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Interaction of *p*-phenylenediamine with the iron-bleomycin complex

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Abstract

The iron-bleomycin complex has been shown to catalyze the oxidation of *p*-phenylenediamine to a stable, purple coloured oxidation product, characterized by an absorption maximum around 520 nm. Molecular oxygen is used for reoxidizing Fe(II)-bleomycin after reduction by *p*-phenylenediamine. An apparent Michaelis constant of 5.2 mM and a catalytic constant of 17.2 min^{-1} were obtained from kinetic studies. ATP, ADP and orthophosphate inhibited the catalytic oxidation of p-phenylenediamine, while AMP was without effect. It is proposed that *p*-phenylenediamine may be used as 'substrate' in kinetic studies involving the 'oxidase' activity of iron-bleomycin.

Introduction

Bleomycin is a glycopeptide antibiotic used in the treatment of certain neoplastic diseases. It is shown to cause degradation of DNA in a reaction depending on ferrous ions and molecular oxygen (Suzuki *et al.* 1969; Sausville *et al.* 1976, 1978). Bleomycin molecules are able to bind both ferrous- and ferric ions, as well as DNA (Sausville *et al.* 1976; Burger *et al.* 1979; Lown *et al.* 1982). The destructive action is proposed to involve a reduction of Fe(III)-bleomycin to Fe(II)-bleomycin by an electron donating compound. Fe(II)-bleomycin then reacts with molecular oxygen, forming a highly oxidizing compound, usually referred to as 'activated bleomycin' (Burger *et al.* 1981). This complex is further transformed to Fe(III)-bleomycin (Burger *et al.* 1981). Iron-bleomycin is thus able to oxidize reducing species in a catalytic manner (Caspary *et al.* 1979; Buettner & Moseley 1992).

P-phenylenediamine (PPD) is a good electron donor, which is oxidized by certain metals, metal complexes and enzymes to a stable, purple coloured product, which is easily monitored in a spectrophotometer (Holmberg & Laurell 1951; Rice 1962; Peisach & Levine 1963). In the present communication it has been examined whether PPD could be catalytically oxidized by the iron-bleomycin complex, and possibly be used in the design of a continuous, sensitive and convenient spectrophotometric method for kinetic studies involving iron-bleomycin 'oxidase' activity.

Materials and methods

Bleomycin was obtained from Lundbeck A/S (Copenhagen, Denmark). Stock solutions were standarized optically at 291 nm, using $\epsilon = 17.0$ mM^{-1} cm^{-1} (Burger *et al.* 1985). *P*-phenylenediamine, ATP, ADP and AMP were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other reagents were of the best commercial grade. Aqueous solutions were made in deionized, glass-distilled water.

Fe(III)-bleomycin was prepared as described by Shields & McGlumphy (1984), keeping a ratio of 2/3 between iron and the antibiotic.

Spectrophotometric measurements were performed in a Pye-Unicam 8800 instrument.

Results and discussion

Bleomycin forms a stable 1:1 complex with Fe(III) (log K = 14.3 at pH 7.2, Lown *et al.* 1982). Addition of Fe(III)-bleomycin to a solution of PPD resulted

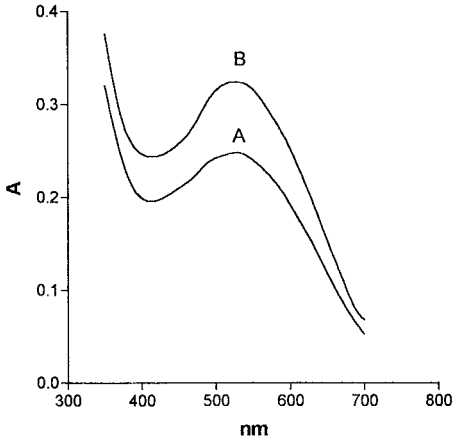


Figure 1. Optical absorption spectrum recorded 10 min (A) and 15 min (B) after addition of 2 μM Fe(III)-bleomycin to 2 mM PPD in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30\,^{\circ}$C).

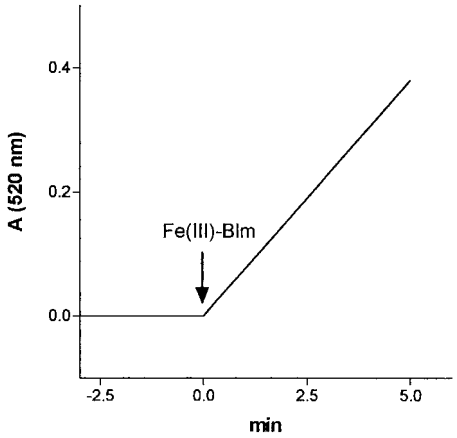


Figure 2. Time course of formation of the purple coloured oxidation product of PPD (recorded at 520 nm), after addition of 3.5 μM Fe(III)-bleomycin to 5 mM PPD in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30\,^{\circ}$C).

in a gradual formation of a purple coloured oxidation product. Figure 1 shows the absorption spectrum in the visible region, recorded 10 and 15 min after mixing 2 μM Fe(III)-bleomycin with 2 mM PPD. The spectrum is characterized by an absorption maximum around 520 nm ($\epsilon = 3.95$ mM^{-1} cm^{-1}). The nature of the purple compound has not been unequivocally established. However, Rice (1962) reported a resemblance between the optical absorption spectrum of the purple product of PPD and that of 'Bandrowski's base', which is a trimer of PPD.

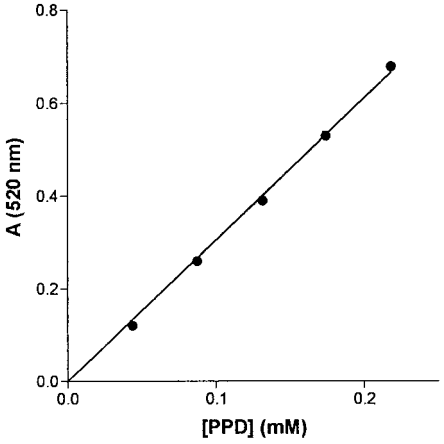


Figure 3. The absorbance readings at steady-state level plotted against PPD concentration. The reaction solution contained 20 μM iron-bleomycin, 0.044–0.2 mM PPD in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30\,^{\circ}$C).

Product formation as a function of time

Figure 2 shows the time course of formation of the product, recorded spectrophotometrically at 520 nm, after addition of Fe(III)-bleomycin to a solution of PPD at pH 6.0. Initially, the product concentration increases linearly with time, making the determination of the initial reaction rate (activity) easy. After a while the reaction rate decreases, and finally a steady-state level is reached. It is calculated that at this stage the amount of PPD oxidized by far exceeds that of iron-bleomycin in the solution. The amount of product formed is proportional to the amount of PPD in the reaction solution (Figure 3), and independent on the concentration of added Fe(III)-bleomycin (not shown). The results demonstrate that iron-bleomycin oxidizes PPD in a catalytic manner.

Linearity in activity and iron-bleomycin concentration

The relationship between Fe(III)-bleomycin concentration and the rate of product formation was tested. A good correlation between activity and iron-bleomycin concentration was obtained, as shown in Figure 4. In the absence of bleomycin the iron ions had no significant oxidizing effect. The Cu(II)-bleomycin complex (log K = 13.8, Lown *et al.* 1982) did not catalyze the oxidation of PPD (Figure 4).

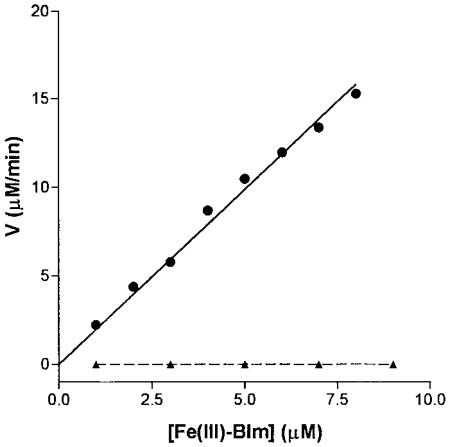


Figure 4. The initial rate of PPD oxidation after addition of Fe(III)-bleomycin (●), or Cu(II)-bleomycin (▲). The reaction solution contained 1–9 μM metal-bleomycin and 1 mM PPD in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30\,^{\circ}$C).

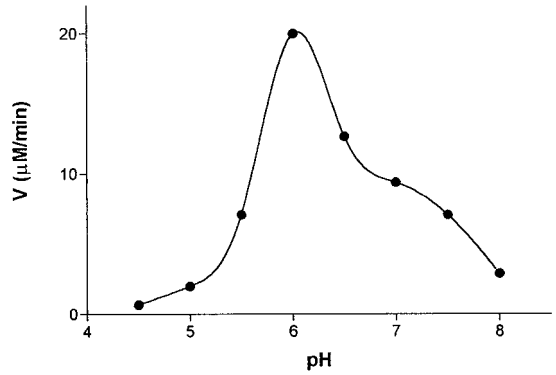


Figure 5. Effect of pH on the initial rate of the iron-bleomycin catalyzed oxidation of PPD. The reaction solution contained 4 μM iron-bleomycin and 2 mM PPD in 0.1 M sodium acetate buffer at pH $\leq$ 6, and in 20 mM Tris-HCl buffer at pH $>$ 6.0 ($T = 30\,^{\circ}$C).

pH-dependence

The effect of pH on the PPD-oxidizing activity of iron-bleomycin was also measured. As shown in Figure 5 the reaction was characterized by a pH optimum at 6 with a shoulder around pH 7. In the absence of iron-bleomycin the autoxidation of PPD was negligible. In the present study the other experiments were performed in acetate buffer, pH 6.0.

Activity dependence on PPD concentration

The rates of product formation as the result of PPD oxidation by iron-bleomycin at various PPD concentrations were measured at 520 nm. Hyperbolical curves were obtained when the activity, *V*, re-expressed as micromolar concentration of PPD oxidized per min, was

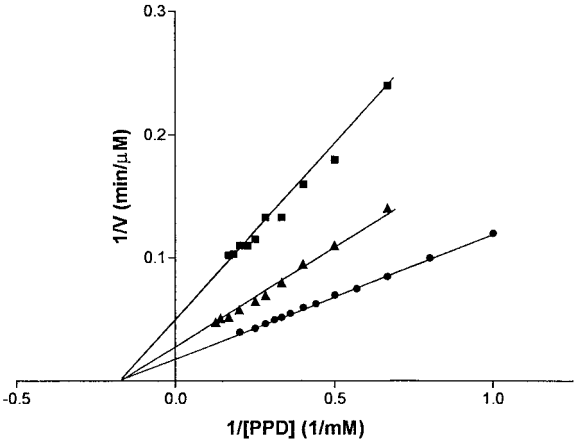


Figure 6. The reciprocal rate of the iron-bleomycin catalyzed oxidation of PPD plotted against the reciprocal PPD concentration. The reaction solution contained 3.5 μM (●), 2 μM (▲) or 1 μM (■) iron-bleomycin, and various concentrations of PPD, ranging from 1 to 8 mM, in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30\,^{\circ}$C).

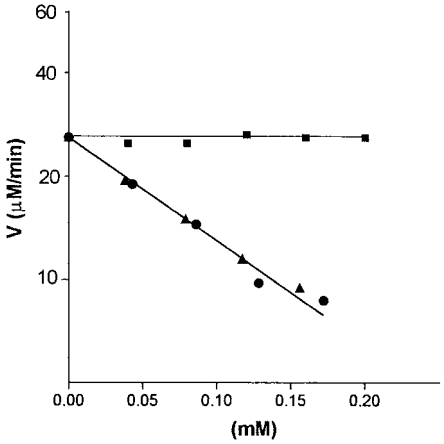
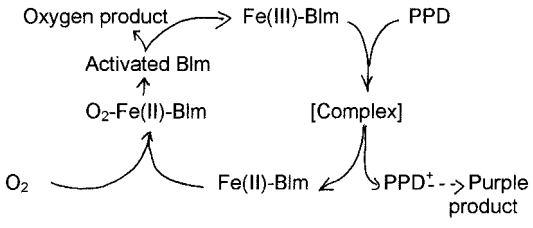


Figure 7. The initial rate of the iron-bleomycin catalyzed oxidation of PPD in the presence of ATP (●), ADP (▲) and AMP (■). The reaction solution contained 3 μM iron-bleomycin, 5 mM PPD and 0.038–0.2 mM nucleotide concentration in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30\,^{\circ}$C).

plotted against PPD concentration; the data giving rise to straight lines, when $1/V$ was plotted against 1/[PPD] (Figure 6). Based on the data obtained the following mechanism for the catalytic activity is proposed:



In this reaction PPD and iron-bleomycin form a complex; the activity reaching a maximum (V_{max}) when all iron-bleomycin molecules are involved in the catalytic process. Molecular oxygen is used for reoxidizing a reduced form of the drug complex back to Fe(III)-bleomycin again. The reoxidation process involves the formation of 'activated bleomycin', which embodies the drug's DNA-cleaving activity (Burger *et al.* 1981). The oxygen species formed after reoxidation of the iron ion is proposed to be a superoxide radical (Caspary *et al*. 1979). From Figure 6 an apparent Michaelis constant, K_m, of 5.2 ± 0.5 mM (mean value $\pm$ SEM ($n = 3$)) was determined for the iron-bleomycin catalyzed oxidation of PPD. A catalytic constant, $k = V_{max}$/[Fe(III)-Blm), was estimated to $17.2 \pm 1.0\ min^{-1}$ (mean value $\pm$SEM ($n = 3$)).

Effect of phosphate containing compounds

Orthophosphate, ATP, ADP and several other phosphate containing compounds bind to iron-bleomycin, enhancing the extent of DNA degradation by Fe(II)-bleomycin. They increase both the release of free nucleic base and that of base propenals, which are DNA cleavage products (Burger *et al*. 1985). AMP does not affect the optical absorption spectrum of Fe(II)-bleomycin, nor does it affect the activity of the drug (Burger *et al.* 1985).

Figure 7 shows that ATP and ADP inhibit the iron-bleomycin catalyzed oxidation of PPD equally well; the inhibition characterized by an I_{50}-value of 0.11 mM. AMP had no effect on the reaction (Figure 7), supporting the suggestion that AMP does not bind to the iron-bleomycin complex (Burger *et al.* 1985). The data indicate that the second phosphate group on the nucleotide molecule is important for the interaction with iron-bleomycin during catalysis. Orthophosphate, which binds weakly to iron-bleomycin (Burger *et al.* 1985), also inhibited the reaction (not shown); the I_{50}-value obtained being 8.2 mM in this case. Preliminary studies suggest that ATP, ADP and orthophosphate inhibit the iron-bleomycin catalyzed PPD oxidation in a competitive manner (R. Løvstad, unpublished data).

Conclusion

Kinetic studies on iron-bleomycin catalytic activity can be, and has been, performed by measuring the rate of decrease in dissolved oxygen by means of a polarographic oxygen electrode (Caspary *et al.* 1979, 1981; Buettner & Moseley 1992). The method is tedious, time-consuming, rather unsensitive, and cannot easily be adopted to small samples. The present study demonstrates that iron-bleomycin possesses catalytic activity in oxidizing PPD, and follows kinetics characteristic of an enzyme. The rate of the stable, purple product formed under standard conditions could easily be followed spectrophotometrically at 520 nm with high accuracy and reproducibility. It is proposed that PPD may be adopted to design continuous and sensitive spectrophotometric assays for kinetic studies involving the 'oxidase' activity of iron-bleomycin.

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References

Buettner GR, Moseley PL. 1992 Ascorbate both activates and inactivates bleomycin by free radical generation. *Biochemistry* **31**, 9784–9788.

Burger RM, Peisach J, Blumberg WE, Horwitz SB. 1979 Iron-bleomycin interactions with oxygen and oxygen analogues. *J Biol Chem* **21**, 10906–10912.

Burger RM, Peisach J, Horwitz SB. 1981 Activated bleomycin. A transient complex of drug, iron, and oxygen that degrades DNA. *J Biol Chem* **25**, 11636–11644.

Burger RM, Horwitz SB, Peisach J. 1985 Stimulation of iron (II) bleomycin activity by phosphate-containing compounds. *Biochemistry* **24**, 3623–3629.

Caspary WJ, Niziak C, Lanzo DA, Friedman R, Bachur NR. 1979 Bleomycin A_2: A ferrous oxidase. *Mol Pharmacol* **16**, 256–260.

Holmberg CG, Laurell C-B. 1951 Investigations in serum copper III. Coeruloplasmin as an enzyme. *Acta Chem Scand* **5**, 476–480.

Lown JW, Joshua AV, Chen H-H. 1982 Reactive oxygen species leading to lipid peroxidation and DNA lesions implicated in the cytotoxic action of certain antitumour antibiotics. In: McBrien DCH, Slater TF, eds. *Free Radicals, Lipid Peroxidation and Cancer*. London: Academic Press; 305–320.

Peisach J, Levine WG. 1963 On the mechanism of ceruloplasmin-catalyzed oxidations. *Biochim Biophys Acta* **77**, 615–628.

Rice EW. 1962 Standardization of ceruloplasmin activity in terms of international enzyme units. *Anal Biochem* **3**, 452–456.

Sausville EA, Peisach J, Horwitz SB 1976. A role for ferrous ion and oxygen in the degradation of DNA by bleomycin. *Biochem Biophys Res Commun* **73**, 814–822.

Sausville EA, Stein RW, Peisach J, Horwitz SB 1978. Effect of chelating agents and metal ions on the degradation of DNA by bleomycin and Fe(II). *Biochemistry* **17**, 2746–2754.

Shields H, McGlumphy C. 1984 The orientation of the ligands in Fe(III)-bleomycin intercalated with DNA. *Biochim Biophys Acta* **800**, 277–281.

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 Antibiot* **22**, 446–448.