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Interactions of Bleomycin Analogues with Deoxyribonucleic Acid and Metal Ions Studied by Fluorescence Quenching†

Cheng-Hsiung Huang,* Louis Galvan, and Stanley T. Crooke

ABSTRACT: Fluorescence quenching has been used to study the interactions of DNA, in the presence and absence of metal ions, with various bleomycin (BLM) analogues, which include the active BLM A_2, BLM B_1', talisomycin A (TLM A), desamido-BLM A_2, the three less active BLM A_2 analogues with a substitution at the α-amino group of the β-aminoalanine, and the two inactive TLM fragments (W_a and W_b). All drugs showed a nonionic type DNA-induced fluorescence quenching of 20–30% but varied in the extent of the ionic type of quenching as follows: TLM A, W_α, and W_b, 50–60%; BLM A_2, 20–25%; BLM B_1' and desamido-BLM A_2, <10%; the three α-amino-substituted BLM A_2 analogues, 0%. Thus, there is no apparent correlation of the overall DNA breakage activity of drugs with the extent of either type of quenching. Thus, DNA–drug interactions as seen by quenching effects are not sufficient for breakage activity. However, the modification of the α-amino group completely eliminated the ionic quenching and reduced the nonionic type. The removal of the terminal amide group of the β-aminoalanine as in desamido-BLM A_2 markedly reduced the ionic quenching, whereas an increase in the positive charge of the C-terminal amine enhanced the ionic quenching. Thus, both the β-aminoalanine and the C-terminal amine may be essential for the ionic type of quenching effect, which may be associated with the DNA double-strand breakage activity of BLM since such activity was very low in desamido-BLM A_2 in contrast to BLM A_2. The binding analysis indicated that at high ionic strength, the removal of the amide or the loss of the positive charge in the terminal amine minimally affected the binding constant but reduced the number of available binding sites by 50%. These residual sites are nonionic in nature. The overall quenching effect showed a base and sequence preference consistent with the reported GC, GT, and TA specificity of the DNA breakage. The quenching effects of DNA and Fe(II) or Cu(II) were additive. β-Mercaptoethanol, which showed a differential effect on Fe(II)- and Cu(II)-induced quenching, had little effect on the DNA-induced quenching.

Bleomycins are a family of glycopeptides, some of which have been clinically used to treat certain human tumors (Umezawa, 1976; Crooke & Bradner, 1976; Muller & Zahn, 1977; Goldberg et al., 1977). The antitumor activity of the bleomycins is thought to be related to the ability of bleomycins to induce single- and double-strand breakage of DNA molecules (Suzuki et al., 1969; Haidle, 1971; Muller et al., 1972; Takeshita et al., 1974).

It has been reported that DNA quenched the fluorescence of bleomycin A_2 (Chien et al., 1977; Strong & Crooke, 1978a) and of talisomycin (Strong & Crooke, 1978a,b). In a previous report (Huang et al., 1979), we have demonstrated that the quenching effects of Fe(II) and Cu(II) ions on the fluorescence of various bleomycin analogues were related to the ability of bleomycin analogues to induce DNA strand breakage. The purpose of the present study was to investigate the interactions of bleomycin analogues with DNA in the presence or absence of externally added metal ions and the relationship between these interactions and the ability of the bleomycin analogues to induce DNA breakage.

Materials and Methods

Chemicals. The following bleomycin analogues and hydrolytic fragments were obtained from Bristol Laboratories: bleomycin (BLM)[1] A_2, BLM B_1', talisomycin (TLM) A, methylsulfonamido-BLM A_2, benzylsulfonamido-BLM A_2, dansylsulfonamido-BLM A_2, and TLM fragments W_a and W_b. The chemistry and biology of the semisynthetic compounds are subjects of a separate report. Desamido-BLM A_2 was a generous gift from Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. PM-2 DNA was isolated according to the procedure of Strong & Hewitt (1975). For fluorescence studies, each PM-2 DNA preparation was dialyzed against the Tris-HCl buffer used in fluorescence assays to reduce the NaCl concentration. Calf thymus DNA, *Micrococcus lysodeikticus* DNA, *Clostridium perfringens* DNA, and yeast tRNA were obtained from Sigma Co. (St. Louis, MO). Chloride salts of Fe(II) and Cu(II) were obtained from Mallinckrodt Co. (St. Louis, MO). Synthetic polynucleotides were purchased from P-L Biochemicals Co. (Milwaukee, WI).

Fluorescence Measurements. Fluorescence was measured with an Amico-Bowman spectrophotofluorometer in a 1-mL cuvette. The reaction buffer (1 mL) contained 2.5 mM

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[1] Abbreviations used: BLM, bleomycin; Na_2EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; TLM, talisomycin.

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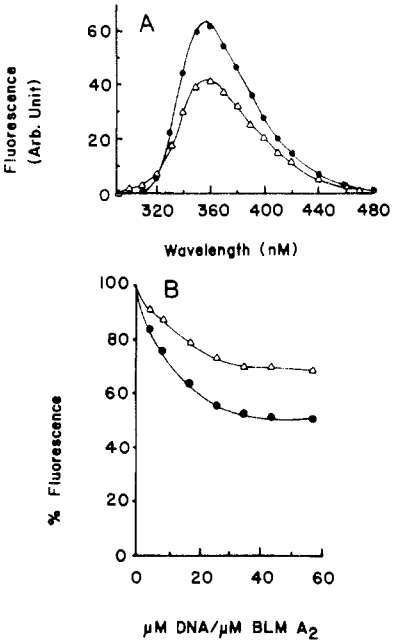

FIGURE 1: (A) Quenching effects of calf thymus DNA on BLM A_2 fluorescence (excitation at 300 nm). (●) Spectrum of 14 μM BLM A_2; (△) spectrum of 14 μM BLM A_2 + 170 μM calf thymus DNA. (B) Quenching effects of DNA at increasing concentrations on BLM A_2 fluorescence (excitation 300 nm; emission 360 nm) measured in the absence (●) or presence (△) of 20 mM Na_2EDTA.

Tris-HCl and 1.2 mM NaCl, pH 8.4 (Chien et al., 1977), and in some experiments Na_2EDTA at various concentrations. Drugs were studied at a concentration of 14 μM. For assays of fluorescence quenching, small volumes of concentrated calf thymus DNA or PM-2 DNA (1.5 mg/mL), and in some experiments freshly prepared solutions of metal ions (1 or 2 mM) or β-mercaptoethanol (1 M), were added to the reaction mixture. The dilution effect was properly corrected. In this report uncorrected fluorescence was assayed. Unless specified otherwise, the fluorescence quenching was studied at 360 nm with an excitation at 300 nm. The fluorescence properties of the bleomycin analogues studied have been described in a previous report (Huang et al., 1979). The possibility of fluorescence quenching due to DNA absorption of the 300-nm excitation light was tested by a comparison with results obtained by using an excitation at 310 nm which is well beyond the DNA absorption range. A comparison indicated that a decrease of the quenching effect by 5–9% was observed with 310-nm excitation and the proportion of the nonionic quenching remained unchanged. Thus, the DNA absorption effect at 300 nm is insignificant.

Binding Studies. The Scatchard analysis (Scatchard, 1949) has been used to obtain binding parameters for various BLM analogues from the DNA-induced fluorescence quenching effect:

$$r_b/c = K_a n - K r_b$$

where r_b is the number of drug molecules bound per DNA phosphate, c is the free drug concentration, K_a is the apparent association constant, and n is the number of drug binding sites per nucleotide phosphate. A plot of r_b/c vs. r_b gives a K_a value from the slope and an n value from the intercept. The fluorescence of BLM analogues in the absence of DNA and metal ion was considered as the fluorescence of free drugs. The residual fluorescence after obtaining maximal quenching by DNA either in the absence or in the presence of metal ion was considered as the fluorescence of bound drugs.

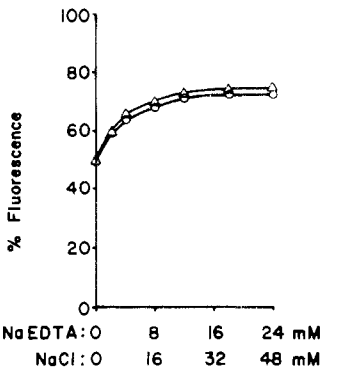

FIGURE 2: Partial reversal by NaCl (△) or Na_2EDTA (○) of the DNA-induced quenching effects of BLM A_2 fluorescence. The fluorescence of 14 μM BLM A_2 was first quenched by 800 μM DNA to 50%, and then NaCl or Na_2EDTA was added subsequently.

Results

DNA-Induced Quenching of Bleomycin A_2 Fluorescence. Figure 1A shows the fluorescence spectrum of BLM A_2 (excitation at 300 nm) and the fluorescence quenching effect of calf thymus DNA. In agreement with previous studies (Chien et al., 1977; Strong & Crooke, 1978a), BLM A_2 has a fluorescence peak at 355–360 nm. The DNA quenching effect was immediate. Figure 1B shows the quenching of BLM A_2 fluorescence induced by increasing concentrations of calf thymus DNA. In the absence of Na_2EDTA, DNA induced a maximal fluorescence quenching of 50% at a [DNA]/[drug] ratio of 50–60, in agreement with previous reports (Chien et al., 1977; Strong & Crooke, 1978a). Similar results (not shown) were obtained for PM-2 DNA, indicating that the fluorescence quenching was independent of the conformational structure of DNA preparations. Figure 1B also shows that in the presence of 20 mM Na_2EDTA the extent of quenching by DNA was reduced by half. Further addition of either Na_2EDTA or 1 M NaCl to the reaction mixture had no further effect on the residual fluorescence (not shown).

Figure 2 shows that addition of either Na_2EDTA or NaCl to a DNA–BLM A_2 mixture partially reversed the fluorescence quenching induced by DNA. The addition of 1 M NaCl had little effect on bleomycin A_2 fluorescence, and 20 mM Na_2-EDTA caused a reduction of fluorescence of less than 7%. After the fluorescence of BLM A_2 was maximally quenched by DNA (to 50%), the addition of either Na_2EDTA or NaCl gradually eliminated the quenching effect until a residual fluorescence of 73% was obtained at a concentration of 18–20 mM Na_2EDTA or 40–50 mM NaCl. The kinetics of the reversal of the quenching effect were similar for Na_2EDTA and NaCl.

The simultaneous addition of Na_2EDTA and NaCl resulted in additive effects but the maximal fluorescence was ~73% of that for unquenched BLM A_2. Thus, it seems that both NaCl and Na_2EDTA acted through a similar mechanism, i.e., increasing ionic strength which interfered with the electrostatic interactions. Thus, at least two types of quenching of BLM A_2 by DNA were observed. One type of quenching (25–30%) was sensitive to both Na_2EDTA and NaCl, probably resulting from electrostatic or ionic types of interaction. The other type of quenching (20–25%) was insensitive to either Na_2EDTA or NaCl. The results obtained when PM-2 DNA was employed were similar to those obtained for calf thymus DNA (not shown).

Quenching of BLM A_2 Fluorescence by tRNA. The specificity of nucleic acids with respect to the fluorescence quenching effect was tested with yeast tRNA. The results (not

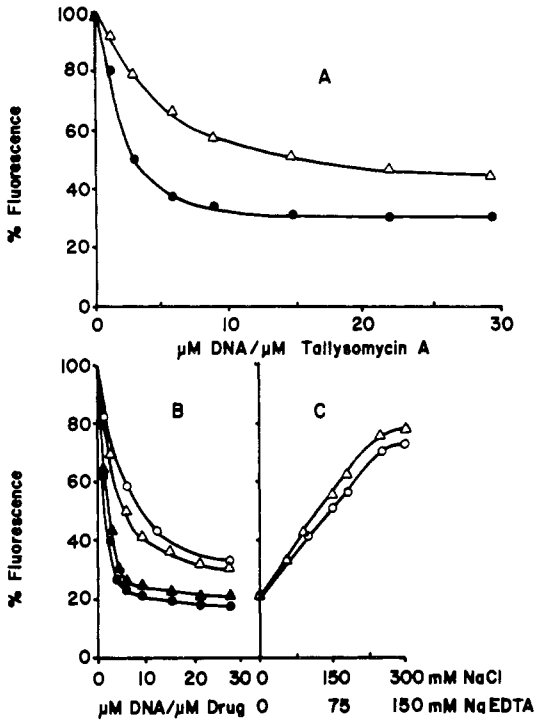

FIGURE 3: Quenching effects of DNA at increasing concentrations on the fluorescence of 14 μM talisomycins A, W_a, and W_b. (A) TLM A in the absence (●) or presence (△) of 20 mM Na_2EDTA. (B) TLM W_a (▲) or W_b (●) without Na_2EDTA and TLM W_a (△) or W_b (○) in the presence of 20 mM Na_2EDTA. (C) Reversal by NaCl (△) or Na_2EDTA (○) of the DNA-induced quenching of TLM W_a fluorescence. Fluorescence of TLM W_a was first quenched by DNA (to 21%), and then NaCl was added. Buffer solutions with increasing concentrations of Na_2EDTA were used to study the effects of Na_2-EDTA.

shown) indicated that tRNA induced a fluorescence quenching of BLM A_2 very similar to that induced by DNA. A maximal quenching of 53% was obtained at a [tRNA]/[drug] ratio of ~70. The quenching was reduced to 30% when 60 mM NaCl or 20 mM Na_2EDTA was added.

Quenching Effect of DNA on the Fluorescence of Bleomycin Analogues. Figures 3, 4, and 5 show the quenching profiles of various other BLM analogues and two hydrolytic fragments of TLM induced by DNA at increasing concentrations. The structural modifications and the fluorescence properties of these compounds have been described (Huang et al., 1979). Figure 3 shows the quenching profiles of TLM A (Figure 3A) and TLM fragments W_a and W_b (Figure 3B), which are the C-terminal hydrolytic half-molecules of TLM A and TLM B, respectively (Konishi et al., 1977). These compounds were extensively quenched by DNA at a relatively low concentration. The maximal quenching induced by DNA of the fluorescence of TLM A (70%), W_a (80%), or W_b (85%) was obtained at a [DNA]/[drug] ratio of less than 5. The presence of 20 mM Na_2EDTA reduced the DNA-induced quenching especially at low [DNA]/[drug] ratios, indicating competition of Na_2EDTA with DNA for the drugs. Figure 3C shows that Na_2EDTA or NaCl reversed the DNA-induced fluorescence quenching of TLM W_a. Compared to that of BLM A_2, the quenching effect was more resistant to either Na_2EDTA or NaCl, requiring as much as 150 mM Na_2EDTA or 300 mM NaCl to reverse the quenching to a fluorescence level of 20–25%. The response of the fluorescence quenching of TLM A to NaCl or Na_2EDTA was equivalent to that of TLM W_a (not shown). Thus, these compounds exhibited two types of quenching, ~20–25% insensitive and ~50–60% sensitive to Na_2EDTA and NaCl.

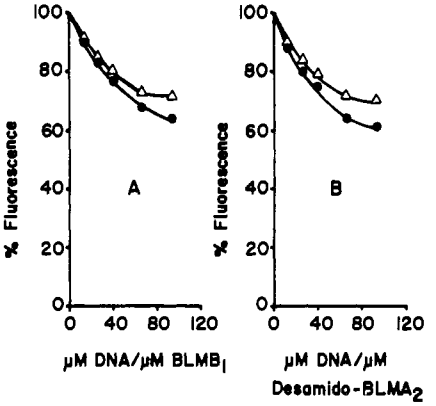

FIGURE 4: Quenching effects of DNA at increasing concentrations on the fluorescence of BLM B_1' (A) and desamido-BLM A_2 (B) in the absence of Na_2EDTA (●) and in the presence of 20 mM Na_2-EDTA (△).

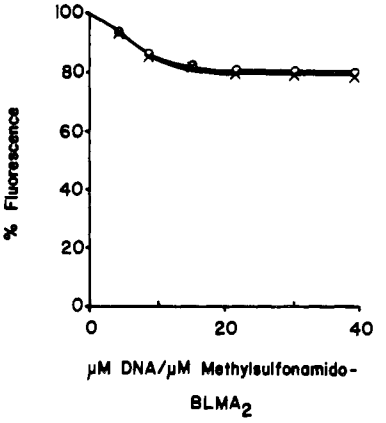

FIGURE 5: Quenching effects of DNA at increasing concentrations on the fluorescence of 14 μM methylsulfonamido-BLM A_2 in the absence (○) and presence (×) of 20 mM Na_2EDTA.

Figure 4 shows the quenching characteristics of BLM B_1' (Figure 4A) and desamido-BLM A_2 (Figure 4B). In BLM B_1', the terminal amine structure is an NH_2 group. In desamido-BLM A_2, the amide group of the β-aminoalanine moiety of BLM A_2 is removed. The fluorescence of each of these compounds was quenched to a maximum of 40% at a [DNA]/[drug] ratio of 80. The presence of 20 mM Na_2EDTA (or 50 mM NaCl) consistantly resulted in reversal of less than 10% of the quenching. Higher concentrations of either Na_2EDTA or NaCl did not cause further reversal. Thus, although the fluorescence of the two compounds was quenched by ~30% by an Na_2EDTA- and NaCl-insensitive process, the fluorescence was quenched by only ~10% by an Na_2EDTA- and NaCl-sensitive process.

Another group of analogues included three BLM A_2 derivatives with substitutions at the α-amino group of the β-aminoalanine moiety, i.e., methylsulfonamido-BLM A_2. A typical DNA-induced fluorescence quenching profile is shown in Figure 5 for methylsulfonamido-BLM A_2. The other two derivatives had similar quenching profiles (not shown). The fluorescence of all three derivatives was quenched to a maximum of 23–28%, and this was insensitive to 0.2 M Na_2EDTA or 1 M NaCl. The peak at 535 nm of dansylsulfonamido-BLM A_2 reacted to the addition of DNA in a manner similar to the 360-nm peak.

Fluorescence Quenching Effect of DNA on Bleomycin·Cu Complexes. Figure 6 shows the quenching effects of DNA at increasing concentrations on the fluorescence of the BLM A_2·Cu complex in comparison to that of Cu-free BLM A_2. The fluorescence intensity of Cu-free BLM A_2 was taken as 100%.

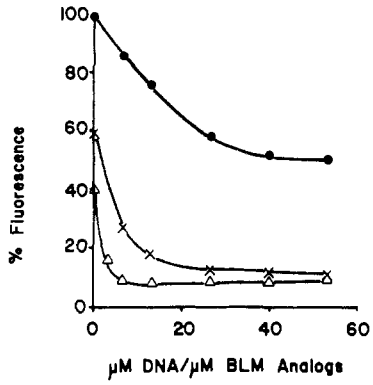

FIGURE 6: Quenching effects of DNA at increasing concentrations on the fluorescence of BLM A_2 (●), BLM A_2·Cu complex (△), and a mixture (×) of 14 μM BLM A_2 and 15 μM Cu(II). The fluoresence of Cu-free BLM A_2 was taken as 100%.

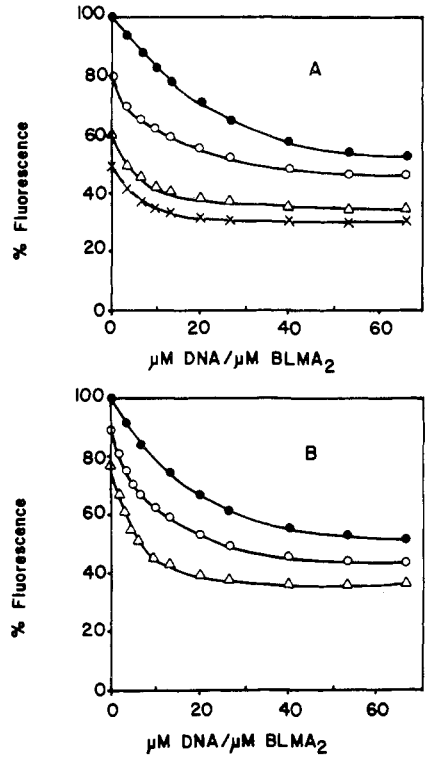

FIGURE 7: Quenching effects of DNA on BLM fluorescence in the presence of externally added Fe(II) or Cu(II). (A) Quenching effects of DNA obtained after the fluorescence of 14 μM BLM A_2 was first quenched by Fe(II) at 0 (●), 1.7 (○), 3.4 (△), and 5.0 μM (×). (B) Quenching effects of DNA obtained after the fluorescence of 14 μM BLM A_2 was first quenched by Cu(II) at 0 (●), 2.5 (○), and 5 μM (△).

On a molar basis, BLM A_2·Cu gave only 40% of the fluorescence of the Cu-free BLM A_2, probably resulting from the fluorescence quenching by Cu(II) ion since in a previous report we have shown that Cu(II) induced an extensive quenching of bleomycin fluorescence. The fluorescence of the BLM A_2·Cu complex was more effectively quenched by DNA than that of Cu-free BLM A_2. A maximal quenching, which resulted in a residual fluorescence of 8%, was obtained at a [DNA]/[drug] ratio of ~7. The enhancement by Cu(II) of the DNA-induced fluoresence quenching was also evident when the fluoresence of Cu(II)-free BLM A_2 was first maximally quenched by Cu(II) and then subsequently quenched by DNA. Detailed studies on the quenching effects of a combination of DNA and metal ions are described in the following section.

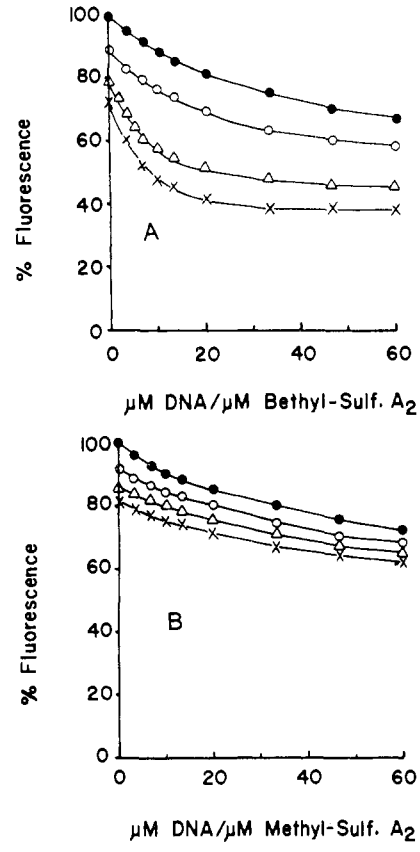

FIGURE 8: (A) Quenching effects of DNA on the fluorescence of 14 μM methylsulfonamido-BLM A_2 in the presence of Cu(II) at 0 (●), 2.5 (○), 5.0 (△), and 7.5 μM (×). (B) Quenching effects of DNA on the fluorescence of 14 μM methylsulfonamido-BLM A_2 in the presence of Fe(II) at 0 (●), 2.5 (○), 10 (△), and 20 mM (×).

Effects of Fe(II) and Cu(II) on DNA-Induced Fluorescence Quenching. The metal ion Fe(II) has been reported to stimulate BLM-induced DNA breakage (Ishida & Takahashi, 1975; Sausville et al., 1976, 1978; Lown & Sim, 1977) whereas Cu(II) was inhibitory (Nagai et al., 1969; Suzuki et al., 1973). Parts A and B of Figure 7 show the effects of Fe(II) and Cu(II), respectively, on the DNA-induced quenching of BLM A_2 fluorescence. As has been reported in our previous communication, both Fe(II) and Cu(II) effectively quenched the fluorescence of BLM A_2. The subsequent addition of DNA to Fe(II)- or Cu(II)-quenched BLM A_2 further quenched the fluorescence. An enhancement by Fe(II) and Cu(II) of DNA-induced quenching was observed especially at low [DNA]/[drug] ratios, and as a result the maximal quenching induced by DNA was obtained at lower [DNA]/[drug] ratios than in the absence of metal ions. The extent of the quenching by a combination of DNA with either Fe(II) or Cu(II) was the same regardless of the order of addition.

The effects of Fe(II) and Cu(II) on BLM analogues other than BLM A_2 were also studied. In general, if the fluorescence of bleomycin analogues was extensively quenched by metal ion, an enhancement of DNA-induced quenching was observed, especially at low [DNA]/[drug] ratios. Figure 8 shows the effect of Cu(II) (Figure 8A) and Fe(II) (Figure 8B) on DNA-induced fluorescence quenching of methylsulfonamido-BLM A_2, which was nearly inactive in causing DNA breakage. We have previously reported that Cu(II) but not Fe(II) interacted strongly with methylsulfonamido-BLM A_2, inducing a fluorescence quenching comparable to that obtained with BLM A_2. Figure 9 clearly shows that Cu(II) enhanced the fluorescence quenching by DNA whereas Fe(II) resulted

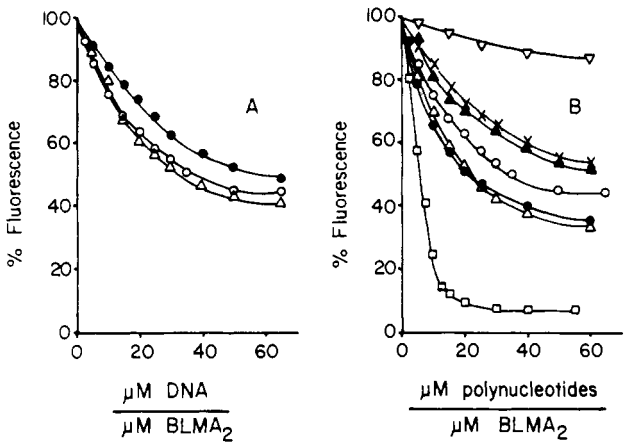

FIGURE 9: Quenching effects of various natural DNAs and synthetic polynucleotides on the fluorescence of BLM A_2. BLM A_2 (7 μM) was titrated with increasing concentrations of nucleic acids. The excitation is 300 nm and the emission is 360 nm. (A) Natural DNAs: (●) *C. perfringens* DNA; (○) calf thymus DNA; (△) *M. lysodeikticus* DNA. (B) Synthetic polynucleotides: (▽) poly(dA)·poly(dT); (▲) poly(dG)·poly(dC); (○) calf thymus DNA; (●) poly(dG,dC); (△) poly(dA-dT)·poly(dA-dT); (□) poly(dG-dC)·poly(dG-dC); (×) poly(dA,dT).

Table I: Scatchard Analyses of the Calf Thymus DNA-Induced Fluorescence Quenching of Various Bleomycin Analogues

BLM analogues	K_a (M^{-1})	n
BLM A_2 (14 μM)	7.98×10^5	0.045
BLM A_2 (14 μM) + NaCl (50 mM)	8.10×10^5	0.028
BLM B_1	8.96×10^5	0.018
desamido-BLM A_2	9.46×10^5	0.022
TLM A	10.02×10^5	0.276
TLM W_a	3.98×10^5	0.357
TLM W_b	3.40×10^5	0.530
BLM A_2 (14 μM)	7.30×10^5	0.035
BLM A_2 (14 μM) + Fe(II) (1.7 μM)	1.00×10^5	0.140
BLM A_2 (14 μM) + Fe(II) (3.4 μM)	1.10×10^5	0.210
BLM A_2 (14 μM) + Fe(II) (5.0 μM)	2.29×10^5	0.175
BLM A_2 (14 μM)	8.30×10^5	0.040
BLM A_2 (14 μM) + Cu(II) (2.5 μM)	1.18×10^5	0.152
BLM A_2 (14 μM) + Cu(II) (5.0 μM)	1.81×10^5	0.210

in no significant enhancement. Since methylsulfonamido-BLM A_2 undergoes only an Na_2EDTA- or NaCl-insensitive fluorescence quenching by DNA, the ehancement by Cu(II) did not depend on the existence of the Na_2EDTA- or NaCl-sensitive type of quenching.

Binding Parameters of BLM Analogues. In Table I we have shown the results of Scatchard analyses (Scatchard, 1949) of the binding of BLM analogues to the calf thymus DNA. The apparent association constant (K_a) for BLM A_2 was (7.3–8.3) × 10^5 M^{-1}, and the number of available binding sites per nucleotide (n) was 0.035–0.045, which is equivalent to 22–29 nucleotides/site. In the presence of NaCl, the K_a was not altered whereas n was reduced to almost half. Desamido-BLM A_2 and BLM B_1', both of which showed a very small ionic type of fluorescence quenching effect, had a K_a similar to that of BLM A_2 but a value of n approaching that of BLM A_2 in the presence of NaCl. Compared to BLM A_2, TLM A had a slightly higher K_a and a value of n sixfold higher, whereas W_a TLM and W_b had a high n value and a low K_a. The addition of either Fe(II) or Cu(II) resulted in a four- to sixfold increase in the n value and a four- to eightfold reduction in K_a.

Base and Sequence Specificity of the Quenching. The fluorescence quenching of BLM A_2 induced by natural and synthetic nucleic acids was tested to examine the base and sequence specificity for binding, and the results are shown in parts A and B of Figure 9. While the natural DNAs with different C+G contents (Figure 9A) varied very little in the extent of quenching of bleomycin induced, the synthetic polynucleotides (Figure 9B) varied significantly. The order according to the maximal extent of quenching was poly(dG-dC)·poly(dG-dC) > poly(dG,dC) = poly(dA-dT)·poly(dA-dT) > *M. lysodeikticus* DNA > calf thymus DNA > *C. perfringens* DNA > poly(dG)·poly(dC) = poly(dA,dT) > poly(dA)·poly(dT).

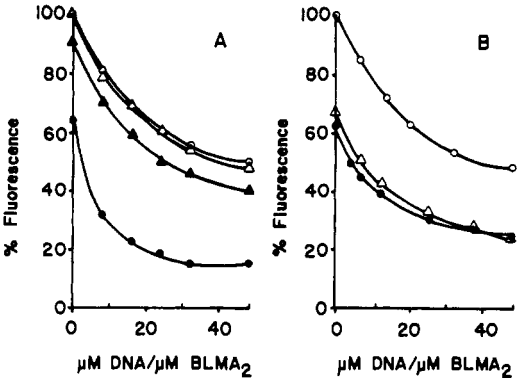

FIGURE 10: (A) Effects of β-mercaptoethanol on the DNA-induced quenching of BLM A_2 fluorescence in the presence or absence of Cu(II): (○) 14 μM BLM A_2 alone; (△) BLM A_2 + 25 mM β-mercaptoethanol (added before DNA addition); (●) BLM A_2 + 10 μM Cu(II); (▲) 10 μM Cu(II) added to a mixture of BLM A_2 and 25 mM β-mercaptoethanol. (B) Effects of β-mercaptoethanol on the DNA-induced quenching of BLM A_2 fluorescence in the presence or absence of Fe(II): (○) 14 μM BLM A_2 + 25 mM β-mercaptoethanol; (△) 14 μM BLM A_2 + 5 μM Fe(II); (●) 5 μM Fe(II) added to a mixture of 14 μM BLM A_2 and 25 mM β-mercaptoethanol.

Effects of β-Mercaptoethanol on the DNA-Induced Fluorescence Quenching. β-Mercaptoethanol facilitates bleomycin-induced DNA breakage (Suzuki et al., 1969; Onishi et al., 1975). We have previously reported that prior addition of β-mercaptoethanol blocked the Cu(II)-induced but not the Fe(II)-induced fluorescence quenching of BLM A_2. The effects of β-mercaptoethanol on the DNA-induced fluorescence quenching of BLM A_2 are shown in Figure 10. β-Mercaptoethanol at 25 mM caused a decrease of only 3–5% of BLM A_2 fluorescence. Figure 10A shows that 25 mM β-mercaptoethanol has little effect on the quenching effects of DNA. Prior addition of 25 mM β-mercaptoethanol reduced the Cu(II)-induced fluorescence quenching and the Cu(II)-induced enhancement of DNA-induced quenching. Addition of β-mercaptoethanol after the addition of Cu(II) failed to affect the quenching effects of Cu(II) and DNA (not shown). Figure 10B shows that β-mercaptoethanol did not affect the quenching effect of Fe(II) and DNA. We have previously demonstrated that β-mercaptoethanol increased the rate but not the final extent of the Fe(II)-induced quenching. Thus, it is clear that β-mercaptoethanol has more significant effects on bleomycin–metal ion interactions than on bleomycin-DNA interactions.

Discussion

Fluorescence quenching by DNA has been reported for BLM A_2 (Chien et al., 1977; Strong & Crooke, 1978a), and several useful kinetic parameters have been determined for the drug–DNA interactions. Chien et al. (1977) attributed the fluorescence properties of bleomycin to the bithiazole rings and, from proton magnetic resonance studies, further noted the involvement of the bithiazole rings and the dimethylsulfonium groups in the amine terminal of BLM A_2 in binding to DNA. There are, in contrast, BLM–metal ion interactions which primarily involve the N-terminal half of the bleomycin

molecule (Dabrowiak et al., 1978a,b; Takita et al., 1978).

We have previously reported (Huang et al., 1979) the ability of various BLM analogues to cause DNA breakage. BLM A_2, BLM B_1', TLM A, and desamido-BLM A_2 were found to be active, inducing breakage of 50% of the DNA molecules at concentrations (EC_{50}) of 78, 135, 80, and 220 ng/mL, respectively. Methylsulfonamido-BLM A_2, dansylsulfonamido-BLM A_2, and benzylsulfonamido-BLM A_2 were markedly less active with EC_{50} = 3900, 2500, and 2500 ng/mL, respectively. Both TLM W_a and W_b were inactive.

The present studies of various bleomycin analogues indicate that there are at least two types of DNA-induced fluorescence quenching. One type is sensitive and the other type is insensitive to changes in ionic strength, probably resulting from ionic and nonionic types of BLM–DNA interaction, respectively. The nonionic type of quenching may result from the partial intercalation of bleomycin bithiazole rings into DNA molecules. The occurrence of such types of intercalation has been previously proposed (Murakami et al., 1976; Povirk et al., 1979), and it has been suggested to provide the observed base and sequence specificity of the BLM-induced DNA breaks (Takeshita et al., 1978).

All bleomycin analogues tested, either active or inactive in causing DNA breakage, exhibited a similar extent of nonionic type of DNA-induced fluorescence quenching (20–30%), whereas the extent of the ionic type of quenching varied with the different BLM analogues. The lack of correlation between the extent of DNA-induced fluorescence quenching and the ability of the analogues to cause DNA breakage may be explained by the following possibilities: (1) the DNA–BLM interactions essential for the overall DNA breakage may result in no or insignificant fluorescence quenching effects; (2) the types of DNA–BLM interactions detectable by fluorescence quenching may not be essential to or may not be the rate-limiting steps of the overall process of DNA breakage. However, it is possible that various types of quenching processes occur and some of them may be related to various types of DNA damage, such as single-strand breaks, double-strand breaks, and alkaline-labile sites. In this regard, it is interesting to note that we have reported a possible correlation between the extent of the Fe(II)-induced fluorescence quenching effect and the drug ability to induce DNA breakage (Huang et al., 1979).

The present study shows that the status of both the N-terminal β-aminoalanine and the C-terminal amine moieties of BLM significantly affects the extent of the ionic type of fluorescence quenching effect of DNA. In the β-aminoalanine moiety, modification of the α-NH_2 group (e.g., by methylsulfonylation) eliminated the ionic type of quenching and the removal of the terminal amide group, as in desamido-BLM A_2, markedly reduced the same type of quenching. The observation of the involvement of β-aminoalanine in the ionic type of BLM–DNA interaction is interesting in that Chien et al. (1977) reported that only the bithiazole and the C-terminal amine of BLM interacted with DNA. The effect of the C-terminal amine on the ionic type of quenching is evident from the observation that BLM B_1' lost most of this type of quenching whereas TLM A, W_a, and W_b, which have a high number of positive charges in the terminal amine (>+2), displayed extensive ionic type quenching. Thus, it is conceivable that the charge may play an important role in the BLM–DNA interactions leading to the ionic type of fluorescence quenching.

Modification of the α-NH_2 of the β-aminoalanine moiety has been shown to eliminate the ability to induce all types of DNA breakage and the Fe(II)-induced fluorescence quenching (Huang et al., 1979). We have recently observed (unpublished experiments) that desamido-BLM A_2 induces primarily single-strand DNA breaks with very few double-strand breaks whereas BLM A_2 gives both types of breaks. Both desamido-BLM A_2 and BLM B_1' lost most of the ionic type of interaction with DNA. Although more studies are needed, it is tempting to suggest that the interaction of DNA with the β-aminoalanine moiety, alone or in conjunction with the C-terminal amine, may be related to the ability to induce double-strand breakage. A correlation between the ionic type of DNA–BLM interaction and the double-strand breakage activity is consistent with the observation of Lloyd et al. (1978) that increasing ionic strength significantly reduces the double-strand breakage activity of BLM, with less effect on the single-strand breaks.

A comparison of the binding parameters obtained from Scatchard analyses suggests that the increase of ionic strength did not affect the apparent association constant (K_a) of BLM A_2 but did reduce the number of available binding sites by 50%. The apparent association constants of both desamido-BLM A_2 and BLM B_1' were similar to that of BLM A_2, but the number of available binding sites was approximately half that of BLM A_2. Thus, if the ionic type of quenching is related to the double-strand breakage activity, we may speculate that the missing binding sites for desamido-BLM A_2 may be responsible for the double-strand breaks.

The apparent association constant of TLM A is only slightly higher than that of BLM A_2 whereas the number of binding sites is sixfold greater than that of BLM A_2. We have recently observed (Mirabelli et al., 1979) that most of the specific sites of double-strand breaks induced by TLM A are different from those induced by BLM A_2. The relationship between this difference and the extensive ionic type of fluorescence quenching of TLM A remains to be studied.

Synthetic polynucleotides with either alternating, double-stranded or random, single-stranded C and G bases induced the most extensive quenching effect, followed by the double-stranded polynucleotides with alternating A and T bases. These observations are consistent with the reports (D'Andrea & Haseltine, 1978; Takeshita et al., 1978) that the BLM mixture cleaves DNA at GC and GT sequences and to a lesser extent at TA sequences. Thus, the specificity of the fluorescence quenching may at least partially reflect the specificity of BLM-induced DNA breakage.

During the course of this study, Kashi et al. (1978) reported some results from fluorescence quenching studies on the interactions between several bleomycin analogues and calf thymus DNA as well as a variety of synthetic polynucleotides. The concluded that (1) the binding site of BLM to DNA is independent from the reaction site of BLM leading to strand scission of DNA, (2) the bithiazole part of BLM preferentially binds to guanine bases in nucleic acids, and (3) the positive charge at the terminal amine facilitates BLM binding to nucleic acids. Our results are consistent with their interpretations.

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Effect of Nonprotein Chromophore Removal on Neocarzinostatin Action†

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ABSTRACT: Evidence is presented for the possible biologic role of the nonprotein chromophore associated with the antitumor, protein antibiotic neocarzinostatin (NCS). Thus, irradiation of NCS at 360 nm, where the chromophore absorbs, is as effective in inactivating its ability to block the growth of *Escherichia coli* as irradiation in the region of protein (tryptophan and tyrosine) absorption. Further, nonprotein chromophore removal by chromatography on Amberlite XAD-7 results in chromophore-free and chromophore-poor fractions whose activities in inhibiting DNA synthesis and cell growth in HeLa cells or in inducing single-strand breaks in supercoiled pMB9 DNA correlated well with their chromophore contents, as measured by their UV–visible absorption and fluorescence spectra. The chromophore-free form of NCS blocks in a dose-dependent and specific manner the in vitro DNA strand scission activity of native NCS. Similarly, the chromophore-free form (macromomycin) of the antibiotic auromomycin inhibits DNA scission by the latter. Chromophore removal from NCS (p*I* 3.3) leads to formation of a protein with a more acidic p*I* (3.2) and a CD spectrum characteristic of a possible biosynthetic precursor form of NCS, referred to as pre-NCS. Inactive, chromophore-free forms of NCS (p*I* 3.2) have also been isolated from clinical NCS and from previously purified and stored NCS by rechromatography on CM-cellulose. Further, a protein-free fraction eluting at a pH of 3.8–4.0 has the spectral characteristics of the nonprotein chromophore. These data raise the possibility that pre-NCS is a chromophore-free (apoprotein) form of NCS.

Neocarzinostatin (NCS)[1] is an acidic antitumor, protein antibiotic whose primary 109 amino acid sequence has been determined (Meienhofer et al., 1972). Work from several laboratories indicates that cellular DNA is a target in the action of NCS and that DNA strand scission is a consequence of NCS action both in vivo and in vitro [reviewed in Goldberg et al. (1980)]. Evidence for the existence of an active, labile form of NCS that places single-strand breaks in linear and supercoiled DNA has been presented (Kappen & Goldberg, 1978a, 1979). It has only recently been appreciated that NCS

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[1] Abbreviations used: NCS, neocarzinostatin; Tris, tris(hydroxymethyl)aminomethane; CM-cellulose, carboxymethylcellulose (CM-23; Whatman).

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