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Bleomycin resistance conferred by a drug-binding protein

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The protein coded by a bleomycin-resistance gene (*ble*) cloned from producing actinomycetes was purified from a culture of a recombinant *E.coli* strain and its action on bleomycin was determined by in vitro assays. The protein binds reversibly in a one to one ratio to bleomycin which can no longer cleave DNA. The bleomycin resistance of cells harboring a *ble* gene could be accounted for by a sequestering effect of the bleomycin-binding protein.

Bleomycin resistance; Bleomycin-binding protein; DNA cleavage

1. INTRODUCTION

Bleomycin and the related antibiotics, phleomycin and tallysomycin, are DNA-breaking compounds which kill both procaryotic and eucaryotic cells at low concentrations [1,2]. Although these antibiotics have never been used as antimicrobial agents, plasmid-mediated resistance to bleomycin is widely spread among clinically important aminoglycoside-resistant Gram-negative [3] and Gram-positive bacteria [4]. Two bacterial resistance genes (*ble* genes), one from the transposon Tn5 and the other from the staphylococcal plasmid pUB110 have been recently characterized [4–6]. We have cloned and sequenced a third gene designated Sh *ble* from the chromosomal DNA of tallysomycin-producing actinomycetes (Drocourt, D., Gatignol, A., Calmels, T. and Tiraby, G., submitted). The proteins encoded by the three genes are highly homologous and hence probably participate in the same biochemical reaction. The Tn5 and Sh *ble* resistance genes have been exploited as dominant markers which can be selected with phleomycin in yeasts [7,8], fungi [9], plant cells [10] and animal cells (Mulsant, P., Gatignol, A., Dalens, M. and Tiraby, G., submitted). The mechanisms of plasmid-determined resistance have been studied for numerous antimicrobial drugs [11,12] and recently reviewed [13] but until now the biochemical basis of the bleomycin resistance has not been elucidated. The ease with which the protein of the Sh gene can be isolated in a purified form from a recombinant *E. coli* strain allowed us to investigate this question. In vitro assays demonstrated that the Sh protein prevents reversibly the action of bleomycin on DNA. The natural fluorescence of the Sh protein being quenched by the addition of bleomycin provides direct experimental evidence that this protein could be a bleomycin-binding protein exerting its resistance activity by forming a complex with the antibiotic.

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Abbreviations: IPTG, isopropyl-β-D-thiogalactoside; BLM, bleomycin; PAGE, polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Isolation of the Sh protein*

E. coli cells of DH5 pIN-III-ompA2 [14] and DH5 pUT50 were grown in a 1 l fermenter in LB medium. The pH was kept constant at 7 by automatic addition of 1 N potassium hydrox-

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ide. After 14 h incubation at 37°C, 2 mM IPTG was added and growth was allowed for an additional 4 h. The cultures were centrifuged and periplasmic proteins extracted by osmotic shock [15]. Proteins collected in water were brought to 20 mM Tris-HCl, pH 6.7, and injected on a Q-Sepharose fast flow column (Pharmacia) previously equilibrated with the same buffer. The resin was abundantly washed and the elution of fixed proteins was performed by sequential steps of increased NaCl concentration in the same buffer. The column effluent was monitored at 280 nm and the UV absorbance vs time was recorded. Three fractions of both extracts were collected at 0.3 M, 0.7 M and 1 M NaCl and analysed for a protective effect on DNA treated with BLM as indicated in the legend of fig.2. Proteins of fraction II of the DH5 pUT50 extract were precipitated by 2 vols of acetone at −20°C for 2 h and resuspended in 10 mM Tris-HCl, pH 7.5. The protein concentration of the purified protein was determined as described by Vernon et al. [16].

2.2. *Bleomycin-mediated strand scission of DNA*

Reaction mixtures (20 μl total volume) contained 10 mM Tris-HCl buffer, pH 7.5. The Sh protein and BLM (1 μM final concentration) (BLM, Roger Bellon, France) were added sequentially. The mixture was maintained for 5 min at 25°C and 0.2 μg of DNA was added followed by 100 μM $FeSO_4$ from a freshly prepared solution and 1 mM dithiothreitol. The reaction was stopped by EDTA (40 mM) after 5 min incubation at 25°C. Following each reaction, 4 μl of a loading solution (Ficoll 15%, Bromophenol blue 0.25%) was added and the resulting solution was applied to a 0.8% agarose gel. After electrophoresis, the gel was stained with ethidium bromide, visualized and photographed.

When specified, heat treatment was carried out by placing the Eppendorf tube containing the reaction mixtures prior to the addition of DNA for 1 h in a boiling water bath. Protease treatment was carried out by addition of 200 μg/ml of proteinase K and incubation at 45°C for 90 min.

3. RESULTS AND DISCUSSION

From the DNA of the tallysomycin-producer strain of *Streptoalloteichus hindustanus* ATCC 31158, we recently cloned a gene (Sh) that confers high bleomycin-type antibiotic resistance in *E. coli* (Drocourt, D., Gatignol, A., Calmels, T. and Tiraby, G., submitted). The Sh structural gene has been cloned on pIN-III-ompA2, an *E. coli* inducible secretion vector [14], to give the plasmid pUT50 (not shown). Polyacrylamide gel electrophoresis of periplasmic proteins from the DH5 strain revealed an additional intense band of the expected 14 kDa size protein only in extracts of cells harboring the pUT50 plasmid (not shown). The separation of the different protein components of periplasmic extracts was performed by fast liquid protein chromatography (FPLC). Because the Sh protein has a very low theoretical isoelectric point (pI = 3.78), the extracts were submitted to an anion-exchange column. As expected, a well resolved major peak present only in extracts from the bleomycin-resistant cells was eluted in a buffer with 0.7 M NaCl (fraction II) (fig.1). SDS-PAGE of the collected fraction II revealed a unique band at the expected position for the Sh protein. The desalting and the concentration of the protein from this fraction were assured by acetone precipitation, a rather drastic procedure to which the Sh protein proved to be insensitive. The resuspended material was used to investigate the protective effect of the Sh protein toward the in vitro DNA-cleaving action of the bleomycin in the presence of ferrous ions. The results of these experiments are presented in fig.2.

Bleomycin (BLM) at concentrations as low as 1 μM completely degrades 0.2 μg of chromosomal, linear or covalently closed circle (CCC) DNA (and is itself inactivated) in 5 min at 25°C in a standard 20 μl reaction mixture as revealed by agarose gel electrophoresis (fig.2A, lanes 4–6). The Sh protein added prior to BLM at concentrations of 6 μM or higher leads to a complete protection of the DNA (fig.2A, lanes 10–12) while at 3 μM protection is only partial (fig.2A, lanes 7–9). These results clearly indicate that the Sh protein prevents BLM-induced DNA breakage in vitro. Indeed when the amount of protein was not sufficient, the DNA was partially cleaved, even when the incubation time of the Sh protein with BLM was prolonged (2 h) or performed at 37°C (not shown). A lack of a necessary co-factor could explain the time-independence of the reaction. We were unable to find co-enzymes or ions that could activate the reaction to render it time-dependent. This hypothesis cannot be completely excluded, although results of experiments which follow indicate that the interaction Sh protein-bleomycin is not enzymatic.

The Sh protein is a very stable polypeptide. It can however be inactivated by boiling for 1 h or by proteinase K (200 μg/ml) treatment at 45°C for 90 min (fig.2B, lanes 6,7). Under the same heat or protease treatment, BLM retains its full DNA-breaking potency (fig.2B, lanes 2,3). When the mixture Sh protein-BLM is treated under the same conditions, BLM recovers the ability to degrade the DNA (fig.2B, lanes 9,10). The reversibility of the inactivating effect implies that no enzymatic

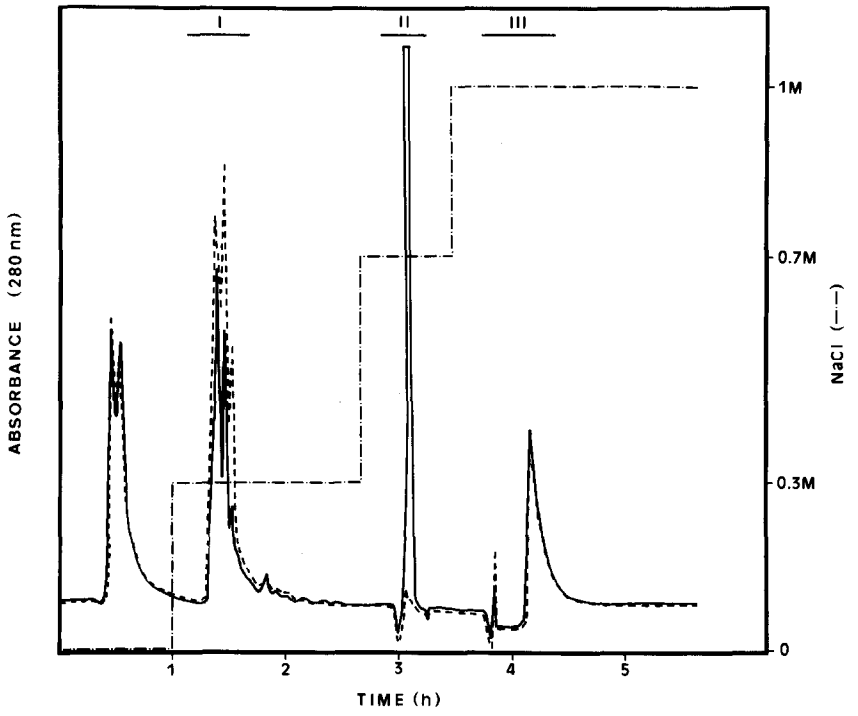

Fig.1. Chromatographic profile on a Q-Sepharose fast flow anion-exchange column of periplasmic extracts from *E. coli* cells harboring two different plasmids. Dashed line, DH5 pIN-III-ompA2; solid line, DH5 pUT50.

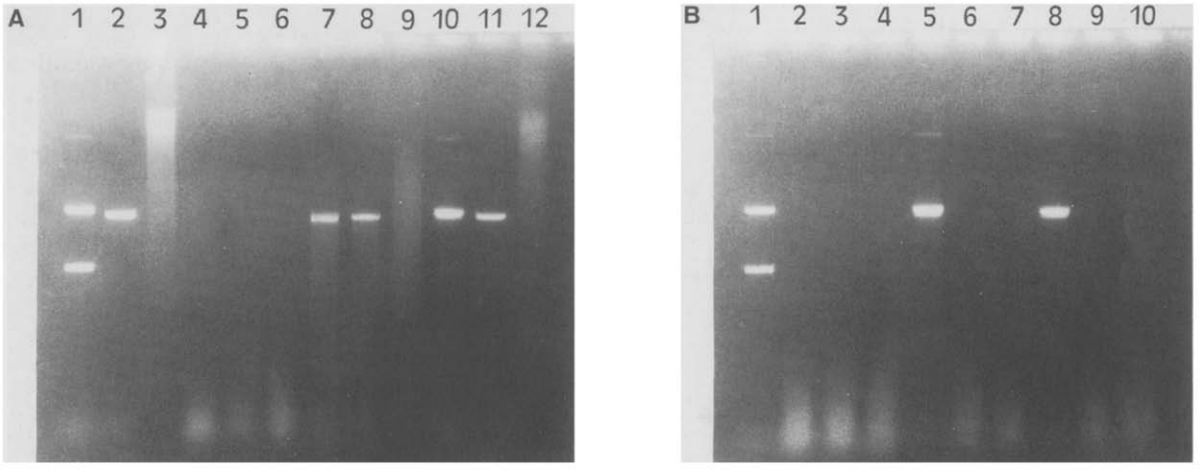

Fig.2. Agarose gel electrophoresis of plasmid and chromosomal DNA treated with BLM alone or in combination with the Sh protein. (A) Inhibition of the BLM-mediated DNA breakage by the Sh protein. Lanes: 1–3, DNA alone; 4–6, BLM + DNA; 7–9, Sh protein (3 μM) and BLM + DNA; 10–12, Sh protein (6 μM) and BLM + DNA. DNAs were from pBR322 (lanes 1,4,7,10), pBR322 linearized by *Bam*HI (lanes 2,5,8,11) and calf thymus DNA (lanes 3,6,9,12). (B) Restoration of the DNA breakage activity of the BLM combined with the Sh protein by a heat or protease treatment. Lanes: 1, control; 2, BLM; 3, heat-treated BLM; 4, protease-treated BLM; 5 and 8, BLM + Sh protein; 6, BLM + heat-treated Sh protein; 7, BLM + protease-treated Sh protein; 9, heat-treated BLM-Sh protein; 10, protease-treated BLM-Sh protein. pBR322 DNA.

modification of the antibiotic molecule has been brought about by the Sh protein and suggests that the protein may exert its inhibitory effect by direct complex formation with BLM.

To determine whether this is the case we have taken advantage of the strong fluorescence emission with a maximum at 350 nm observed when the Sh protein is excited at 280 nm in aqueous solutions. This is probably due to the 5 tryptophan residues of the molecule. The fluorescence of the Sh protein in solution is progressively quenched by addition of increasing concentrations of metal-free BLM or BLM-Fe(II) even after correction of the inner filter effect. When the reciprocal of the measured decrease of the fluorescence intensity is plotted versus the reciprocal of added BLM concentrations, a straight line is obtained (with a regression coefficient always greater than 0.99) from which a half-saturating concentration ($K_{1/2}$) is calculated (fig.3). At low ionic strength (no NaCl), an identical complex presenting a $K_{1/2}$ of 55 nM (± 10) for a concentration of 400 nM Sh protein is observed both with metal-free BLM and BLM-Fe(II). When the ionic strength is increased to 100 mM NaCl, metal-free BLM no longer decreases the fluorescence. Fluorescence quenching is, however, still observed with BLM-Fe(II). In contrast to the results at low ionic strength, the double reciprocal plot exhibits a biphasic character. At low concentrations of BLM-Fe(II), the $K_{1/2}$ is found at 50 nM (± 10) for 400 nM protein. At concentrations of the antibiotic equal or higher than that of the protein a $K_{1/2}$ of 220 (± 70) nM is obtained. The large error associated with this value is due to the fact that BLM itself exhibits low fluorescence which can possibly be quenched by the protein. A half-saturating concentration of 200 nM BLM-Fe(II) for 400 nM protein is best interpreted by considering that one BLM molecule interacts with one protein molecule at physiological ionic strength.

From these data it appears that the Sh protein is a BLM-binding protein which displays two types of interactions. One is purely electrostatic between the basic antibiotic and the acidic protein at low BLM concentrations. The other involves the formation, with a high affinity, of a complex between the binding protein and the BLM in a chelated form with Fe(II) in a one to one ratio. Because of the specificity of this complex, it is likely that the binding takes place on the part of the molecule that chelates metal ions (the reactive site) rather than on the bithiazole moiety (DNA-binding site). Experiments with other chelated forms of BLM are in progress and should clarify this point. Studies from a number of laboratories indicate that Fe(II)

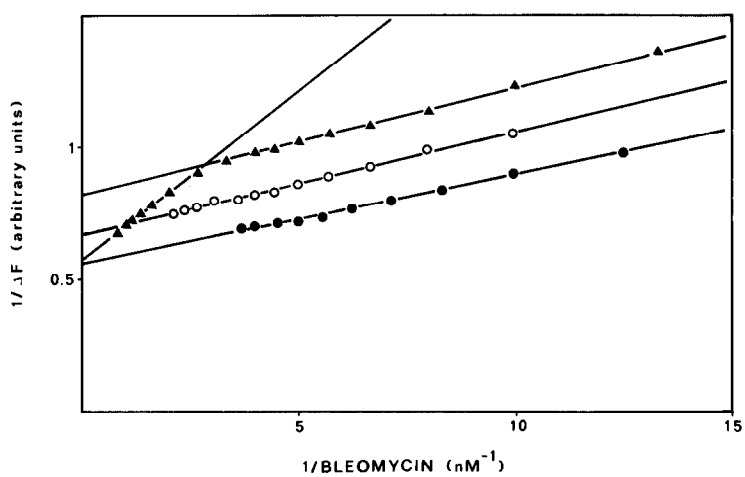

Fig.3. Lineweaver-Burk representation of the reciprocals of fluorescence decrease intensities (ΔF) versus bleomycin concentrations. BLM or BLM-Fe(II) (up to 1.5 μM) was added to 2 ml of Sh protein (0.4 μM) in 1 mM Tris-HCl, pH 7.5. Experiments were carried out with a JY3 Jobin & Yvon spectrofluorimeter, λ excitation = 280 nm, λ emission = 350 nm, slit width at excitation = 10 nm, slit width at emission = 10 nm. BLM (●—●) and BLM-Fe(II) (○—○) with the protein at low ionic strength, BLM-Fe(II) with the protein in 100 mM NaCl (▲—▲).

and O_2 combine with BLM to form activated BLM which upon interaction with DNA produces two types of monomeric products, free base and base propenal [17,18]. The manner by which a BLM-binding protein coded by the Sh gene and very likely by the Tn5 and the pUB110 *ble* genes interferes with the activation of the BLM remains to be determined.

The observation that in procaryotic and eucaryotic cells resistance levels to BLM or phleomycin are proportionately correlated with expected expression levels of the Tn5 and the Sh gene, even at high levels (unpublished), is better understood in the light of the discovered stoichiometric relationship between the *ble* protein and the drug. BLM has never been used as an antimicrobial antibiotic and thereby, the occurrence of bleomycin-resistant pathogenic bacteria cannot be accounted for by the selective pressure exerted by this drug. The Sh *ble* gene cloned from an actinomycete producer of a bleomycin-type antibiotic presumably participates in the self resistance of producing cells to the antibiotic and consequently one can assume that the bleomycin-binding effect described here is the primary function of the *ble* genes. Nevertheless, it still remains possible that the *ble* proteins display some other unrelated properties in a similar way to the chloramphenicol acetyltransferase of the only type I which enzymatically inactivates chloramphenicol and also sequesters fusidic acid and crystal violet by binding to these hydrophobic compounds [19].

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