SELF-INACTIVATION OF Fe(II)-BLEOMYCIN

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Fe(II)-Bleomycin is activated in air to form an electron paramagnetic resonance (EPR)-active species, termed "activated bleomycin[1]", that cleaves DNA, when present. When DNA is absent, the potential DNA cleavage activity is lost and the drug becomes self inactivated. A method is described for the preparation and purification of this self-inactivated product from bleomycin A_2, together with some of its physical properties. It is shown that the loss of DNA cleavage activity parallels an alteration of bithiazole fluorescence, attributed to chemical change at this residue. EPR evidence is brought forth that the Cu(II) binding site of inactivated bleomycin in not altered, nor is the ability to form a species with Fe(II) and O_2 having the identical spectroscopic signature as activated bleomycin.

The bleomycins constitute a family of glycopeptide antibiotics that cleave DNA[2]. These drugs are bifunctional molecules, which bind to DNA through the terminal peptide moiety containing an intercalating bithiazole, and which bind to transition metal ions in a separate region of the molecule[3]. Early studies demonstrated that the drug required metal ions for DNA cleavage activity[4]. With Fe(II), DNA scission is O_2-dependent and is preceded by a drug activation process in which Fe(II) first forms a 1 : 1 complex with bleomycin which then subsequently reacts with O_2 producing O_2-Fe(II)-bleomycin[5,6]. This ternary complex autoxidizes, forming a species, termed "activated bleomycin", which is kinetically competent to cleave DNA[1,7]. The activated complex, characterized by its unique electron paramagnetic resonance (EPR) spectrum[1], contains iron as Fe(III)[8] and at least one atom of oxygen originally derived from O_2[1]. Activated bleomycin is formally an Fe(V) complex which requires two reducing equivalents to be discharged to the catalytically inactive species, Fe(III)-bleomycin[9]. In this way, the redox properties of activated bleomycin resemble activated complexes of various peroxidases and the putative activated form of cytochrome P-450[10,11].

In the presence of DNA, activated bleomycin initiates a series of reactions leading to the formation of specific DNA breakdown products[12]. In air, base propenal formation[12~14], stoichiometric with[15] and subsequent to[16] DNA strand scission, occurs. Concomitant with attack on DNA, activated bleomycin is reduced to Fe(III)-bleomycin, with the rate of reduction equal to the rate of oxidative lesion formation in DNA[1]. Subsequent addition of Fe(II)[17] or reduction of Fe(III) *in situ*[18,19] leads to the reactivation of the drug, which is capable of continuing DNA breakdown to a point which is believed to be limited by the ability of the drug to bind to DNA[5].

If, however, DNA is not present, activated bleomycin undergoes a self-inactivation process such that the ability of the drug to cleave DNA is not fully restored upon subsequent addition of Fe(II) and O_2[1]. The rate of self-inactivation is the same as the rate of attack on DNA, if present, and is concomitant with the formation of a species with the same optical and EPR properties as Fe(III)-bleomycin.

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In this communication, we describe the conversion of bleomycin A_2, one of the isomers found in the commercial preparation Blenoxane, into a product that retains the ability to generate the species formed with Fe(II) and O_2 having the EPR properties of activated bleomycin, and yet is incapable of cleaving DNA. The inactive form exhibits altered fluorescence properties compared to bleomycin A_2 which indicate an alteration of the bithiazole residue. It is suggested that the alteration or destruction of the bithiazole residue reduces binding to DNA and this is largely responsible for the loss of DNA cleavage activity.

Materials and Methods

Materials

Bleomycin sulfate (Blenoxane™) was a gift from Bristol Drugs. Calf thymus DNA was obtained from Sigma and was dissolved in 20 mM sodium phosphate, pH 7.0. The concentration was calculated on the basis of E_{260} 6.6 (mM nucleotide)$^{-1}$ cm^{-1}. Analytical grade ferric and ferrous ammonium sulfate (Baker) were dissolved in distilled water before use. All reactions were carried out at room temperature, unless otherwise noted, in 20 mM sodium phosphate buffer, pH 7.0.

Purification of Bleomycin A_2

Bleomycin A_2 was purified from Blenoxane. Chelex-treated, distilled, and deionized water was used throughout the purification. The drug (80 mg) was dissolved in 3 ml H_2O and was applied to a 14×400 mm CM-Sephadex C-25 column† equilibrated with 50 mM ammonium formate, pH 6.5. After washing the column with 200 ml of the same buffer, the various bleomycin isomers were eluted by linear gradient up to 1.0 M ammonium formate, pH 6.5, monitoring column eluates at 254 nm. Peak fractions were combined, lyophylized, and minimal amounts of water were added to dissolve crystalline salts and drug. Desalting was carried out on a 14×400-mm Sephadex G-10 column, monitoring bleomycin elution visually from its light yellow color. The identity as well as the purity of the isolated bleomycins was based on chromatographic elution profiles and by TLC on pre-coated Silica gel 60 F_{254} plates from EM Laboratories, according to the method of Roy *et al.*[20]. Bleomycin A_2 was characterized by an Rf of 0.37 in the solvent methanol - 10% ammonium acetate (w/v) - 10% ammonium hydroxide (10:9:1). A millimolar extinction coefficient of 15.1 was reported (as an unpublished observation in ref 21).

Effect of Fe(II) upon Bleomycin A_2

Eight 10-μl aliquots of 20 mM Fe(II) were added sequentially, with 15 minutes incubation time between additions, to a stirred, 1 ml solution of 0.2 mM bleomycin A_2, either in the absence or in the presence of 2.6 mM DNA. After each incubation, 20 μl of the reaction mixture was applied to a TLC plate and was developed in the same solvent used for bleomycin purification. An UV lamp was used to visualize spots[20].

Preparation of Bleomycin A_2 Oxidation Product

Eight 20-μl aliquots of 20 mM Fe(II) were added sequentially to 2 ml of a gently stirred solution of 0.2 mM bleomycin A_2, with 15 minutes incubations between additions. The product and untreated bleomycin A_2 were separated on Silica gel 60 TLC plates (20×20 cm) from EM Laboratories, developed in methanol - 10% (w/v) ammonium acetate (10:9). In this solvent the Rf of bleomycin A_2 was 0.41. The product band (Rf 0.35) showed a yellow fluorescence as compared to a blue fluorescence for the parent compound, and was scraped from the plate. The product was eluted from the silica gel with 10 mM HCl and, after centrifugation, the supernatant was neutralized with 0.1 M NaOH. This solution was used for optical spectral and fluorescence studies.

† In order to remove bound metal ion, the resin was hydrated overnight in 5 mM ammonium formate, pH 6.5, containing 1 mM EDTA. It was subsequently washed three times by decantation with 1 mM EDTA, adjusting the pH to 6.5 with acetic acid. Finally, the resin was equilibrated with 50 mM ammonium formate, pH 6.5, before being packed in the column.

Assay of Bleomycin Activity

Bleomycin activity was judged from the yield of base propenal which results from DNA breakage when Fe(II) is added aerobically. Base propenal was assayed by the thiobarbituric acid procedure described previously[12]. The reaction mixture contained 0.08 mM bleomycin A_2, 0.5 mM DNA and 0.08 mM Fe(II). Aliquots (0.4 ml) were added to 0.6 ml of 40 μM 2-thiobarbituric acid containing 1 mM EDTA. Mixtures were heated at 100°C for 15 minutes and subsequently cooled to room temperature. The adduct formed with 2-thiobarbituric acid was quantitated based on the extinction coefficient at 532 nm (E_{mM} 160 cm^{-1})[12].

Fluorescence Studies

A Perkin-Elmer fluorometer, Model MPF-3, was used for fluorescence and kinetic measurements[22,23]. Titration of bleomycin A_2 with Fe(II) was carried out under anaerobic conditions in a quartz Thunberg fluorescence cell fitted with rubber septum stoppers. The cell, containing 0.02 mM bleomycin A_2 in 20 mM phosphate buffer (pH 7.0) in the central cuvette and Fe(II) in the side arm, was thoroughly degassed with highly purified argon. The fluorescence was measured before and after mixing with Fe(II) and after subsequent mixing of the solution with air. Fluorometric studies of Cu(II), Fe(III) and Zn(II) binding were carried out aerobically. Bleomycin solutions were titrated with metal ion and the fluorescence was recorded 10 minutes after additions, the time needed in some cases to achieve equilibrium.

EPR Spectroscopy

Activated bleomycin (g=2.26, 2.17, 1.94) and Fe(III)-bleomycin (g=2.45, 2.18, 1.89) were identified using EPR spectroscopy[1,17]. Activation of the bleomycin A_2 oxidation product was carried out as follows: Ten individual 10-μl aliquots of 50 mM Fe(II) were added to a 1-ml stirred solution containing 0.5 mM bleomycin A_2 in 20 mM phosphate buffer, pH 7.0, with 15 minutes incubation between each addition. After the final addition and incubation, the solution was centrifuged for 10 minutes to remove iron precipitates. Subsequent steps were carried out at 0°C. A 10-μl aliquot of 50 mM Fe(II) was added to the supernatant to re-initiate the activation reaction[1]. A 200-μl aliquot was diluted with 200 μl ethylene glycol, required for the formation of a glass for low temperature EPR study, and the sample was quickly transferred to an EPR tube and frozen in liquid N_2 (~40 seconds). As a control, Fe(II) was added to untreated bleomycin A_2 which was diluted as above and frozen for EPR examination.

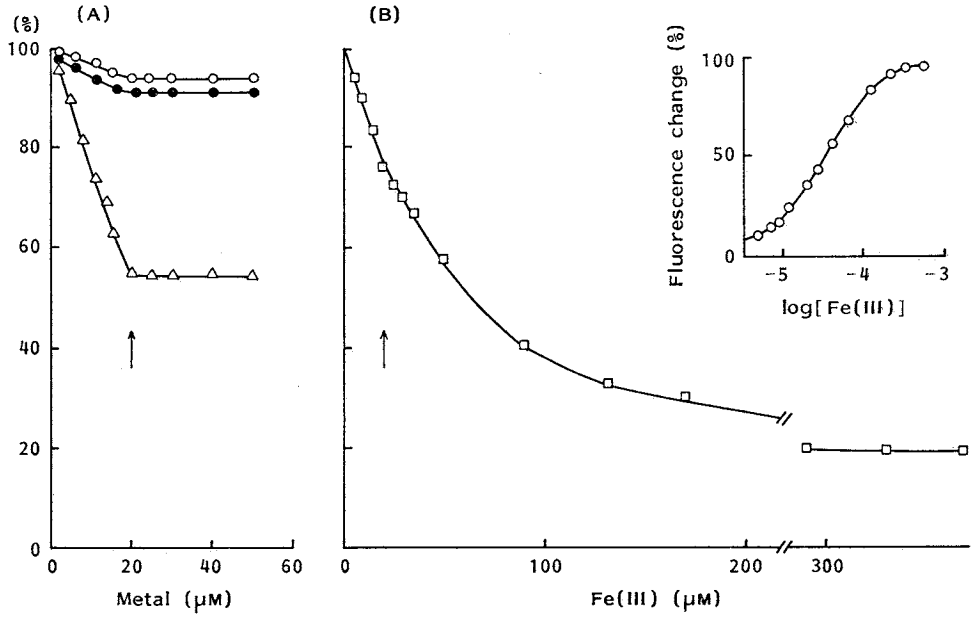
The EPR spectrum of the Cu(II) complex of bleomycin A_2 and the catalytically inactive oxidation product were obtained by the addition of 0.4 mM Cu(II) instead of Fe(II) to a bleomycin solution originally in 20 mM HEPES, pH 7.3. Spectra were recorded as described previously[1].

Results

The fluorescence of bleomycin A_2 with an emission maximum at 353 nm (excitation at 300 nm), is assigned to the 2,4-bithiazole group[23]. Fig. 1 shows the fluorescence properties of the drug when it is titrated with different transition metal ions. Under metal ion saturating conditions, the fluorescence quenching order is Cu(II) $\gg$ Fe(II) $>$ Zn(II). Tight metal binding[24] is confirmed by the sharp breaks in the titration curves and the extinction coefficient for the drug[21] is verified. The result of a titration with Fe(III) is shown in Fig. 1B and it is evident from the shape of the curve that it binds far weaker to the drug than the other metal ions. A log plot of the binding data is shown as an insert to Fig. 1B. The association constant for Fe(III), 3.2×10^4, is smaller by three orders of magnitude than for Fe(II), 2.7×10^7 [24], and supports the view that Fe(II) can replace bleomycin-bound Fe(III).

When a 2-fold molar excess of Fe(II) is added anaerobically to bleomycin A_2, the fluorescence is partially quenched (Fig. 2A, curve b). When this solution is aerated, a further change in fluorescence

Fig. 1. The effect of metal ions on the fluorescence of bleomycin A_2.



The fluorescence intensity at 350 nm (excitation at 300 nm) is plotted against metal ion concentration, with the fluorescence of metal-free drug taken as 100%.

In (A), a 2.5-ml solution of 20 μM bleomycin A_2 in 20 mM phosphate buffer, pH 7.0, was titrated with Zn(II) ○, Fe(II) ●, or Cu(II) △. Fe(II) addition was carried out in an anaerobic Thunberg fluorescence cuvette, equipped with a rubber stoppered septum used for the anaerobic introduction of metal ion. In (B), Fe(III) was added to 20 μM bleomycin.

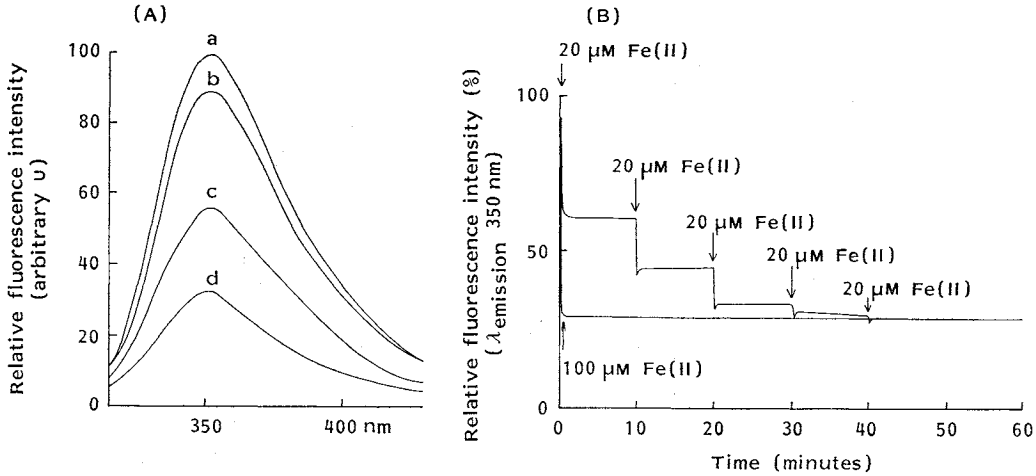
The vertical arrow in (A) and (B) indicates stoichiometric equivalence of drug and metal ion. The insert in (B) is a plot of the alteration of fluorescence as a function of ligand free Fe(III) concentration. The overall fluorescence change is the difference in fluorescence for metal-free drug and that at high Fe(III) concentrations.

is observed (Fig. 2A, curve d). The fluorescence yield of this product is less than that of an untreated bleomycin A_2 solution to which a 2-fold molar excess of Fe(III) was added (Fig. 2A, curve c), suggesting that an alteration of the fluorophore had taken place.

In a separate study (Fig. 2B), the time course of alteration of fluorescence brought about by the aerobic addition of five aliquots of 20 μM Fe(II) is compared to a single addition of 100 μM Fe(II) to a solution of 20 μM bleomycin. The fluorescence yield at the end of five additions does not differ significantly from that where a single addition of 100 μM Fe(II) is made, but it is less than that observed when 100 μM Fe(III) is added *de novo* to bleomycin A_2 (Fig. 1B). The total change in fluorescence in each step is complete in about 2 minutes (if oxygen levels are reduced, the time course for the fluorescence change after a single addition of Fe(II) can be prolonged to as much as 15 minutes). It is suggested from these studies that the Fe(II) binding reaction, activation with O_2, and displacement of Fe(III) formed from activated bleomycin is completed within 2 minutes at room temperature.

When Fe(II) is added to bleomycin A_2 in air, partial self-inactivation of the drug occurs when DNA is absent[1,12] and a loss of DNA cleaving activity can be demonstrated when DNA is added after re-addition of Fe(II) to a partially inactivated sample. Data from such a study is shown in Fig. 3 where Fe(II), equimolar with bleomycin, is allowed to react aerobically with the drug. A subsequent

Fig. 2. Fluorescence changes of bleomycin A_2 caused by aerobic reaction with Fe(II).



(A) The effect of Fe(II) on the fluorescence at 350 nm of bleomycin A_2. Trace **a** is a 20-μM bleomycin A_2 solution (OD at 290 nm, 0.31) studied anaerobically in 20 mM phosphate buffer, pH 7.0. In **b**, anaerobic Fe(II) (40 μM) is added. Trace **d** is obtained after the introduction of air, allowing 10 minutes for equilibrium to take place. Trace **c** is obtained by adding Fe(III) (40 μM) to a fresh sample of 20 μM bleomycin A_2. The excitation wavelength was 300 nm.

(B) A comparison of fluorescence change brought about by aerobic addition of Fe(II) to 20 μM bleomycin A_2 in phosphate buffer. At the times indicated by vertical arrows, 20 μM Fe(II) was added and the fluorescence emission at 350 nm was followed. For comparison, 100 μM Fe(II) was added to a separate sample of 20 μM Fe(II). Data were normalized to 100% for the untreated drug. It should be noted that soon after the introduction of Fe(II), at least after the first introduction, the fluorescence decreases to a minimum to which it rises to a higher value at equilibrium. It is possible that a short lived intermediate is formed having fluorescence properties different from that of more stable reaction products.

addition of Fe(II) is made, and the catalytic activity is measured as base propenal release from added DNA[12]. Each subsequent addition of Fe(II) up to a total of 6 equivalents causes roughly a 14%-loss of DNA cleaving activity. After 10 additions of Fe(II), only 6% of the original activity remains.

Concomitant with the loss of ability of bleomycin A_2 to cleave DNA, there is a conversion of the drug to a product with a decreased Rf on TLC. This conversion which occurs in aerobic reactions with Fe(II) is prevented when DNA is present (data not shown). Each addition of Fe(II) causes a loss of bleomycin A_2 with the appearance of a product that does not migrate in methanol - 10% ammonium acetate (w/v) - 10% ammonium hydroxide (10 : 9 : 1), (the Rf for bleomycin A_2 here is 0.37). In methanol - 10% (w/v) ammonium acetate (10 : 9) the product migrates with an Rf of 0.36 while bleomycin A_2 has an Rf of 0.47 in this system.

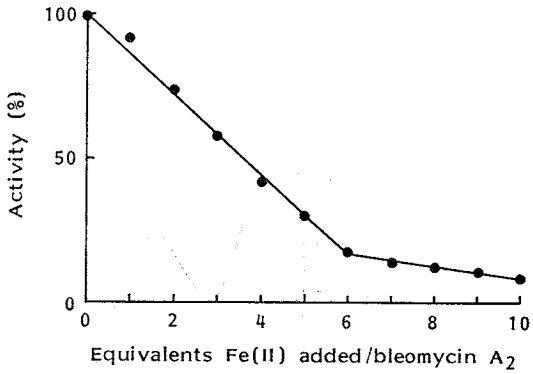
In Fig. 4A, we compare the optical spectra of bleomycin A_2 and the inactivated product recovered after TLC (Fig. 4B) arbitrarily adjusting the absorption of the latter at 290 nm to be similar to that of untreated bleomycin A_2. Although the shoulder near 245 nm is the same for both samples, the absorption near 290 nm for the product is broadened. Also, the ability of inactivated bleomycin to release base propenal from DNA with Fe(II) is only 7% that observed for bleomycin A_2, for solutions having comparable absorptions at 290 nm.

Fig. 4C is the fluorescence emission spectrum of the bleomycin A_2 solution, whose optical spectrum is given in Fig. 4A, taken with an excitation wavelength of 300 nm. The fluorescence maximum is at 353 nm. When the fluorescence spectrum of the inactivated product whose spectrum is given in

Fig. 4B is examined, an emission is also observed at 353 nm, but with only 10% of the fluorescence intensity of that in Fig. 4C. Excitation at 394 nm, though, produces the fluorescence emission shown in Fig. 4D. When DNA is added to bleomycin A_2, the fluorescence of the drug (Fig. 4C) is partially quenched, which indicates that binding to DNA occurs[22,23]. With the inactive product (Fig. 4D), no such quenching by DNA is seen.

In Fig. 5A, we compare the EPR spectrum of the Cu(II) adduct of the inactivated product with that of Cu(II)-bleomycin A_2. It can be readily seen that the spectra are identical, suggesting that the equatorial ligands to Cu(II)[24~26] are the same in both complexes. When Fe(II) is added to the inactivated product at 0°C, the resulting EPR spectral lineshape (Fig. 5B) is identical to that seen when Fe(II) is added to bleomycin A_2 never treated with Fe(II). This suggests that even though the inactivated drug has a markedly reduced DNA cleavage activity, it retains its ability to activate oxygen in the same way as the active drug.

Fig. 3. The effect of preincubation of bleomycin A_2 with Fe(II) on its ability to cleave DNA.



An 0.4-ml aliquot of an 80-μM bleomycin solution was assayed for DNA cleavage activity by base propenal formation with 80 μM Fe(II) and 0.5 mM DNA in 10 mM phosphate buffer, pH 7.0. Incubation was carried out for 15 minutes before the addition of thiobarbituric acid reagent[12]. Fe(II), 80 μM, was added to the original solution and another aliquot was withdrawn after 30 minutes to assay for DNA cleavage activity. This was repeated for 10 individual additions of Fe(II) to the original bleomycin solution. The plot shows the percent of remaining DNA cleavage activity, measured as base propenal release taking 100% for the drug before initial incubation with Fe(II) in the absence of DNA. Drug activity of untreated bleomycin, as assayed by base propenal formation, was inhibited less than 5% by 1 mM Fe(III).

Discussion

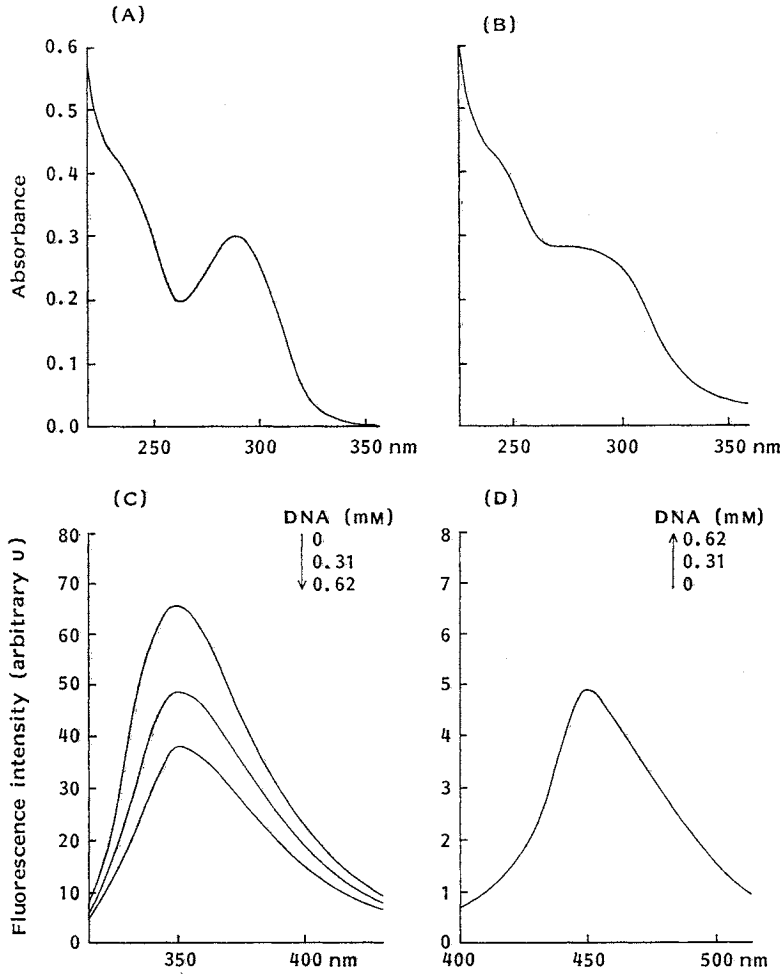
In the absence of DNA, activated bleomycin, formed from the autoxidation of O_2-Fe(II)-bleomycin[1,7], is partially self-inactivated. Subsequent addition of DNA and re-addition of Fe(II) yields less DNA cleavage products compared to the yield from activated drug not initially cycled with Fe(II). Other studies have shown that base propenal release accompanying DNA cleavage[15] requires O_2 subsequent to drug activation, either with Fe(II) and O_2 or with Fe(III) and peroxide[1]. In contrast, partial inactivation of the drug does not require O_2 beyond that necessary for activation of Fe(II)-bleomycin[27]. In this way, drug self-inactivation may resemble a reaction of activated bleomycin with DNA that takes place when O_2 is limited[12,14,28].

Early studies addressed to drug self-inactivation by treatment with Fe(II) suggest that there is the release of a fragment from the molecule[3] while a change in the ^{13}C NMR spectrum also suggested an alteration in the pyrimidine structure[29]. GUTTERIDGE and SHUTE[30] suggest that the polarity of bleomycin is altered when it is self-inactivated, and this is borne out from the changes in Rf we observe for the product.

Evidence from spectroscopic data and chromatographic behavior suggest that the structure of bleomycin is altered when it is inactivated, though no alteration in metal binding properties can be demonstrated. Fe(III)-bleomycin generated from Fe(II)-bleomycin is reported to have the same *pK*'s, determined by optical titration, as the complex formed by *de novo* addition of Fe(III) to the drug[17] suggesting that no alteration in ionizable groups associated with metal binding occur. Further, the EPR of the Cu(II) complex of inactivated drug, indicates identical metal coordination (Fig. 5).

A major difference, however, is observed in the optical and fluorescence properties of the mole-

Fig. 4. A comparison of optical and fluorescence properties of bleomycin A_2 with that of the oxidation product.

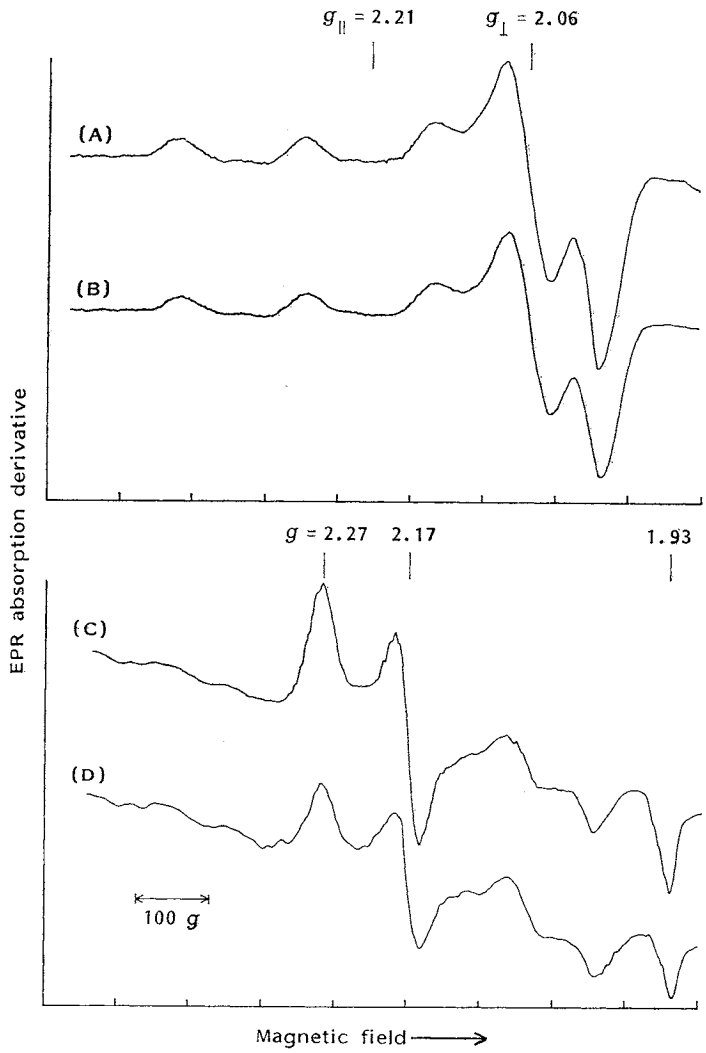


Trace (A) is the UV spectrum of 20 μM bleomycin A_2 and trace (B) of the oxidation product formed by aerobic addition of Fe(II) and separation by TLC. The absorption at 290 nm of the solution in (B) was arbitrarily adjusted to be similar to that in (A). Measurements were carried out in 10 mM phosphate buffer, pH 7. Trace (C) is the fluorescence spectrum studied with an excitation wavelength of 300 nm of the bleomycin A_2 solution whose optical spectrum is given in trace (A). (D) is the fluorescence spectrum of the product having the optical spectrum shown in trace (B) using an excitation wavelength of 394 nm. In (C) and (D), the effects of DNA addition (concentrations are given in the figure) on the fluorescence properties of bleomycin A_2 and of the oxidation product are indicated.

cule when it becomes inactivated. Notable in these is a broadening of the 290 nm absorption band (Figs. 4A and 4B) and the diminution of the fluorescence emission near 350 nm as well as the formation of a new fluorescent species (Fig. 4D). In addition to spectral and fluorescence change, the ability of the bleomycin molecule to release base propenal from DNA when Fe(II) is added is impaired (Fig. 3).

This result is to be compared with the recent report by Morii *et al.*[31], of a photo-transformed Cu(II)-bleomycin that exhibits a broadened optical absorption near 290 nm, similar to the one we observe for Fe(II)-inactivated bleomycin. The fluorescence emission maximum for the photo-transformed product is shifted to 410. In this, as well as in our own study, an alteration in bithiazole

Fig. 5. A comparison of EPR spectra of Cu(II) and Fe(II) reacted with bleomycin A_2 and its inactivated product.



Upper traces: Trace (A) is the EPR spectrum of the Cu(II) complex formed with the inactive product formed by multiple additions of Fe(II) to bleomycin A_2. Trace (B) is the spectrum of Cu(II) with authentic bleomycin A_2.

Lower traces: A similar comparison of spectra of product formed subsequent to Fe(II) addition to Fe(II)-inactivated bleomycin A_2 (C) and authentic bleomycin A_2 (D). Samples were diluted 1 : 1 with ethylene glycol. A control sample of activated bleomycin A_2 was prepared by adding Fe(II) to an aerobic drug solution that had never been pretreated with Fe(II), diluting with ethylene glycol and freezing for EPR examination. Spectra were recorded at 77 K on a Varian E-12 spectrometer as described previously[17] with a microwave power of 10 mW and a modulation amplitude of 20 G. The g values are indicated in the figure.

structure is suggested. Unlike the self-inactivated product reported here, there is no report of reduction of DNA cleavage activity of the photo-transformed product.

In summary, we describe the preparation of an inactivated product of bleomycin with a markedly reduced catalytic potential for base propenal release from DNA with Fe(II) and O_2. An EPR study shows that Cu(II) ligation to the inactivated drug is unchanged and, further, that a paramagnetic

species can be produced from Fe(II) and O_2 having the same EPR spectrum as activated bleomycin (Fig. 4), a molecule shown to contain Fe(III) and at least a single atom of oxygen derived from O_2[1,8]. It is suggested that reduction of catalytic activity is a consequence of alteration of bithiazole structure involved in the binding of the drug to DNA[32].

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