BioMetals 1998, **11**, 199–202

Stimulatory effect of ascorbate on iron transfer from bleomycin to apotransferrin

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Received 29 January 1998; accepted for publication 3 February 1998

The chemotherapeutic agent, bleomycin, forms a 1:1 complex with both Fe(III) and Fe(II). The rate of ferric ion transfer from bleomycin to apotransferrin is rather slow. However, when ascorbate was added to Fe(III)–bleomycin prior to exposure to apotransferrin, the transfer rate was markedly increased. Ascorbate readily reduces Fe(III)–bleomycin to Fe(II)–bleomycin. A second order rate constant of 2.4 mM^{-1} min^{-1} was estimated for this reaction. Fe(II)–bleomycin immediately combines with O_2, generating the so-called 'activated bleomycin' complex. The data suggest that a reduced form of iron–bleomycin more readily donates its iron ion to apotransferrin. Reoxidation of ferrous ions, and Fe(III)–transferrin formation occur rapidly.

Keywords: ascorbate, bleomycin, iron ions, transferrin

Introduction

The bleomycins are a family of glycopeptide antibiotics, used in the treatment of selected neoplastic diseases, causing a degradation of deoxyribonucleic acid (DNA) (Suzuki *et al*. 1969) in a reaction shown to depend on both ferrous ions and oxygen (Sausville *et al*. 1976, 1978, Takita *et al*. 1978). Bleomycin molecules form complexes with both ferric and ferrous ions (Sausville *et al*. 1976, Burger *et al*. 1979, Lown *et al*. 1982). It is proposed that the mechanism of destruction involves reduction of Fe(III)–bleomycin–DNA to Fe(II)–bleomycin–DNA, which in turn combines with molecular oxygen, forming a highly oxidizing compound, usually referred to as 'activated bleomycin' (Burger *et al*. 1981).

Apotransferrin is a plasma protein with a strong affinity for ferric ions at physiological pH (2 Fe(III)/molecule; log K_1 = 22.7, log K_2 = 22.1; Martin *et al*. 1987). A previous study showed that the rate of ferric ion transfer from bleomycin to apotransferrin was rather slow (Løvstad 1989). In the present communication it has been examined whether ascorbate, an effective Fe(III)–bleomycin reducing agent (Sausville *et al*. 1978, Buettner & Moseley 1992) might increase the rate of iron transfer to apotransferrin.

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Materials and methods

Bleomycin was obtained from Lundbeck A/S (Copenhagen, Denmark); human apotransferrin, ascorbic acid, bathophenanthroline disulfonic acid, cysteine HCl, Hepes buffer from Sigma Chemical Company (St. Louis, MO, USA); $(NH_4)_2Fe(SO_4)_2.6H_2O$, sodium acetate from E. Merck AG (Darmstadt, Germany); and Sepharose CL-6B from Pharmacia AB (Uppsala, Sweden). All aqueous solutions were made in deionized, glass-distilled water. Fe(III)–bleomycin was prepared as described by Shields & McGlumphy (1984), keeping a ratio of $^3/_4$ between iron and the antibiotic. Bleomycin stock solution concentrations were determined using ε_{291} = 17.0 mM^{-1} cm^{-1} (Burger *et al*. 1985). The concentration of apotransferrin was determined at 280 nm (ε = 91.2 $mM^{-1}cm^{-1}$, Carver *et al*. 1982),

and the concentration of Fe(III)–transferrin at 460 nm ($\varepsilon = 2.5$ mM^{-1} cm^{-1}, Johnson *et al.* 1967).

In gel filtration experiments (Figure 4) the amount of iron in each fraction was measured spectrophotometrically at 533 nm by chelating iron with bathophenanthroline in the presence of cysteine as a ferric ion reducing agent ($\varepsilon = 22.4$ mM^{-1} cm^{-1}, Smith *et al.* 1952). The solution contained 0.8 ml of each fraction, 0.1 ml sodium acetate buffer (1 M), pH 5.0, 0.05 ml bathophenanthroline (5 mM) and 0.05 ml cysteine (0.1 M).

Spectrophotometric measurements were performed in a Pye Unicam 8800 instrument. A Clark-type oxygen electrode, connected to an MSE Spectroplus instrument, was used for measuring oxygen uptake.

Results and discussion

At neutral pH Fe(III)–bleomycin is a yellow coloured 1:1 low-spin complex (log $K = 14.9$, Lown *et al.* 1982), characterized by an absorption band at 384 nm ($\varepsilon = 3.3$ mM^{-1} cm^{-1})(Burger *et al.* 1979). Ascorbate rapidly reduces Fe(III)–bleomycin, causing a decrease in the 384 nm absorption band. An ascorbate free radical is probably formed as a result of the one-electron transfer reaction (Buettner & Moseley 1992):

$$\text{Fe(III)–Blm} + \text{A} \xrightarrow{k} \text{Fe(III)–Blm} + \text{A}^{\bullet} \qquad (1)$$

where Blm = bleomycin and A = ascorbate. Figure 1 shows the optical absorption spectrum before and after reduction by ascorbate. When apotransferrin was added to ascorbate reduced iron–bleomycin the characteristic pink colour of Fe(III)–transferrin gradually appeared in the reaction solution. Figure 1 shows that the absorption spectrum, recorded after the reaction was finished, was that of Fe(III)–transferrin with a maximum around 460 nm. It is calculated, that at this time all the iron was bound to transferrin.

Figure 2 shows that the initial rate of reduction of the 384 nm absorption band, V, increases linearly with Fe(III)–bleomycin concentration. The second order rate constant, $k = V/([\text{Fe(III)–Blm}][\text{A}])$, was estimated to 2.4 mM^{-1} min^{-1}. In the reaction mixture molecular oxygen was consumed due to its combination with Fe(II)–bleomycin:

$$\text{Fe(II)–Blm} + \text{O}_2 \rightarrow \text{O}_2\text{–Fe(II)–Blm} \qquad (2)$$

The complex generated is further transformed to the 'activated bleomycin' (Burger *et al.* 1981, Buettner & Moseley 1992). As shown in Figure 2 the initial rate of oxygen uptake is equal to that of Fe(III)–bleomycin reduction; the data suggesting that oxygen is immediately taken up by Fe(II)–bleomycin.

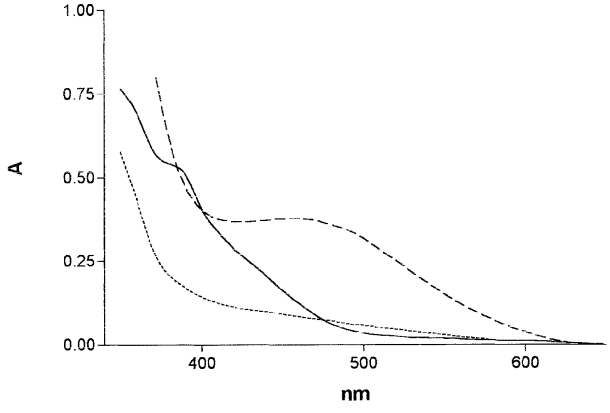

Figure 1. Optical absorbance spectrum of Fe(III)–bleomycin before (——) and after (·····) reduction with 1 mM ascorbate. The solution contained 0.15 mM Fe(III)–bleomycin in 40 mM Hepes buffer, pH 7.4 (T = 30°C). The dashed line indicates the spectrum obtained at steady-state after addition of 0.1 mM apotransferrin to the ascorbate-containing reaction mixture.

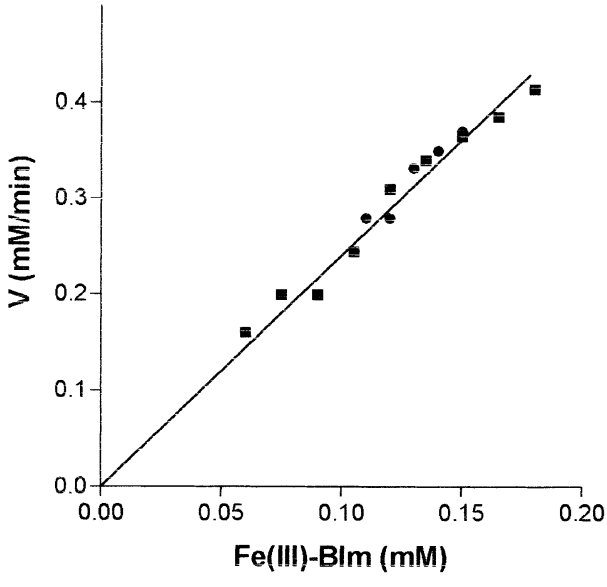

Figure 2. Initial rate of Fe(III)–bleomycin reduction by 1 mM ascorbate at different Fe(III)–bleomycin concentrations (■) (0.06–0.18 mM), and the initial rate of oxygen consumption (●) during the reaction, using 40 mM Hepes buffer, pH 7.4 (T = 30°C).

Figure 3 shows the increase in the 460 nm absorption, occurring when apotransferrin was added to iron–bleomycin; the change being due to formation of Fe(III)–transferrin, which absorbs light more strongly than iron–bleomycin at this wavelength (Figure 1). The concentration of transferrin bound ferric ions (Figure 3) was calculated from the equation:

$$C = (A_{460} - C_0\varepsilon')/(\varepsilon - \varepsilon')$$

where C_0 is the total iron ion concentration, and ε and ε' the absorption coefficients at 460 nm for Fe(III)–transferrin and iron–bleomycin, respectively. The experiment, performed in the absence and presence of ascorbate, clearly demonstrates that ascorbate markedly stimulates the iron ion transfer from bleomycin to apotransferrin. This is probably because a reduced form of the iron–bleomycin complex is less stable than Fe(III)–bleomycin, and more readily donates its iron ion to apotransferrin. The reoxidation of ferrous ions, and Fe(III)–transferrin formation is very rapid, and is not significantly affected by the presence of ascorbate (Carver *et al.* 1982).

Gel filtration experiments confirm that ascorbate increases the amount of iron transferred to transferrin (Figure 4). When a mixture of iron–bleomycin and transferrin was passed over a Sepharose CL-6B column, immediately after mixing, more iron was detected in the transferrin fraction after separation, when ascorbate was added to Fe(III)–bleomycin before apotransferrin.

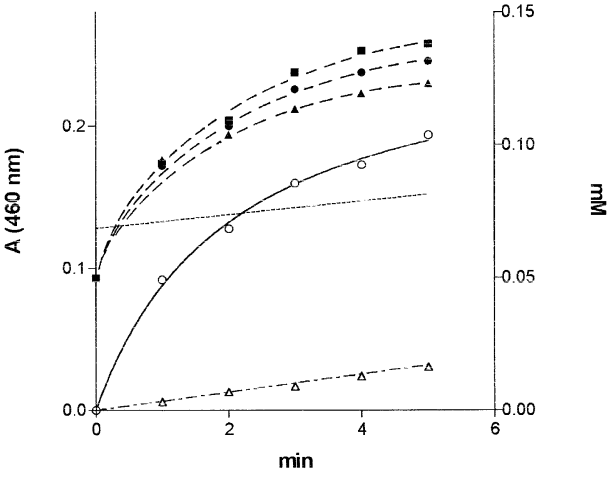

Figure 3. Time course of the 460 nm absorbance when 0.1 mM apotransferrin was added to 0.15 mM Fe(III)–bleomycin in the absence (·····) and presence (------) of ascorbate (■, 1.4 mM; ●, 1 mM; ▲, 0.6 mM), using 40 mM Hepes buffer, pH 7.4 (T = 30°C). Air was continuously administered to the reaction solution in order to keep molecular oxygen in excess. Open circles indicate formation of Fe(III)–transferrin in the mixture receiving 1 mM ascorbate; open triangles indicate formation of Fe(III)–transferrin in the mixture without ascorbate.

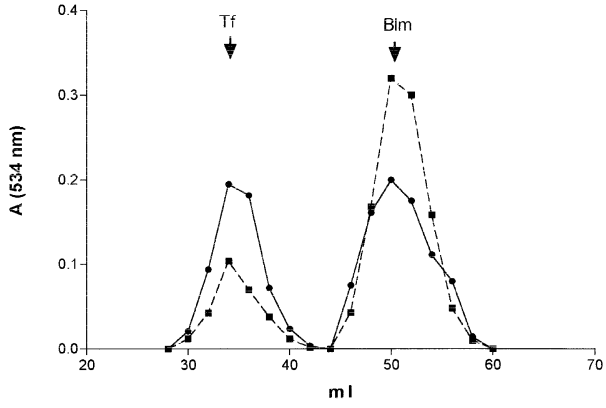

Figure 4. Distribution of iron ions between transferrin (Tf) and bleomycin (Blm) after separation of the two components on a Sepharose CL-6B column (1.3 × 50 cm) following addition of 0.1 mM apotransferrin to a solution of 0.15 mM iron–bleomycin, with (——) or without (-----) 1 mM ascorbate, in 40 mM Hepes buffer, pH 7.4 (T = 22°C). 1 ml of the reaction solution was administered on the column immediately after mixing apotransferrin with iron–bleomycin. Eluent: 40 mM Hepes buffer, pH 7.4; flow rate: 20 ml h^{-1}. The iron content in each fraction was detected at 533 nm as Fe(II)–bathophenanthroline (Materials and methods). Transferrin and bleomycin were identified spectrophotometrically at 280 and 291 nm, respectively, as well as by elution diagrams obtained when they were administered separately.

Transferrin is an iron transport protein in human plasma. Studies in several mammalian species indicate that apotransferrin is synthesized in a variety of tissues (Morgan 1974), although in man the major source of this protein is the liver. If Fe(III)–bleomycin should appear in an environment containing apotransferrin *in vivo*, the presence of ascorbate, and possibly other reducing agents, might promote a 'detoxification' of the iron–drug complex by facilitating a transfer of iron to apotransferrin. Interestingly, Buettner & Moseley (1992) showed that prolonged incubation of iron–bleomycin with ascorbate, in the absence of DNA, resulted in the formation of a red-ox inactive bleomycin, incapable of nicking DNA. This might also be a mechanism by which ascorbate protects cells from bleomycin toxicity, as proposed by the authors.

Acknowledgements

This work was supported by grants from 'Nansen-fondet' and 'Dr.med. Einar og hustru Ebba Langfeldts Fond'. The skilful technical assistance of Ing. Marit Haug is gratefully acknowledged.

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