Homma, Y., Imaki, J., Nakanishi, O., & Takenawa, T. (1988) *J. Biol. Chem. 263*, 6592-6598.
Imai, A., & Gershengorn, M. C. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8540-8544.
Laemmli, U. K. (1970) *Nature 227*, 680-685.
Low, M. G., & Weglicki, W. B. (1983) *Biochem. J. 215*, 325-334.
Majerus, P. W., Wilson, D. B., Connolly, T. M., Bross, T. E., & Neufeld, E. J. (1985) *Trends Biochem. Sci. (Pers. Ed.)* 10, 168-171.
Marban, E., Kitakaze, M., Chacko, V. P., & Pike, M. M. (1988) *Circ. Res. 63*, 673-678.
Martell, A. E. (1971) in *Stability Constants of Metal-Ion Complexes*, Suppl. 1, pp 733-735, Burlington House, London.
Martin, T. F. J., Lucas, D. O., Bajjalieh, S. M., & Kowalchyk, J. A. (1986) *J. Biol. Chem. 261*, 2918-2927.
McDonald, L. J., & Mamrack, M. D. (1988) *Biochem. Biophys. Res. Commun. 155*, 203-208.
McDonough, P. M., Goldstein, D., & Brown, J. H. (1988) *Mol. Pharmacol. 33*, 310-315.
McMillian, M. K., Soltoff, S. P., Lechleiter, J. D., Cantley, L. C., & Talamo, B. R. (1988) *Biochem. J. 255*, 291-300.
Monaco, M. E. (1987a) *J. Biol. Chem. 262*, 147-151.
Monaco, M. E. (1987b) *J. Biol. Chem. 262*, 13001-13006.
Murayama, T., & Ui, M. (1987) *J. Biol. Chem. 262*, 5522-5529.
Nakamura, T., & Ui, M. (1985) *J. Biol. Chem. 260*, 3584-3593.
Organisciak, D. T., & Noell, W. K. (1976) *Exp. Eye Res. 22*, 101-113.
Poggioli, J., Sulpice, J. C., & Vassort, G. (1986) *FEBS Lett. 206*, 292-298.
Raaflaub, J. (1956) *Methods Biochem. Anal. 3*, 301-325.
Renard, D., Poggioli, J., Berthon, B., & Claret, M. (1987) *Biochem. J. 243*, 391-398.
Rhee, S. G., Suh, P.-G., Ryu, S.-H., & Lee, S. Y. (1989) *Science 233*, 546-550.
Ryu, S. H., Suh, P.-G., Cho, K. S., Lee, K.-Y., & Rhee, S. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6649-6653.
Schwertz, D. W., & Halverson, J. B. (1989) *Arch. Biochem. Biophys. 269*, 137-147.
Schwertz, D. W., Halverson, J. B., Palmer, J. W., & Feinberg, H. (1987) *Arch. Biochem. Biophys. 253*, 388-398.
Segel, I. H. (1975) in *Enzyme Kinetics*, pp 242-271, Wiley, New York.
Suh, P.-G., Ryu, S. H., Choi, W. C., Lee, K.-Y., & Rhee, S. G. (1988) *J. Biol. Chem. 263*, 14497-14504.
Toner, M., Vaio, G., McLaughlin, A., & McLaughlin, S. (1988) *Biochemistry 27*, 7435-7443.
Williams, S. E., & Schacht, J. (1986) *J. Antibiot. 39*, 457-462.
Wilson, D. B., Bross, T. E., Hofmann, S. L., & Majerus, P. W. (1984) *J. Biol. Chem. 259*, 11718-11724.

Bleomycin·Iron Can Degrade DNA in the Presence of Excess Ethylenediaminetetraacetic Acid in Vitro†

Larry R. Solomon,‡ Rama D. Beerelli, and Pope L. Moseley*
Department of Internal Medicine, Pulmonary Division, University of Iowa College of Medicine, Iowa City, Iowa 52242
Received May 16, 1989; Revised Manuscript Received August 2, 1989

ABSTRACT: The antineoplastic drug bleomycin, when complexed to Fe(II), causes both single- and double-stranded lesions in DNA in vitro. EDTA is commonly used to inhibit the reaction of bleomycin·Fe with DNA, presumably by removing the metal cofactor. In this study, we utilized a simple assay involving the conversion of supercoiled plasmid DNA to the nicked or linear forms to further investigate the ability of bleomycin·Fe to degrade DNA in the presence of EDTA. We found that a 1:1 complex of bleomycin and Fe can degrade plasmid DNA even in the presence of a 10^6 molar excess of EDTA over bleomycin. Furthermore, we found that the half-life for inactivation of bleomycin·Fe by excess EDTA is about 1.5 h. Finally, we demonstrate that excess bleomycin associated with the outer plasma membranes of cells can damage DNA after the cells are lysed in buffers containing EDTA and SDS. These results suggest that EDTA may not be an efficient inhibitor of the reaction of bleomycin·Fe with DNA.

Bleomycins, a group of glycopeptide antibiotics isolated from *Streptomyces verticillus* (Umezawa et al., 1966), are commonly used for the treatment of several different carcinomas (Bennett & Reich, 1979; Sikic et al., 1985). When complexed to a reduced transition metal such as Cu(I) or Fe(II), bleomycins have been shown to degrade DNA both in vitro and in vivo. This degradation includes both single-stranded lesions and double-stranded breaks in the DNA backbone (Hecht, 1986). The single-stranded lesions or "nicks" are caused by both direct breaks in the DNA backbone and release of bases from the deoxyribose moiety. Regions in which the bases are removed are then susceptible to alkaline lysis (Lloyd et al., 1978). Double-stranded breaks in the DNA are thought to be due to two independent breaks on opposite strands of the DNA (Mirabelli et al., 1982), although it is likely that one single-stranded nick will increase the chance of a second break on the opposite strand (Keller & Oppenheimer, 1987). The ability of bleomycin to degrade DNA is thought to be the basis for its antineoplastic activity [for a review, see Sikic (1986)].

† This work was supported by NIH Clinical Investigator Award HL-01366. L.R.S. was a postdoctoral fellow supported by the University of Iowa Institutional Interdisciplinary Cardiovascular Training Grant (NIH HL07121).
* To whom correspondence should be addressed.
‡ Present address: Department of Pathology, University of Tennessee Medical Center, Memphis, TN 38163.

0006-2960/89/0428-9932$01.50/0

Since bleomycin-mediated degradation of DNA requires the presence of a metal cofactor, primarily Fe(II), chelating agents such as ethylenediaminetetraacetic acid (EDTA),[1] have often been used as the standard method to stop the reaction (Sausville et al., 1978a; Fisher et al., 1985; Grimwade & Beerman, 1987; Beckman et al., 1987; Moseley & Chalkley, 1987). While attempting to study the effects of bleomycin on specific genes in intact cells, we became aware of the possibility that EDTA may not efficiently inhibit bleomycin·Fe(II)-induced DNA degradation. If EDTA does not effectively inhibit the reactions, cells exposed to bleomycin and then lysed in the presence of EDTA may undergo DNA strand scission by bleomycin·Fe after the cells are lysed. This postlytic damage could result in the overestimation of the amount of DNA damage thought to occur in vivo. Thus, the purpose of the present studies was to further analyze the efficiency by which EDTA can inhibit the reaction of bleomycin·Fe with DNA. To accomplish this goal, we employed an assay wherein we measure the ability of bleomycin·Fe(II) to convert supercoiled pBR322 DNA to the nicked and linear forms of the molecule in the presence and absence of EDTA. Since a single nick in the supercoiled plasmid molecule will result in its conversion to a nicked molecule, this represents a very sensitive assay for studying DNA degradation (Kross et al., 1982; Povirk et al., 1977).

Materials and Methods

Materials. Blenoxane, a clinical preparation of bleomycins containing approximately 70% bleomycin A_2, was obtained from Bristol Meyers (Syracuse, NY). $FeSO_4$ was obtained from Mallinckrodt. Agarose–ME was obtained from FMC Marine Colloids. Ultrapure Tris base was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). [^{3}H]Thymidine (60 Ci/mmol) was obtained from ICN Biochemicals. All other chemicals were reagent grade.

Cell Culture. CV-1 cells, obtained from the American Type Culture Collection, Bethesda, MD, were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Hyclone) in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were passaged 1:4 every 4 days using trypsin–EDTA. When desired, cells were treated with bleomycin as described below.

Preparation of Bleomycin·Fe(II). Stock solutions of bleomycin were prepared by dissolving Blenoxane in sterile deaerated water to a concentration of about 5 mM (~15 units/mL). The stock solution was then diluted to 1 mM using an extinction coefficient of $\epsilon_{291} = 1.7 \times 10^4$ (Burger et al., 1985). These solutions were stable at 4 °C for at least 1 month. $FeSO_4$ solutions at concentrations of 10–20 mM were prepared in sterile deaerated water immediately before use. A stock bleomycin·Fe(II) solution was prepared by mixing the $FeSO_4$ and bleomycin solutions so that the final concentration of each was 0.5 mM. This solution was stored on ice and used within a few hours of its preparation. For actual reactions, the stock bleomycin·Fe(II) solution was diluted with sterile water to 5× working solutions.

Plasmid Preparation. Escherichia coli strain HB101 harboring the plasmid pBR322 was grown in M9Ca supplemented with 5–10 μCi/mL [^{3}H]thymidine and 100 μg/mL ampicillin. Plasmid was prepared from 500-mL cultures by using the alkaline lysis method as previously described (Maniatis et al., 1982). The plasmid was further purified by equilibrium centrifugation in CsCl gradients.

Assay Conditions. Twelve microliters of a solution containing ^{3}H-labeled pBR322 (25 μg/mL, ~4000 cpm) in 31.25 mM Tris-HCl (pH 7.8) and various concentrations of EDTA was placed in 400-μL Eppendorf tubes. The reaction was initiated by addition of 3 μL of a 5× bleomycin·Fe(II) solution. O_2 was provided by the DNA solution. This addition led to a final plasmid concentration of 20 μg/mL and a Tris-HCl concentration of 25 mM. The EDTA and bleomycin·Fe(II) concentrations were as described in the text and figure legends. The reaction was allowed to proceed for 20–30 min at room temperature and then immediately loaded on an agarose gel as described below.

To determine the rate at which the bleomycin·Fe(II) complex loses activity in the presence or absence of EDTA, 1 μM bleomycin·Fe(II) was mixed with an equal volume of either 500 mM EDTA or water. No precautions were taken to use deaerated solutions for these experiments; thus the reaction mixture would contain bleomycin·Fe(II) as well as the products of its reactions with oxygen. At the times described in the figure legends, 3 μL of the mixture was added to 12 μL of DNA/Tris solution as described above. To ensure that the final EDTA concentration was the same for all reactions, 62.5 mM EDTA was included in the DNA/Tris mixture for control reactions that involved preincubation in the absence of EDTA. Thus, in all reactions, the final conditions were 20 μg/mL pBR322, 25 mM Tris (pH 7.8), 50 mM EDTA, and 100 nM bleomycin·Fe. The dilutions of the bleomycin·Fe solution were staggered so that all reactions would be completed at the same time. Reactions with DNA proceeded 20–30 min before analysis by gel electrophoresis.

Treatment and Analysis of CV-1 Cells. Confluent 35-mm tissue culture dishes of CV-1 cells were washed twice with serum-free DMEM and then incubated for 1 h in 0.5 mL of DMEM containing bleomycin·Fe at the indicated concentrations. Cells were then processed as described in the figure legends and text. To determine the residual activity of bleomycin·Fe on the cell surface, cells were incubated for 15 min with 0.5 mL of PBS (140 mM NaCl/50 mM phosphate, pH 7.4) containing 1 μg of ^{3}H-labeled pBR322. The PBS solution was removed and spun at 3000 rpm in an Eppendorf microfuge to pellet any cells that were removed from the dish. The supernatant solution was transferred to a clean 1.5-mL Eppendorf tube to which 1 mL of 95% ETOH was added. The DNA was precipitated for 2 h at −20 °C and collected by centrifugation. The DNA was then dissolved in 30 μL of 10 mM Tris, pH 7.8, and 1 mM EDTA. This procedure typically resulted in >75% recovery of the initial plasmid DNA. The radioactivity was quantitated by liquid scintillation counting, and 5000 cpm was run on an agarose gel.

To analyze residual bleomycin·Fe activity in cell lysates, a modification of the procedure of Hirt (1967) was employed. Briefly, treated cells were washed 2 times with PBS and lysed in 0.5 mL of 25 mM Tris (pH 7.8), 10 mM EDTA, and 1% SDS containing 2 μg of ^{3}H-labeled pBR322 by gently rocking the dishes for 15 min at room temperature. The lysate was then carefully poured into 1.5-mL Eppendorf tubes to which 150 μL of 5 M NaCl was added. The resulting solution was mixed by gentle inversion and kept at 4 °C overnight. Following centrifugation at 15000*g* for 30 min at 4 °C, the supernatant solution was transferred to a clean tube and treated with 100 μg/mL RNase A (Sigma) for 30 min at 37 °C followed by 100 μg/mL proteinase K (Boehringer Mannheim) for 30 min at 37 °C. The solution was then extracted 2 times

[1] Abbreviations: PBS, phosphate-buffered saline; EthBr, ethidium bromide; EDTA, ethylenediaminetetraacetate; DMEM, Dulbecco's modified Eagle's medium; TE, 10 mM Tris-HCl (pH 7.8)/1 mM EDTA.

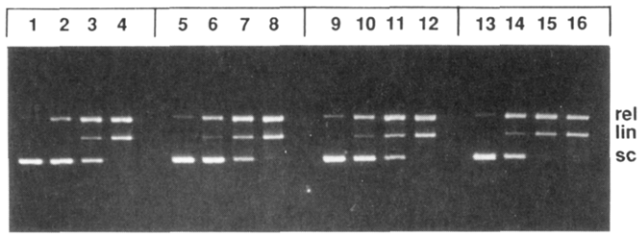

FIGURE 1: Agarose gel analysis of bleomycin·Fe-mediated damage to supercoiled DNA in the presence of EDTA. Bleomycin·Fe was added to supercoiled pBR322 DNA in the presence of various concentrations of EDTA and incubated as described under Materials and Methods. Shown is the EthBr fluorescence of the reaction products after electrophoresis on 1% agarose gels. The final EDTA concentration was 0 mM (lanes 1–4), 5 mM (lanes 5–9), 10 mM (lanes 9–12), and 50 mM (13–16). For each set of samples at a given EDTA concentration, the final bleomycin·Fe concentration was, from left to right, 0, 25, 50, and 100 nM, respectively. The positions of the supercoiled (sc), linear (lin), and relaxed (rel) plasmid molecules are shown.

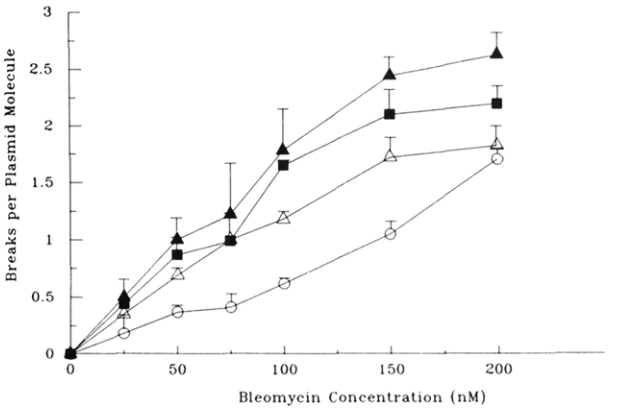

FIGURE 2: Quantitative effects of bleomycin·Fe on supercoiled DNA in the presence of EDTA. Reactions of bleomycin·Fe with supercoiled ^{3}H-labeled pBR322 in the presence of various concentrations of EDTA were subjected to electrophoresis on 1% agarose gels. Gel slices containing the supercoiled, linear, and relaxed plasmid molecules were excised and quantitated as described under Materials and Methods. Shown is a plot of the total number of breaks per supercoiled plasmid molecule against the concentration of bleomycin·Fe(II) used in the reactions in the absence of EDTA (○), or in the presence of 5 mM EDTA (△), 10 mM EDTA (■), and 50 mM EDTA (▲). The points represent the mean ± the standard deviation of five separate experiments.

with phenolchloroformisoamyl alcohol (1:1:0.01) and 1 time with chloroform. The DNA was then precipitated by adding 2 volumes of ethanol and incubating at −70 °C for at least 30 min. Following centrifugation, the pelleted DNA was dissolved in 400 μL of TE. The DNA was then reprecipitated by adding 100 μL of 10 M ammonium acetate and 1.0 mL of ethanol. Following centrifugation, the DNA pellet was washed with 70% ethanol, dried, and dissolved in 100 μL of TE. The radioactivity in each sample was quantitated by scintillation counting, and 5000 cpm was loaded onto agarose gels.

Agarose Gel Electrophoresis and Quantitation. DNA damage was analyzed by electrophoresis on 1% agarose gels in TAE (40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA) for 4 h at 80 V using a 25-cm gel. Following electrophoresis, the gels were stained in 0.5 μg/mL ethidium bromide and photographed on a Fotodyne UV transilluminator. Gel slices corresponding to the supercoiled, linear, and nicked forms of the DNA were excised from the gels and melted directly in plastic scintillation vials by adding 1.0 mL of H_2O and placing in a microwave oven for 3 min on high. After being cooled, 10 mL of Scintiverse E (Fisher Scientific) scintillation cocktail was added, and the radioactivity was quantitated by liquid scintillation counting. For each sample, the amount of radioactivity was determined from all three forms of DNA. The number of nicks per supercoiled molecule was determined as previously described (Povirk et al., 1977; Povirk & Houlgrave, 1988). All results were corrected for the number of nicks in the untreated plasmid DNA.

RESULTS

Degradation of Supercoiled pBR322 DNA by Bleomycin in the Presence of EDTA. The ability of bleomycin to degrade supercoiled plasmid DNA in a concentration-dependent manner was examined in the presence of 0–50 mM EDTA as described under Materials and Methods. Figure 1 shows an ethidium bromide stained agarose gel depicting the damage caused by 0–100 nM bleomycin·Fe in the presence of 0, 5, 10, and 50 mM EDTA. It is clear from this picture that, regardless of the EDTA concentration, the supercoiled DNA is converted to nicked and linear DNA as the bleomycin·Fe concentration increases from 0 to 100 nM. Furthermore, at a given bleomycin·Fe concentration, the extent of conversion of supercoiled molecules to nicked plasmid appears to increase with increasing EDTA concentration.

To quantitate the results observed in Figure 1, pBR322 DNA, uniformly labeled with [^{3}H]thymidine, was used in the reactions. Following agarose gel electrophoresis, the bands corresponding to supercoiled, nicked, and linear DNAs were excised, melted, and counted. The number of breaks per supercoiled molecule was quantitated by using a Poisson distribution as previously described (Povirk et al., 1977; Povirk & Houlgrave, 1988). Figure 2 shows the relationship between bleomycin·Fe and DNA damage at the various concentrations of EDTA. Note that concentrations of EDTA from 5 to 50 mM either do not protect the DNA at all or actually augment the bleomycin-induced damage. Furthermore, qualitative experiments showed that bleomycin·Fe in the presence of lower concentrations of EDTA induced DNA damage similarly to that observed for the controls (data not shown). Finally, control experiments showed that EDTA and Fe in the absence of bleomycin did not damage DNA in this system and bleomycin in the absence of added Fe(II) caused very little damage in the presence or absence of EDTA (data not shown).

In these experiments, the plasmid concentration was 20 μg/mL, which corresponds to approximately 7 nM pBR322. In the absence of EDTA, approximately 150 nM bleomycin·Fe(II) was necessary to introduce an average of one break per plasmid molecule which corresponds to about a 20-fold molar excess of bleomycin·Fe to pBR322. However, the actual extent of damage is underestimated because this assay does not detect alkaline-labile lesions introduced to the DNA. Furthermore, because one break in a supercoiled plasmid is sufficient to convert it to a nicked plasmid, the assay underestimates the actual damage resulting from bleomycin·Fe concentrations that cause greater than one break per plasmid.

Rate of Inactivation of Bleomycin·Fe(II) by Excess EDTA. The experiments described above involved adding bleomycin·Fe solutions to DNA in the presence of EDTA. It is possible that the reaction of bleomycin·Fe with DNA occurs at a faster rate than the release of Fe from the bleomycin. Therefore, it was important to determine the rate of inactivation of bleomycin·Fe with excess EDTA. To accomplish this goal, bleomycin·Fe(II) was mixed with excess EDTA for 0–5 h prior to addition to DNA (see Materials and Methods). Figure 3 shows the results of these studies. Compared to control samples of bleomycin·Fe alone, bleomycin·Fe incubated with EDTA for extended periods prior to the addition to DNA does show a decrease in

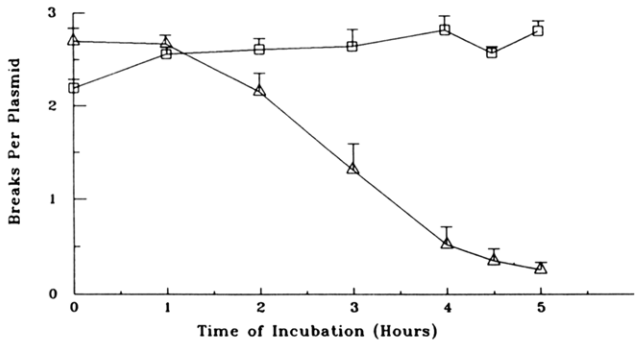

FIGURE 3: Time course for the biological inactivation of bleomycin·Fe by EDTA. Bleomycin·Fe(II) (1 μM) was mixed with an equal volume of either water or 500 mM EDTA. At the indicated times, 3 μL of the mixture was added to 12 μL of ^{3}H-labeled pBR322 and allowed to react, and the resulting damage was quantitated as described under Materials and Methods. The final reaction conditions were 100 nM bleomycin·Fe, 20 μg/mL pBR322, 50 mM EDTA, and 25 mM Tris·HCl. Shown is a plot of the number of breaks per plasmid molecule following incubation of the bleomycin·Fe with either water (□) or EDTA (△) for the indicated time. The points represent the mean ± the standard deviation of four separate experiments.

ability to damage the DNA. However, even after a 5-h incubation with a 10^6-fold molar excess of EDTA, the bleomycin·Fe still retains the ability to nick the plasmid DNA. Therefore, it appears that the rate of inactivation of the bleomycin·Fe by EDTA is very much slower than the rate of the reaction of the bleomycin·Fe with DNA.

Figure 3 shows that the inactivation of bleomycin·Fe by EDTA lags during the first hour of the incubation. This lag period is probably due to the fact that the initial number of breaks per plasmid molecule is underestimated because the bleomycin·Fe concentration is large enough to cause many breaks in each plasmid. Therefore, if we consider only the linear portion of this curve, the half-life for the inactivation of the biological activity of the bleomycin·Fe complex in the presence of excess EDTA is about 1.5 h.

Activity of Bleomycin·Fe(II) in EDTA-Containing Lysates from Treated Cells. Many laboratories have lysed cells in detergent solutions containing EDTA to stop the reaction of bleomycin·Fe with intracellular DNA. Since the results presented above make it unlikely that the EDTA could stop the reaction of residual bleomycin·Fe with the DNA, we thought it prudent to test the residual bleomycin·Fe activity in cell lysates. To this end, cells were treated with various concentrations of bleomycin·Fe, washed twice with PBS, and subsequently lysed in a solution containing EDTA, SDS, and supercoiled plasmid DNA. The plasmid DNA was purified from the cell lysates and analyzed for damage as described under Materials and Methods. The results of the experiments are shown in Figure 4A. As the concentration of bleomycin·Fe used to treat the cells was increased, the damage to the plasmid DNA also increased as evidenced by the decrease in supercoiled DNA. Since this DNA was introduced to the system in lysate buffer after the cells were washed with PBS, it is clear that residual bleomycin·Fe is capable of damaging DNA in the cell lysates. However, it was not clear whether the residual bleomycin·Fe was internal or on the cell surface.

In order to determine whether or not the residual bleomycin·Fe was on the cell surface, treated cells were washed with PBS and then incubated with PBS containing the supercoiled plasmid DNA. Following a brief centrifugation to remove any unattached cells, the plasmid DNA was purified from the PBS and analyzed for the extent of damage. As seen with the whole cell lysates, figure 4B shows that the damage to the plasmid DNA increased with the concentration of bleomycin·Fe used

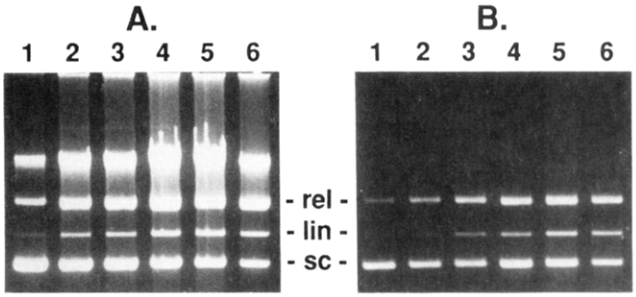

FIGURE 4: Agarose gel analysis of residual bleomycin·Fe activity associated with treated cells. Monolayers of CV-1 cells were treated with various concentrations of bleomycin·Fe, washed twice with PBS, and then either lysed in 0.5 mL of 25 mM Tris·HCl (pH 7.8), 10 mM EDTA, and 1% SDS containing 2 μg of supercoiled pBR322 DNA or incubated with 0.5 mL of PBS containing 2 μg of pBR322 DNA. The plasmid DNA was recovered from each fraction as described under Materials and Methods and subjected to electrophoresis on a 1% agarose gel. Shown is the EthBr-stained agarose gel. Panel A represents the damage occurring in cell lysates whereas panel B represents the damage to supercoiled plasmid associated with bleomycin·Fe from the surface of treated cells. Lanes 1–6 in each panel represent damage occurring after the cells were treated for 1 h with 0, 10, 25, 50, 75, and 100 μM bleomycin·Fe, respectively. The positions of the relaxed (rel), linear (lin), and supercoiled (sc) plasmids are indicated. The band above "rel" in panel A represents fragmented chromasomal DNA isolated in the cell lysates.

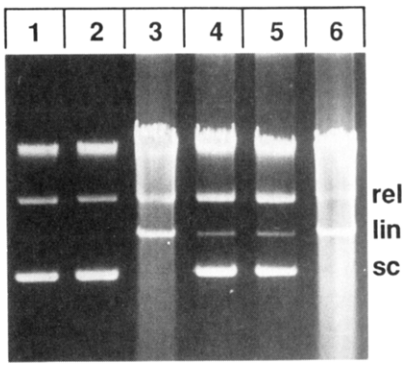

FIGURE 5: Removal of residual bleomycin·Fe from treated cells. CV-1 cells on 35-mm dishes were treated with 10 μM bleomycin·Fe for 1 h in serum-free DMEM, washed with PBS and/or trypsin as described below, and lysed in the presence of supercoiled pBR322 DNA as described uner Materials and Methods. Shown is a picture of an EthBr-stained agarose gel depicting the damage to the supercoiled DNA recovered from lysates of treated cells (lanes 1–5). Lanes 1 and 2 are controls wherein the cells were not treated with bleomycin·Fe(II) and either washed with PBS (lane 1) or washed briefly with trypsin followed by PBS (lane 2) prior to lysis. Lanes 3–5 represent the plasmid DNA recovered from lysates of treated cells after they were washed 4 times with 2 mL of PBS (lane 3), washed briefly 1× with 2 mL of 0.1% (w/v) trypsin followed by 2 mL of PBS (lane 4), or removed from the dish with trypsin and collected by centrifugation (lane 5). Lane 6 represents the residual bleomycin·Fe found in the trypsin-containing supernatant from the treatment in lane 5 after the supernatant was incubated with CV–1 cells. The position of the relaxed (rel), linear (lin), and supercoiled (sc) plasmids are indicated. The band above "rel" fragmented chromosomal DNA isolated in the cell lysates.

to treat the cells. These results strongly suggest that residual bleomycin·Fe on the surface of treated cells is not readily removed by washing the monolayer with PBS; however, the possibility that internal bleomycin·Fe escapes back through the cell membrane during incubation with the plasmid DNA cannot be excluded.

The results presented above demonstrate that washing treated monolayers 2 times prior to lysis is not sufficient to eliminate residual bleomycin·Fe that could potentially damage intracellular DNA. We therefore attempted to find conditions that would eliminate this residual activity. Figure 5 shows

the results of these attempts. Washing bleomycin·Fe-treated monolayers 4 times with PBS does not effectively remove residual bleomycin·Fe from the cell surface (lane 3). However, the bleomycin·Fe-mediated damage to the supercoiled DNA can be substantially reduced if the treated cells are either washed briefly with a trypsin-containing solution prior to lysis (lane 4) or removed from the dish with trypsin, pelleted, and resuspended in the lysis solution containing supercoiled plasmid (lane 5). To show that the trypsin itself does not directly inactivate the bleomycin·Fe, supercoiled DNA was added to the supernatant solution from trypsinized treated or untreated cells. Lane 2 of Figure 5 shows that the trypsin-containing supernatant from untreated cells has no DNA hydrolytic activity whereas lane 6 shows that the DNA is substantially degraded in the trypsin-containing supernatant from treated cells. Thus, it is possible to remove the residual bleomycin·Fe from cell monolayers with trypsin although the trypsin itself is not an inhibitor of bleomycin·Fe.

Discussion

In this report, we have demonstrated that bleomycin·Fe retains the ability to degrade DNA in the presence of excess EDTA. Some of this activity is present even after the bleomycin·Fe is incubated for several hours with a large excess of EDTA prior to reaction with the DNA, suggesting that the off-rate for Fe from bleomycin is probably very slow. Finally, residual bleomycin on the surface of treated cells is capable of degrading DNA after the cells are lysed in buffers containing EDTA and SDS. This result suggests the necessity for extensively washing cells prior to lysis.

When bleomycin·Fe is added to the DNA in the presence of excess EDTA, we assume that the free Fe is rapidly chelated. However, the Fe bound to bleomycin will be chelated at a rate dependent on the off-rate of the Fe from the bleomycin. If this rate is slower than the rate of reaction of bleomycin·Fe with DNA, then the DNA will be nicked regardless of the presence of excess chelator. Similar results have been reported by Aronovitch et al. (1987) in that the removal of Fe(III) from the ternary complex of DNA–bleomycin·Fe(III) is very slow, even in the presence of excess chelators.

It is not clear why the damage to DNA from a given concentration of bleomycin·Fe increases with increasing EDTA concentration. Preliminary results using this system suggest that the damage to DNA from bleomycin·Fe can be augmented by increases in ionic strength (Solomon and Moseley, unpublished observations). Although this is in contrast to previous observations using a different system (Sausville et al., 1978b), it is possible that the increase in ionic strength associated with elevated EDTA concentrations is responsible for the increased bleomycin·Fe-induced DNA damage.

Many investigators use EDTA to stop the reaction of bleomycin·Fe with both naked DNA and isolated nuclei (Fisher et al., 1985; Grimwade & Beerman, 1987; Beckman et al., 1987). If the reaction of bleomycin·Fe with DNA is faster than the inactivation of the bleomycin·Fe by EDTA, it is likely that the reaction is complete prior to the addition of EDTA. Thus, the results for most in vitro analyses would likely be the same regardless of whether the EDTA was used to stop the reaction or not.

In contrast to the in vitro data, the extent of damage to genomic DNA in experiments involving bleomycin·Fe treatment of cultured cells can be influenced by residual DNA on the cell surface prior to lysing treated cells. Since DNA damage can occur after cell lysis, it is possible that the intracellular DNA damage reported by many investigators is overstated. Thus, it is extremely important that residual bleomycin·Fe be completely removed from the cell surface prior to lysis. Iqbal et al. (1975) have previously described the possibility of artifactual bleomycin-induced DNA damage and methods to correct for this damage.

Although our results show that trypsin effectively removes residual bleomycin from the surface of treated cells, the mechanism of removal is not clear. It is possible that the trypsin treatment simply loosens cell/cell and cell/substratum contacts sufficiently to allow trapped bleomycin to escape. In addition, it is possible that the bleomycin could bind to cell surface proteins that are cleaved by the trypsin treatment.

Radiolabeled bleomycin has been shown to bind to cell membranes in a saturable manner that can be completed with unlabeled bleomycin (Roy & Horwitz, 1984; Moseley, submitted for publication), suggesting the presence of specific receptors on the cell surface for bleomycin. Thus, the residual bleomycin activity demonstrated on the surface of treated cells could be due, at least in part, to bleomycin bound to these receptors. Due to the large amount of DNA released upon lysis of the cells, it is not practical to quantitate the released bleomycin based on degradation of supercoiled plasmid DNA. However, it is unlikely that a specific receptor for bleomycin would be present in a concentration sufficient to cause the observed damage to the supercoiled plasmid. Thus, the proposed receptor for bleomycin is probably only one source of the residual bleomycin activity.

Although it is clear that bleomycin·Fe degrades DNA in vitro, it is not clear whether this is the primary mechanism for bleomycin toxicity in vivo. Berry et al. (1985a,b) have shown that DNA damage by individual bleomycin congeners in permeabilized cells does not necessarily correlate with cell growth inhibition. Furthermore, Stamato et al. (1987) describe a bleomycin-sensitive mutant of Chinese hamster ovary cells that does not sustain greater DNA damage than the parent cells upon treatment with bleomycin. Finally, preliminary results in this lab have shown that physiologically relevant concentrations of bleomycin lead to increased turnover of arachidonic acid in human lung fibroblasts (Moseley and Peterson, submitted for publication). These results suggest the possibility of alternative mechanisms for bleomycin cytotoxicity.

Acknowledgments

We thank Gary W. Hunninghake for editorial assistance in the preparation of the manuscript.

Registry No. EDTA, 60-00-4; $FeSO_4$, 7720-78-7; blenoxane, 9041-93-4.

References

Aronovitch, J., Godinger, D., Goldstein, S., & Czapski, G. (1987) *J. Antibiot. 40*, 1344–1348.

Beckman, R. P., Agostino, M. J., McHugh, M. M., Sigmund, R. D., & Beerman, T. A. (1987) *Biochemistry 26*, 5409–5415.

Bennett, J. E., & Reich, S. D. (1979) *Ann. Intern. Med. 90*, 945–948.

Berry, D. E., Chang, L., & Hecht, S. M. (1985a) *Biochemistry 24*, 3207–3214.

Berry, D. E., Kilkuskie, R. E., & Hecht, S. M. (1985b) *Biochemistry 24*, 3214–3219.

Burger, R. M., Horwitz, S. B., & Peisach, J. (1985) *Biochemistry 24*, 3623–3629.

Fisher, M. L., Kuroda, R., & Sakai, T. T. (1985) *Biochemistry 24*, 3199–3207.

Grimwade, J. E., & Beerman, T. A. (1987) *Mol. Pharmacol. 30*, 358–363.

Hecht, S. M. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 2784–2791.
Hirt, B. (1967) *J. Mol. Biol. 26*, 365–369.
Iqbal, Z. M., Kohn, K. W., Ewig, R. A., & Fornace, A. J., Jr. (1975) *Cancer Res. 36*, 3834–3838.
Keller, T. J., & Oppenheimer, N. J. (1987) *J. Biol. Chem. 262*, 15144–15150.
Kross, J., Henner, W. D., Hecht, S. M., & Haseltine, W. A. (1982) *Biochemistry 21*, 4310–4318.
Lloyd, R. S., Haidle, C. W., & Hewitt, R. R. (1978) *Cancer Res. 38*, 3191–3196.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mirabelli, C. K., Ting, A., Huang, C.-H., Mong, S., & Cooke, S. T. (1982) *Cancer Res. 42*, 2779–2785.
Moseley, P. L., & Chalkley, R. (1987) *J. Lab. Clin. Med. 110*, 618–623.
Povirk, L. F., & Houlgrave, C. W. (1988) *Biochemistry 27*, 3850–3857.
Povirk, L. F., Wubker, W., Kohnlein, W., & Hutchinson, F. (1977) *Nucleic Acids Res. 4*, 3573–3580.
Roy, S. N., & Horwitz, S. B. (1987) *Cancer Res. 44*, 1541–1546.
Sausville, E. A., Peisach, J., & Horwitz, S. B. (1978a) *Biochemistry 17*, 2740–2746.
Sausville, E. A., Stein, R. W., Peisach, J., & Horwitz, S. B. (1978b) *Biochemistry 17*, 2746–2754.
Sikic, B. I. (1986) *Cancer Surveys 5*, 81–91.
Sikic, B. I., Rozencweig, M., & Carter, S. K. (1985) *Bleomycin Chemotherapy*, Academic Press, New York.
Stamato, T. D., Peters, B., Patil, P., Denko, N., Weinstein, R., & Giaccia, A. (1987) *Cancer Res. 47*, 1588–1592.
Umezawa, H., Maeda, K., Takeuchi, T., & Okami, Y. (1966) *J. Antibiot. 19*, 200–209.

Function of Threonine-55 in the Carbamoyl Phosphate Binding Site of *Escherichia coli* Aspartate Transcarbamoylase†

Wei Xu and Evan R. Kantrowitz*
Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167
Received June 28, 1989; Revised Manuscript Received August 17, 1989

ABSTRACT: Carbamoyl phosphate is held in the active site of *Escherichia coli* aspartate transcarbamoylase by a variety of interactions with specific side chains of the enzyme. In particular, the carbonyl group of carbamoyl phosphate interacts with Thr-55, Arg-105, and His-134. Site-specific mutagenesis was used to create a mutant version of the enzyme in which Thr-55 was replaced by alanine in order to help define the role of this residue in the catalytic mechanism. The Thr-55 → Ala holoenzyme exhibits a 4.7-fold reduction in maximal observed specific activity, no alteration in aspartate cooperativity, and a small reduction in carbamoyl phosphate cooperativity. The mutation also causes 14-fold and 35-fold increases in the carbamoyl phosphate and aspartate concentrations required for half the maximal observed specific activity, respectively. Circular dichroism spectroscopy has shown that saturating carbamoyl phosphate does not induce a conformational change in the Thr-55 → Ala holoenzyme as it does for the wild-type holoenzyme. The kinetic properties of the Thr-55 → Ala catalytic subunit are altered to a greater extent than the mutant holoenzyme. The mutant catalytic subunit cannot be saturated by either substrate under the experimental conditions. Furthermore, as opposed to the wild-type catalytic subunit, the Thr-55 → Ala catalytic subunit shows cooperativity for aspartate and can be activated by *N*-(phosphonoacetyl)-L-aspartate in the presence of low concentrations of aspartate and high concentrations of carbamoyl phosphate. As deduced by circular dichroism spectroscopy, the conformation of the Thr-55 → Ala catalytic subunit in the absence of active-site ligands is distinctly different from the wild-type catalytic subunit. Furthermore, carbamoyl phosphate causes an alteration in the circular dichroism spectrum of the Thr-55 → Ala catalytic subunit as opposed to the mutant holoenzyme. For the Thr-55 → Ala catalytic subunit, the K_D of carbamoyl phosphate and the K_i of PALA are both increased approximately 50-fold compared to the wild-type catalytic subunit. These data suggest that in the wild-type holoenzyme Thr-55 plays an important part in the binding of carbamoyl phosphate, has a small catalytic function in polarizing the carbonyl, and is important in the conformational change that occurs upon binding of carbamoyl phosphate which in turn is important for the ordered binding of aspartate. In addition, Thr-55 also plays a role in stabilizing the native conformation of the wild-type catalytic subunit.

Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the condensation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and L-aspartate in the committed step of the pyrimidine biosynthesis pathway. The enzyme, used as a model system to study the molecular mechanisms of allosteric regulation [see reviews by Allewell (1989); Gerhart (1970), Jacobson and Stark (1973), Kantrowitz and Lipscomb (1988), Kantrowitz et al. (1980a,b), and Schachman (1974)], consists of three regulatory subunits and two catalytic subunits. The holoenzyme exhibits homotropic cooperativity for both

† This work was supported by Grants DK1429 and GM26237 from the National Institutes of Health.
* To whom correspondence should be addressed.

0006-2960/89/0428-9937$01.50/0