Quantitative study of kanamycin action on different functions of *Escherichia coli* ribosomes

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1. INTRODUCTION

Kanamycin, an aminoglycoside antibiotic, was reported to inhibit peptide synthesis via blocking of translocation [1–3]. This occurs owing to fixing of peptidyl-tRNA to the acceptor (A) site [3]. Two molecules of the antibiotic can be bound tightly to the ribosome, one to the 30 S and another to the 50 S subunit [2]. The inhibition of translocation was attributed to the kanamycin molecule which interacts with the large ribosomal subunit [2]. It is known also that kanamycin induces codon misreading [4] and affects the peptidyl-transferase reaction [5] (reviews [6,7]).

The results of a more detailed study of kanamycin action on particular functions of ribosomes are presented here: We show that kanamycin:

(i) Stabilizes the binding of peptidyl-tRNA to the A site of isolated 30 S subunits;
(ii) At high concentrations, stabilizes the binding of poly(U) to 70 S ribosomes;
(iii) Contrary to [5], does not affect peptidyl-transferase activity of ribosomes.

2. MATERIALS AND METHODS

Isolated 30 S and 50 S subunits, enriched Ac-[^{14}C]Phe-tRNA^{Phe} and [^{14}C]Phe-tRNA^{Phe} (1400 pmol/A_{260} unit), poly[^{3}H](U) (20 000 M_r) and unlabeled one (30 000 M_r) were prepared as in [8–10]. Equilibrium binding and association constants of tRNA with 30 S subunits and 70 S ribosomes, as well as poly[^{3}H](U) with 70 S ribosomes, were determined as in [8–10].

$\bar{\nu}^{\Sigma}$ = no. tRNA molecules bound/ribosome (or 30 S subunit);
$\bar{\nu}^{D}$ and $\bar{\nu}^{A}$ = the same for the D and A sites, respectively:
K_a^D and K_a^A = association constants of tRNA for D and A sites;
$\bar{n}$ = the number of poly([^{3}H]U) molecules bound/ribosome.

Elongation factor G was prepared as in [11]. The assay for (Phe)$_2$-tRNA^{Phe} synthesis was performed as in [8]. Puromycin reaction was done as in [12]. All antibiotics were from Serva (Heidelberg).

3. RESULTS

In a control experiment we checked the inhibitory activity of kanamycin. The pre-translocation complex was made as in [13]; namely, a sufficient amount of total deacylated tRNA was added to Ac-Phe-tRNA^{Phe} to shift the binding of the latter exclusively to the A site. Then GTP, puromycin and various amounts of EF-G were added simultaneously to the incubation mixtures. Kanamycin effectively suppresses the factor-dependent yield of Ac-Phe-puromycin, i.e., translocation, provided that nearly stoichiometric amounts of EF-G are used (fig.1). However, the more excess of EF-G is present, the less inhibitory effect of kanamycin is found. It could be explained, for instance, by the competition between EF-G and essential molecule of the antibiotic for the binding site of the latter, when both are added simultaneously.

Fig.2A shows the titration of 30 S · poly(U) complexes by Ac-Phe-tRNA^{Phe} (in inverse coordi-

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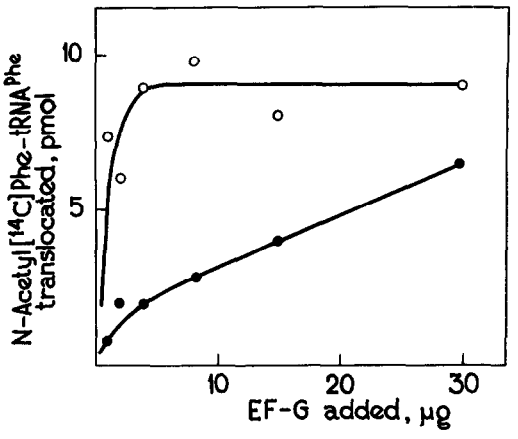

Fig.1. Kanamycin action on EF-G-dependent translocation of Ac-Phe-tRNA^{Phe}. Mixtures contained in 100 μl buffer I: 10 pmol 30 S subunits, 12 pmol 50 S subunits, 5 μg poly(U), 100 pmol Ac-[^{14}C]Phe-tRNA^{Phe} and 2000 pmol total unfractionated tRNA. After 30 min incubation at 24°C (the first step), GTP and puromycin with final concentrations $3 \cdot 10^{-4}$ M, 10^{-4} M kanamycin (if indicated) and 0–30 μg of EF-G were added, in 40 μl buffer I, to each mixture. Then incubation was continued for an additional 30 min, and amounts of Ac-[^{14}C]Phe-puromycin were determined in the absence (—○—) and presence of kanamycin (—●—). Buffer I: 0.02 M Tris–HCl (pH 7.3); 0.01 M $MgCl_2$; 0.02 M NH_4Cl; 0.001 M EDTA.

nates). Two molecules of this analogue of peptidyl-tRNA can be bound per subunit (—o—). As was shown in [8], the more stable binding takes place at the D, and the less stable at the A site of subunit. We see from fig.2A, that kanamycin strongly stimulates the overall binding (—△—); moreover, it overcomes, to a great extent, the inhibitory action of tetracyline (—▲—). We ascribe the stimulatory effect observed to the A-site binding only, because kanamycin:

(i) Restores the binding of peptidyl-tRNA to the tetracycline-sensitive, i.e., A site;
(ii) Does not affect the D-site binding on 70 S ribosomes [3,14].

With this assumption, we can compute the binding isotherm of peptidyl-tRNA for the A site separately, as in [8]. Kanamycin increases 20-fold the affinity constant of peptidyl-tRNA to the A site of the 30 S . poly(U) complex (fig.2).

Fig.2C demonstrates the kinetics of binding of Ac-Phe-tRNA^{Phe} to 70 S ribosomes, when the amount of the former is not sufficient to saturate both D and A sites. Comparing the curves without and with tetracycline, we see that the D sites are filled completely ($\bar{\nu}^D = 1$), whereas the equilibrium $\bar{\nu}^A$ value is equal to 0.4. This results from the fact that the affinity of peptidyl-tRNA for the D site is 20–50-fold higher than for the A site [10,16,17]. Kanamycin stimulates the A-site binding (cf. —○— and —●—); it is easy to calculate from experimental data that the K_a^A-value is increased 3-fold (from $4.3 \cdot 10^7$–$1.3 \cdot 10^8 M^{-1}$).

To re-examine the influence of kanamycin on the peptidyl-transferase activity of ribosomes, we used the test-system in [8]. In the first stage of the experiment, two molecules of Phe-tRNA^{Phe} were bound to the 30 S . poly(U) complex in the absence or presence of kanamycin; then the reaction of transpeptidation was triggered by the addition of 50 S subunits. To stop the peptide bond synthesis immediately, EDTA equimolar to [Mg^{2+}] in incuba-

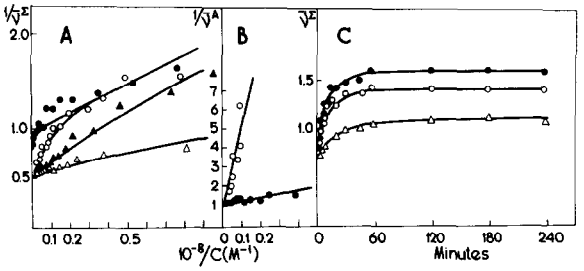

Fig.2. Kanamycin action on the binding of Ac-Phe-tRNA^{Phe} to the 30 S subunits and 70 S ribosomes. (A) Incubation mixtures contained in 250 μl buffer I (with 20 mM Mg^{2+}): 10 pmol 30 S subunits, 15 μg poly(U) and 9–200 pmol Ac-[^{14}C]Phe-tRNA^{Phe}. After 2 h incubation at 24°C $\bar{\nu}^{\Sigma}$ values were determined by the nitrocellulose filter technique. Mixtures contained: $2 \cdot 10^{-5}$ M tetracycline (—●—); 10^{-4} M kanamycin (—△—); both antibiotics (—▲—); no antibiotics (—○—). (B) A plot of $1/\bar{\nu}^A$ vs $1/C$ without (—○—) and with (—●—) kanamycin (calculated from the data in (A). (C) Kinetics of binding of Ac-Phe-tRNA^{Phe} to 70 S ribosomes. Mixtures contained in 250 μl of buffer I (with 20 mM Mg^{2+}): 10 pmol 30 S subunits, 12 pmol 50 S subunits, 10 μg poly(U) and 21 pmol Ac-[^{14}C]Phe-tRNA^{Phe} in the absence (—○—) or presence of 10^{-4} M kanamycin (—●—). Mixtures were incubated at 24°C and $\bar{\nu}^{\Sigma}$-values were determined after indicated times; (—△—) control in the presence of $2 \cdot 10^{-5}$ M tetracycline.

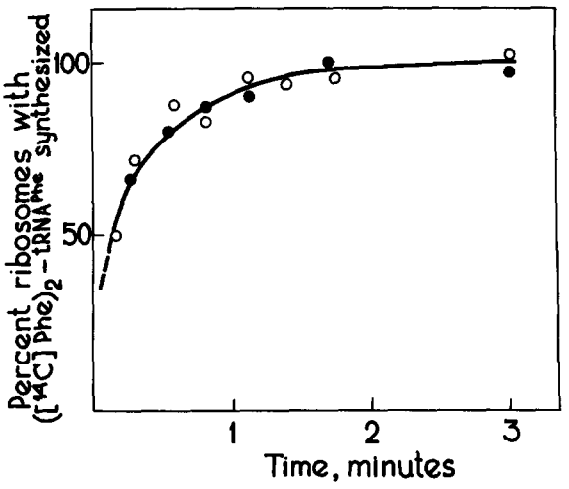

Fig.3. Kanamycin action on peptide bond synthesis. Two mixtures contained in 300 μl buffer I (with 20 mM Mg^{2+}): 150 pmol 30 S subunits, 75 μg poly(U) and 570 pmol [^{14}C]Phe-tRNA^{Phe} in the absence or presence of 10^{-4} M kanamycin. After 30 min incubation at 0°C, when $\bar{\nu}^{\Sigma}$-values became close to 2 (not shown), 180 pmol 50 S subunits was added to each mixture, and kinetics of $(Phe)_2$-tRNA^{Phe} synthesis was measured without (—○—) or with (—●—) kanamycin.

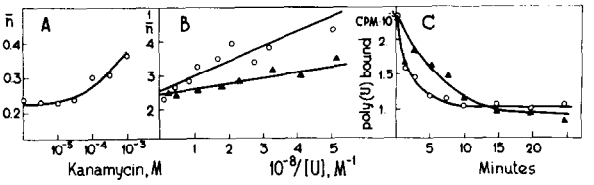

Fig.4. Kanamycin action on poly(U) . 70 S ribosome interaction. (A) Stimulation of binding. Mixtures contained in 3 ml buffer I: 20 pmol 30 S subunits, 25 pmol 50 S subunits, 25 pmol poly([^{3}H]U) and kanamycin with different final concentrations. After 10 min incubation at 30°C, $\bar{n}$-values were determined using alkali-treated nitrocellulose filters as described in [9]. (B) Measurement of binding isotherms (in inverse coordinates). Mixtures contained in 0.2—10 ml buffer I: 20 pmol 30 S subunits, 25 pmol 50 S subunits and 25 pmol poly([^{3}H]U) in the presence (—▲—) or absence (—○—) of 10^{-4} M kanamycin. Incubation was for 30 min at 30°C. (C) Kinetics of exchange of ^{3}H-labeled poly(U) with the unlabeled poly(U). Mixtures contained in 0.4 ml buffer I: 20 pmol 30 S subunits, 25 pmol 50 S subunits and 17 pmol poly([^{3}H]U) with or without kanamycin (final conc. $3 . 10^{-4}$ M). After 10 min incubation at 0°C, 60 pmol unlabeled poly(U) was added to each mixture, and the kinetics of exchange was measured in the absence (—○—) or presence (—▲—) of the antibiotic.

tion mixtures was added at indicated times, as in [15]. Diphenylalanines form very fast in such a system, even at 0°C: $(Phe)_2$-tRNA^{Phe} was synthesized in 50% ribosomes after 8 s, and the reaction was completed in all ribosomes after 1 min (fig.3). Kanamycin has no effect on either kinetics or yield of dipeptides formed. This result is in contrast with the data in [5].

Finally, we verified whether kanamycin contributes to mRNA . ribosome interaction. This was reasonable because translocation had been postulated to depend on both relative affinities of peptidyl-tRNA for the D and A sites, and the affinity of mRNA for the ribosome [17,18]. Kanamycin stimulates the binding of poly ([^{3}H]U) to 70 S ribosomes (fig.4A) because of the increase of the affinity constant (fig.4B) 3—10-fold (in different experiments). In addition, kanamycin reduces the exchange rate of ^{3}H-labeled poly(U) for unlabeled poly(U) (Fig.4C). However, this effect of the antibiotic on the mRNA . ribosome complex seems to be unspecific, because the largest stimulation of the binding is observed at 10^{-3} M kanamycin, at which concentration several additional molecules were found to bind to both ribosomal subunits [2].

4. CONCLUSION

Two molecules of kanamycin bind tightly to the ribosome, one to the small, and another to the large subunit [2]. However, the precise role of each in affecting particular ribosomal functions (section 1) was not known.

Summarizing:

(i) Kanamycin increases 20-fold the affinity of peptidyl-tRNA for the A site of 30 S subunits. This is a strong evidence that the molecule of the antibiotic bound to the small subunit is responsible for the fixing of peptidyl-tRNA to the A site of 70 S ribsome and, therefore, for the inhibition of translocation;

(ii) At high concentrations (10^{-4}—10^{-3} M) kanamycin stabilizes the 70 S . poly(U) complex. It leads to additional fixing of the peptidyl-

tRNA . mRNA complex to the A site of ribosome;

(iii) The role of a kanamycin molecule bound to the 50 S subunit is unclear; but, definitely, it does not interfere with the peptidyl-transferase activity of ribosomes.

REFERENCES

[1] Modolell, J. and Vazquez, D. (1977) Eur. J. Biochem. 81, 491–497.
[2] Misumi, M., Nishimura, T., Komai, T. and Tanaka, N. (1978) Biochem Biophys. Res. Commun. 84, 358–365.
[3] Misumi, M. and Tanaka, N. (1980) Biochem. Biophys. Res. Commun. 92, 647–654.
[4] Tanaka, N., Sashikata, K., Nishimura, T. and Umezawa, H. (1964) Biochem. Biophys. Res. Commun. 16, 216–220.
[5] Suzuki, J., Kunimoto, T. and Hori, M. (1970) J. Antibiot. 23, 99–101.
[6] Vazquez, D. (1979) in: Inhibitors of Protein Biosynthesis, Springer-Verlag, Berlin.
[7] Nierhaus, K. and Wittmann, H.G. (1980) Naturwissenschaften 67, 234–250.
[8] Kirillov, S.V., Makhno, V.I. and Semenkov, Yu.P. (1980) Nucleic. Acids Res. 8, 183–196.
[9] Katunin, V.I., Semenkov, Yu.P., Makhno, V.I. and Kirillov, S.V. (1980) Nucleic. Acids Res. 8, 403–421.
[10] Odintsov, V.B. and Kirillov, S.V. (1978) Nucleic Acids Res. 5, 3871–3879.
[11] Arai, K., Kawakita, M. and Kaziro, H. (1972) J. Biol. Chem. 247, 7029–7037.
[12] Leder, P. and Bursztyn, H. (1966) Biochem. Biophys. Res. Commun. 25, 233–247.
[13] Watanabe, S. (1972) J. Mol. Biol. 67, 443–457.
[14] Semenkov, Yu.P., Makarov, E.M., Makhno, V.I. and Kirillov, S.V. (1982) FEBS Lett., 144, 125–129.
[15] Thompson, R., Dix, D. and Ecclestone, J. (1980) J. Biol. Chem. 255, 11088–11090.
[16] Peters, M. and Yarus, M. (1979) J. Mol. Biol. 134, 471–491.
[17] Holschuh, Reisner, D. and Gassen, H.G. (1981) Nature 293, 675–677.
[18] Holschuh, K. and Gassen, H.G. (1980) FEBS Lett. 100, 169–172.