EFFECTS OF CATIONS, POLYAMINES AND OTHER AMINOGLYCOSIDES ON GENTAMICIN C_2

BINDING TO RIBOSOMES FROM SENSITIVE AND RESISTANT *ESCHERICHIA COLI* STRAINS

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Gentamicin C_2 interacts cooperatively with ribosomes from a sensitive *Escherichia coli* strain in a multiphasic way with several classes of sites. It is shown that this binding is highly-dependent on Mg^{++} and natural endogenous polyamine concentrations. The differences observed between ribosomes from sensitive and resistant strains may be explained by the absence of specific cooperative gentamicin interactions with resistant ribosomes. The effects of other aminoglycoside antibiotics are discussed in terms of structure-activity relationships.

Gentamicin (Gm) is an aminoglycoside antibiotic produced by *Micromonospora purpurea*. It induces multiphasic disturbances of translocation of the *Escherichia coli* mRNA by endogenous polysomes.[1,2] We have shown previously — as did MISUMI *et al.*[2] with kanamycin — that each step of the inhibition was related to successive binding sites for Gm on the ribosomes (S-ribosomes)[3]; the strongest binding site (one Gm molecule per 70 S ribosome) is responsible for the inhibition of translocation observed with low Gm concentrations. Subsequently, the cooperative binding of 4 to 5 Gm molecules in several affinity-type sites, located on the 50 S subunit are responsible for the increased translational activity observed at higher Gm concentrations. Finally, saturation of the ribosome by Gm in numerous, cooperative low affinity-type sites leads to a complete change in structure, thus preventing any translational activity and leading probably to the bactericidal effect.

With altered ribosomes (R-ribosomes) from a Gm resistant *E. coli* strain, Gm still interacts[3] (L6 modification)[4] but the multiclass and the cooperative aspects of the interaction are not observed. It had previously been shown that Gm was able to inhibit the translational activity of resistant ribosomes during one phase, but could neither reverse the inhibition, nor induce a significant rate of misreading[5].

The aim of the present work was to investigate the nature of these complex interactions, and to try to understand at a molecular level the difference between binding of Gm to R- and S-ribosomes. Hence we have studied the effects of ionic strength, Mg^{++}, natural polyamines, and other aminoglycoside antibiotics on the binding of gentamicin C_2 (GmC_2) to these receptors.

Materials and Methods

Chemicals

[^{3}H]Gentamicin C_2 ([^{3}H]GmC_2) was synthesized as previously described[6].

Preparation of Ribosomes

E. coli AB 2834-5 Gm resistant, a gift from Dr. BÖCK, and MRE 600 Gm sensitive were grown

Fig. 1.

(A) Effect of NH_4Cl on the binding of [^{3}H]GmC_2 to *E. coli* MRE 600 (○) and AB 2834-5 (△) 70 S ribosomes in 10 mM Tris-HCl, pH 7.6, and 10 mM $Mg(OAc)_2$.

(B) Effect of $Mg(OAc)_2$ on the binding of [^{3}H]GmC_2 to *E. coli* MRE 600 (○) and AB 2834-5 (△) 70 S ribosomes in 10 mM Tris-HCl, pH 7.6, and 100 mM NH_4Cl.

(C) Effect of spermidine on the binding of [^{3}H]GmC_2 to *E. coli* MRE 600 (○) and AB 2834-5 (△) 70 S ribosomes in 10 mM Tris-HCl, pH 7.6, 10 mM $Mg(OAc)_2$, and 100 mM NH_4Cl.

Standard conditions for equilibrium dialysis were used (ribosomes 0.24 μM).

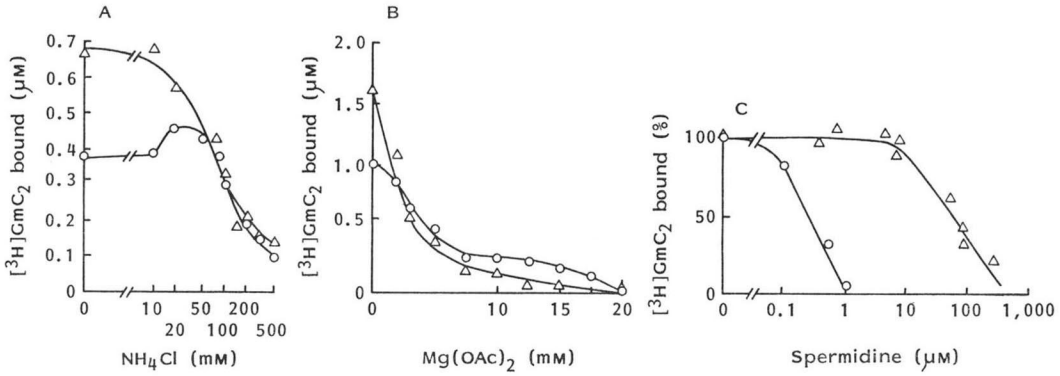
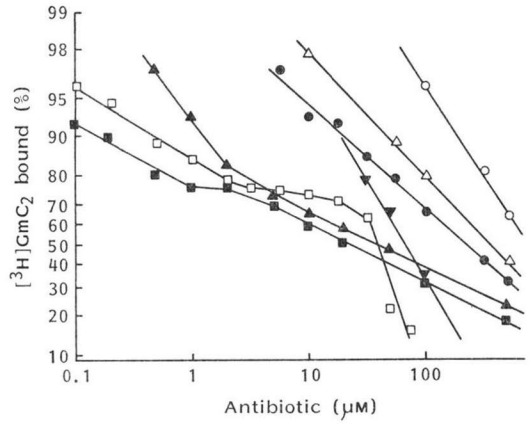


Fig. 2. Effects of other aminoglycosides on the binding of [^{3}H]GmC_2 to *E. coli* MRE 600 ribosomes under standard conditions.

Ribosomes 0.24 μM, [^{3}H]GmC_2=0.54 μM. Plot of logit - log ($B/B_{Initial}-B$) *versus* log (antibiotic).

○ Amikacin, △ netilmicin, ● kanamycin A, ▼ sisomicin, ▲ tobramycin, □ Gm C_2, ■ ribostamycin.



in Tryptic soy broth at 37°C. The S 30 preparation and the ribosome purification were as previously described[7].

Binding Experiments

Binding experiments were performed by equilibrium dialysis[8] 4 hours, at 25°C. Unless otherwise specified, the experiments were performed in a standard buffer which consisted of 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 10 mM Tris-HCl, pH 7.6.

Results

The effects of NH_4^+, Mg^{++} and polyamines on the ribosomal binding of GmC_2 were studied using 70 S ribosomes extracted from both aminoglycoside sensitive and resistant *E. coli* strains. The resistant strain possesses an altered L6 protein due to a mutation in the *rplF* gene.

Increasing concentrations of these cations were used to displace the GmC_2-ribosome complexes. The concentration of radioactive GmC_2 was chosen in order to fill the high affinity site of the ribosomes with approximately 2 molecules per ribosome under standard conditions. The results are shown in Fig. 1. For each cation tested, a greater stability of the GmC_2 complex with wild-type ribosomes was indicated.

The displacement of [^{3}H]GmC_2 bound to *E. coli* MRE 600 ribosomes by other aminoglycoside antibiotics is presented in Fig. 2 (logit representation). Under such equilibrium conditions (standard buffer, 2 Gm molecules per ribosome) the results permit the classification of the competitors into two

groups. (1) GmC_2, tobramycin and ribostamycin were able to displace bound [^{3}H]GmC_2 successively in two ways; 25% were displaced with low IC_{50} (1 μM), and the remaining molecules were further displaced with high IC_{50} (10~100 μM). (2) The other molecules tested displaced the radioactive GmC_2 from the ribosomes according to a linear logit - log plot and with high IC_{50} (about 100 μM).

As expected, streptomycin was unable to displace GmC_2 bound to ribosomes (not shown).

Discussion

The effect of ionic strength (Fig. 1A) on Gm binding shows that the ribosome-Gm complex is not simply an electrostatic type. Although the interaction between ribosomes (negatively charged) and a highly polycationic molecule could be mainly of an ion-pair type, the bell-shaped effect of changing ionic strength indicates that a preferential conformational state does exist in the thermodynamic equilibrium with S-ribosomes. Conversely the stability of the complexes with R-ribosomes is directly linked with the ionic strength. Without NH_4Cl, the capacity of these latter ribosomes for GmC_2 is almost two times higher than the S-ribosomes.

In standard conditions using 70 S ribosomes, Mg^{++} concentration is 10 μM, thus allowing the association of subunits. Fig. 1B indicates that lowering Mg^{++} concentration induces an increase of Gm binding. This may be explained in part by the capacity of separate subunits to bind more GmC_2 than 70 S ribosomes[2]. However, this cannot account for more than a two-fold increase, whereas one observes a three to four-fold capacity increase. Gm probably interacts with Mg^{++} sites not only in the whole ribosomal structure but also with rRNA-Mg^{++} sites (manuscript in preparation).

A difference in stability has been observed between S- and R-ribosome-Gm complexes in relation to the dissociation in the presence of high Mg^{++} concentration; the wild-type complexes are resistant to dissociation and remain quite stable over a wide range of Mg^{++} concentrations, while the complexes obtained with Gm-R-ribosomes are completely dissociated in the same Mg^{++} range. The same experiment was performed under standard conditions, at a [^{3}H]Gm concentration of 100 μM where complexes containing up to 45 Gm molecules per ribosome can be formed; in the presence of 20 mM Mg^{++}, about 10 molecules tightly bound to S-ribosomes were not displaced, while similar mutant ribosome complexes were fully dissociated (results not shown). This confirms the existence of a specific conformational state of the complex formed between S-ribosomes and a few GmC_2 molecules.

The natural endogenous polyamines (spermidine and putrescine) are known to interact strongly with macromolecules. COHEN and LICHTENSTEIN[9] have shown that 12 to 15% of the polyamines of *E. coli* were located in the ribosomes, this amount being sufficient to neutralize 8% of the ribosomal phosphate. It is well known that these low molecular weight substances can enhance protein synthesis, both in prokaryotic and in eukaryotic cell-free systems[10]. This effect has been attributed to an increased translational fidelity[11,12]. Their interaction with ribosomes and RNA thus contributes to maintain the specificity of the codon/anticodon interaction. More recently, GOLDEMBERG and ALGRANATI[13] have shown that the inhibition of polypeptide synthesis by streptomycin requires a normal endogenous level of polyamine. They observed a similar behavior with other aminoglycosides which induce misreading (neomycin and kanamycin), but could not explain why inhibition of protein synthesis by Gm did not show this strong polyamine dependence.

Aminoglycosides and polyamines display opposite effect in relation to polypeptide synthesis, although they are both strong polycations capable of interacting with ribosomes and ribosomal RNA. We demonstrate here some unexpected results concerning the effect of polyamines on Gm binding which are in contrast with our previous results obtained with tobramycin. In the latter case, polyamines, at low concentrations, were not able to displace tobramycin from its high affinity sites[14]. In contrast, GmC_2 is removed from its high affinity sites by very low polyamine concentrations. This effect seems to concern the large subunit, since AB 2834-5 ribosome-Gm complexes are dissociated only at high polyamine concentrations. The effect of Gm on the accuracy of translation and on the role of polyamines, seems therefore to be due to its cooperative binding to the 50 S subunit.

This could explain the activity of Gm on protein synthesis observed on polyamine auxotrophic

mutants[13]; when these bacterial strains unable to synthesize putrescine are submitted to a polyamine starvation, they show a decreased growth rate and a concomitant lower amino acid incorporation into proteins, both *in vivo* and *in vitro*. This reduction is accompanied by changes at the ribosomal level, in particular the presence of defective 30 S particles. The inhibition of protein synthesis observed with neomycin, kanamycin and kasugamycin was markedly enhanced in polyamine supplemented bacteria, whereas Gm caused the same degree of inhibition in putrescine starved and unstarved cells. This different behavior shown by Gm could be a consequence of the multiple sites of action of this antibiotic on both ribosomal subparticles[5].

The effect of other aminoglycosides on GmC_2 binding shows that the number and the *pKa*'s of the amino groups determine the capacity of aminoglycosides to bind to ribosomes. On the other hand, putrescine and spermidine, with 2 or 3 amino groups, are capable of displacing Gm more efficiently than other aminoglycosides; this may be due to the structural flexibility of these molecules.

Fig. 2 shows the displacement of [^{3}H]GmC_2 from *E. coli* MRE 600 ribosomes by other 2-deoxystreptamine aminoglycosides. [^{3}H]GmC_2 at the concentration of 0.5 μM which was used in these experiments. At this concentration, Gm is presumed to bind preferentially to the high affinity binding sites, but the shape of the displacement curves shows that in fact, it also interacts with low affinity sites. Thus, [^{3}H]GmC_2 is displaced by non radioactive GmC_2 in a biphasic way; 20 to 30 percent of the complexes are first displaced with an EC_{50} <1 μM, then, after a plateau has been reached, the remaining drug is completely displaced with an EC_{50} of about 50 μM.

Streptomycin was not able to displace Gm from its high affinity sites on ribosomes at any of the concentrations tested. This confirms that streptomycin does not interfere with the 2-deoxystreptamine aminoglycoside sites on the ribosome[2,14,15].

The biphasic displacement shows that, under these conditions, [^{3}H]GmC_2 is removed successively from at least two classes of sites. The other aminoglycosides can be divided into two families; those able to displace [^{3}H]GmC_2 from its high as well as from its low affinity sites (ribostamycin and tobramycin), and those able to displace it only from its low affinity sites (sisomicin, kanamycin A, netilmicin, amikacin). Ribostamycin and Gm have previously been shown to interfere moderately with binding at the high affinity site of tobramycin[14]. Conversely, tobramycin and ribostamycin interfere with the high affinity sites for Gm. This indicates that the high affinity binding sites of aminoglycosides are not located at the same place on ribosomes. In contrast, the multiple low affinity binding sites are probably common to all aminoglycosides.

The *pKa*'s and the spatial arrangement of the amino groups seem to be important for the ability of aminoglycosides to bind to high or low affinity sites; kanamycin A, which does not possess a 2′-NH_2 is a less potent competitor than tobramycin, and amikacin which is a 1-acyl derivative of kanamycin A, is the worst competitor. The influence of the spatial arrangement is quite clear in the behavior of sisomicin. This antibiotic is considered to be closely related to Gm, although the presence of the 4′-5′ double bond gives the A ring a conformation quite different from that of Gm. This change could increase the distance between the 6′ and the 2′-NH_2 residues, thus preventing the binding of sisomicin to Gm high affinity sites.

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