Accelerated Publications

Copper(II) Facilitates Bleomycin-Mediated Unwinding of Plasmid DNA†

Mark J. Levy‡ and Sidney M. Hecht*
Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901
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ABSTRACT: The unwinding of plasmid DNA by bleomycin A_2 (BLM A_2) was investigated by use of two-dimensional gel electrophoresis. It was found that Cu^{2+} ions greatly facilitated the unwinding of topoisomers of plasmid DNA by BLM A_2 at concentrations where cupric ions alone had no effect on DNA supercoiling. The concentration of BLM A_2 required for observable unwinding was reduced at least 100-fold in the presence of equimolar Cu^{2+}. A plot of $[Cu^{2+}]$ vs extent of DNA unwinding in the presence of 10^{-4} M BLM A_2 gave a curve consistent with the action of cupric ions on BLM in an allosteric fashion, possibly rearranging the drug into a conformation that facilitates DNA unwinding. The participation of the metal center in enhancing DNA unwinding via direct ionic interaction with one or more negatively charged groups on the DNA duplex also seems possible. Further analysis of the structural factors required for BLM-mediated DNA unwinding was carried out with Cu^{2+} + BLM demethyl A_2, the latter of which differs from BLM A_2 only in that it lacks a methyl group, and associated positive charge, at the C-terminus. Cu(II)·BLM demethyl A_2 was found to be much less effective than Cu(II)·BLM A_2 as a DNA unwinding agent, emphasizing the strong dependence of this process on the presence of positively charged groups within the BLM molecule. These findings constitute the first direct evidence that the metal center of BLM can participate in DNA interaction, as well as in the previously recognized role of oxygen binding and activation.

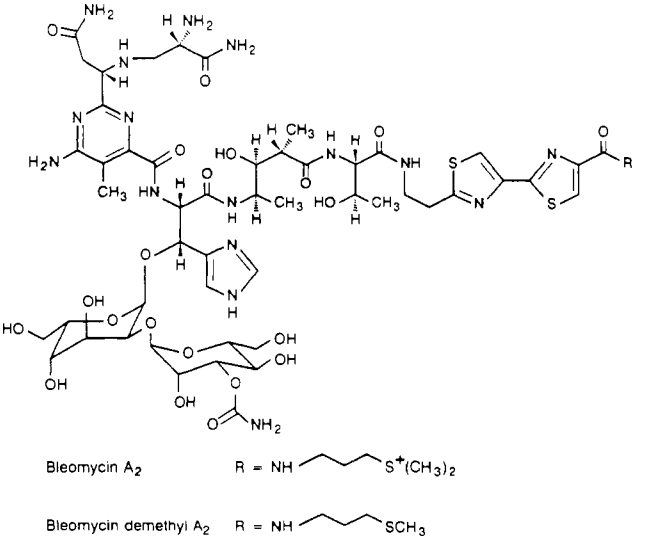
The bleomycins (BLM's)[1] are structurally related, glycopeptide-derived antibiotics shown to be useful clinically in the treatment of certain malignancies (Hecht, 1979; Umezawa, 1980; Povirk, 1983). The antitumor effects of the bleomycins appear to be related to their ability to mediate DNA degradation (Suzuki et al., 1969; Umezawa, 1979), a process that requires the initial binding of any one of several metal ions by bleomycin (Ishida & Takahashi, 1975; Sausville et al., 1978; Chang & Meares, 1984; Ehrenfeld et al., 1984, 1985, 1987; Kuwahara et al., 1985). The bound metal ion promotes DNA cleavage either by binding and activation of oxygen (Sausville et al., 1978; Burger et al., 1981; Ehrenfeld et al., 1984, 1985, 1987) or else by transduction of light energy (Chang & Meares, 1982, 1984; Suzuki et al., 1984, 1985).

Previous studies of bleomycin–DNA interaction have suggested that bleomycin can partially or fully intercalate into double-stranded DNA helices (Povirk et al., 1979, 1981; Chen et al., 1980; Lin & Grollman, 1981; Booth et al., 1983). While the effect of chelated metal ions on the extent of BLM–DNA binding has been investigated (Kasai et al., 1978; Huang et al., 1980; Roy et al., 1981), the influence of those metal ions on intercalative binding has remained unclear, with NMR experiments showing little difference between BLM and metallobleomycins (Glickson et al., 1981).

Reported herein is the facilitation of BLM-mediated DNA unwinding by Cu^{2+}, in what appears to be one of the first examples of participation of a metal ion as an allosteric effector. Also demonstrated for the first time is the role of the metal center in a metallobleomycin in determining the nature of BLM–DNA interaction, as well as oxygen activation.

Experimental Procedures

ϕX174 replicative form DNA and agarose were purchased from Bethesda Research Laboratories. Bleomycin was obtained from Bristol Laboratories through the courtesy of Dr. William Bradner; it was fractionated as described previously (Chien et al., 1977; Oppenheimer et al., 1979) to provide bleomycin A_2 and bleomycin demethyl A_2. Calf thymus topoisomerase I was a gift from Dr. Robert Hertzberg, Smith Kline & French Laboratories.



Preparation of Topoisomers. The topoisomer populations needed for two-dimensional gel electrophoresis were produced by calf thymus topoisomerase I relaxation of supercoiled DNA in the presence of ethidium bromide, according to the method of Fisher et al. (1985).

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* Address correspondence to this author at the Department of Chemistry.
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[1] Abbreviations: BLM, bleomycin; EDTA, ethylenediaminetetraacetic acid; cccDNA, covalently closed circular DNA.

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Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was performed by running tube gels containing the effector as the first dimension (Espejo & Lebowitz, 1976) and slab gels without effector as the second dimension. Bleomycin A_2 and $CuCl_2$ were premixed in 100 μL of H_2O, then added to 12 mL of 0.8% agarose in 40 mM Tris–OAc buffer, pH 7.8, containing 1 mM EDTA, and poured into 5 mm × 40 cm glass tubes. Two micrograms of ϕX174 plasmid DNA containing a distribution of topoisomers was loaded onto the tube gels in 40 mM Tris–OAc buffer, pH 7.8, containing 1 mM EDTA, 30% glycerol, and 0.03% bromophenol blue, and run at 60 V for 20 h. Bleomycin migrated in a direction opposite that of DNA, but at a rate less than or equal to that of the tracking dye. The bromophenol blue was only permitted to reach the halfway point of the gel, ensuring that the (metallo)BLM did not run past the DNA (Espejo & Lebowitz, 1976). The top half of the tube gel, which contained the DNA, was first excised, then sealed into a 20 × 28 cm slab gel (0.8% agarose in 40 mM Tris–OAc buffer, pH 7.8, containing 1 mM EDTA), and run in the orthogonal direction for 18 h at 50 V. The gels were stained for 1–3 h (1 μg/mL ethidium bromide) and illuminated with UV light. Some gels were destained in 1 mM aqueous $MgSO_4$ for 1 h to facilitate visualization of faint bands.

Results and Discussion

Bleomycin-mediated DNA unwinding was studied by use of a sensitive two-dimensional agarose gel electrophoresis technique, which combined existing two-dimensional methods (Lee et al., 1981; Wang et al., 1982) with a tube gel procedure (Espejo & Lebowitz, 1976) to facilitate the use of high concentrations of drug in a relatively small volume. In this manner, we were able to extend the study of Fisher et al. (1985), who first utilized two-dimensional gels as probes of BLM analogue–DNA interaction. The parameter that is observed in these experiments is the change in the number of supercoils in a population of plasmid DNAs containing a variety of linkage numbers. Because certain types of agents such as intercalators are known to decrease the duplex winding number β (Waring, 1970; Wang et al., 1982; Scovell, 1986), thereby altering the electrophoretic mobility of DNA (Keller, 1975; Lee et al., 1981), their interaction with DNA can be studied conveniently by use of two-dimensional gel electrophoresis.

In our experimental system, bleomycin A_2 alone had no effect on the behavior of ϕX174 DNA at concentrations below 10^{-4} M (data not shown). As illustrated in Figure 1 (upper panel), a single negative supercoil was removed in the presence of 10^{-4} M BLM A_2. Remarkably, when 10^{-4} M BLM A_2 was employed in the presence of at least 1 equiv of $CuCl_2$, 12–13 negative supercoils were removed from the same plasmid DNA (Figure 1, middle panel). $CuCl_2$ and $Cu(ClO_4)_2$ had the same effect in facilitating DNA helix unwinding by BLM A_2. Two-dimensional gel electrophoresis using equimolar amounts of Cu^{2+} and BLM A_2 indicated that the minimum concentration of Cu(II)·BLM A_2 needed to remove one supercoil from ϕX174 DNA was 1×10^{-6} M, i.e., ~100 times lower than that required to achieve the same effect with BLM A_2 alone. Cupric ion alone caused no unwinding of DNA when tested at concentrations up to 10^{-3} M; higher concentrations of Cu^{2+} actually appeared to *add* negative supercoils, suggesting that BLM did not simply act to lower the effective concentration at which Cu^{2+} was able to unwind DNA. The addition of 10^{-4} M $ZnCl_2$ + 10^{-4} M BLM A_2 to plasmid DNA produced no more DNA unwinding than that observed with BLM A_2 alone (data not shown).

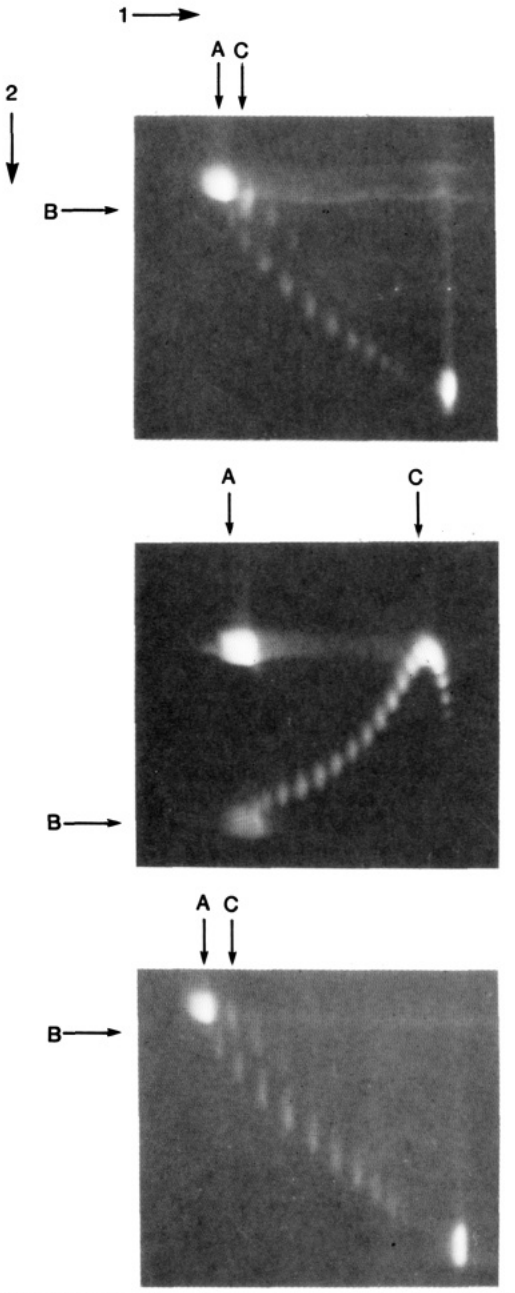

FIGURE 1: Two-dimensional agarose gel electrophoresis of ϕX174 DNA in the presence of bleomycin. The first dimension was run in a 5 mm × 40 cm 0.8% agarose tube gel containing 1×10^{-4} M BLM A_2 (upper panel), 1×10^{-4} M Cu(II)·BLM A_2 (middle panel), or 1×10^{-4} M Cu(II)·BLM demethyl A_2 (lower panel). Band A corresponds to nicked circular DNA, band B to DNA that lacked supercoils in the presence of effector (i.e., in the first dimension), and band C to the DNA topoisomer that was fully relaxed in the absence of effector (i.e., in the second dimension). The number of negative supercoils removed was equal to the number of bands present between bands B and C; this was 1, 13, and 1 for the DNAs in the upper, middle, and lower panels, respectively. The nicked DNA present in these gels was the result of adventitious cleavage during the preparation of topoisomer populations, rather than a consequence of (metallo)BLM action.

The role of Cu^{2+} was also assessed by measuring DNA unwinding in the presence of constant (10^{-4} M) BLM A_2, with varying concentrations of $CuCl_2$. As shown in Figure 2, it was found that the extent of unwinding increased steadily as a function of [Cu^{2+}] over the concentration range from $\sim 5 \times 10^{-6}$ M to $\sim 10^{-4}$ M. Increasing the concentration of Cu^{2+} beyond that of BLM A_2 had no additional effect on DNA unwinding.

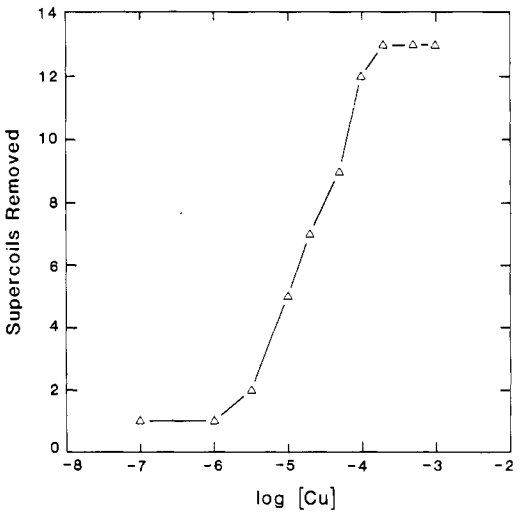

FIGURE 2: Removal of negative supercoils from topoisomers of ϕX174 DNA in the presence of 1×10^{-4} M BLM A_2 as a function of $CuCl_2$ concentration. Unwinding was determined by two-dimensional gel electrophoresis as described under Experimental Procedures.

Although the role of the metal ion in metallobleomycins has been thought to involve oxygen activation (or *hν* transduction) to generate the reactive species responsible for DNA strand scission (Sausville et al., 1978; Burger et al., 1981; Ehrenfeld et al., 1984, 1985, 1987; Meares, 1984), the present results suggest strongly that the metal center is also capable of participating in mediating BLM–DNA interaction. A few previous studies with linear DNA have measured the affinity of BLM for DNA; the binding constants and number of drug molecules bound to DNA were not a strong function of the presence or absence of Cu^{2+} (Kasai et al., 1978; Povirk et al., 1981; Roy et al., 1981). Although these data may possibly not be applicable to supercoiled DNA, they suggest that the present observation can best be explained by a *qualitative* alteration in the nature of BLM binding upon addition of Cu^{2+}. The pattern of enhancement of DNA unwinding suggests that Cu^{2+} may be acting in a manner analogous to an allosteric effector by altering the conformation of BLM in a fashion conducive to DNA unwinding. Another example of the ability of a metal ion to promote a specific type of DNA binding by organizing a conformationally unrestrained ligand has recently been reported (Griffin & Dervan, 1987).

Additional information about the nature of the process by which Cu^{2+} facilitated BLM-mediated DNA unwinding was obtained by use of bleomcyin demethyl A_2. Two-dimensional agarose gels that employed BLM demethyl A_2 at a concentration of 1×10^{-4} M in the presence of a slight molar excess of $CuCl_2$ resulted in the removal of only 1—2 negative supercoils from ϕX174 DNA (Figure 1, lower panel). DNA unwinding by Cu(II)·BLM demethyl A_2 was thus not significantly greater than that obtained with metal-free bleomycin A_2. The same result was obtained with 1×10^{-4} M BLM demethyl A_2 and a 10-fold molar excess of $CuCl_2$, indicating that a low affinity of Cu(II) for BLM demethyl A_2 did not cause the limited unwinding observed for this BLM congener. These findings are consistent with earlier observations that underscored the importance of the number and spatial distribution of positive charges on bleomycin group antibiotics to their DNA binding characteristics (Kasai et al., 1978; Huang et al., 1980; Kross et al., 1982).

The accumulated results seem consistent with a scheme in which Cu^{2+} acts to organize the metal binding ligands of BLM in a fashion conducive to DNA unwinding. Given the observations made for Cu(II)·BLM demethyl A_2, it seems reasonable to suggest that the metal center per se may also contribute to DNA interaction via an ionic interaction with the DNA duplex. Intercalators are known to remove negative supercoils from cccDNA (Waring, 1970), and it is tempting to conclude that the observed unwinding is due to intercalation of the bithiazole moiety of BLM between adjacent DNA base pairs. Mechanistically, the addition of Cu^{2+} could be thought to facilitate intercalation either by making the bithiazole moiety more accessible to DNA (e.g., via ligand organization) or else by ionic stabilization of a BLM–metal–DNA ternary complex. This scheme would be consistent with earlier suggestions (Takeshita et al., 1978; Povirk et al., 1979, 1981; Chen et al., 1980; Lin & Grollman, 1981; Kross et al., 1982) about the nature of BLM–DNA binding and with the observation that BLM unwound DNA but phleomycin did not (Povirk et al., 1979, 1981).[2] It should be noted, however, that certain steroidal diamines have also been reported to mediate DNA unwinding (Waring & Chisholm, 1972; Gourêvitch et al., 1981; Gourêvitch & Puigdomênech, 1986) by what is believed to be an ionic mechanism. Indeed, we found that the steroidal diamine malouetine removed 3 negative supercoils from DNA when employed at 1×10^{-5} M concentration. Thus, it is also possible that the DNA unwinding mediated by BLM A_2 may be primarily or exclusively ionic in nature. Clearly, carefully defined experiments using additional congeners of BLM may provide insight into the mechanistic details of DNA unwinding by bleomycin.

Two additional observations may be made regarding the proposed participation of the metal center of bleomycin in mediating BLM–DNA interaction. First, the scheme is entirely consistent with the recent observation by Sugiyama et al. (1986) that congeners of BLM known to differ (solely) in their metal chelation geometries (Oppenheimer et al., 1979, 1982) exhibited strikingly different strand selectivities for cleavage of a DNA oligonucleotide. It further predicts that alteration of the metal binding domain of BLM might be expected to lead to BLM congeners with substantially different DNA binding characteristics. Second, the recognition that the metal binding/oxygen activation domain of BLM may also participate qualitatively in DNA interaction suggests that models of BLM that consist solely of a DNA binding molecule covalently attached to a DNA cleaving molecule may lack a characteristic feature associated with BLM function.

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Registry No. BLM A_2, 11116-31-7; BLM demethyl A_2, 41089-03-6; BLM, 11056-06-7; Cu, 7440-50-8; O_2, 7782-44-7.

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[2] Phleomycin contains a thiazolinylthiazole moiety (Ikekawa et al., 1964; Takita et al., 1972), rather than a planar bithiazole, and would logically be thought to be incapable of intercalation. However, it should also be noted that phleomycin is typically employed as a mixture of congeners differing at the C-terminus; the present results suggest strongly that individual phleomycins could differ dramatically in their interactions with DNA.

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