.

Also studied was the ability of Cu(II)·BLM to effect cleavage of cccDNA in the absence of O_2 or a reducing agent, but in the presence of the oxygen surrogate iodosobenzene. As shown in Figure 10, substantial conversion of cccDNA to nicked circular DNA and linear duplex DNA was observed, and the extent of damage was proportional to the concentration of Cu(II)·BLM present. In separate experiments it was shown that Fe(II)·BLM would not effect strand scission under these conditions.

The effect of Cu(II) concentration on DNA cleavage by bleomycin was studied to permit comparison of the results obtained here with earlier reports. As illustrated in Figure 11, at constant concentrations of bleomycin, DNA, and dithiothreitol, the extent of DNA degradation was shown to depend on the presence of Cu(II) and to increase (from 22 to 38%) as the Cu(II) concentration was raised from 0.1 to 5 μM. A slight increase in solubilization of DNA radiolabel (to 41%) was noted as the concentration of Cu(II) was increased to 25 μM, and sharply decreased DNA degradation was observed at higher Cu(II) concentrations. Repetition of this experiment at a higher (85 vs. 40 μM) concentration of dithiothreitol gave results that were quite similar to those shown in Figure 11 (data not shown).

DISCUSSION

While it has been demonstrated convincingly that Fe(II)·BLM mediates oxidative degradation of DNA in cell-free

Table IV: DNA Strand Scission by $Cu^{I}Cl$ + BLM B_2 in the Presence of Dithiothreitol[a]

additions	form I DNA (%)[b]	form II DNA (%)[b]	form III DNA (%)[b]
10 μM BLM B_2 + 60 μM Cu(I)[c] + 50 μM dithiothreitol		55	45
10 μM BLM B_2 + 60 μM Cu(I)[c]	81	16	3
10 μM BLM B_2 + 50 μM dithiothreitol	29	51	20
5 μM BLM B_2 + 30 μM Cu(I)[c] + 50 μM dithiothreitol	8	60	32
5 μM BLM B_2 + 30 μM Cu(I)[c]	87	11	2
5 μM BLM B_2 + 50 μM dithiothreitol	47	40	13
1 μM BLM B_2 + 3 μM Fe(II) + 50 μM dithiothreitol	61	33	6
60 μM Cu(I)[c] + 50 μM dithiothreitol	91	9	
50 μM dithiothreitol	89	11	

[a] Demetalized reaction mixtures (50 μL total volume) containing 50 mM sodium cacodylate buffer, pH 7.0, 500 ng of SV-40 form I DNA, 100 μM Na-EDTA, and 50 μM dithiothreitol were treated successively with metal ion and BLM B_2 as indicated below and incubated at 4 °C for 30 min. The reaction mixtures were then analyzed by gel electrophoresis on a 1.2% horizontal agarose gel, containing 1 μg/mL ethidium bromide. The bands were visualized by UV and the percent strand scission determined by densitometry. [b] Relative to untreated DNA. [c] Added from a 100% dimethyl sulfoxide solution.

Table V: Production of Thymine and Thymine Propenal Concomitant with Bleomycin-Mediated DNA Degradation[a]

additions	DNA degradation (%)	[^{3}H]thymine propenal (cpm)	[^{3}H]thymine (cpm)
2.5 μM Cu(II)·BLM B_2, 20 μM dithiothreitol	64	*b*	315
2.5 μM BLM B_2, 20 μM dithiothreitol	15	*b*	110
2.5 μM Cu(II), 20 μM dithiothreitol	1	*b*	120
1 μM BLM B_2, 3 μM Fe(II), 20 μM dithiothreitol	61	1820	670

[a] Reaction mixtures (220 μL total volume) containing 50 mM sodium cacodylate buffer, pH 7.0, 10 μM *E. coli* [^{3}H]thymine-labeled DNA (New England Nuclear, 3.92 mCi/mg), 100 μM Na-EDTA, and 20 μM dithiothreitol were demetalized by batch chromatography on Chelex-100 resin (preequilibrated in 50 mM sodium cacodylate, pH 7.0) at 4% for 10 min. The demetalized reaction mixtures were treated with metal and then bleomycin B_2 as indicated below, and then incubated at 4 °C for 10 min. Fifty-microliter aliquots were removed after 0 and 10 min, treated with 15% TCA, and applied to glass fiber disks for determination of radioactivity (1). Twenty-microliter aliquots of each reaction mixture were removed after 10 min and analyzed for [^{3}H]thymine and [^{3}H]thymine propenal by HPLC (2); the samples were coinjected with authentic unlabeled samples of these pyrimidines. (1) The aliquots (50 μL) were added to 1.0 mL of 15% trichloroacetic acid, and the combined solution was treated with carrier calf thymus DNA (final DNA concentration was 50 μg/mL). The solution was maintained at 4 °C for 1.5 h, then isolated by filtration on glass fiber disks, and washed with portions of cold 15% TCA. (2) The samples were analyzed by injection onto a Rainin C_{18} 3-μm particle size column; elution was effected at 25 °C by a 20-min 100% H_2O to 100% CH_3OH concave gradient. The column was monitored for A_{254}, and forty 0.5-mL fractions were collected and analyzed for radioactivity. [b] Not measurable above [^{3}H] DNA background.

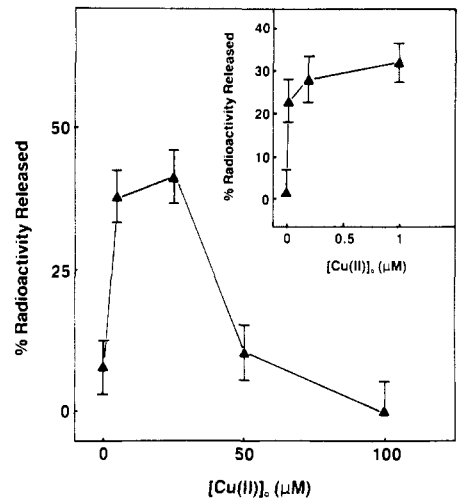

FIGURE 11: Effect of copper concentration on the Cu(II)·BLM-mediated degradation of DNA. Reaction mixtures (0.35 mL total volume) contained 50 mM sodium cacodylate (pH 7.0), 2.9 μM [^{3}H]DNA, 40 μM dithiothreitol, 3.4 μM bleomycin A_2, and the indicated amount of $Cu(OAc)_2$. Dithiothreitol was added to the buffered DNA solution, and the combined solution was incubated at 4 °C for 4 h prior to addition of bleomycin. The reactions were initiated by the addition of Cu(II); the extent of DNA degradation was determined after 15 min.

systems in the presence of O_2, much less is known about the behavior of other metallobleomycins in vitro or about the nature of the species responsible for DNA degradation in vivo. As intracellular DNA strand scission represents the putative therapeutic locus of action of bleomycin, we sought to define better the nature of the metallobleomycin complex responsible for this transformation.

One intriguing observation made by Umezawa (1973) was that both metal-free bleomycin and Cu(II)·BLM were equally as active in inhibiting the growth of bacterial and animal cells. Additionally, metal-free BLM and Cu(II)·BLM both inhibited the growth of mammalian cells and were equally active as antimicrobial agents. Although the similar activity profiles of these species have been suggested to be due to the removal of Cu(II) from Cu(II)·BLM in situ (Takahashi et al., 1977; Umezawa, 1977, 1979), and lack of BLM-mediated DNA degradation obtained in the presence of Cu(II) has been noted (Antholine et al., 1982; Asakura et al., 1975; Sausville et al., 1976; Shirakawa et al., 1971; Suzuki et al., 1969; Takahashi et al., 1977; Umezawa, 1974; Umezawa et al., 1973), two factors prompted us to investigate the nature of this metallobleomycin further. One of these was the observation that Cu(I)·BLM, which can be formed either by admixture of Cu(I) and BLM or by reduction of Cu(II)·BLM (Oppenheimer et al., 1981), was redox active (Dabrowiak & Santillo, 1979; Sugiura, 1979), an observation also made for Fe(II)·BLM and Co(III)·BLM, both of which are now known to effect DNA strand scission [Chang & Meares, 1982; see Sausville et al. (1978a,b) and references cited therein] under defined conditions. The other factor was the finding that Cu(I)·BLM and Cu(I)·BLM·CO bound to calf thymus DNA as effectively as the respective Fe(II) complexes, as judged by fluorescence quenching of the bithiazole moiety of BLM (Oppenheimer et al., 1981).

Cu(I)·BLM has been characterized previously by UV and visible spectroscopy (Oppenheimer et al., 1981). Presently, ^{1}H and ^{13}C NMR spectroscopy of Cu(I)·BLM has been employed to provide additional data concerning the nature of this complex.

The ability to obtain direct correspondence between carbon resonances in the metal complex and those in metal-free

bleomycin would permit definitive assignments to be made for Cu(I)·BLM complexes if unambiguous assignments of the ^{13}C natural abundance spectra were available. To date, the ^{13}C spectrum of bleomycin has been analyzed on the basis of spectral data derived from an extensive range of derivatives and precursors (Naganawa et al., 1977), which have been augmented by selective biosynthetic incorporation of ^{13}C-labeled precursors (Nakatani et al., 1980). Resonances have been designated on the basis of similarity to spectral data provided by "chemical shift maps" (Naganawa et al., 1977) and values calculated from the presumed structures. Although these criteria are adequate in most cases, they cannot by themselves provide unambiguous assignments of the ^{13}C resonances, and more rigorous methods must be used if detailed analysis of the data is to be conducted.

The connectivity of the resonances for the noncarbohydrate moieties of bleomycin can be readily assigned in the ^1H NMR spectrum via their homonuclear coupling constants. As a consequence, a direct determination of the correlation of the ^{13}C resonances to the known ^1H resonances would allow unambiguous assignment of the ^{13}C spectrum. Two-dimensional ^1H–^{13}C correlated spectroscopy provides such a direct means of establishing the correspondence between ^1H and ^{13}C resonances (Freeman & Morris, 1978). In addition, it has been found that for Cu(I)·BLM there is a rapid exchange of Cu(I) between free and bound forms. An important consequence of the rapid exchange rate of Cu(I) is that a direct correlation can be made between the resonances in the metal-free spectrum with the corresponding resonances in the Cu(I) complex. This is not the case for species in slow exchange, e.g., the Zn(II) complex, where this direct means of establishing unambiguous correspondence is precluded.

As described above, the ^{13}C resonances for Cu(I)·BLM have been assigned unambiguously (see Table I). New assignments included the resonance at 75.7 ppm which had previously been assigned to carbon 5 of mannose (Naganawa et al., 1977) but clearly corresponds to C^β of hydroxyhistidine. The propionamide and β-aminoalaninamide methine carbons were shown to resonate at 62.4 and 55.2 ppm, respectively, which is the reverse of the assignments made previously (Fukuoka et al., 1980; Naganawa et al., 1977).

The formation of Cu(I) and Cu(I)–CO complexes with bleomycin effected substantial changes in the ^{13}C chemical shifts of a number of resonances (Table I). The chemical shift observations were consistent with participation of the β-aminoalaninamide and pyrimidinyl moieties in binding of Cu(I). The case for participation of the imidazole was not as clear on this basis. However, the absence of major changes in chemical shifts for all of the imidazole ^{13}C resonances upon complexation with divalent metals has been noted previously in systems where convincing evidence exists for the direct participation of imidazole as a ligand (Oppenheimer et al., 1979a). Thus, the absence of major shifts does not preclude the participation of the imidazole as a ligand in the Cu(I) complex. This observation, however, serves to point out the limitations of using ^{13}C chemical shifts as the sole criterion for establishing metal binding sites. The observation of the α-NH proton of β-hydroxyhistidine at 9.30 ppm in the ^1H NMR spectrum of Cu(I)·BLM [9.36 ppm for Cu(I)·BLM·CO] represents the first time that this proton has been observed in a bleomycin–metal complex. Its presence precludes participation of the pyrimidinyl carboxamide nitrogen in the coordination of Cu(I) to bleomycin. Previous studies have noted the absence of the α-NH resonance in Zn(II) and Fe(II)·BLM·CO complexes (Oppenheimer et al., 1979b), an observation that is consistent either with the participation of the carboxamide nitrogen in coordination of divalent metals or with the possibility that the proton had been rendered too acidic to be observable by ^1H NMR under conditions where the metal complex is stable.

Increasing the pD from 7.2 to 10 had only negligible effects on chemical shifts or line width in the ^1H NMR spectra of the Cu(I)·BLM complexes. There was no indication of the effects resulting from titration of the primary amine (pK_a = 7.8) that are observed in metal-free bleomycin. Thus, we confirm the results of potentiometric titrations which indicated the absence of titratable groups in this pH range (Oppenheimer et al., 1981). Above pD 10, there was a broadening of some resonances, especially those of the β-hydroxyhistidine and pyrimidinyl propionamide moieties. In addition the imidazole ring protons began to shift upfield with increasing pD. The magnitude of the shift for the H-4 resonance was about 0.30 ppm and for H-2 about 0.35 ppm. Unlike the Zn(II)·BLM complex where a clear titration of the imidazole coordinated to the Zn was observed (Oppenheimer et al., 1979a), the increasing line width at higher pD made measurement difficult for the Cu(I) complex. The results are still suggestive of Cu(I) coordination to imidazole and consistent with the general affinity of Cu(I) for imidazole coordination.

The chemical shifts of the resonances of the pyrimidine ring are influenced by the binding of cationic species. Protonation of the pyrimidine/secondary amine at pH 2.7 led to only small shifts of the 5-methyl resonance (<0.05 ppm) (Chen et al., 1977; Oppenheimer et al., 1979a), whereas the 4-amine had an extrapolated shift of 0.6 ppm (N. J. Oppenheimer, unpublished results). The binding of Cu(I) appeared to mimic protonation. Little effect was observed for the 5-methyl group, whereas the 4-amine resonances shifted 0.5 ppm, without coordination to the 6-carboxamide nitrogen. In contrast, large downfield shifts (>0.3 ppm) were observed for the 5-methyl protons in the ^1H NMR spectra of divalent metal ion complexes. In these cases, however, some evidence pointed to a possible additional coordination site at the deprotonated nitrogen of the β-carboxamide. Occupancy of this coordination site would lead to a juxtaposition of the deshielding region of the carboxamide carbonyl and the methyl, thus accounting for the observed deshielding of the 5-methyl resonance. The similarity of the large shift observed for the 4-amino group in both the Cu(I) and divalent metal complexes, on the other hand, primarily reflects the effects of coordination at N-1 and is not sensitive to coordination, or lack of coordination, at the α-NH group of β-hydroxyhistidine.

As noted above, the addition of Cu(I) to bleomycin had dramatic effects on the line widths of a number of ^1H resonances (Figures 5 and 6; Table II). Similar line broadening effects have been reported for the N-terminal bithiazole ^1H resonances of blenoxane following addition of Cu(II) (Kasai et al., 1978) and for Fe(II)·BLM complexes (Antholine et al., 1981). The broadening observed for bleomycin in the presence of paramagnetic metals can result from a number of causes. Potentially weak coordination of the metal is possible, either intramolecularly or intermolecularly. Also, the bithiazole may possibly undergo base stacking, thus bringing resonances of the metal-free compound into proximity with the paramagnetic complex.

For the diamagnetic Fe(II)·BLM·CO complex there is evidence of a possible weak association between metal and the bithiazole that may be either intramolecular or intermolecular in nature (Oppenheimer et al., 1979b). The observed broadening can be completely reversed by addition of thiols.

It should be noted that the metal complexes were prepared in that study in the presence of other potential exogenous ligands such as ammonia, which have been noted to alter the spectral properties of Fe(III)·BLM (Sugiura, 1980).

In the presence of dithiothreitol, Cu(I)·BLM effected degradation of [^{3}H]DNA at least to the same extent as Fe(II)·BLM (Figure 8). Moreover, activated Cu·BLM was also shown to be capable of effecting DNA strand scission, as may be judged by conversion of supercoiled PM-2 DNA and SV-40 form I DNA to the nicked circular and linear duplex forms (Figure 9). Similar results were obtained when the metal ion and bleomycin were added to the reaction separately or as a preformed metallobleomycin complex (data not shown). As shown in Tables III and IV, strand scission of SV-40 DNA was also observed when dithionite (which had been used in the NMR studies) was substituted for dithiothreitol or when Cu(I)Cl was employed. In common with the DNA degradation mediated by Fe(II)·BLM, Cu(I)·BLM-mediated DNA degradation was also found to be O_2 dependent (data not shown). However, at comparable extents of degradation of *E. coli* [^{3}H]DNA, Cu(I)·BLM produced little thymine and no detectable thymine propenal (Burger et al., 1980; Giloni et al., 1981), relative to that produced by Fe(II)·BLM (Table V). This observation, and the finding that the DNA degradation obtained with Cu(I)·BLM could not have been to contaminating Fe, is sufficient to establish Cu(I)·BLM ($+O_2$) as an active metallobleomycin complex.

Recently, we have shown that in the presence of oxygen surrogates such as iodosobenzene, both Fe(III)·BLM and Cu(II)·BLM were able to effect the transfer of oxygen to *cis*-stilbene (Murugesan et al., 1982) and other olefins (N. Murugesan, unpublished results). Cu(II)·BLM + C_6H_5IO was also shown to effect strand scission when supercoiled pBR325 DNA was employed as a substrate. We have now extended this finding to supercoiled pBR322 DNA and SV-40 DNA, both of which undergo strand scission in proportion to the amount of Cu(II)·BLM present (Figure 10). Although we initially found that 5 μM BLM B_2 + 25 μM Fe(III) would not effect detectable degradation of [^{3}H]DNA in the presence of 100 μM C_6H_5IO (Murugesan et al., 1982), subsequent experiments employing 25 μM Fe(III)·BLM + C_6H_5IO in the same assay system have provided unequivocal evidence for DNA degradation (G. Ehrenfeld, unpublished results). Moreover, as shown in Figure 10, Fe(III) BLM + C_6H_5IO also effected conversion of SV-40 form I DNA to form II and form III DNA. Due to the more sensitive nature of this assay system, strand scission could be observed at 5 μM Fe(III)·BLM. Also illustrated clearly in this figure was the fact that more strand scission was obtained with Cu(II)·BLM than with Fe(III)·BLM (cf. Figure 8).[7] Under the assumption that DNA degradation in the aerobic system can proceed via the same activated intermediate produced from Fe(III)·BLM + C_6H_5IO, activation could presumably be represented as in eq 1. One important implication of eq 1 is that bleomycin would

$$\left.\begin{array}{l}\mathrm{Fe(II)} + \mathrm{BLM} + \mathrm{O_2} + \mathrm{e^-} \\ \mathrm{Fe(III)} + \mathrm{BLM} + \mathrm{C_6H_5IO}\end{array}\right\rangle \text{activated Fe·BLM} \quad (1)$$

necessarily be capable of functioning in DNA degradation as a monooxygenase, consistent with the observed products of olefin oxidation (Murugesan et al., 1982). An interesting point worthy of investigation is the properties of the activated BLM produced by anaerobic activation relative to the short-lived species described by Burger et al. (1981).

The experiments carried out with Cu(II)·BLM + C_6H_5IO are important in that they demonstrate the oxygen-transfer properties of activated Cu(II)·BLM (as well as its ability to degrade DNA) in a system that lacks any reducing agent. The possible involvement of contaminating metals is thus remote, reinforcing the conclusion that Cu(II)·BLM participates in formation of an active complex in the presence of oxygen surrogates such as C_6H_5IO; in analogy with eq 1, the available evidence suggests strongly that Cu(I)·BLM participates in the formation of an active complex under aerobic conditions (eq 2). This analysis suggests that in addition to their role in

$$\left.\begin{array}{l}\mathrm{Cu(I)} + \mathrm{BLM} + \mathrm{O_2} + \mathrm{e^-} \\ \mathrm{Cu(II)} + \mathrm{BLM} + \mathrm{C_6H_5IO}\end{array}\right\rangle \text{activated Cu·BLM} \quad (2)$$

maintaining Fe(II)·BLM and Cu(I)·BLM in their reduced forms, reducing agents such as dithiothreitol may also provide a source of electrons for formation of the active metallobleomycins per se. In the absence of a reducing agent, the requisite electron would have to be acquired from within the assay system [e.g., by disproportionation of two Fe(II)·BLM molecules (Kuramochi et al., 1981)], which would account for the substantial enhancement of DNA degradation by bleomycin observed in the presence of modest concentrations of dithiothreitol.

Antholine et al. (1982) have described the interaction of glutathione and cysteine with certain metallobleomycin complexes. Since cytochrome P-450, with which bleomycin appears to share some mechanistic similarities, may also have S as a metal ligand (White & Coon, 1980), it seemed of interest to investigate the possible binding of dithiothreitol to Cu(I)·BLM. The ^{1}H NMR study of the interaction of dithiothreitol with Cu(I) was inconclusive. The results demonstrated the ability of dithiothreitol to sequester metal ions from bleomycin, but no spectroscopic evidence was found for the formation of a ternary complex. It is clear, however, that at the high concentrations used for the NMR experiments the solubility product for Cu(I)–dithiothreitol was exceeded.[8] Since the lower concentrations used for the DNA cleavage studies are not accessible to observation by ^{1}H NMR, we cannot exclude the direct participation of dithiothreitol as a ligand during the cleavage reaction.

The participation of other sulfur ligands such as dithionite is unlikely. We observed no difference in the ^{1}H NMR spectra of the Cu(I)·BLM complex prepared by addition of Cu(I)·OAc in acetonitrile to bleomycin in the absence of dithionite and that derived from in situ reduction of Cu(II)·BLM with dithionite. Furthermore, increasing the concentration of dithionite had no effect on the spectral parameters.

Several workers have described the lack of activity of Cu(II)·BLM in the presence and absence of dithiothreitol. In fact Cu(II) has been used to quench bleomycin-mediated processes. These reports seemed inconsistent with our findings and prompted a comparison of the experimental conditions employed. As one key difference was the concentration of Cu(II), the effect of variable [Cu(II)] was investigated. As

[7] Although excluded from these reaction mixtures to simplify mechanistic considerations, O_2 had little or no effect on the extent of DNA degradation mediated by metallobleomycins + C_6H_5IO under these conditions.

[8] Similar results, indicating sequestering of Cu(I) following reduction of Cu(II)·BLM by thiols, have been reported on the basis of fluorescence experiments (Antholine et al., 1982).

shown in Figure 11, degradation of [^{3}H]DNA by Cu(II)·BLM was strongly dependent on the concentration of Cu(II) when 40 μM dithiothreitol was employed. At the Cu(II) concentrations employed for the present study ($\leq$25 μM), substantial DNA degradation was observed. However, at concentrations comparable to those used in previous studies (>50 μM), little or no DNA degradation was obtained, consistent with the findings reported in the literature. A possible explanation of the lack of DNA cleavage at high Cu(II) concentration may relate to the well-documented [see, e.g., Eichhorn et al. (1971)] ability of this metal ion to bind to and unwind dsDNA. Given the large molar excess of Cu(II) relative to DNA nucleotides, it would not be surprising if the resulting Cu(II)–DNA complexes were refractory to degradation by Cu(I)-BLM. Regardless of the reason(s) for the apparent inactivity of Cu(I)·BLM at high concentrations of metal ion, Cu(I)·BLM does degrade DNA under the experimental conditions defined here and should not be ignored when interpreting the results of DNA cleavage experiments involving bleomycin and mixtures of metals [see, e.g., Freedman et al. (1982)].

The findings outlined here have two important implications for future studies of bleomycin mechanism of action. First, under the assumption that bleomycin mediates its therapeutic effect as part of a metal complex, it seems possible that Cu, rather than Fe, may be the relevant metal, if only because of the higher affinity of Cu for bleomycin. Second, Cu(I)·BLM clearly has a geometry distinct from Fe(II)·BLM and deglyco-Fe(II)·BLM, both of which have also been shown to effect DNA strand scission [Aoyagi et al., 1982; Oppenheimer et al., 1982; see Sausville et al., (1978a) and references cited therein]. Therefore, the design of additional bleomycin analogues active in DNA degradation clearly does not depend on adherence to a single geometry for metal ion coordination.

Registry No. Cu(I)·BLM, 72348-90-4; Cu(I)·BLM·CO, 77145-93-8; thymine, 65-71-4; dithiothreitol, 3483-12-3; sodium dithionite, 7775-14-6; monooxygenase, 9038-14-6.

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Lamprey Fibrinogen γ Chain: Cloning, cDNA Sequencing, and General Characterization†

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Received June 22, 1984

ABSTRACT: A cDNA library from lamprey liver was constructed in pBR322 and screened with a synthetic mixed oligonucleotide probe, the sequence of which was based on a partial amino acid sequence of the lamprey fibrinogen γ chain determined by conventional procedures. Among the positive clones was one containing a 600-base insert that covered the carboxy-terminal third of the chain and another with a 1950-base insert that stretched more than full length. The two inserts were sequenced by the Maxam–Gilbert procedure. The DNA sequencing was corroborated by reference to the amino acid sequences of five cyanogen bromide peptides that compose the carboxy-terminal 130 amino acids, as well as to a number of tryptic peptides from elsewhere in the molecule. The clone with the smaller insert (6G) contained 594 nucleotides (not counting G and C tails), 435 of which are coding and correspond to residues 264–408 of the γ chain. The remaining 159 nucleotides included the terminator codon followed by a noncoding segment. The larger clone (2E) coded for 408 amino acids that could be readily aligned with the 411-residue human γ chain. A 24-residue signal peptide adjacent to the proposed amino terminal was also inferred. The amino acid sequence of the fibrinogen γ chain has been differentially conserved during evolution, the lamprey and human sequences being more than 70% identical in certain key regions but dropping to less than 25% in other sections, including the segment thought to be a part of the "coiled coils". Overall, the resemblance amounts to 50% identity. Of the 10 cysteines found in mammalian chains, 9 are at identical positions, but the tenth, which in mammalian fibrinogens is a part of the interdimeric bridging, is absent in the lamprey.

Vertebrate blood coagulation centers on the conversion of a soluble plasma protein, fibrinogen, into an insoluble gel, fibrin. In all species examined, from fish to mammals, the fibrinogen molecule consists of three pairs of nonidentical chains interconnected by a network of disulfide bonds. Because the lamprey is one of the most primitive of extant vertebrates, considerable attention has been focused on its fibrinogen (Doolittle, 1965; Murtaugh et al., 1974; Doolittle & Wooding, 1974; Cottrell & Doolittle, 1976). Although the fundamental plan of lamprey fibrinogen is the same as that found in mammals, there are some unique and interesting features, including the ability to clot upon the exclusive removal of its fibrinopeptide B, a moiety that in lampreys contains carbohydrate (Doolittle & Cottrell, 1974). There is no immunological cross-reactivity between lamprey and human fibrinogens, and the amino acid compositions of the various chains are recognizably different (Doolittle et al., 1976). Aspects which have been conserved throughout vertebrate evolution include the existence of two thrombin-released fibrinopeptides, a polymerization scheme that is inhibited by Gly-Pro-Arg peptides (Laudano & Doolittle, 1980), and a factor XIII catalyzed stabilization system (Doolittle & Wooding, 1974; Murtaugh et al., 1974).

We have pursued the study of lamprey fibrinogen over the years in the hope not only that clues would be revealed about the evolution of the molecule but also that the mechanisms of action involved in fibrin formation might be inferred from

† This work was supported by a grant from the National Institutes of Health (HL 26873).

0006-2960/85/0424-0092$01.50/0