Catalytic Release of Deoxyribonucleic Acid Bases by Oxidation and Reduction of an Iron·Bleomycin Complex†

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ABSTRACT: The kinetics and stoichiometry of several reactions involving bleomycin, iron, DNA, oxygen, and sulfhydryls were examined in order to assess their possible role in degradation of DNA by bleomycin. Oxidation of Fe(II) in the presence of bleomycin resulted in an iron(III)·bleomycin complex, having an optical absorption spectrum with a broad shoulder at 320–400 nm, which was stable for several hours. If Fe(II) was allowed to oxidize before bleomycin addition, the complex did not form. The complex was reduced by dithiothreitol 5 times faster than unchelated Fe(III), and reduction of the complex was inhibited by high concentrations of DNA. However, stopped-flow studies showed that, when sufficient DNA was present to bind most of the iron(II)·bleomycin, its rate of oxidation by molecular oxygen was 60 times faster than that of unbound iron(II)·bleomycin. Under the same conditions, oxidation of each mole of DNA-bound iron(II)·bleomycin released 0.18 mol of thymine. Treatment of pyrimidine-labeled *Escherichia coli* DNA with bleomycin and high concentrations of Fe(II) and 2-mercaptoethanol resulted in the release of up to 2.4 mol of pyrimidines (of which 60% were thymine) per mol of bleomycin. This result implies that each Fe·bleomycin complex went through several cycles of oxidation and reduction and that bleomycin usually was not inactivated in the base-release reaction. In supercoiled pDR3709 DNA, one base was released per single-strand break (measured in alkali), eliminating the possibility of multiple base release during a single bleomycin–DNA interaction. Thus, the iron·bleomycin complex acts as a catalyst which, after being reduced by sulfhydryls, binds to DNA in a way which facilitates both the oxidation of the chelated Fe(II) and the degradation of the DNA backbone by the products of this oxidation.

Recent studies of the degradation of DNA by bleomycin (Sausville et al., 1978a,b) strongly suggest that a critical step in the reaction is the oxidation of an Fe(II) ion which is chelated by a bleomycin molecule bound to DNA. Most of the final products of the reaction have been identified (Haidle et al., 1972; Sausville et al., 1978b; Takeshita et al., 1978), but the precise sequence of events proceding and following Fe(II) oxidation remains speculative.

Since both Fe(II) (Goto et al., 1970) and iron(II)·bleomycin (Sausville et al., 1976) oxidize rapidly at neutral pH, even in the absence of DNA, the kinetics of oxidation and reduction of iron and its complexes with bleomycin and DNA must be important determinants of the efficiency of DNA degradation by bleomycin. A related problem is the fate of the oxidized iron(III)·bleomycin·DNA complex and the possibility that both the bleomycin and the iron (through reduction by sulfhydryls) may be recycled to produce an active iron(II)·bleomycin complex. The question of whether bleomycin is chemically changed during its reaction with DNA or whether it is merely a catalyst is of crucial importance in elucidating the mechanism of the reaction. In this study, these questions have been addressed by attempting to isolate and study separately the kinetics and stoichiometry of several possible reaction steps in the degradation of DNA by bleomycin, iron, oxygen, and sulfhydryls. The results support the view that bleomycin degrades DNA by selective catalysis of the oxidation and reduction of chelated Fe.

Experimental Procedure

Drugs. Either purified bleomycin A_2 or Blenoxane, the clinical mixture (both from Bristol), was used in all experiments. Concentrations were determined from the A_{290} by assuming an extinction coefficient of 14 000 M^{-1} (Dabrowiak et al., 1978). Iron(III)·bleomycin was prepared by mixing equimolar amounts of bleomycin and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, each at a concentration of at least 2 mM, in distilled water, pH 5, and incubating the mixture for 5 min at 22 °C, with frequent vortexing to assure complete oxidation. Experiments with iron(III)·bleomycin were performed within 3 h after its preparation.

DNA. Calf thymus DNA (Sigma) was purified by four phenol extractions, followed by dialysis against 0.1 M EDTA, pH 8, precipitation by 95% ethanol–1 M sodium acetate (7:3), and dialysis against 20 mM Tris, pH 8.

Labeled *Escherichia coli* DNA was prepared by growing AB2497 (*thy*⁻) cells in K medium (Howard-Flanders & Theriot, 1966). For [[^{3}H]cytosine,[^{14}C]thymine]DNA, 1 μCi/mL 5-[^{3}H]cytidine (20 Ci/mmol) and 0.015 μCi/mL [^{14}C]thymine (46 Ci/mol) were added; for [[^{3}H]thymine]-DNA, 0.05 or 0.1 μCi/mL [^{3}H]thymine (15 Ci/mmol) was added. Unlabeled thymine was added to a total thymine concentration of 4 μg/mL. DNA was isolated from overnight cultures by using the procedure of Marmur (1961) and dialyzed against 20 mM Tris, pH 8. The A_{260}/A_{280} ratio was at least 1.9, and the RNA contamination, determined by alkline hydrolysis of [[^{3}H]cytosine,[^{14}C]thymine]DNA (Povirk et al., 1978), was less than 5%. The DNA concentration determined from its radioactivity and the specific activity of thymine in the medium accounted for 90% of the total A_{260} of the preparation. Diphenylamine-DNA determinations (Dische, 1930) also agreed to within ±10%. Krasin & Hutchinson (1977) have verified that, for AB2497 cells, the specific activity of thymine in the DNA is the same as that in the growth medium. Thus, two independent estimates of the specific activity of thymine in DNA were obtained, one, which was used in base-release calculations, from the specific activity of thymine in the medium and one from the radioactivity and concentration (determined from the A_{260}) of the DNA. These estimates always agreed to within 10% with the latter giving a slightly lower estimate. The specific activity of cytosine in [[^{3}H]cytosine,[^{14}C]thymine]DNA was deter-

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mined from the $^3H/^{14}C$ ratio of the DNA.

The miniplasmid pDR3709 was obtained from Dr. A. Sancar, Department of Therapeutic Radiology, Yale University. This plasmid is a fragment of pBR322, having a length of 1700 ± 100 base pairs and retaining the sequences coding for ampicillin resistance. The plasmid was transfected into *E. coli* AB2487 *thy⁻,recA⁻* by using the method of Cohen et al. (1972) with selection on plates containing 100 μg/mL ampicillin. These cells were grown in K medium containing 5 μg/mL thymine and 0.3 μCi/mL [^{3}H]thymine. Chloramphenicol was added to 180 μg/mL to induce plasmid production, and supercoiled pDR3709 was isolated by the method of Blair et al. (1972).

Transition metal content of some DNA preparations was estimated by extracting the solution with an equal volume (1.5 mL) of chloroform containing 4 μg/mL dithizone (Epps, 1966; Bünemann & Dattagupta, 1973), a potent chelator of divalent ions. Samples were shaken vigorously for 4 min, and the A_{595} of the chloroform phase was measured. Of the metals which strongly inhibit DNA degradation by bleomycin (Sausville et al., 1978a), copper(II), zinc(II) and cobalt(II), in both the presence and absence of 1.2 mM DNA, all produced a decrease in the A_{595} of 0.3 ± 0.05 per 10^{-5} M cation added to the aqueous phase (20 mM Tris, pH 8); this constant was used in calculating estimates of metal content. Nickel(II) required 20-fold higher concentrations to produce comparable absorbance changes and probably was not quantitatively extracted.

Reduction Assays. The reaction mixtures contained, in 0.7–2.0 mL, 0.2 mM iron(III)·bleomycin A_2, 0–6 mM DNA, and 20 mM Tris, pH 8. The solution was continuously bubbled with nitrogen for the duration of the experiment. After 5 min of bubbling, concentrated sulfhydryl compounds were added to a final concentration of 0.2 mM (0.1 mM in the case of dithiothreitol). At various times thereafter, 0.1-mL samples were removed and added to 0.9 mL of Ellman's reagent (0.5 mg/mL 5,5′-dithiobis(nitrobenzoic acid), 0.5 mg/mL Na_2EDTA, 10 mM NaCl, and 10 mM Tris, pH 8) in order to determine the remaining concentration of sulfhydryls (Sleigh, 1976). The samples were incubated for 2 min at 22 °C, and the A_{412} [ϵ_{412} = 90 000/mol of sulfhydryls (Ellman, 1959)] was read against a reagent blank.

In other experiments, loss of iron(III)·bleomycin was measured spectrophotometrically. Reaction mixtures including sulfhydryl compounds were prepared as above, bubbled for 2 min with nitrogen, and sealed, and the A_{380} was recorded as a function of time.

Stopped-Flow Oxidation Measurements. Kinetics of oxidation were measured on a Durrum stopped-flow spectrophotometer in a cell with 2-cm optical path length. First, one channel of the mixing apparatus was flushed 3 times with 10 mL of buffer (20 mM Tris, pH 8) which had been bubbled with nitrogen for 5 min. Fe(II) or iron(II)·bleomycin (2.5 mL; 10^{-4} M) in 20 mM Tris, with or without calf thymus DNA, was prepared anaerobically by bubbling the solution without Fe(II) with nitrogen for 5 min and then adding Fe(II) in a small volume of nitrogen-saturated distilled water. This solution was bubbled for an additional 5 min and immediately loaded into the syringe of the flushed channel of the mixing apparatus. The other syringe contained fully aerated buffer. The two solutions were mixed rapidly (within 10 ms), and the A_{350} [for Fe(II)] or A_{380} [for iron(II)·bleomycin] was recorded as a function of time.

Base Release. In several experiments, the release of free bases from labeled DNA was measured by using descending paper chromatography. Specific reaction conditions are given in the figure legends. In all cases, the reaction mixture was dried onto paper strips 10 μL at a time and then developed in 95% ethanol–1 M sodium acetate, 7:3, 8–18 h. The radioactivity chromatographing at the position of thymine and/or cytosine was determined by liquid-scintillation counting, as described previously (Povirk et al., 1978).

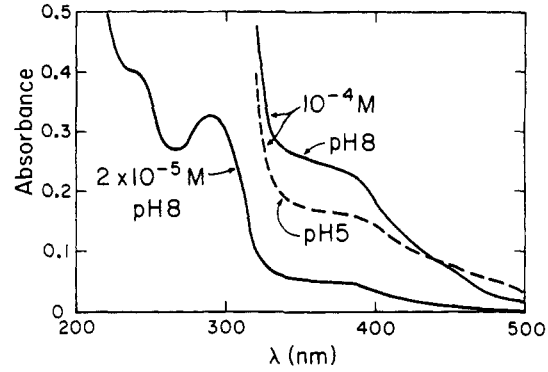

FIGURE 1: The ultraviolet–visible absorption spectrum of freshly prepared iron(III)·bleomycin A_2 in distilled water, pH 5, or in 20 mM Tris, pH 8. The complex was prepared by oxidation of iron(II)·bleomycin in distilled water.

DNA Strand Breakage. In base-release experiments with the plasmid pDR3709, strand breakage was measured simultaneously. Five microliters of the reaction mixture, after addition of EDTA, was added to 20 μL of 0.15 M NaOH and incubated for 30 min at 37 °C to denature the DNA. Ten microliters of this solution was layered atop a 5–20% alkaline sucrose gradient (0.5 M NaCl, 0.05 M NaOH, and 0.01 M Na_2EDTA, pH 12) and centrifuged for 160 min at 45 000 rpm in a Beckman SW50.1 rotor at 18 °C. Thirty-two equal-volume fractions were collected and their radioactivity was counted. Strand breakage was determined from the loss of rapidly sedimenting form I DNA.

Results

Fe(III)·Bleomycin Complex. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, dissolved in aerated distilled water at pH 5, was stable to oxidation for several hours. If the solution was deoxygenated and an equimolar quantity of bleomycin was added, a pink-violet iron(II)·bleomycin complex (Sausville et al., 1978b) was formed, which, upon aeration, oxidized to a bright yellow complex, presumably iron(III)·bleomycin. The absorbance spectrum of the complex (Figure 1) had a shoulder at 235 nm, similar to copper(II)·bleomycin and zinc(II)·bleomycin (Dabrowiak et al., 1978), and also had a broad shoulder at 320–400 nm, not present in other bleomycin complexes. The absorbance at 320–400 nm was 2–3 times greater than that of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ allowed to oxidize at pH 8 in the absence of bleomycin (not shown). The iron(III)·bleomycin spectrum was stable for several hours but showed a decrease of 10–15% in absorbance at 320–400 nm after 1 day at pH 8 and 22 °C.

Several experiments indicated that iron(III)·bleomycin was a stable long-lived complex and that any exchange between free and bound Fe(III) was very slow. Addition of excess $CuCl_2$ to a solution of iron(III)·bleomycin resulted in a slow, exponential loss of absorbance at 380 nm, probably due to replacement of Fe(III) by Cu(II) at the bleomycin chelation site. For 10^{-5} M iron(III)·bleomycin in distilled water, the time constant for replacement by a 10-fold excess of Cu(II) was 20 min; for 10^{-4} M iron(III)·bleomycin in 20 mM Tris, pH 8, the time constant for replacement by a twofold excess of Cu(II) was 80 min (data not shown). Since binding of

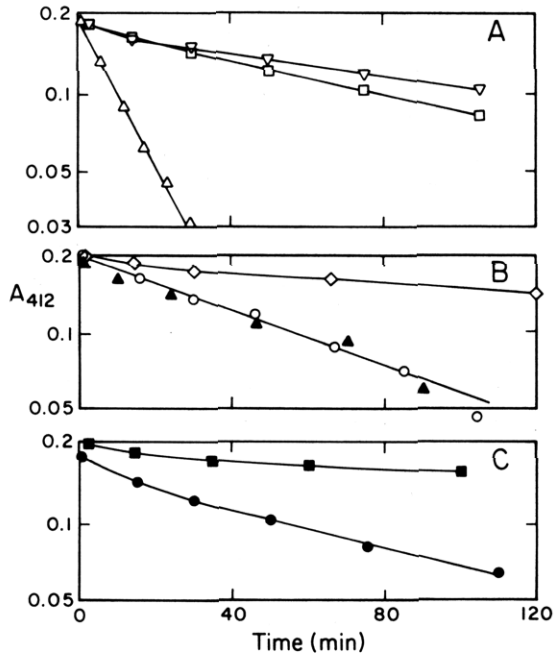

FIGURE 2: Reduction of 0.2 mM Fe(III) or iron(III)·bleomycin A_2 in nitrogen-saturated buffer measured by the oxidation of an equimolar concentration of sulfhydryls, assayed with Ellman's reagent. The A_{412} is approximately equal to the sulfhydryl concentration in millimolarity. (A) Reduction of iron(III)·bleomycin A_2 by dithiothreitol in the presence of 0 (△), 3 (□), or 6 (▽) mM DNA. (B) Reduction of Fe(III) in the presence of 0.2 mM bleomycin A_2 (▲), 4 mM DNA (◇), or neither bleomycin nor DNA (○). (C) Reduction of iron(III)·bleomycin A_2 by glutathione in the presence of 0 (●) or 4 mM DNA (■).

Cu(II) to metal-free bleomycin is very rapid, the lifetime of iron(III)·bleomycin at pH 8 must be at least 80 min and possibly much longer. Also, if bleomycin was added to a solution of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ which had already oxidized to Fe(III), there was no strong absorbance at 320–400 nm. This result does not completely exclude the possibility that a complex may be formed from Fe(III) and bleomycin but does indicate that the complex either is formed very slowly or is much different from the complex formed by oxidation of iron(II)·bleomycin.

Reduction of the Complex. In aerated solution, sulfhydryls slowly autoxidize in a reaction catalyzed by trace metal contaminants (Jocelyn, 1972). In deoxygenated (nitrogen-flushed) solution with or without DNA or bleomycin, no oxidation of sulfhydryls (assayed by using Ellman's reagent) was observed for at least 3 h, except when Fe(III) was present. Thus, this oxidation assay could be used to study the kinetics of reduction of Fe(III) or iron(III)·bleomycin by sulfhydryls. Iron(III)·bleomycin (0.2 mM) was reduced rather rapidly by 0.1 mM dithiothreitol, following an exponential time course with a time constant $\tau = 17$ min; kinetics of sulfhydryl loss exactly paralleled those of the decrease in A_{380} (not shown) of the complex. The end product of this reduction had a spectrum similar to that described by Sausville et al. (1976) for iron(II)·bleomycin, with an absorbance at 476 nm. Aeration of the solution restored the iron(III)·bleomycin spectrum (data not shown).

Since the reduction of Fe(III) alone [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ allowed to oxidize at pH 8 and then flushed with nitrogen] was 5 times slower ($\tau = 80$ min; Figure 2B) than that of iron(III)·bleomycin, the rapid reduction of iron(III)·bleomycin must have resulted from a direct interaction between dithiothreitol and Fe(III)·bleomycin, without prior dissociation of Fe(III). Addition of bleomycin to Fe(III) did not change

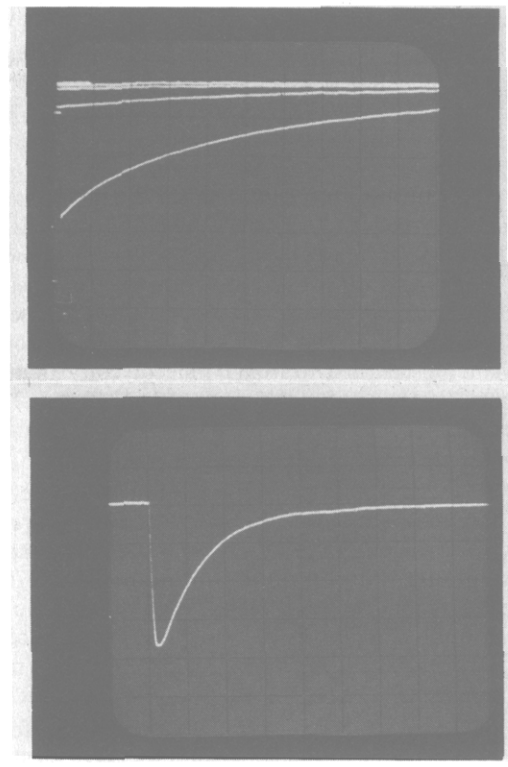

FIGURE 3: Kinetics of oxidation of iron(II)·bleomycin A_2 by O_2. A nitrogen-saturated solution of 10^{-4} M iron(II)·bleomycin was rapidly mixed with a solution of aerated buffer, and the A_{380} was recorded. In both cases, the y axis was set at 0.02 A_{380} units/division and the final absorbance was approximately 0.24. In the top photo, no DNA was present, and the x axis was set at 5 s/division. In the bottom photo, the iron(II)·bleomycin solution also contained 2 mM DNA and the x axis was set at 0.5 s/division.

the rate of Fe(III) reduction, again suggesting that at pH 8, iron(III)·bleomycin can only be formed by oxidation of iron(II)·bleomycin. At pH 9 (not shown), the reduction of iron(III)·bleomycin was even faster ($\tau = 4$ min), which may explain the greater activity of bleomycin at this pH. These results contrast with those of Sleigh (1976), showing that chelation by the related drug phleomycin completely inhibited reduction of Cu(II) by sulfhydryls.

High concentrations of DNA decreased the rate of reduction of Fe(III) by a factor of 3 and of iron(III)·bleomycin by a factor of 5, probably by binding both these species and decreasing their accessibility to dithiothreitol (Figure 2A,B). It is not known whether the sulfhydryl loss seen in the presence of DNA results from reduction of DNA-bound iron(III)·bleomycin or from reduction of residual unbound iron(III)·bleomycin. Glutathione, a likely candidate for the in vivo cofactor of bleomycin, also reduced iron(III)·bleomycin but at a slower rate than dithiothreitol (Figure 2C). Kinetics of iron(III)·bleomycin reduction by 2-mercaptoethanol (not shown) were similar to those of glutathione.

Oxidation of the Complex. Kinetics of oxidation were measured by mixing a deoxygenated solution of iron(II)·bleomycin with aerated buffer in a stopped-flow spectrophotometer and recording the A_{380} ($\epsilon_{380} = 2400$ for iron(III)·bleomycin; $\epsilon_{380} = 550$ for iron(II)·bleomycin). Comparison of the ΔA_{380} in Figure 3 with that observed in other reactions on this instrument indicated that, while there may have been some oxidation during preparation and loading, most of the iron(II)·bleomycin was still in reduced form at the time of mixing. Only about half the total absorbance change for the reaction is seen, due to incomplete removal of reacted

material from the absorbance cell.

Kinetics of iron(II)·bleomycin oxidation followed a single exponential with a time constant $\tau = 35 \pm 1$ s. In the presence of a high concentration of DNA, τ decreased to 0.56 ± 0.02 s (Figure 3). Since studies with the copper(II)·bleomycin analogue indicate that about 80% of the complex would be bound to DNA at this concentration (results to be published elsewhere), the simplest explanation of the kinetic data is that iron(II)·bleomycin is much more susceptible to oxidation when bound to DNA. Although not necessarily identical, the products of the reaction in both the presence and absence of DNA had a visible spectrum characteristic of iron(III)·bleomycin. In contrast to iron(II)·bleomycin, oxidation of unchelated Fe(II) was 5 times slower ($\tau = 195 \pm 3$ s) in the presence of DNA than in its absence ($\tau = 40 \pm 2$ s; data not shown).

It should be noted that some oxidations of Fe(II) or iron(II)·bleomycin may not have occurred by direct reaction with O_2. The reduced forms of oxygen, $O_2^{-}\cdot$, H_2O_2, and OH·, are also capable of oxidizing Fe(II) (Goto et al., 1970) and may be capable of oxidizing iron(II)·bleomycin. However, since an initial oxidation by O_2 is required to produce these species, the kinetics observed spectrophotometrically must at least approximate the rate of oxidation of the complex by O_2.

Stoichiometry of DNA Base Release. By performing base-release experiments under conditions where most of the Fe(II)·bleomycin is expected to be bound to DNA, we could assess the effeciency of base release by the oxidation reaction. Mixtures of bleomycin, [[^{3}H]Thy]DNA, and Fe(II) were prepared under nitrogen bubbling, then vortexed to oxygenate the solution, and subjected to paper chromatography. When Fe(II) was included as the limiting reactant, results at two different bleomycin concentrations gave a ratio of 0.18 mol of thymine released per mol of Fe(II) oxidized (Figure 4). Assuming that thymine accounts for half the total base release (Povirk et al., 1978), these data would indicate that about 36% of the oxidations of DNA-bound iron(II)·bleomycin resulted in the release of a base.

When excess Fe(II) was added to a limiting quantity of bleomycin (Figure 4), a more complicated stoichiometry was observed. Although base release plateaued at roughly a one to one ratio of Fe(II) and bleomycin, the plateau values indicated an efficiency of base release per bleomycin molecule of 0.09–0.14, considerably lower than that observed when Fe(II) was the limiting reactant. The dose–response curve (Figure 4B) had an initial toe, which could be explained by removal of a given quantity of bleomycin from the pool of active molecules by complexation with traces of metal ions (~ 4 μM) contaminating the DNA preparation. This explanation is supported by the finding that an additional 2-propanol precipitation of the DNA, following overnight incubation in the presence of EDTA, improved the yield of released bases in the presence of limiting bleomycin, while not affecting the yield in the presence of limiting Fe(II) (Figure 4A). This same precipitation procedure reduced the content of dithizone-extractable transition metals in an exhaustively dialyzed sample of 1.2 mM calf thymus DNA from 13 to 5 μM, indicating that the procedure removed some, but not all, metal contaminants. Thus, although the specificities of dithizone and bleomycin for chelating various ions may differ, the data suggest that the metal ion content of the DNA preparations may be sufficient to account for the toe in the dose–response curve. However, metal contamination would not explain the downward concavity of the curve seen at higher bleomycin concentrations (Figure 4B). Since a considerable

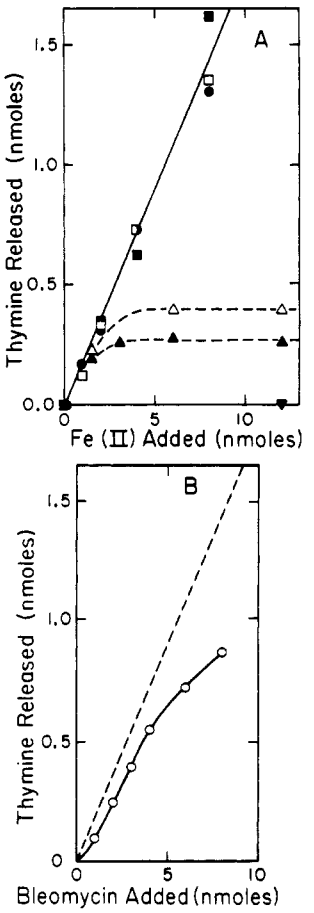

FIGURE 4: Release of [^{3}H]thymine [including its minor product (Povirk et al., 1978)] from DNA by Fe(II) and bleomycin A_2, in 20 mM Tris, pH 8. All reaction mixtures contained 1.2 mM *E. coli* DNA (120 nmol of nucleotides and 41 000 or 63 000 cpm total). (A) Bleomycin [0 (▼), 3 (▲, △), 11 (●), or 22 (■, □) nmol] and the indicated amounts of Fe(II) were added. (B) Fe(II) (12 nmol) and the indicated amounts of bleomycin were added. The dashed line shows thymine release for the case of excess bleomycin and limiting Fe(II). In both cases, DNA, bleomycin, and Fe(II) were added in that order, with 5 min of nitrogen bubbling after each addition to assure complete mixing of the components before oxidation. The solutions were then oxygenated by vortexing, 3 times at 5-min intervals, and subjected to paper chromatography. Closed symbols: results using DNA isolated by the procedure of Marmur (1961). Open symbols: same procedures except the DNA was reprecipitated with 2-propanol after overnight incubation in 0.3 M sodium acetate–10^{-4} M EDTA, pH 7.

excess of Fe(II) was present, it is unlikely that any significant uncomplexed bleomycin remained, and since the dose–response curve in the case of limiting Fe(II) remained linear, it is unlikely that base release was saturating due to lack of substrate. Thus, it must be postulated that base release by a given quantity of iron(II)·bleomycin was either enhanced by excess bleomycin or inhibited by excess Fe(II); the latter seems more likely (see Discussion).

Unless bleomycin were chemically altered during iron(II)·bleomycin oxidation, addition of sulfhydryls to the reaction mixture would be expected to result in the cycling of iron·bleomycin through a series of oxidations and reductions, possibly resulting in the successive release of several DNA bases. Greater than stoichiometric base release was in fact seen (Table I) with both mercaptoethanol and dithiothreitol. Assuming that purines accounted for 13% of the bases released (Povirk et al., 1978), the total estimated base release would be 1.15 times higher than the values given for pyrimidines only in Table I, or a maximum of 2.82 bases per bleomycin. The necessity of rather high concentrations of Fe(II) may have been

Table I: Stoichiometry of DNA Base Release by Bleomycin[a]

reducing agent	reaction mixtures: Fe(II) (μM)	reaction mixtures: bleomycin[d] (nmol)	base release: Thy[e] (nmol)	base release: Cyt[e] (nmol)	base release: bases/bleomycin[f]	base release: Cyt/Thy[f]
80 mM BME[b]	100	0	0.02	0.005		
	100	0.09	0.10	0.065	1.58	0.75
	100	0.26	0.34	0.22	2.06	0.67
	100	0.79	1.09	0.78	2.34	0.71
60 mM BME[b]	100	0	0.006	0.002		
	100	0.26	0.27	0.18	1.70	0.67
	200	0	0.021	0.009		
	200	0.26	0.41	0.26	2.46	0.64
1 mM DTT[c]	100	0	0.021	0.008		
	100	0.26	0.137	0.080	0.72	0.62
5 mM DTT[c]	100	0	0.027	0.020		
	100	0.26	0.30	0.210	1.78	0.70

[a] [[^{3}H]Cyt,[^{14}C]Thy]DNA (22 nmol of nucleotides total in 75 μL) was treated with bleomycin, Fe(II), and sulfhydryls, with bleomycin being added last. After 3 h at 22 °C, samples were subjected to paper chromatography. The amount of thymine released was calculated from the ^{14}C radioactivity chromatographing as thymine and the known specific activity of thymine in the bacterial growth medium (521 cpm/nmol). The specific activity of cytosine (1485 cpm/nmol) was determined from the ^{3}H/^{14}C ratio of the DNA. [b] 2-Mercaptoethanol in 40 mM Tris, pH 8. [c] Dithiothreitol in 20 mM Tris, pH 8. [d] Blenoxane, the clinical mixture. [e] Including the minor products of thymine and cytosine. [f] Pyrimidines only, including minor products, corrected for release at zero bleomycin concentration.

due to competition for Fe(II) ions between bleomycin and the high concentrations of sulfhydryls required to sustain the reaction long enough to oxidize and reduce each Fe·bleomycin several times (Jocelyn, 1972).

The high concentrations of Fe(II) and sulfhydryls did not cause any loss in the specificity of DNA degradation. The cytosine/thymine ratio (Table I) was nearly the same as that seen earlier in experiments using lower Fe(II) and sulfhydryl concentrations (Povirk et al., 1978) and is smiliar to that expected from the sequence specificity of bleomycin-induced DNA breakage, in the absence of added Fe(II) (Takeshita et al., 1978). Furthermore, similar experiments with [[^{3}H]-Gua,[^{14}C]Thy]DNA showed that the rate of guanine release was about 0.05 times the rate of thymine release, even at high concentrations of Fe(II) and mercaptoethanol (not shown), in agreement with previous base-release studies (Povirk et al., 1978; Takeshita et al., 1978). Therefore, it is likely that nearly all the base release resulted from bleomycin–DNA interactions rather than from the generation of free radicals during oxidation of unbound iron(II)·bleomycin, since the latter would be expected to result in indiscriminant base release (Ward & Kuo, 1976). Thus, either a single bleomycin–DNA interaction results in the release of two to three bases or, more likely, bleomycin is capable of releasing a base without itself being inactivated.

To test the possibility of multiple base release in a single reaction, base release and strand breakage were compared by measuring both end points simultaneously on different aliquots of a single sample of bleomycin-treated plasmid pDR3709 [[^{3}H]Thy]DNA. For each strand break, 0.49 thymine was released (Figure 5). Under similar though not identical conditions, thymine was found to account for 47% of the total base release (Povirk et al., 1978). Thus, it appears likely that a single base was released in the formation of each strand break (measured in alkali). Since it is extremely unlikely that one bleomycin–DNA interaction could cause strand breaks on different molecules, each of the two to three bases released per bleomycin (Table I) must have resulted from an independent DNA–bleomycin interaction. Therefore, bleomycin action is catalytic.

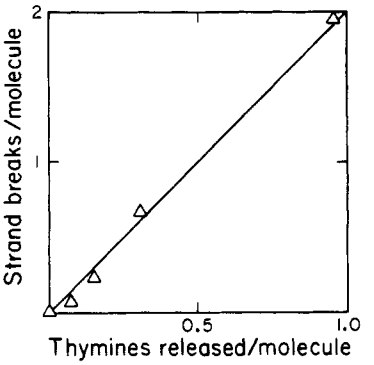

FIGURE 5: Strand breakage and thymine release in pDR3709 DNA. [[^{3}H]Thy]DNA (328 000 cpm; 0.3 mM in nucleotides) in 0.1 mL of 20 mM Tris, pH 8, was treated with 0–0.4 μM bleomycin A_2, 1 μM Fe(II), and 20 mM 2-mercaptoethanol for 30 min at 37 °C. EDTA (25 μL; 0.25 M) was then added to stop the reaction. After 15 min at 22 °C, 5 μL of each sample was removed and assayed for strand breakage by using alkaline sucrose gradients; the remainder of the sample was subjected to paper chromatography. The number of thymines released was determined from the fraction of total ^{3}H radioactivity chromatographing as thymine (excluding the thymine minor product) by assuming 850 thymines per pDR3709 molecule.

Discussion

The results presented here are consistent with the major points of the model of bleomycin-induced DNA degradation proposed by Sausville et al. (1976, 1978a). Their demonstrations of requirements for Fe(II) and O_2 for the reaction and of the formation of a one to one complex between Fe(II) and bleomycin (Sausville et al., 1978b) suggest the oxidation of DNA-bound iron(II)·bleomycin as a crucial reaction step. This proposal is supported by the finding that, while the iron(II)·bleomycin complex oxidizes in solution, the oxidation is 60 times faster when the complex is bound to DNA (Figure 3); this kinetic factor would contribute strongly to the efficiency of DNA degradation by such a mechanism. In addition, this result suggests an interaction between DNA and the chelating portion of the iron(II)·bleomycin complex. While the chelating groups of metal-free bleomycin appear not to bind to DNA (Chien et al., 1977), the opposite could easily be true for iron(II)·belomycin, since Fe(II) chelation would give a net +1 charge to the chelation site and probably alter its three-dimensional structure considerably. Fluorescence studies indicate that the affinity of the copper(II)·bleomycin analogue for DNA is considerably greater than that of metal-free bleomycin (Kasai et al., 1978; our unpublished experiments).

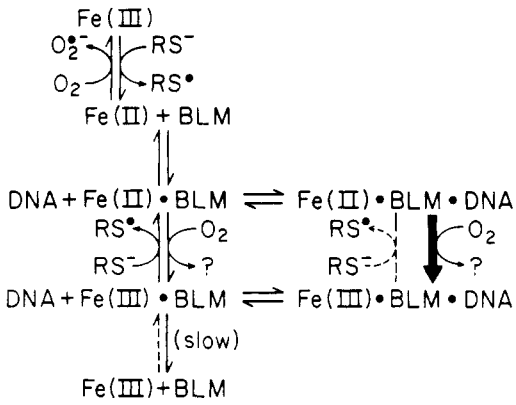

FIGURE 6: Proposed sequence of reactions leading to DNA degradation by bleomycin. An iron(II)·bleomycin complex must first be formed by chelation of Fe(II) produced from Fe(III) by reaction with sulfhydryls. Once formed, the iron·bleomycin complex cycles between oxidation by O_2 and reduction by sulfhydryls. When DNA is present, there is a high probability that the oxidation step will occur when the complex is bound to DNA, due to the enhanced oxidation rate of the bound complex (indicated by thick arrow). This oxidation eventually leads to degradation of the deoxyribose of DNA, in a reaction which may be mediated by a highly sequestered radical. The reactions shown by dashed arrows have not been demonstrated; they occur slowly, if at all. BLM = bleomycin.

Assuming that DNA degradation does result from oxidation of iron(II)·bleomycin·DNA, the greater than stoichiometric base release observed upon treatment of DNA with bleomycin, Fe(II) and sulfhydryls (Table I), combined with the finding that only one base is released per strand break (Figure 5), shows that the oxidized iron(III)·bleomycin complex can dissociate from DNA, be reduced to iron(II)·bleomycin, bind to DNA, and again oxidize, eventually releasing up to three DNA bases by successive oxidation–reduction cycles. The conclusions that 1 base is released per strand break (Figure 5) and that 0.36 base is released per oxidation of iron(II)·bleomycin·DNA (Figure 4) are based on the assumption that half the bases released were thymine. The assumption seems a reasonable one, since every quantitative study of base release from natural DNA by bleomycin, performed under a variety of conditions (Povirk et al., 1978; Takeshita et al., 1978; Sausville et al., 1978b), has yielded similar results; i.e., thymine accounts for about half of the total base release, and cytosine, adenine, and guanine are released in progressively lower amounts. Thus, the specificity appears to be relatively independent of reaction conditions.

Active iron(II)·bleomycin could be regeneraed either by direct reduction of iron(III)·bleomycin (Figure 2) or by replacement of Fe(III) by Fe(II) at the chelation site. However, the iron(III)·bleomycin complex appears to be fairly stable, and the fact that excess Fe(II) did not increase the amount of base release beyond that seen at the one to one ratio of Fe(II) and bleomycin (Figure 4) suggests that any such replacement is slow compared to the oxidation of the excess free Fe(II). Moreover, the rate of reduction of iron(III)·bleomycin is faster, at least at low sulfhydryl concentrations, than that of unchelated Fe(III) (Figure 2). Therefore, direct reduction of iron(III)·bleomycin to iron(II)·bleomycin appears to be the more likely route for bleomycin recycling.

The results suggest a specific sequence of reactions, as summarized in Figure 6. This reaction sequence is not greatly different from that suggested by Sausville et al. (1978a) except that they propose dissociation of Fe(III) from iron(III)·bleomycin·DNA, before its reduction back to Fe(II). This dissociation may well occur, once bleomycin is inactivated; Takita et al. (1978) have reported that reaction of bleomycin with Fe(II) and sulfhydryls eventually leads to destruction of the pyrimidine ring, one of the strong chelating groups of bleomycin. However, the kinetic data presented here indicate that the Fe·bleomycin complex can remain intact through several oxidation–reduction cycles, before bleomycin is inactivated.

Recent evidence has shown that the damage produced in DNA by bleomycin [with Fe(II) as a cofactor] is quite different from that produced by Fe(II) alone. Fe(II) alone produces strand breaks randomly in DNA, leaving 3′-phosphate termini, while bleomycin produces breaks at specific sequences, leaving fragments of altered electrophoretic mobility with unknown 3′ termini (D'Andrea & Haseltine, 1978; Takeshita et al., 1978); bleomycin produces one double-strand break for every ten single-strand breaks, while Fe(II) produces no measurable double-strand breaks (Povirk et al., 1977; Hutchinson & Povirk, 1979; Lloyd et al., 1978). Thus, it is unlikely that bleomycin acts merely by increasing the local Fe(II) concentration near DNA; a more specific mode of action is indicated. On the other hand, there is a certain lack of specificity in the action of bleomycin, in that the products of the reaction are not always the same. Alkali-labile bonds as well as true single-strand breaks are produced (Povirk et al., 1977; Ross & Moses, 1978), and the breaks have at least two different types of 3′ termini (D'Andrea & Haseltine, 1978). Both free bases and minor products of bases are released (Povirk et al., 1977), and only about one-third of the oxidations of iron(II)·bleomycin·DNA (Figure 4) results in release of a base (or minor product). Furthermore, although bleomycin can act more than once, it is eventually inactivated by its own oxidation–reduction reactions (Onishi et al., 1975). All these results would be consistent with a mechanism having as an intermediate a free radical, produced at a specific site in the iron·bleomycin·DNA complex, but having finite probabilities for attacking different parts of the deoxyribose, attacking bleomycin itself, or escaping into solution.

The exact nature of the postulated radical is uncertain. The primary product of oxidation of an Fe(II) complex by O_2 would be expected to be the superoxide radical, $O_2^{\cdot-}$ (Goto et al., 1970). However, there is no evidence for direct damage to DNA by $O_2^{\cdot-}$ (Scholes, 1978). Rather, $O_2^{\cdot-}$-generating species are thought to damage DNA through production of highly reactive OH· radicals, which are formed from $O_2^{\cdot-}$ in a series of reactions having H_2O_2 as an intermediate (Morgan et al., 1975; Lown & Sim, 1976). A similar scheme has been proposed for iron(II)·bleomycin (Lown & Sim, 1977), wherein O_2 is successively reduced by 3 equiv of iron(II)·bleomycin to $O_2^{\cdot-}$, to H_2O_2, and finally to OH· plus OH^-. This scheme would require diffusion of $O_2^{\cdot-}$ and H_2O_2 between iron(II)·bleomycin complexes and would predict the scavengers of these intermediates should inhibit DNA degradation by bleomycin. However, studies testing such scavengers have yielded contradictory results (Lown & Sim, 1977; Sausville et al., 1978b). The result that 0.36 base is released per Fe(II) oxidized (Figure 4) indicates that, if such a three-step reduction of O_2 to OH· occurs, then iron(II)·bleomycin must be very nearly 100% efficient in its use of the $O_2^{\cdot-}$ and H_2O_2 produced. This is, every $O_2^{\cdot-}$ must very rapidly react with two more iron(II)·bleomycin complexes before they can be oxidized by O_2, which is present at a much higher concentration. In such a reaction, 3 mol of Fe(II) would be oxidized per mol of O_2 consumed. However, this prediction is contradicted by recent data of Horwitz et al. (1979) indicating that, in the presence of excess DNA and bleomycin, 1 mol of O_2 is consumed for each mol of Fe(II) oxidized, implying that very

little OH· is produced. While it would be desirable to perform both oxygen consumption and base-release measurements simultaneously, the experimental conditions used by Horwitz et al. (1979) were very similar to those used in the base-release measurements (Figure 4A), and the combined results at least suggest that a single one-step oxidation of iron(II)·bleomycin by O_2 is capable of releasing a DNA base and that production of OH· is not required.

Horwitz et al. (1979) also found that, in the presence of excess DNA and limiting bleomycin, oxygen consumption was a complex function of initial Fe(II) concentration. When more Fe(II) than bleomycin was present, the amount of O_2 consumed was 40–60% less than the amount of Fe(II) added, implying that significant and variable amounts of H_2O_2 and/or OH· were produced. Interference of these products with the oxidation of iron(II)·bleomycin·DNA by O_2 may explain the complex stoichiometry of base release seen in the presence of excess Fe(II) and limiting bleomycin (Figure 4B).

If DNA degradation occurs via a one-step oxidation of iron(II)·bleomycin·DNA by O_2, some form of O_2^{-}· would presumably be the ultimate agent of DNA damage. Despite the apparent unreactivity of free O_2^{-}· the possibility of DNA degradation by a one-step oxidation of iron(II)·bleomycin by O_2 cannot be excluded. An O_2^{-}· radical produced as part of an Fe coordination complex might be more reactive than free O_2^{-}· in solution. Furthermore, oxidation of the complex to iron(III)·bleomycin would give the chelating portion of bleomycin a net +2 charge, so that it may bind strongly to DNA phosphates and, being rather bulky, sequester the radical long enough to greatly increase its chances of reacting with DNA. Finally, intercalation of the bithiazole rings between DNA bases (Povirk et al., 1979) at the iron(II)·bleomycin binding site would unwind and extend the DNA backbone, which may make the deoxyribose more accessible to radical attack.

The finding that bleomycin can act more than once strongly suggests that bleomycin is usually not chemically altered during its reaction with DNA, thus placing an important constraint on proposals for the molecular mechanism. This result agrees with that of Müller & Zahn (1976), showing that bleomycin, after a reaction with poly(dA-dT) which released 0.84 mol of thymine per mol of bleomycin, still retained 95% of its antiproliferative activity against L5178Y cells. These results do not exclude the possibility that a radical may exist transiently on some part of the bleomycin molecule itself.

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