DIFFERENTIAL BINDING OF STREPTOMYCIN TO RIBOSOMES OF POLYAMINE-DEFICIENT BACTERIA GROWN IN THE ABSENCE AND PRESENCE OF PUTRESCINE

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1. Introduction

Streptomycin and other aminoglycoside antibiotics are known to interfere with bacterial protein synthesis through interaction with 30 S subunits and 70 S ribosomes [1–6]. Since the pioneer work of Gorini's group on streptomycin-resistant and -dependent mutants [7,8] our knowledge of the mechanism of action of this antibiotic has increased considerably [6,9,10].

Although it has been shown that ribosomal protein S_{12} is important for the specific binding of streptomycin to ribosomes of susceptible strains of *Escherichia coli* [11] and proteins S_3, S_4 and S_5 are able to modify this interaction [12,13], it is not yet clear which ribosomal proteins contribute to the structure of the binding site.

In work with streptomycin-sensitive polyamine-auxotrophic mutants we have shown that streptomycin caused a marked inhibition of protein synthesis in polyamine-supplemented cells, whereas bacteria starved for polyamines were less sensitive to the inhibitory action of the antibiotic [14]. These results might be due to the presence of defective 30 S subunits in bacteria submitted to polyamine starvation [15,16]. Under these conditions the small ribosomal particles are incorrectly assembled [16], contain a reduced amount of S_1 protein [17] and the 16 S RNA is under-methylated [18].

In this, dealing with the polyamine requirement for streptomcyin action, we describe in vivo and in vitro studies on the interaction of the antibiotic with ribosomes of a polyamine-auxotrophic strain of *E. coli* grown in the absence and presence of putrescine.

2. Materials and methods

Streptomycin sulphate was a generous gift from Lepetit (Argentina); dihydrostreptomycin sulphate was purchased from Sigma and magnesium-Titriplex from Merck. L-[^{14}C]Phenylalanine (527 Ci/mol) and L-[^{14}C]valine (281 Ci/mol) were obtained from New England Nuclear and [^{3}H]dihydrostreptomycin sesquisulphate (1.8 Ci/mmol) from the Radiochemical Centre (Amersham).

The polyamine-auxotrophic *E. coli* BGA 8 and MA 255, which are streptomycin-sensitive and -resistant strains, respectively, were described in [14]. Growth medium and culture conditions were as in [19]. The streptomycin sensitive strain *E. coli* MRE 600 was kindly provided by Dr M. García-Patrone and grown in the same minimal medium used for the other strains. Steady-state cultures of bacteria grown in the absence or presence of putrescine were slowly cooled and harvested at mid-logarithmic phase ($A_{490} \sim 0.25$). Cells were collected by centrifugation, washed with a buffer solution containing 10 mM Tris–HCl (pH 7.8), 5 mM magnesium acetate, 60 mM NH_4Cl and 6 mM 2-mercaptoethanol (standard buffer) and stored at −70°C.

Pellets of frozen cells grown in the absence or presence of putrescine were thawed, resuspended in 2.5 vol. 'standard buffer' containing 5 mM Mg-Titriplex and disrupted by sonication. After two successive centrifugations at 10 000 × *g* for 10 min and 30 000 × *g* for 30 min to obtain the S-30 extracts, ribosomes were separated by sedimentation at 150 000 × *g* for 150 min. The ribosomal pellet was resuspended in a small volume of 'standard buffer' (to ~400 A_{260}

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units/ml) and dialysed overnight against the same buffer. The ribosomal suspension was centrifuged at 10 000 × *g* for 15 min to discard the aggregated material and the supernatant liquid was quickly frozen at −70°C. The ribosomes were not washed with salt solutions of high concentration in order to avoid changing their polyamine content.

For the in vitro assay of antibiotic binding, ribosomes and [^{3}H]dihydrostreptomycin at the concentrations indicated in each experiment were incubated for 5 min at 37°C in a solution containing 20 mM Tris–HCl buffer (pH 7.5), 70 mM KCl and 5 mM magnesium acetate (binding buffer) in 0.2 ml final vol. After addition of 3 ml cold binding buffer, the mixture was filtered through nitrocellulose membranes (Millipore type HA, 0.45 μm pore size, or Schleicher and Schüll BA 85), pre-soaked in binding buffer. The filters were washed twice with 3 ml of the same solution, then dried. Bound radioactivity was measured in a scintillation spectrometer. Control experiments have indicated that under the conditions used, 80% of the ribosomes were retained by the nitrocellulose filters.

For the in vivo studies, steady-state cultures of polyamine-starved and -supplemented bacteria, at $A_{490} \sim 0.25$, were fractionated into two aliquots. Streptomycin (20 μg/ml) was added to one of the aliquots and incubation was continued for 40 min. All samples were centrifuged and the bacterial pellets were washed with 'standard buffer'. After resuspension in 10 mM Tris–HCl buffer (pH 7.8) containing 5 mM magnesium acetate and 50 mM KCl, cells were sonicated for 1 min. Cell extracts were centrifuged at 10 000 × *g* for 10 min and aliquots of the supernatant fluids were layered on top of 15–45% (w/v) linear sucrose gradients made up in 10 mM Tris–HCl (pH 7.5), 5 mM magnesium acetate, 50 mM KCl. Centrifugation was for 125 min at 45 000 rev./min in a Spinco SW 50.1 rotor. Ribosomal profiles were analysed by monitoring at 254 nm with an ISCO ultraviolet analyser.

Protein synthesis in vivo was measured as in [14] and polypeptide synthesis in vitro, directed by MS2 phage RNA, was done as in [20].

3. Results and discussion

The addition of streptomycin to cultures of *E. coli* BGA 8 produced different effects depending on the presence of polyamines in the culture medium. Thus, putrescine-starved bacteria maintained a steady low rate of duplication, whereas polyamine-supplemented cells stopped growth almost completely ~30–40 min after the addition of the antibiotic (fig.1). When protein synthesis was measured, the results obtained were in agreement with this differential behaviour [14]. The in vivo [^{14}C]phenylalanine incorporation into peptides was markedly reduced by streptomycin when bacteria were cultivated in the presence of polyamine, but it was almost unaltered in putrescine-depleted cells (table 1). Furthermore, the polypeptide synthesis programmed by MS2 phage RNA was significantly inhibited by the in vitro addition of streptomycin only when the cell-free systems were obtained from bacteria grown in the presence of putrescine (table 1).

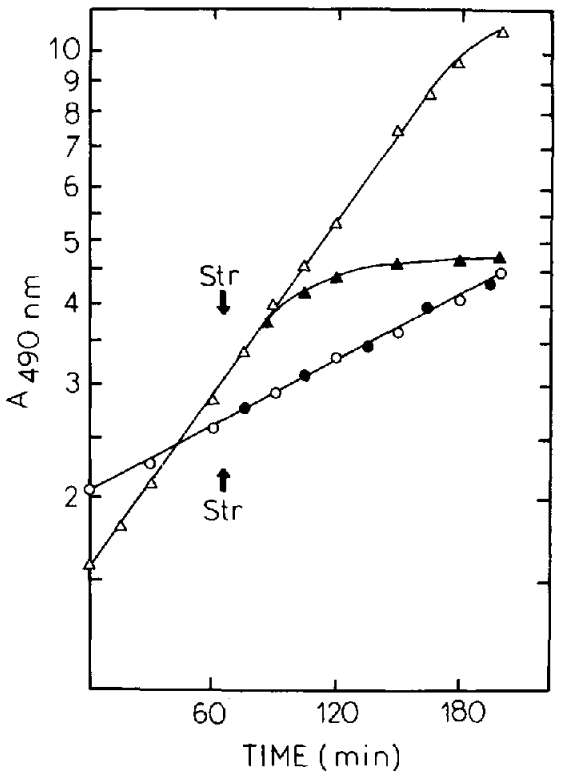

Fig.1. Effect of streptomycin on the growth rate of *E. coli* BGA 8. Bacteria were cultivated in the absence or presence of putrescince as in section 2 with the addition of streptomycin (Str) (20 μg/ml) at the indicated time. Cell growth was followed at 490 nm: (○) and (●) cultures without putrescine in the absence and presence of antibiotic, respectively; (△) and (▲) cultures containing putrescine in the absence and presence of streptomycin, respectively.

Table 1
Streptomycin inhibition of in vivo and in vitro protein synthesis in bacteria and cell-free systems obtained from cultures carried out in the absence and presence of putrescine

Conditions	Putrescine added	Streptomycin added	Amino acid incorp. (cpm)	Inhib. (%)
In vivo	–	–	9606	–
	–	+	8530	11.2
	+	–	25 697	–
	+	+	7401	71.2
In vitro	–	–	9152	–
	–	+	7539	17.6
	+	–	17 910	–
	+	+	8704	51.4

E. coli BGA 8 was grown as indicated in each case and in vivo protein synthesis was measured after 90 min incubation in the presence of [^{14}C]phenylalanine. Polypeptide synthesis in vitro was determined by [^{14}C]valine incorporation into trichloroacetic acid-insoluble material directed by MS2 phage RNA in S-30 extracts. The inhibition caused by streptomycin (20 μg/ml) added either in vivo or in vitro was calculated with respect to the corresponding control assays carried out in the absence of antibiotic

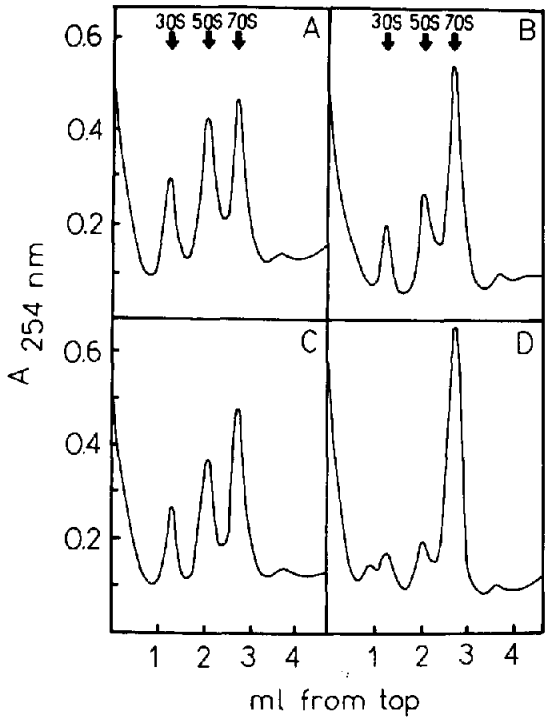

Fig.2. Ribosomal profiles obtained after addition of streptomycin to cells of *E. coli* BGA 8 grown in the absence and presence of putrescine. Polyamine-depleted cultures, without (A) or with (C) the addition of streptomycin (20 μg/ml), and putrescine-supplemented cultures in the absence (B) or presence (D) of antibiotic were harvested, bacteria were lysed and cell-extracts were analysed as in section 2.

These results indicate that the multiple effects of streptomycin on sensitive bacterial strains require a normal intracellular level of polyamines [14]. In fact, under conditions of polyamine starvation, defective ribosomal particles are formed [16] which might not be appropriate targets for streptomycin, becoming resistant to the action of the drug. To test this hypothesis we have studied in vivo and in vitro the interaction of the antibiotic with ribosomes from polyamine-depleted and supplemented bacteria.

Steady-state, polyamine-starved and -supplemented cultures of *E. coli* BGA 8 growing exponentially were incubated with and without streptomycin, and the cell extracts obtained from them were analysed by sucrose gradient centrifugation. In polyamine-depleted bacteria no differences were evident in the ribosomal patterns corresponding to cultures carried out in the absence or presence of streptomycin, except for a small increase in subunit association evoked by the antibiotic (fig.2A,C). However, the action of streptomycin on polyamine-supplemented bacteria was more striking, not only in the enhanced association effect but also in the deformation of 30 S particles with the appearance of an additional peak sedimenting at ~20 S (fig.2D). Controls corresponding to bacteria grown in the presence of putrescine without streptomycin or with the antibiotic added in vitro to cell extracts gave the profile shown in fig.2B. Therefore the antibiotic was able to induce a conformational change in 30 S subunits only in polyamine-supplemented bacteria (fig.2). The different ribosomal patterns observed with polyamine-starved and unstarved cells were not due to a reduced streptomycin uptake under conditions of polyamine deprivation, as shown in [14,21]. The double peak corresponding to 30 S ribosomal particles (fig.2D) seems to be the result of the streptomycin-effective action, since it has also been observed when *E. coli* MRE 600, a strain not requiring polyamines, was treated with the antibiotic (not shown). The direct binding of the antibiotic to ribosomes prepared from bacteria cultivated in the absence and presence of putrescine has been studied by using radioactive dihydrostreptomycin.

Binding of antibiotic to ribosomes of streptomycin-

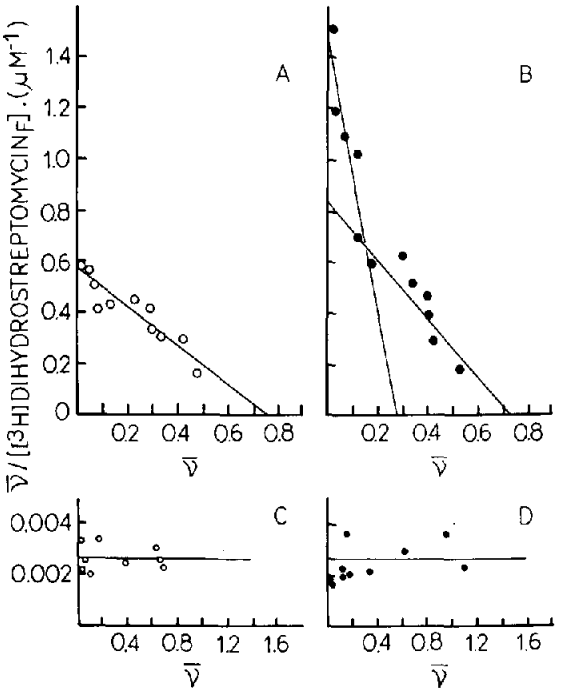

Fig.3. Scatchard plots of [^{3}H]dihydrostreptomycin binding to ribosomes of streptomycin-sensitive and -resistant polyamine-auxotrophic strains: (A) and (B) correspond to polyamine-starved and unstarved *E. coli* BGA 8, respectively; (C) and (D) to polyamine-depleted and supplemented *E. coli* MA 255, respectively. Best lines fitting the experimental points were calculated by the least squares regression method. Assays were run in quadruplicate.

sensitive cells of *E. coli* BGA 8 was almost independent of temperature in other strains [1,22]; it was complete within 1 min and Mg^{2+} was an absolute requirement.

The binding of [^{3}H]dihydrostreptomycin to ribosomes of the polyamine auxotrophic strains *E. coli* BGA 8 (streptomycin-sensitive) and *E. coli* MA 255 (streptomycin-resistant) has been measured at different concentrations of antibiotic. In the case of MA 255 there was almost no binding in the antibiotic concentration range which was effective for ribosomes of *E. coli* BGA 8, while at much higher dihydrostreptomycin levels a non-specific binding was observed.

Fig.3 shows the Scatchard plots [23] corresponding to the binding of antibiotic to ribosomes of *E. coli* BGA 8 and MA 255. A significantly different behaviour was observed for the binding of [^{3}H]dihydrostreptomycin to ribosomal particles of putrescine-starved and unstarved BGA 8. Two types of antibiotic interaction were observed with ribosomes from putrescine-supplemented bacteria: a high affinity for the antibiotic ($K_d = 1.9 \times 10^{-7}$ M, $n = 0.3$) and a low affinity ($K_d = 0.9 \times 10^{-6}$ M, $n = 0.74$). The high affinity constant is very similar to that derived using the filtration method [1]. The above heterogeneity was not detected in the ribosomal particles from putrescine-starved bacteria, which showed a binding affinity defined by $K_d = 1.4 \times 10^{-6}$ M ($n = 0.77$), almost identical to the low affinity constant of the ribosomes of polyamine-unstarved cells. Considering that only 80% of ribosomal particles were retained on the nitrocellulose filters, the corrected *n*-values increased to 0.37 and 0.92–0.96, respectively. Several authors have reported only one type of binding site for streptomycin in ribosomes of sensitive bacteria; however, our results indicate a heterogeneity in the type of interaction in particles from polyamine-supplemented bacteria, in agreement with suggestions of a dual action of streptomycin on ribosomes. This hypothesis has been proposed after studies of hydrogen–tritium exchange [24] and the use of fluorescent or spin-labeled probes [25].

However, [^{3}H]dihydrostreptomycin did not bind specifically to ribosomes from the resistant strain *E. coli* MA 255 as shown in the corresponding Scatchard plots (fig.3C,D).

Working with polyamine-requiring bacteria we have been able to detect some features of streptomycin action which were not evident earlier. We now propose three types of interaction between ribosomal particles and streptomycin:

(1) Non-specific binding detected in streptomcyin-resistant strains as *E. coli* MA 255;
(2) Specific binding with high and low affinity interactions as observed in the streptomycin-sensitive polyamine-auxotrophic strain BGA 8 cultivated in the presence of putrescine, where the antibiotic evokes inhibition of growth and protein synthesis;
(3) Specific binding with only a low affinity interaction as shown by polyamine-depleted *E. coli* BGA 8.

In these cells, behaving in other aspects as a resistant strain, the growth rate and protein synthesis were not altered by the presence of the drug.

We can conclude that correctly assembled ribosomal particles formed in the presence of normal intracellular levels of polyamines are the appropriate

targets for streptomycin, because they can undergo the conformational changes necessary for the antibiotic action [10,26].

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