SUBSTITUTION REACTIONS OF IRON-BLEOMYCIN-DNA WITH EDTA, DETAPAC OR DESFERRIOXAMINE

J. ARONOVITCH, D. GODINGER, S. GOLDSTEIN† and G. CZAPSKI†

Department of Molecular Biology, School of Medicine and †Department of Physical Chemistry, The Hebrew University-Hadassah Medical School, Jerusalem 91904, Israel

(Received for publication January 7, 1987)

Bleomycin (BLM) acts as an antitumor agent by virtue of its ability to cleave DNA[1~3]. BLM-induced DNA-damage requires the presence of molecular oxygen and ferrous ions[4,5], which may be replaced by ferric ions and a reducing agent[6].

Reaction mixtures for the assay of DNA degradation were usually prepared with all the components except iron or the reducing agent. The reaction was initiated by adding the missing component and terminated by the addition of ethylenediaminetetraacetic acid (EDTA) to a final concentration of 1 ~ 10 mM[4,6~10]. This procedure was adopted although SAUSVILLE *et al.*[6] have shown that a 30-fold excess of EDTA over BLM at neutral pH only slowed down DNA scission but did not stop it, and that the presence of a reducing agent increased the activity of EDTA.

In the present study we measured the rates of the substitution reactions of BLM-Fe(III) and of the ternary DNA=BLM-Fe(III) complex, with EDTA, diethylenetriaminepentaacetic acid (DETAPAC) and desferrioxamine (DESFERAL), which are strong chelators of iron. We also measured the time required to stop ascorbate oxidation and DNA degradation by BLM-Fe(III) and the effect of reducing agents on the substitution reaction and on the degradation of DNA. We find that putting a rapid stop to the catalytic activity of BLM-Fe(II) at a neutral pH requires the simultaneous addition of an iron chelator and a reducing agent.

Electrophoretic studies and the preparation of BLM-iron chelates and calf thymus DNA solutions were previously described[11]. The substitution reaction of BLM-Fe(III) with EDTA, DETAPAC or DESFERAL,

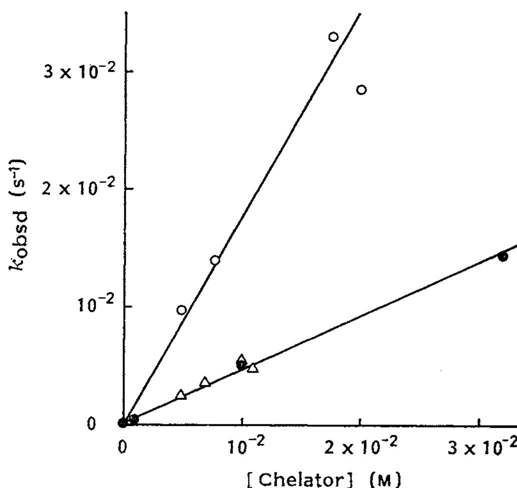
$$\text{BLM-Fe(III)} + \text{chelator} \rightarrow \text{chelator-Fe(III)} + \text{BLM} \quad (1)$$

was followed by change in absorbance at 365 nm. The kinetics of the reactions were followed under pseudo first order conditions ([chelator]$_0$ ≫ [BLM-Fe(III)]$_0$). The solutions contained 0.1 ~ 0.18 mM BLM-Fe(III), 1 ~ 100 mM chelator and 1 mM phosphate buffer at pH 6.8. The change in absorbance at 365 nm in the case of EDTA and DESFERAL was first order with respect to [BLM-Fe(III)] and k_{obsd} was linearly dependent on [chelator]. The rate constant of reaction (1) was determined by plotting k_{obsd} as a function of [chelator] yielding $k_1 = (0.45 \pm 0.08)\ M^{-1}s^{-1}$ for both EDTA and DESFERAL (Fig. 1). This value is similar to that determined for the substitution reaction of BLM-Cu(II) with EDTA[12]. In the case of DETAPAC, we observed two processes: One was first order with respect to [BLM-Fe(III)] and k_{obsd} was linearly dependent on [DETAPAC]. The second process was second order and the rate constant, which is most probably k_1, was determined to be $(1.75 \pm 0.25)\ M^{-1}s^{-1}$ (Fig. 1). The second process was very slow, with $T_{1/2} = 10$ minutes and was independent of [DETAPAC]. In the presence of 0.92 mM DNA, the rate of the sub-

Fig. 1. The observed rate constant of the reaction of 0.18 mM BLM-Fe(III) with various chelators as a function of [chelator].

Aerated solutions in 1 mM phosphate buffer at pH 6.8.

● EDTA, △ DESFERAL, ○ DETAPAC.



stitution reaction with 10 mM of the chelators was slowed down considerably. More than 1 hour was required to complete the reaction. The kinetics seemed to be very complicated and more than one process may have been involved.

Ascorbate, at concentrations exceeding that of oxygen, added with the chelator accelerated the rate of the substitution reaction both in the absence and in the presence of DNA. In fact, the reaction was too rapid to be followed spectrophotometrically. However, when Fe(II) was added to air saturated solutions containing BLM and 10 mM of the chelator, the oxidation of BLM-Fe(II) by oxygen was much faster than the substitution reaction. The yellow color of BLM-Fe(III) appeared immediately and subsequently the slow substitution reaction of BLM-Fe(III) with the chelator proceeded. We also found that the rate constant of the oxidation of BLM-Fe(II) by O_2 is much higher than that of the reduction of BLM-Fe(III) by ascorbate (unpublished results) and therefore, the concentration of ascorbate had to exceed that of O_2 (0.24 mM) in order to maintain significant concentrations of BLM-Fe(II). The rate of the substitution reaction of iron-BLM with the various chelators in the presence of 1 mM ascorbate was the same, whether in air or in argon saturated solutions, indicating that O_2 is not involved in the substitution reaction. When we incubated DNA with BLM-Fe(III) and 1 mM ascorbate for 30 minutes and then added the chelator, the kinetics and $T_{1/2}$ of the substitution reaction were the same as with BLM-Fe(III) in the absence of DNA and ascorbate. Since under these conditions ascorbate was fully oxidized and the DNA was degraded, it seems that DNA degradation products do not bind BLM.

The spectrophotometric experiment supported the assumption that it is easier to sequester Fe(II) from BLM than Fe(III), and the difference is even greater when BLM is bound to DNA. We have previously studied[11] the BLM-Fe(III) catalyzed oxidation of ascorbate in air saturated solutions. By measuring the decay of the catalytic activity of BLM-Fe(III) after the addition of DETAPAC we could indirectly measure the substitution reaction with great sensitivity (Fig. 2). It can be seen that when 10 μM BLM-Fe(III) was incubated with 100 μM DETAPAC, more than 2 hours were required to inhibit the

Fig. 2. The effect of DETAPAC on BLM-Fe(III) catalyzed oxidation of ascorbate.

Reaction mixtures contained 0.1 mM ascorbate, 10 μM BLM-Fe(III) in 1 mM phosphate buffer at pH 6.8. (a) Effect of preincubation of BLM-Fe(III) with 100 μM DETAPAC for various times: 0, 5, 30, 60 and 120 minutes. (b) Effect of increasing amounts of DETAPAC: 0, 10, 40, 160 and 640 μM, added together with ascorbate. Ascorbate oxidation was followed at 266 nm.

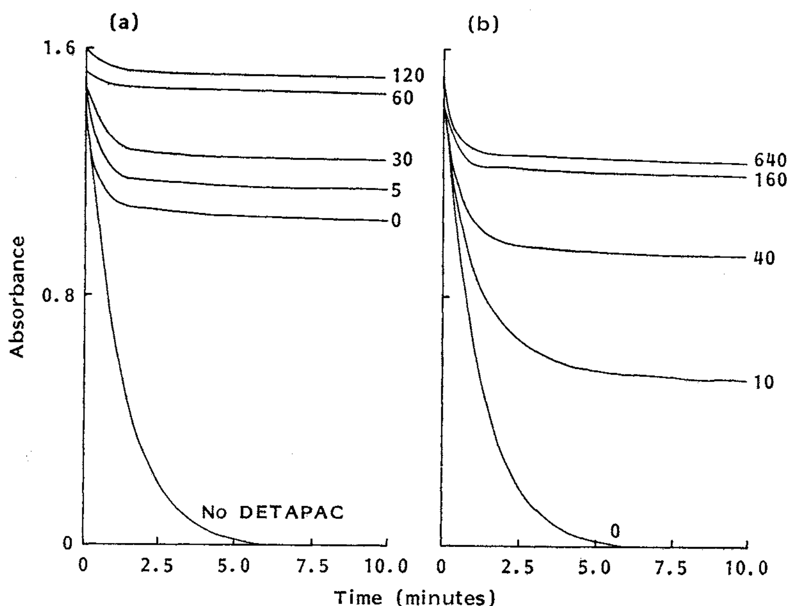
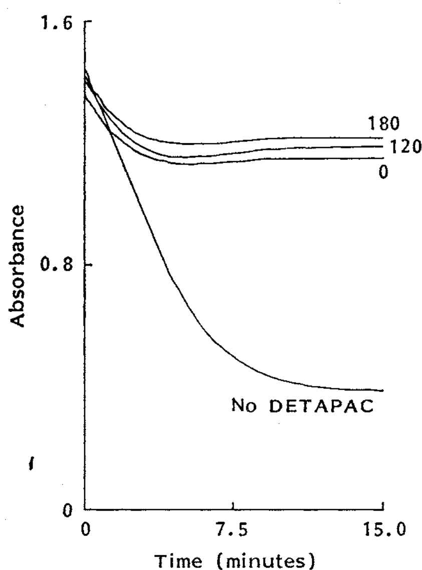


Fig. 3. The effect of preincubation of DNA=BLM-Fe(III) with DETAPAC on the oxidation of ascorbate.

Reaction mixtures containing: 0.25 mM DNA, 10 μM BLM-Fe(III), and 100 μM DETAPAC in 1 mM phosphate buffer at pH 6.8 were incubated for 0, 120 and 180 minutes. Then 0.1 mM ascorbate and 0.1 M NaCl were added and the change in absorbance at 266 nm was followed.



activity of the catalyst (Fig. 2a), whereas only about 2 minutes were required to stop the oxidation of ascorbate when DETAPAC was added to BLM-Fe(III) together with ascorbate (Fig. 2b).

The ternary complex DNA=BLM-Fe(III) is relatively inactive as a catalyst for the oxidation of ascorbate[11]. High ionic strength (I) increases the oxidative activity of BLM-Fe(III) in the presence of DNA, probably because the binding constant of BLM-Fe(III) to DNA decreases as I is increased[11]. A similar effect of I was observed in the case of BLM-Co(III)[13]. In order to test the effect of DETAPAC on the catalytic activity of the ternary complex DNA=BLM-Fe(III), we incubated 0.25 mM DNA and 10 μM BLM-Fe(III) with 100 μM DETAPAC at low ionic strength (0.001) for various periods of time, then added 100 μM ascorbate and measured its oxidation rate at increased I (0.1). Fig. 3 shows that up to 3 hours of preincubation with DETAPAC, in the absence of ascorbate, had little effect on the catalytic activity of the DNA=BLM-Fe(III) complex, confirming that the sequestration of Fe(III) from the complex with DNA is slow compared to the reaction with BLM-Fe(III) (Fig. 2a). We assume that at low ionic strength an equilibrium between DNA=BLM-Fe(III) and DETAPAC-Fe(III) is achieved. Since the relatively rapid substitution reaction with BLM-Fe(II) is unaffected by the presence of DNA it appears that Fe(III) binds to BLM in a way which affects the environment of the iron as the complex binds to DNA, while the environment of Fe(II) in complex with BLM is unaffected by the binding to DNA. These conclusions are in agreement with earlier data showing that the reduction of BLM-Fe(III) by CO_2^- decreased as [DNA] increased, while the oxidation of DNA=BLM-Fe(II) by CO_2^- was independent of [DNA][14].

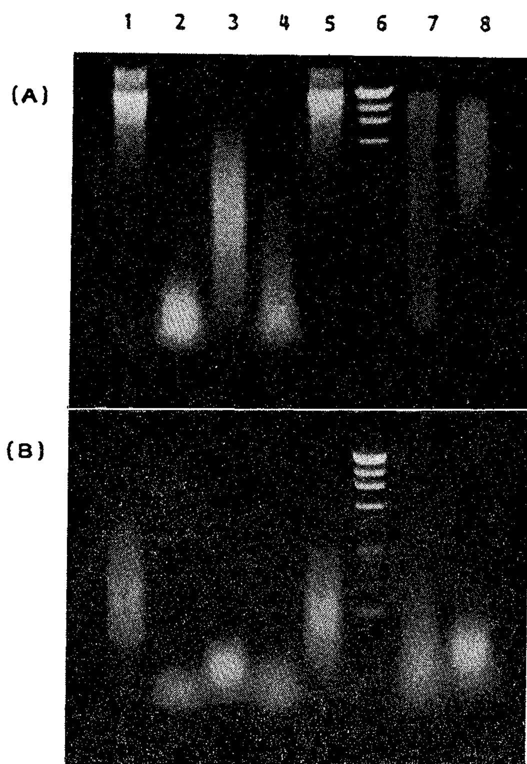
The substitution reaction between DNA=BLM-Fe(III) and DETAPAC was also followed by measuring the time required to stop the formation of DNA single and double strand breaks in the presence of ascorbate at high I[11]. Using a reaction mixture which contained 0.25 mM DNA, 0.5 mM ascorbate and 1 μM BLM-Fe(III) we found that DETAPAC 0.1~10 mM slowed down the formation of DNA double strand breaks but did not stop the reaction completely. With a [DETAPAC] - [BLM-Fe(III)] ratio of 100:1, more than 20 minutes were required to stop DNA scission. EDTA at 1 mM concentration was only slightly less active than DETAPAC. In order to stop the reaction without delay, both 10 mM DETAPAC and 5 mM ascorbate were required. Ascorbate by itself (>2 mM) also inhibited the reaction[11]. This may be attributed to the competition between O_2 and ascorbate for DNA=BLM-Fe(II). Indeed, the addition of high concentrations of ascorbate to DNA=BLM-Fe(III) yielded a mixture of ferric and ferrous ternary complexes. Comparing the effects of DETAPAC and DETAPAC together with ascorbate or with KI on the formation of DNA single and double strand breaks (Fig. 4), we found that DNA damage was prevented only when DETAPAC was added together with ascorbate. KI was less effective than ascorbate.

We have shown that the removal of Fe(III) from BLM bound to DNA with the commonly used iron chelators is a very slow process, whereas the substitution reaction with BLM-Fe(II) is much faster and is unaffected by the presence of

Fig. 4. DNA degradation induced by BLM-Fe(III); prevention by DETAPAC and reducing agents.

Reaction mixtures contained: 0.25 mM DNA, 2 μM BLM-Fe(III), 0.1 M NaCl and 0.5 mM ascorbate in 1 mM phosphate buffer, pH 6.8. DETAPAC and KI when added were 10 mM. Reaction mixtures were incubated for 5 minutes at 30°C and the reaction was terminated by the addition of a stopping mixture which contained 10 mM DETAPAC and 5 mM ascorbate.

DNA electrophoretic migration profiles for detection of A; double strand breaks, B; single strand breaks. Lanes: 1; untreated DNA, 2; no addition, 3; DETAPAC, 4; ascorbate, 5; DETAPAC and ascorbate, 6; λ DNA *Hin*d III digest, 7; KI, 8; DETAPAC and KI.



DNA. This probably reflects differences in the structures of the ternary complexes of DNA=BLM-iron. The practical implication of this study is that in order to prevent DNA degradation by BLM or to stop it efficiently, both an iron chelator and a high concentration of a reducing agent have to be used.

Acknowledgments

This work was done under the auspices of Contract 1409 of Council of Tobacco Research, a grant by the Israel Academy of Science, and partially a grant from GSF Neuherberg.

References

1) SUZUKI, H.; K. NAGAI, H. YAMAKI, N. TANAKA & H. UMEZAWA: On the mechanism of action of bleomycin: Scission of DNA strands *in vitro* and *in vivo*. J. Antibiotics 22: 446~448, 1969
2) HALIDE, C. W.: Fragmentation of deoxyribonucleic acid by bleomycin. Mol. Pharmacol. 7: 645~652, 1971
3) UMEZAWA, H.: Advances in bleomycin studies. *In* Bleomycin: Chemical, Biochemical, and Biological Aspects. *Ed.*, S. M. HECHT, pp. 24~36, Springer Verlag, New York, 1979
4) SAUSVILLE, E. A.; J. PEISACH & S. B. HORWITZ: A role for ferrous ion and oxygen in the degradation of DNA by bleomycin. Biochem. Biophys. Res. Commun. 73: 814~822, 1976
5) EKIMOTO, H.; H. KURAMOCHI, K. TAKAHASHI, A. MATSUDA & H. UMEZAWA: Kinetics of the reaction of bleomycin-Fe(III)-O_2 complex with DNA. J. Antibiotics 33: 426~434, 1980
6) SAUSVILLE, E. A.; J. PEISACH & S. B. HORWITZ: Effect of chelating agents and metal ions on the degradation of DNA by bleomycin. Biochemistry 17: 2740~2746, 1978
7) SAUSVILLE, E. A.; R. W. STEIN, J. PEISACH & S. B. HORWITZ: Properties and products of the degradation of DNA by bleomycin and iron (II). Biochemistry 17: 2746~2754, 1978
8) HORWITZ, S. B.; E. A. SAUSVILLE & M. TAKESHITA: A simple assay for bleomycin activity: Degradation of radioactive DNA to acid-soluble products. *In* Bleomycin: Chemical, Biochemical, and Biological Aspects. *Ed.*, S. M. HECHT, pp. 346~347, Springer-Verlag, New York, 1979
9) SUGIURA, Y.; T. SUZUKI, J. KUWAHARA & H. TANAKA: On the mechanism of the hydrogen peroxide-, superoxide-, and ultraviolet light-induced DNA cleavages on inactive bleomycin-iron(III) complex. Biochem. Biophys. Res. Commun. 105: 1511~1518, 1982
10) GUTTERIDGE, J. M. C.; A. P. C. BEARD & G. J. QUINLAN: Catalase enhances damage to DNA by bleomycin-iron(II): the role of hydroxyl radicals. Biochem. Int. 10: 441~449, 1985
11) ARONOVITCH, J.; D. GODINGER, A. SAMUNI & G. CZAPSKI: Ascorbic acid oxidation and DNA scission catalyzed by iron and copper chelates. Free Rad. Res. Comms. 2: 241~258, 1987
12) SOLAIMAN, D.; L.A. SARYAN & D.H. PETTERING: Studies on the chemical reactivity of copper bleomycin. J. Inorg. Biochem. 17: 75~94, 1982

13) CHANG, C.-H. & C. F. MEARES: Cobalt-bleomycin and deoxyribonucleic acid: sequence-dependent interactions, action spectrum for nicking, and indifference to oxygen. Biochemistry 23: 2268~2274, 1984

14) GOLDSTEIN, S. & G. CZAPSKI: Oxidation-reduction reactions of iron bleomycin in the absence and presence of DNA. Free Rad. Res. Comms. 2: 259~270, 1987