Biochemistry

 Volume 24, Number 18 August 27, 1985

Accelerated Publications

Copper–Bleomycin Has No Significant DNA Cleavage Activity

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Received April 29, 1985

ABSTRACT: In contrast to a very recent report [Ehrenfeld, G. M., Rodriguez, L. O., Hecht, S. M., Chang, C., Basus, V. J., & Oppenheimer, N. J. (1985) *Biochemistry 24*, 81–92], the present careful reexamination demonstrated that copper–bleomycin systems have no significant DNA cleavage activity. In the presence of dithiothreitol, the bleomycin–Cu(II) complex showed little activity for DNA degradation. The DNA strand scission by the Cu(I)–bleomycin–dithiothreitol system was remarkably depressed by deferoxamine rather than by bathocuproine, suggesting the effect of trace amounts of contaminating iron in the experiments. It seems highly unlikely that the DNA breakage activity due specifically to the Cu(I)–bleomycin complex system is substantially strong. Our results indicate that the metal really relevant to the DNA cleavage by bleomycin is iron not copper.

The antitumor drug bleomycin is a clinically used metalloglycopeptide antibiotic, and its therapeutic activity is attributed to the ability to cleave DNA. The degradation reaction of DNA by bleomycin is greatly activated with ferrous ion and molecular oxygen (Sausville et al., 1978a,b; Sugiura et al., 1982). Although copper–bleomycin had been regarded to be an inactive metallobleomycin for DNA breakage (Takahashi et al., 1977; Sausville et al., 1978a; Antholine et al., 1982), a very recent report pointed out that (1) the bleomycin–Cu(II)–dithiothreitol system has substantial DNA-cleaving activity comparable to the corresponding Fe(III) complex system and (2) copper rather than iron may be the relevant metal in cancer chemotherapy by bleomycin (Ehrenfeld et al., 1985). It is of special importance in connection to the action mechanism of bleomycin whether copper–bleomycin systems can induce really strong DNA strand scission or not, because Cu(II) has higher affinity for bleomycin than Fe(II) in vivo and in vitro (Kanao et al., 1973; Sugiura et al., 1979). In regard to this subject, several significant conflicts exist among some research groups. For example, Kilkuskie et al. (1984) reported that Cu(II)–bleomycin as well as Fe(III)–bleomycin produced the nicks in SV40 DNA following activation with NADPH–cytochrome P-450 reductase. By contrast, Scheulen et al. (1981) showed that the DNA cleavage was effectively induced by the bleomycin–Fe(III) complex plus NADPH–cytochrome P-450 reductase system, but not by the corresponding Cu(II) complex system.

Herein, we carefully reexamined DNA degradation by the bleomycin–copper complex systems and clarified no significant DNA cleavage activity for the Cu(II) and Cu(I) complex species of bleomycin even in the presence of dithiothreitol.

Experimental Procedures

Chromatographically purified bleomycin A_2 and deferoxamine were kindly provided by Nippon Kayaku and Ciba-Geigy, respectively. 4,7-Diphenyl-2,9-dimethyl-1,10-phenanthroline (bathocuproine) disulfonic acid disodium salt and cuprous acetate were purchased from Dojin Chemicals and Alfa Products, respectively. The 1:1 bleomycin–metal complexes were prepared by mixing the antibiotic and cupric sulfate (or ferrous sulfate) at equimolar amounts in sodium cacodylate buffer (pH 7.0). The bleomycin–Fe(III) complex was obtained by oxidation of the corresponding Fe(II) complex under aerobic conditions. The Cu(I) solution was freshly prepared by anaerobic addition of $Cu^{I}OAc$ in 2 mM HCl solution and checked by the formation of a characteristic orange-colored Cu(I) complex [λ_{max} = 480 nm (ϵ = 13 500)] with bathocuproine. To avoid contamination by foreign metal ions, fully deionized, distilled water was used throughout the experiments, and the labware was acid washed and thoroughly rinsed.

The DNA cleavage activity was determined on purified phage ϕX174 RF DNA by using 1% agarose gel electrophoresis which contained ethidium bromide (0.5 μg/mL). Typical

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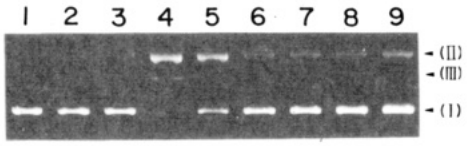

FIGURE 1: Agarose (1%) gel electrophoretic patterns of ethidium bromide stained ϕX174 RF DNA after treatment with bleomycin A_2 (BLM)–Cu(II) complex (lanes 2 and 3), BLM–Fe(III) complex (lanes 4 and 5), BLM alone (lanes 6 and 7), and controls (lanes 1, 8, and 9). The samples contained 0.5 μg of DNA, 50 mM sodium cacodylate buffer (pH 7.0), and the following additions: lane 1, none; lane 2, 20 μM Cu(II)–BLM and 50 μM dithiothreitol (DTT); lane 3, 20 μM Cu(II)–BLM, 50 μM DTT, and 100 μM sodium ethylenediaminetetraacetic acid (NaEDTA); lane 4, 1 μM Fe(III)–BLM and 50 μM DTT; lane 5, 1 μM Fe(III)–BLM, 50 μM DTT, and 100 μM NaEDTA; lane 6, 20 μM BLM and 50 μM DTT; lane 7, 20 μM BLM, 50 μM DTT, and 100 μM NaEDTA; lane 8, 20 μM Cu(II) and 50 μM DTT; lane 9, 20 μM Cu(II), 50 μM DTT, and 100 μM NaEDTA. The reaction mixtures were incubated at 25 °C for 30 min under aerobic conditions.

reaction mixtures containing 50 mM sodium cacodylate buffer (pH 7.0) and 500 ng of ϕX174 RF DNA were treated, as indicated in the figure captions, with 0–20 μM bleomycin–Cu(II) complex plus 50 μM dithiothreitol, 1 μM bleomycin–Fe(III) complex plus 50 μM dithiothreitol, or 30 μM bleomycin and 60 μM Cu(I) plus 50 μM dithiothreitol under aerobic conditions. The reaction samples were incubated at 25 °C for 30 min, and then the DNA was precipitated by addition of cold ethanol.

Results and Discussion

Figure 1 shows the typical results for the bleomycin–Cu(II) complex-mediated strand scission of covalently closed, supercoiled (form I) ϕX174 RF DNA in the presence of dithiothreitol. The experimental condition of lane 3 was the same as the condition which Ehrenfeld et al. (1985) reported to be most effective. The repeated and careful experiments established that the effect of the bleomycin–Cu(II) complex plus dithiothreitol system (lanes 2 and 3) on DNA cleavage was negligibly small and comparable to the bleomycin plus dithiothreitol system (lanes 6 and 7) and the controls (lanes 1, 8, and 9). Under the same experimental conditions, the bleomycin–Fe(III) complex plus dithiothreitol system (lanes 4 and 5) remarkably stimulated the DNA strand breaks to form open-circular (form II) and linear (form III) DNAs even at a lower concentration (1 μM) than that (20 μM) of the bleomycin–Cu(II) complex. In contrast with the results obtained by several workers, Ehrenfeld et al. (1985) observed marked activity of DNA breakage by the bleomycin–Cu(II) complex in the presence of dithiothreitol. They interpreted that one key point for the discrepancy may be the different concentrations of Cu(II) employed in the experiments and that substantial DNA degradation and little (or no) DNA cleavage were obtained at [Cu(II)] $\leq$ 25 μM and [Cu(II)] > 50 μM, respectively. When we examined the DNA-cleaving reaction by the complex systems containing 20 μM bleomycin, 50 μM dithiothreitol, and various concentrations of Cu(II) in this study, the low Cu(II) concentrations (1–25 μM) as well as the high Cu(II) concentrations (50–1000 μM) gave no significant activity for DNA degradation. Similar results were also obtained by the experiments using plasmid pBR322 DNA, 2-mercaptoethanol, bleomycin B_2, and tris(hydroxymethyl)-aminomethane hydrochloride (Tris·HCl) buffer (pH 7.0) instead of ϕX174 RF DNA, dithiothreitol, bleomycin A_2, and sodium cacodylate buffer (pH 7.0) (data not shown).

The high Fe(III)/Fe(II) redox potential [+129 mV vs. a normal hydrogen electrode (NHE)] of the bleomycin–iron complex is clearly responsible for the high DNA cleavage activity of the bleomycin–iron complex system (Melnyk et al., 1981). On the other hand, the Cu(II)/Cu(I) redox potential (−327 mV) (Ishizu et al., 1981) of the bleomycin–copper complex is considerably lower than that of the corresponding iron complex. Reductants such as NAD(P)H (−320 mV), glutathione (−230 mV), and ascorbate (−58 mV) do not have sufficiently low redox potentials to drive the reduction from Cu(II)–bleomycin to Cu(I)–bleomycin. Indeed, Freedman et al. (1982) have demonstrated that (1) no reductions of the bleomycin–Cu(II) complex with excess ascorbate or NAD(P)H could be recognized optically or magnetically and (2) the reduction for the bleomycin–Cu(II) complex with excess dithiothreitol or glutathione is slow and also the removal of copper from the drug to thiol was detected.

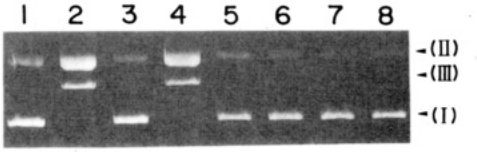

FIGURE 2: Strand scission of ϕX174 RF DNA by Cu(I) and BLM plus DTT in the absence or presence of metal-specific chelators. Individual reaction mixtures contained 0.5 μg of DNA, 50 mM sodium cacodylate buffer (pH 7.0), and the following additions: lane 1, none; lane 2, 60 μM Cu(I), 30 μM BLM, and 50 μM DTT; lane 3, 60 μM Cu(I) and 30 μM BLM; lane 4, 60 μM Cu(I), 30 μM BLM, 50 μM DTT, and 100 μM bathocuproine; lane 5, 60 μM Cu(I), 30 μM BLM, 50 μM DTT, and 100 μM deferoxamine; lane 6, 30 μM BLM and 50 μM DTT; lane 7, 60 μM Cu(I), 50 μM DTT, and 100 μM bathocuproine; lane 8, 60 μM Cu(I) and 50 μM DTT. The reaction samples were incubated at 25 °C for 30 min under aerobic conditions and then analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide.

Figure 2 represents the experimental results for DNA breakage by Cu(I)–bleomycin–dithiothreitol systems under aerobic conditions. Seemingly, this complex system (lane 2) appears to have high activity for DNA degradation, consistent with the previous observation by Ehrenfeld et al. (1985). Of particular importance is the fact, herein, that the DNA cleavage activity of the present bleomycin–Cu(I) complex system was more significantly depressed by deferoxamine (lane 5), an iron(III)-specific chelator, than by bathocuproine (lane 4), a copper(I)-specific chelator. Deferoxamine is known to inhibit strongly DNA strand scission of the bleomycin–Fe(III) complex system, and on the contrary, this chelating agent has little effect on DNA breakage by the Mn(II) (Burger et al., 1984) and Vo(IV) (Kuwahara et al., 1985) complex systems of bleomycin. 2,9-Dimethyl-1,10-phenanthroline (neocuproine) has also suppressed powerfully the substantial conversion of cccDNA to nicked circular DNA and linear duplex DNA by the 4′-(9-acridinylamino)methanesulfon-*m*-anisidide–Cu(I)–oxygen system (Wong et al., 1984). Therefore, it seems highly unlikely that the DNA-cleaving activity due specifically to the bleomycin–Cu(I) complex is essentially strong, although Cu(I)–bleomycin is oxidized by O_2 and can produce an active oxygen species such as the hydroxyl radical (Sugiura, 1979; Oppenheimer et al., 1981). In the presence of reducing agent, the effect of trace amounts of contaminating iron in the experiments cannot be neglected for the DNA cleavage by bleomycin. As shown in lanes 3 and 4 of Figure 3, indeed, even trace amounts (0.3 and 0.06 μM) of iron clearly showed high DNA breakage activity of bleomcyin in the presence of dithiothreitol. The addition of deferoxamine evidently inhibited the DNA strand scission by this complex system (see lane 5).

In conclusion, copper–bleomycin systems do not induce significantly DNA cleavage even in the presence of dithiothreitol. The metal really relevant to the DNA degradation

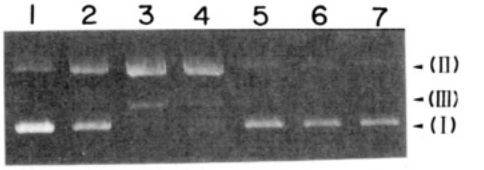

FIGURE 3: Strand scission of ϕX174 RF DNA by trace amounts of iron in the presence of BLM and DTT (lanes 3 and 4). Individual reaction mixtures contained 0.5 μg of DNA, 50 mM sodium cacodylate buffer (pH 7.0), and the following additions: lane 1, none; lane 2, 0.3 μM $FeSO_4$ and 30 μM BLM; lane 3, 0.3 μM $FeSO_4$, 30 μM BLM, and 50 μM DTT; lane 4, 0.06 μM $FeSO_4$, 30 μM BLM, and 50 μM DTT; lane 5, 0.06 μM $FeSO_4$, 30 μM BLM, 50 μM DTT, and 50 μM deferoxamine; lane 6, 0.06 μM $FeSO_4$ and 30 μM BLM; lane 7, 0.3 μM $FeSO_4$ and 50 μM DTT. The reaction samples were incubated at 25 °C for 30 min under aerobic conditions and then analyzed by 1% agarose gel electrophoresis.

of bleomycin is iron not copper.

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Regulation of the Kinetics of the Interaction of *Escherichia coli* RNA Polymerase with the λP_R Promoter by Salt Concentration†

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Received May 13, 1985

ABSTRACT: The rate of formation of transcriptionally competent open complexes between *Escherichia coli* RNA polymerase (RNAP) and the λP_R promoter is extraordinarily sensitive to the nature and concentration of the electrolyte ions in the solution. The pseudo-first-order time constant of open complex formation τ_{obsd}, determined in excess RNAP at 25 °C as a function of NaCl concentration, is proportional to the concentration product $[Na^+]^{12}[RNAP]^{-1}$. Consequently, τ_{obsd} is *far* more sensitive to changes in the salt concentration than to changes in the concentration of RNAP. The origin of this effect is the release of the thermodynamic equivalent of 12 monovalent ions in the process of closed complex formation at the λP_R promoter. In more complex ionic mixtures, ion-specific stoichiometric effects on τ_{obsd} are observed. These are *not* ionic strength effects but are instead both valence and species specific. Both the association and dissociation rate constants of RNAP at the λP_R promoter are strongly salt dependent, varying (in NaCl) as $[Na^+]^{-12}$ and $[Na^+]^8$, respectively. Consequently, the equilibrium constant characterizing open complex formation at this promoter varies with $[Na^+]^{-20}$. Electrostatic interactions and counterion release are the major contributors to the binding free energy driving open complex formation in a dilute salt solution. Since the in vivo ionic environment of *E. coli* (and other cells) is highly variable, these large salt effects are almost certainly of physiological significance. Variations in the intracellular concentrations of inorganic and organic ions, including polyamines, must exert both global and also promoter-specific regulatory effects on the initiation of transcription, as well as on numerous other protein–nucleic acid interactions.

Numerous physical and chemical variables act to regulate the rate of open complex formation and transcription initiation at a promoter. For the P_R promoter of bacteriophage λ, these include the concentration of free RNA polymerase (RNAP) holoenzyme (Hawley & McClure, 1980; Roe et al., 1984), the extent of repression by the cI gene product (λ repressor) or by cro protein (Johnson et al., 1981; Ptashne et al., 1980; Shea & Ackers, 1985), the temperature (Roe et al., 1984, 1985),

† This work was supported by NIH Grant GM23467.

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