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# OXY RADICAL FORMATION DURING REDOX CYCLING OF THE BLEOMYCIN–IRON (III) COMPLEX BY NADPH-CYTOCHROME P-450 REDUCTASE

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**Abstract**—Bleomycin was aerobically incubated with $FeCl_3$, NADPH, isolated rat-liver microsomal cytochrome P-450 reductase and methional. The conversion of methional to ethene, which indicates oxy radicals, was determined. Ethene formation depended on oxygen, NADPH, $FeCl_3$ and the enzyme. About equimolar concentrations of bleomycin and $FeCl_3$ resulted in optimal ethene formation. Dimethyl sulfoxide, mannitol, glycerol, glutathione and glutathione disulfide inhibited ethene formation. These results indicate that oxy radicals are formed after reduction of the bleomycin–Fe-complex by NADPH-cytochrome P-450 reductase.

The molecular mechanism by which bleomycin, a glycopeptide antibiotic, is effective against tumor cells is not yet known. Bleomycin can tightly be bound to DNA and can form a cytochrome-like complex with various metals (for review see [1]). For example, it has been demonstrated that a reduced bleomycin–iron-complex activates molecular oxygen leading to the formation of oxy radicals and DNA breakage (for review see [1]). Several groups performed the reduction of the bleomycin–Fe-complex by reducing chemicals or by the superoxide-forming system, xanthine oxidase/hypoxanthine [2–8]. Our group found that the bleomycin–Fe(III)-complex can also be reduced by isolated NADPH-cytochrome P-450 reductase, resulting in DNA strand breaks, malondialdehyde and base release, when DNA was incubated with NADPH, the enzyme, bleomycin, $FeCl_3$ and oxygen [9, 10]. However, it is not clear whether free oxy radicals are responsible for the damage of DNA which depends on bleomycin, Fe(III) and NADPH-cytochrome P-450 reductase. We used methional to trap oxy radicals if formed in this system. Oxy radicals, such as the hydroxyl radical and organic alkoxy and peroxy radicals, react with methional forming ethene (ethylene) which was measured [11, 12]. The formation of ethene from methional has been shown to be a valuable probe for the study of highly reactive oxy radicals in various biochemical systems [11–16]. We found that ethene formation from methional induced by bleomycin and Fe(III) depended on oxygen, NADPH and NADPH-cytochrome P-450 reductase.

## MATERIALS AND METHODS

Commercially available Bleomycinum Mack® containing 55–70% bleomycin $A_2$ and 25–32% bleomycin $B_2$ from Mack (Illertissen, F.R.G.) was used. The amount of bleomycin is always given in μg/ml due to the different molecular weights of the bleomycins present in the preparation. NADPH and cytochrome *c* (from horse heart) were purchased from Boehringer (Mannheim, F.R.G.). $FeCl_3 \cdot 6H_2O$, dimethyl sulfoxide (DMSO), glycerol and all standard chemicals were obtained from Merck (Darmstadt, F.R.G.). Methional (β-methylthiopropanal), glutathione (GSH) and mannitol were from Sigma (Munich F.R.G.), whereas glutathione disulfide (GSSG) was from Serva (Heidelberg, F.R.G.). The gases including the calibration gas were delivered by Linde (Berlin, F.R.G.). All reagents were of the purest grade available. Methional and DMSO were freshly distilled before use.

NADPH-Cytochrome P-450 reductase was isolated according to a standard procedure [17] as previously described [9]. The enzyme activity was tested with cytochrome *c* as the substrate. In general, the reductase was incubated with bleomycin, $FeCl_3$, NADPH and methional (1 mM) in 20 mM sodium–potassium phosphate buffer pH 7.5 at 37° under aerobic conditions. In one experiment incubations were performed under nitrogen. Methional was added after a 5-min pre-incubation at 37° and the reaction was initiated by adding the enzyme. Incubation (total volume, 1 ml) was carried out under shaking in a 25-ml Erlenmeyer flask (total gas volume about 38 ml) sealed with Teflon-coated silicon septum as previously described [18]. Gas samples of 2 ml were removed from the atmosphere above the incubation mixture with a syringe [18]. The volume of 2 ml was replaced either by 2 ml air or by 2 ml nitrogen/oxygen mixture. The gas sample was injected in a gas chromatograph equipped with a flame-ionization detector. Ethene was separated at 70° on the same alumina column which previously served to separate ethane and *n*-pentane [19]. For standardization a calibration gas containing ethene in nitrogen was used. The

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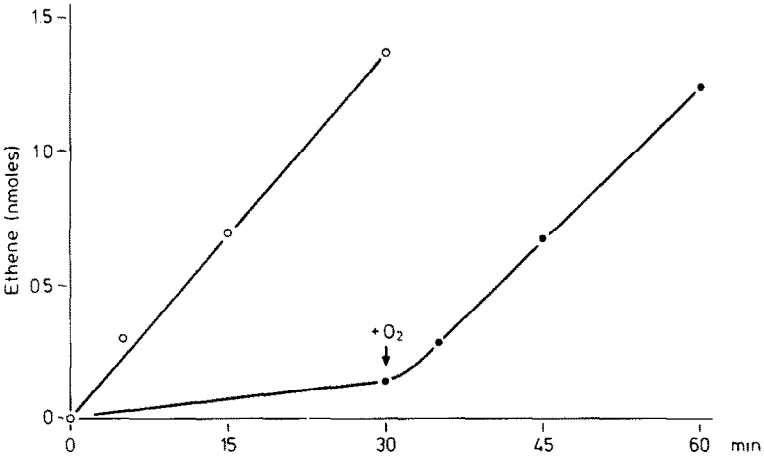


Fig. 1. Time curves of ethene originating from methional (1 mM) formed during incubation with bleomycin (50 μg/ml), $FeCl_3$ (100 μM), NADPH (500 μM) and NADPH-cytochrome P-450 reductase (0.2 U/ml). Total ethene formed by a 1-ml incubation mixture within 30 min is shown. O——O, aerobic incubation; ●——●, incubation under 100% nitrogen for 30 min, then addition of oxygen (final atmospheric concentration 20% $O_2$/80% $N_2$).

total amount of ethene formed by a 1-ml incubation mixture was calculated, taking into account the slightly different volumes of the flasks. The laboratory air was routinely checked for ethene. All experiments were performed at least in duplicate.

## RESULTS

Figure 1 demonstrates that methional is converted to ethene by bleomycin, $Fe^{3+}$, NADPH and NADPH-cytochrome P-450 reductase. It also shows that ethene formation is negligible in the absence of oxygen (under nitrogen).

That under these conditions ethene formation is dependent on the amount of enzyme present can be seen from Fig. 2. Furthermore, ethene is not formed without bleomycin (Fig. 3, Table 1). With bleomycin concentrations between 5 and 100 μg/ml ethene formation increases exponentially (Fig. 3). A similar quantitative relationship is observed with different concentrations of $FeCl_3$ used (Fig. 4). Figure 4 also shows that in the presence of bleomycin higher $FeCl_3$ concentrations result in an inhibition of ethene formation, whereas in the absence of bleomycin higher

Table 1. Effect of different inhibitors on ethene formation originating from methional formed during 30 min incubation

| | % Ethene (mean ± S.D., N ≥ 3) |
|---|---|
| Complete system (100 μg/ml bleomycin, 100 μM $FeCl_3$, 500 μM NADPH, 0.2 U/ml NADPH-cytochrome P-450 reductase, 1 mM methional) | 100 |
| −NADPH | 0 |
| −Bleomycin | 2 ± 3 |
| −$FeCl_3$ | 25 ± 5 |
| +DMSO (10 mM) | 65 ± 5 |
| +Mannitol (50 mM) | 62 ± 8 |
| +Glycerol (50 mM) | 61 ± 9 |
| +GSH (1 mM) | 78 ± 11 |
| +GSH (10 mM) | 21 ± 4 |
| +GSSG (1 mM) | 85 ± 9 |
| +GSSG (10 mM) | 25 ± 5 |

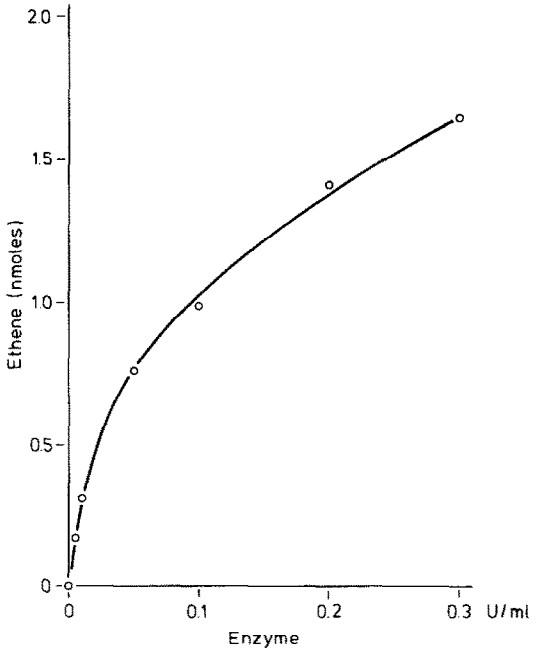


Fig. 2. Enzyme dependence of ethene originating from methional (1 mM) formed during incubation with bleomycin (50 μg/ml), $FeCl_3$ (100 μM), NADPH (500 μM) and various amounts of NADPH-cytochrome P-450 reductase. Total ethene formed by a 1-ml incubation mixture within 30 min is shown.

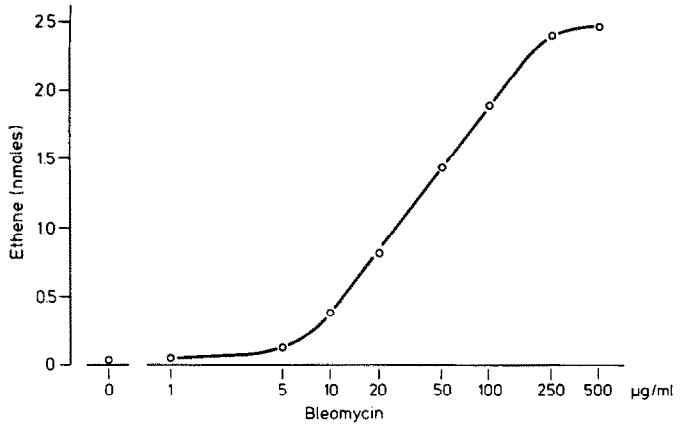


Fig. 3. Dependency of ethene originating from methional (1 mM) on the bleomycin concentration present during incubation with $FeCl_3$ (100 µM), NADPH (500 µM) and NADPH-cytochrome P-450 reductase 0.2 U/ml). Total ethene formed by a 1-ml incubation mixture within 30 min is shown.

amounts of $FeCl_3$ stimulate ethene formation. With bleomycin, but without $FeCl_3$ 25% of the maximal ethene formation is still observable. But Fig. 4 demonstrates conclusively that this part is also dependent on NADPH-cytochrome P-450 reductase.

Compounds like DMSO, mannitol, glycerol, glutathione and glutathione disulfide are able to inhibit ethene formation (Table 1).

## DISCUSSION

The relatively high gas volume of the incubation vessel compared to the volume of the incubation mixture guaranteed that all ethene formed was measurable in the gas phase [18]. Because the amount of ethene formed during incubation of bleomycin, $FeCl_3$, NADPH and NADPH-cytochrome P-450 reductase increased linearly with increasing methional concentrations up to 50 mM (data not shown), we chose 1 mM methional for all experiments. This concentration was high enough to measure the exact amounts of ethene even under incubation conditions without bleomycin or without oxygen (1–2% of the maximal value). We used phosphate buffer to prevent the oxy radicals formed being trapped, as is possible with Tris.

Our data clearly demonstrate that the bleomycin–Fe(III) complex itself is not able to convert significant amounts of methional to ethene even in the presence of oxygen. This agrees with our previous results on DNA damage by bleomycin and $FeCl_3$, which also depended on NADPH-cytochrome P-450 reductase [9, 10]. It has been known for some time that only the reduced bleomycin–Fe-complex is able to activate oxygen (for review see [1]). The presence of oxygen is a prerequisite for the formation of ethene from methional induced by the bleomycin–Fe(II)-complex. This is in agreement with a number of previous studies which showed a strict dependence of DNA damage on oxygen [7–10, 20, 21]. However,

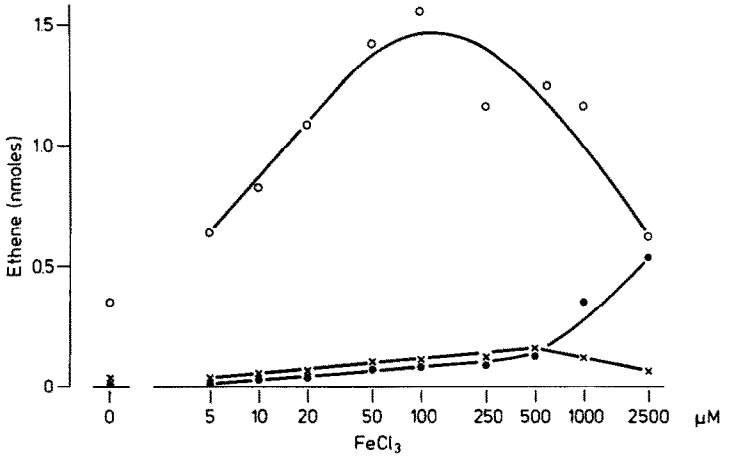


Fig. 4. Dependency of ethene originating from methional (1 mM) on the concentration of $FeCl_3$ formed during incubation with NADPH (500 µM) and NADPH-cytochrome P-450 reductase (0.2 U/ml). ○——○, plus bleomycin (50 µg/ml); ●——●, minus bleomycin; ×——×, plus bleomycin (50 µg/ml), minus reductase. Total ethene formed by a 1-ml incubation mixture within 30 min is shown.

it must be noted that about 25% of ethene were formed in the absence of $FeCl_3$. This is probably due to a contamination with iron ions present in the phosphate buffer used. If we extrapolate the curve shown in Fig. 4, we can calculate that about 2 $\mu$M Fe has already been present in the incubation mixture without the addition of $FeCl_3$. In our previous experiments [9, 10] we were not faced with this problem, because we took iron-free Tris buffer which we could not use here. In the absence of bleomycin only extremely high concentrations of $FeCl_3$ converted significant amounts of methional to ethene. This indicates that iron ions alone are not very efficient in activating oxygen even in the presence of the reductase. Iron ions probably have to be chelated in order to be good substrates for this enzyme [22].

Based on a mean molecular weight of the bleomycins used of about 1000 it can be deduced from our experiments that equimolar concentrations of bleomycin and $FeCl_3$ are almost optimal in the activation of oxygen, which results in the formation of ethene from methional.

The so-called hydroxyl radical-trapping agents (DMSO, mannitol and glycerol) inhibited ethene formation by only up to 40%, although added in concentrations 10–50-fold higher than methional. We suggest that either these inhibitors are not specific for hydroxyl radical trapping or that another oxy radical is formed which converts methional to ethene. On the other hand, glutathione which reacts besides others with hydroxyl radicals was a much better inhibitor of ethene formation. The fact that glutathione disulfide, which has been suggested to trap superoxide radicals [23] also inhibits ethene formation in our system agrees with the previously suggested scheme of activation of oxygen by bleomycin–Fe(II) in which the hydroxyl radical is formed via superoxide [9]. But under the aerobic conditions applied superoxide could also be involved in the reduction of bleomycin–Fe(III) by NADPH-cytochrome P-450 reductase.

Although our data give no conclusive evidence that hydroxyl radicals are formed during redox cycling of the bleomycin–Fe(III) complex, catalysed by NADPH-cytochrome P-450 reductase, they indicate that any highly reactive oxy radical occurs.

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## REFERENCES

1. R. M. Burger, J. Peisach and S. B. Horwitz, *Life Sci.* **28**, 715 (1981).
2. W. J. Caspary, D. A. Lanzo and C. Niziak, *Biochemistry* **20**, 3868 (1981).
3. D. L. Melnyk, S. B. Horwitz and J. Peisach, *Biochemistry* **20**, 5327 (1981).
4. J. M. C. Gutteridge and X.-Ch. Fu, *FEBS Lett.* **123**, 71 (1981).
5. J. W. Lown and S.-K. Sim, *Biochem. biophys. Res. Commun.* **77**, 1150 (1970).
6. E. A. Sausville, J. Peisach and S. B. Horwitz, *Biochemistry* **17**, 2740 (1978).
7. L. F. Povirk, *Biochemistry* **18**, 3989 (1979).
8. W. E. Antholine and D. H. Petering, *Biochem. biophys. Res. Commun.* **90**, 384 (1979).
9. M. E. Scheulen, H. Kappus, D. Thyssen and C. G. Schmidt, *Biochem. Pharmac.* **30**, 3385 (1981).
10. M. E. Scheulen and H. Kappus, in *Oxygen Radicals in Chemistry and Biology* (Eds. W. Bors, M. Saran and D. Tait), p. 425. Walter de Gruyter, Berlin (1984).
11. I. Yamazaki, in *Free Radicals in Biology* III (Ed. W. A. Pryor), p. 183. Academic Press, New York (1977).
12. W. A. Pryor and R. H. Tang, *Biochem. biophys. Res. Commun.* **81**, 498 (1978).
13. S. J. Klebanoff and H. Rosen, *J. exp. Med.* **148**, 490 (1978).
14. M. Saran, W. Bors, Ch. Michel and E. F. Elstner, *Int. J. Radiat. Biol.* **37**, 521 (1980).
15. Ch.C. Winterbourn, *FEBS Lett.* **136**, 89 (1981).
16. Ch.C. Winterbourn, *Biochem. J.* **210**, 15 (1983).
17. T. Omura and S, Takesue, *J. Biochem., Tokyo* **67**, 249 (1970).
18. H. Kappus and H. Muliawan, *Biochem. Pharmac.* **31**, 597 (1982).
19. J. G. Filser, H. M. Bolt, H. Muliawan and H. Kappus, *Archs Toxicol.* **52**, 135 (1983).
20. R. M. Burger, J. Peisach and S. B. Horwitz, *J. biol. Chem.* **256**, 11636 (1981).
21. J. Kuramochi, K. Takahashi, T. Takita and H. Umezawa, *J. Antibiot., Tokyo* **34**, 576 (1981).
22. L. A. Morehouse, C. E. Thomas and S. D. Aust, *Arch. Biochem. Biophys.* **232**, 366 (1984).
23. Ch.A. Pritsos and R. S. Pardini, *Res. Commun. Chem. Pathol. Pharmac.* **42**, 271 (1983).