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Bleomycin A_2: A Ferrous Oxidase

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SUMMARY

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The bleomycins are polypeptides produced by *Streptomyces verticullus* and are used in chemotherapy of various malignancies. We find that bleomycin A_2 catalyzes the oxidation of Fe^{++} to Fe^{+++} and reduces oxygen. The reaction follows classical Michaelis-Menten kinetics (Vmax = 0.27 μmole/min/ml, Km = 1.8 mM). One mole of bleomycin A_2 turns over approximately 5,000 moles Fe^{++}/min. This catalytic activity of bleomycin A_2 is heat insensitive and strongly dependent on pH, buffer and ionic strength, and is unaffected by DNA. This enzyme-like action may be involved in the bleomycin degradation of DNA.

INTRODUCTION

Bleomycin A_2, a potent antimicrobial and antitumor agent (1), is a polypeptide with a molecular weight of approximately 1400 produced by *Streptomyces verticullus*. It was discovered by Umezawa in 1966 (2, 3). While its mechanism of action has yet to be determined, it is known that bleomycin A_2 binds to DNA and causes DNA strand breakage (4–12). Kohn has suggested that bleomycin-induced DNA degradation may be associated with cell toxicity (11). Certain metal ions (Cu^{++}, Zn^{++} or Co^{++}) inhibit bleomycin-induced DNA breakage (13), but others (Fe^{++}, Mg^{++}) are necessary for this activity (8, 9). It appears that Fe^{++}, oxygen and bleomycin are required for the breakage of DNA (8, 9). Lown and Sim (8) have suggested that superoxide and hydroxyl radicals may play a role in this drug's ability to break DNA.

MATERIALS AND METHODS

Bleomycin A_2 was obtained from the Natural Products Branch, Division of Cancer Treatment, NCI, NIH. Purity was checked by thin layer chromatography and by ^{13}C nuclear magnetic resonance spectroscopy. Ten microliters of a bleomycin A_2 solution (2.8 mg/ml) were spotted on a silica gel 60 plate (0.25 mm thickness, E. M. Laboratories, Inc.). The plate was chromatographed in 10% ammonium acetate:methanol (1:1) according to the method of Umezawa *et al.* (3). One spot was observed under shortwave u.v. light (2537 Å). The R_f value was 0.33 (published value for bleomycin A_2 is 0.40 (3)). The ^{13}C nuclear magnetic resonance spectrum of bleomycin A_2 confirmed that any impurity, if present, was less than 1%. All peaks were assigned to bleomycin carbons (14). No extraneous peaks were present. Since the signal/noise was 120/1 and extraneous peaks would be observable at a signal/noise of 1/1, we concluded that

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our sample of bleomycin A_2 was essentially pure.

The oxygen content of reaction mixtures was measured with a Clark electrode in a Yellow Springs Model 53 Instrument. Reaction mixtures (final volume, 1 ml) contained 0.1 M sodium maleate buffer (pH 6.2), 10 μM bleomycin A_2 (when used) and 500 μM $FeSO_4$. Buffer and water were aerated by bubbling with air for 3 min at 37°. Bleomycin A_2 was added to the reaction vessel, the electrode was inserted and the system was equilibrated for 1 min while being monitored for oxygen content. Fifty microliters of $FeSO_4$ (10 μM) was injected into the reaction vessel and the change in oxygen content was determined. $FeSO_4$ solutions were made fresh daily in water that had been sparged with N_2. Rates of oxygen consumption were calculated from the initial linear electrode response (the value used for the dissolved oxygen content in the reaction mixture was 0.16 μmole).

Visible-u.v. spectra were obtained on a Cary 14 recording spectrophotometer.

RESULTS AND DISCUSSION

While investigating the interaction of Fe^{++} and bleomycin A_2, we observed that bleomycin A_2 stimulated an Fe^{++}-dependent consumption of oxygen. Although Fe^{++} was spontaneously transformed to Fe^{+++} in the presence of oxygen, the addition of 10 μM bleomycin A_2 to $FeSO_4$ accelerated the process. This reaction required Fe^{++}, bleomycin A_2 and oxygen. The rate of the oxygen consumption was directly proportional to the bleomycin A_2 concentration (Fig. 1). Preheating the bleomycin A_2 at 100° for up to ten minutes did not affect its ability to stimulate oxygen uptake.

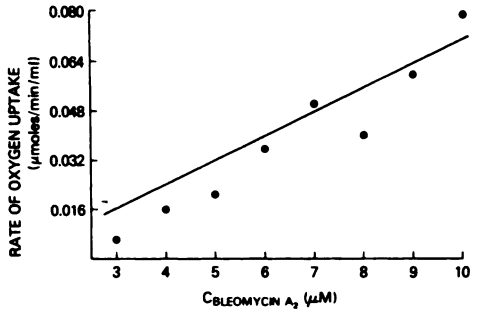


FIG. 1. *Oxygen consumption as a function of bleomycin A_2 concentration*

Fe^{++} (500 μM) was added to reaction mixtures containing the concentrations of bleomycin A_2 indicated. Points represent one determination. The experiment was repeated and similar results were obtained.

We tested the effects of pH, ionic strength and buffer on the stimulation of oxygen uptake by bleomycin A_2. We found that sodium maleate or succinate suppressed the endogenous rate of Fe^{++}-induced oxygen uptake. The bleomycin A_2 stimulation had a pH optimum between pH 6 and 7 in 0.1 M maleate or succinate buffer.

When increasing concentrations of Fe^{++} were added to reaction mixtures containing 10^{-5} M bleomycin A_2, the initial rates of oxygen consumption increased until saturation was reached. These experimental data followed Michaelis-Menten kinetics (Fig. 2). A kinetic analysis of these data by Lineweaver Burk plot (Fig. 2) yielded a Km = 1.8 mM and Vmax = 0.27 μmole/min/ml. Initially one mole of bleomycin A_2 catalyzed approximately 5,000 moles Fe^{++}/min. The specific activity of bleomycin A_2 is 27 μmole O_2 consumed/min/μmole bleomycin A_2.

One of the products of this catalytic process is Fe^{+++} chelated to bleomycin A_2 (Fig. 3). The visible-u.v. spectrum of 400 μM bleomycin A_2 + 400 μM Fe^{++} in air was identical to the spectrum of −400 μM bleomycin A_2 + 400 μM Fe^{+++} under nitrogen or air. These spectra, in turn, were different from the sum of the spectra of 400 μM Fe^{+++} and 400 μM bleomycin A_2 measured in separate cuvettes. These data indicated that the catalyst-substrate complex (bleomycin A_2-Fe^{++}) reacted with oxygen to form a bleomycin A_2-Fe^{+++} complex. If some form of oxygen were present in this complex, it did not affect the visible spectrum since the spectrum of Fe^{+++} and bleomycin A_2 under nitrogen is identical to the product in air or oxygen. The univalent oxidation of ferrous ion and the concurrent consumption of oxygen suggested superoxide was formed in this reaction.

Since Fe^{++} was oxidized to Fe^{+++} in the presence of bleomycin A_2 and oxygen, we would expect that the superoxide radical was formed. While we have been able to detect this radical species when Fe^{++} was added to a phosphate buffer, we have not

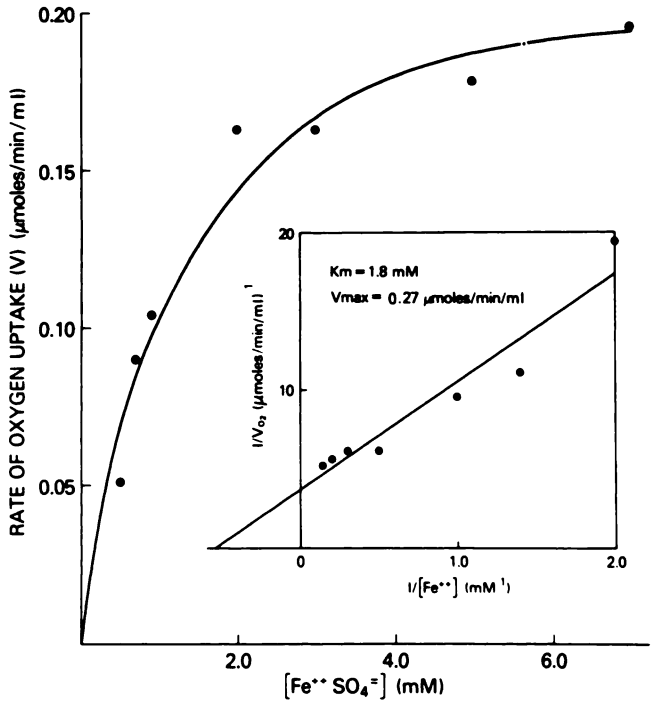


FIG. 2. *Michaelis-Menten plot of oxygen consumption versus Fe^{++} concentration*
The insert is a Lineweaver-Burk plot of the same data. Measurements were made as described in MATERIALS AND METHODS. 0.1 M maleate buffer pH 6.2 and 10μM bleomycin A_2 were used.

been able to detect O_2^- in the presence of Fe^{++}-bleomycin A_2 in a maleate buffer. The assays we used for O_2^- detection were the spectroscopic monitoring of ferricytochrome C reduction, of nitro blue tetrazolium reduction or of epinephrine oxidation (15), electron spin resonance (16, 17) and inhibition of oxygen consumption by SOD[2] or catalase. With this latter assay, if O_2^- and H_2O_2 were formed, an apparent inhibition of oxygen consumption would be expected since oxygen was re-formed according to the following reactions:

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O$$

We observed this inhibition in a Fe^{++} phosphate but not in a Fe^{++}-bleomycin A_2 system. Our inability to observe inhibition in the presence of bleomycin A_2 may have been due to a rather slow dissociation of the iron-bleomycin A_2-oxygen complex leading to small amounts of O_2 being reformed in comparison to the amount of oxygen utilized at any time point.

Bleomycin A_2 possesses catalytic activity in oxidizing ferrous ion and follows kinetics characteristic of an enzyme. In light of these data, we suggest that bleomycin A_2, since it is a polypeptide, may be considered an enzyme and be classified as a ferrous oxidase. Since this activity is heat resistant, it appears unlikely that this catalytic activity could be due to a high molecular weight impurity. While a low molecular weight impurity (such as EDTA) may be heat resistant, such a species would have to be present at concentrations less than 1% of the bleomycin concentration used or 10^{-7} M. Such concentration would not be compatible with the amount of oxygen consumed (18, 19).

Based on our data, we propose the following mechanism for the catalytic activity of bleomycin A_2:

[2] The abbreviation used is: SOD, superoxide dismutase.

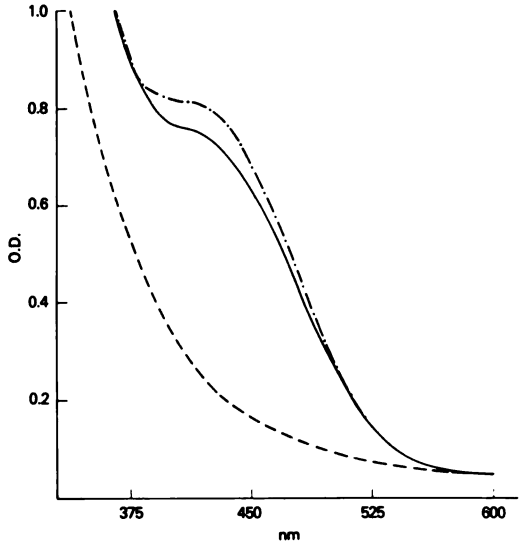
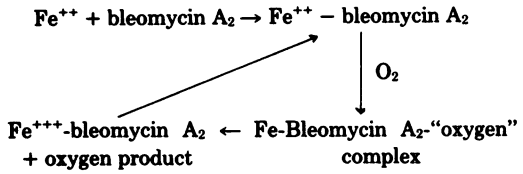


FIG. 3. *Visible-u.v. spectra of iron and bleomycin A_2*

In one of these experiments 400 μM Fe^{+++} was placed in one cuvette and 400 μM bleomycin A_2 in another. These were placed in tandem in a Cary 14 recording spectrophotometer and the additive spectrum (- - -) was recorded. When the two reactants were mixed in the same cuvette under N_2, the solution turned pale yellow and the spectrum indicated (-·-·-) was obtained. This spectrum is identical to the spectrum recorded when 400 μM Fe^{++} and 400 μM bleomycin A_2 were mixed in an aerated solution (——). 0.1 M sodium succinate, pH = 6.2, was used in these experiments.



In this reaction, ferrous ion and bleomycin A_2 form a complex which in the presence of oxygen causes oxygen reduction and the oxidation of Fe^{++} to Fe^{+++}.

Even though our attempts to detect the superoxide radical were unsuccessful, its presence is strongly suggested. Its presence is also supported directly by the work of Lown and Sim (8) who found that SOD inhibited DNA strand breaks caused by bleomycin and Fe^{++}. These authors attributed the cause of the DNA lesion to the hydroxyl radical.

While it is known that various agents such as EDTA lead to accelerated rates of Fe^{++} oxidation (18), the enzyme-like nature of the bleomycin A_2-Fe^{++} reaction has not previously been reported. The catalytic mechanism suggested by our data coupled with the ability of bleomycin A_2 to bind DNA may explain the anti-tumor properties of this compound.

Preliminary experiments show that, when calf thymus DNA is added to a system containing ferrous sulfate and bleomycin A_2 under conditions where DNA is known to bind to bleomycin A_2, the DNA does not inhibit the catalytic effect of the bleomycin A_2.

The significance of our finding that bleomycin A_2 acts catalytically to oxidize ferrous ions to form reduced and potentially reactive oxygen species may not so much lie in the increased kinetics that any catalyst implies. Rather, bleomycin A_2 may be able to bind DNA and chronically and continuously cause metal ion oxidation and oxygen reduction at this important target molecule in the cell, leading to DNA degradation and damage and ultimately cell death.

REFERENCES

1. For general review see *GANN Monograph on Cancer Research*, (ed. S. Carter, T. Ichitiawa, G. Mathe, H. Umezawa), University of Tokyo Press, Tokyo, **19**, 1976.
2. Umezawa, H., Maeda, K., Takeuchi, T. T. & Okami, Y. (1966) New Antibiotics, Bleomycin A and B. *J. Antibiotics*, Ser. A **19**, 200–209.
3. Umezawa, H., Suhara, Y., Takita, T. & Maeda, K. (1966) Purification of bleomycins. *J. Antibiotics*, Ser A **19**, 210–215.
4. Sausville, E., Peisach, J. & Horwitz, S. (1978) Effect of chelating agents and metal ions on the degradation of DNA by bleomycin. *Biochemistry*, **17**, 2740–2746, 1978.
5. Sausville, E., Stein, R., Peisach, J. & Horwitz, S. (1978) Properties and products of the degradation of DNA by bleomycin and iron (II). *Biochemistry*, **17**, 2746–2754.
6. Onishi, T., Shimada, K. & Takagi, Y. (1973) Effects of bleomycin on *Escherichia coli* strains with various sensitivities to radiations. *Biochim. Biophys. Acta*, **312**, 248–258.
7. Ishida, R. & Takachashi, T. (1975) Increased DNA chain breakage by combined action of bleomycin and superoxide radical. *Biochem. Biophys. Res. Comm.*, **66**, 1432–1438.
8. Lown, J. & Sim, S. (1977) The mechanism of the

bleomycin-induced cleavage of DNA. *Biochem. Biophys. Res. Comm.*, **77**, 1150–1157.

9. Sausville, E., Peisach, J. & Horwitz, S. (1976) A role for ferrous ion and oxygen in the degradation of DNA by bleomycin. *Biochem. Biophys. Res. Comm.*, **73**, 814–822.
10. Iqbal, Z., Kohn, K., Ewig, R. & Fornace, A., Jr. (1976) Single-strand scission and repair of DNA in mammalian cells by bleomycin. *Cancer Res.*, **36**, 3834–3838.
11. Kohn, K. & Ewig, R. (1976) Effect of pH on the bleomycin-induced DNA single strand scission in L1210 cells and the relation to cell survival. *Cancer Res.*, **36**, 3839–3841.
12. Suzuki, H., Nagai, K., Yamaki, H., Tanaka, N. & Umezawa, H. (1969) On the mechanism of action of bleomycin: Scission of DNA strands *in vitro* and *in vivo*. *J. Antibiotics*, **22**, 446–448.
13. Nagai, K., Yamaki, H., Suzuki, H., Tanaka, N. & Umezawa, H. (1969) The combined effects of bleomycin and sulfhydryl compounds on the thermal denaturation of DNA. *Biochim. Biophys. Acta*, **179**, 165–171.
14. Naganawa, H., Muraoka, Y., Takita, T. & Umezawa, H. (1977) Chemistry of bleomycin XVIII carbon-13 NMR studies. *J. Antibiotics*, **30**, 388–396.
15. Fridovich, I. in *Free Radicals in Biology* (ed. W. Pryor), Academic Press, N.Y., 1976, 239–277.
16. Knowles, P., Gibson, J., Pick, F. & Bray, R. (1969) Electron-spin-resonance evidence for enzymic reduction of oxygen to a free radical, the superoxide ion. *Biochem. J.*, **111**, 53–58.
17. Harbour, J. & Bolton, J. (1975) Superoxide formation in spinach chloroplasts: Electron spin resonance detection by spin trapping. *Biochem. Biophys. Res. Comm.*, **64**, 803–807.
18. Udenfriend, S., Clark, C., Axelrod, J. & Brodie, B. (1954) Ascorbic acid in aromatic hydroxylation. I. A model system for aromatic hydroxylation. *J. Biol. Chem.*, **208**, 731–739.
19. McCord, J. & Day, E. (1978) Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. *FEBS. Lett.*, **86**, 139–142.