EFFECTS OF AMINOGLYCOSIDE ANTIBIOTICS ON INITIATION OF VIRAL RNA-DIRECTED PROTEIN SYNTHESIS

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Streptomycin, kanamycin, gentamicin, and kasugamycin were observed to inhibit f2 phage RNA-directed protein synthesis in an *Escherichia coli* system. When the reaction mixture was allowed to translate viral RNA for 5 minutes before addition of antibiotics, the synthesis was completely blocked by streptomycin or thiopeptin; but significant synthesis continued in the presence of kasugamycin, kanamycin and gentamicin. The results suggested that kasugamycin, kanamycin and gentamicin may inhibit initiation, and streptomycin may interfere with both chain initiation and elongation. Thiopeptin may affect chain elongation. The binding of fMet-tRNA to 70S ribosomes in the presence of f2 RNA was inhibited by streptomycin, kanamycin, gentamicin, and kasugamycin. The release of fMet-tRNA from the initiation complex was significantly induced by streptomycin, kanamycin, and gentamicin; but not by kasugamycin. The results indicated that the inhibition by kasugamycin of 30S initiation complex formation may result in the apparent inhibition of 70S initiation complex formation. And the apparent inhibition by streptomycin, kanamycin, and gentamicin of 70S initiation complex formation may be caused by breakdown of the complex and inhibition of ribosomal dissociation. The effects of streptomycin, kanamycin, and gentamicin were more significant with the initiation complex formed on washed 70S ribosomes than with the complex formed on unwashed 70S ribosomes. The ribosomal proteins washed out in 1 M NH_4Cl protected the target sites from the antibiotic actions. The T factor- and messenger-dependent binding of Ala-tRNA to the ribosomes was significantly affected by streptomycin, but not by kanamycin, gentamicin, and kasugamycin.

The site of action of aminoglycoside antibiotics has been identified as the 30S ribosomal subunit[1~6,12], but the nature of disturbances induced in the translation mechanism seems to be complicated. The results reported by various investigators appear to be controversial[7~11].

LUZZATTO *et al.*[7] observed the accumulation of monosomes in *E. coli* cultures treated with streptomycin, and suggested that the antibiotic interrupts the ribosomal cycle at the initiation of protein synthesis. *In vitro* streptomycin did not prevent the binding of fMet-tRNA to the 30S subunit-mRNA complex and the subsequent joining of the 50S subunit, but caused release of the fMet-tRNA bound in the 70S ribosomes[9~11]. In contrast, another aminoglycoside kasugamycin was demonstrated to block formation of the 30S initiation complex[12].

The activity of aminoglycoside antibiotics, including streptomycin, kanamycin, gentamicin and kasugamycin, on initiation of viral RNA-directed protein synthesis

has been compared in an *in vitro* system, and the results are presented in this publication.

Materials and Methods

E. coli tRNA, ATP, GTP, PEP and pyruvate kinase were the products of Boehringer. ^{14}C-Valine and -methionine, and ^{3}H-alanine and -methionine were purchased from Daiichi Chemical Co. Streptomycin, kanamycin, kasugamycin and gentamicin were supplied by National Institute of Health, Tokyo. Thiopeptin was kindly given by Dr. H. SAKAI, Fujisawa Pharmaceutical Co. Puromycin was the product of Lederle. Folinic acid was purchased from General Biochemicals.

The preparation of S-30 fraction, ribosomes and initiation factor from *E. coli* Q 13 followed the method of OHTA *et al.*[18]. The ribosomes were washed in 0.1 M NH_4Cl or 1 M NH_4Cl, containing 50 mM Tris-HCl, pH 7.5, 10 mM Mg $(AcO)_2$ and 6 mM 2-mercaptoethanol. The former is termed unwashed ribosomes and the latter the washed ribosomes in this paper. RNA of f2 phage was prepared by the phenol extraction method of NATHANS *et al.*[19] *E. coli* tRNA was acylated with ^{14}C-methionine or ^{3}H-alanine by the method of HERSHEY and THACH[20]. *E. coli* tRNA was fractionated by BD cellulose chromatography. *E. coli* $tRNA_F^{Met}$ was kindly given by Dr. S. NISHIMURA, National Cancer Center, Tokyo. *E. coli* T factor was generously supplied by Dr. Y. KAZIRO, Institute of Medical Science, University of Tokyo.

Results

The f2 phage RNA-directed protein synthesis was studied in an *E. coli* system. The incorporation of valine was inhibited by aminoglycoside antibiotics. At the concentration of 2×10^{-5} M, 95 % inhibition was observed with streptomycin, 76 % inhibition with kanamycin, 78 % inhibition with gentamicin, and 65 % inhibition with kasugamycin, when the antibiotics were added at the start of protein synthesis (Fig. 1).

Fig. 1. Inhibition by aminoglycoside antibiotics of f2 phage RNA-directed protein synthesis.

The reaction mixture contained: 50 mM Tris-HCl, pH 7.5, 160 mM NH_4Cl, 8 mM $Mg(AcO)_2$, 6 mM 2-mercaptoethanol, 3 mg protein/ml S-30 fraction of *E. coli* Q 13 extracts, 500 μg/ml of f2 RNA, 150 μg/ml *E. coli* tRNA, 0.2 μCi/ml ^{14}C-valine (165 μCi/μmole), 0.025 mM amino acids except valine, 2 mM ATP, 5 mM PEP, 20 μg/ml pyruvate kinase, and 0.1 mM GTP; 0.2 ml in each tube. It was incubated at 37°C for 30 minutes. The TCA-insoluble radioactivity was determined with correction for the values obtained in parallel mixtures without messenger.

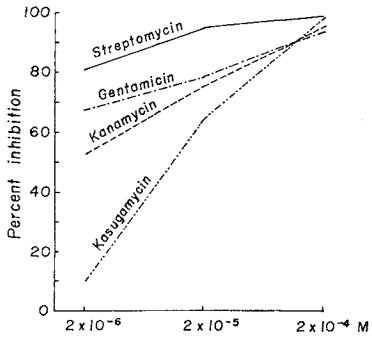


Fig. 2. Kinetics of inhibition by antibiotics of f2 RNA-directed protein synthesis.

The reaction mixture was as described in the legend of Fig. 1.

↓ Addition of antibiotics

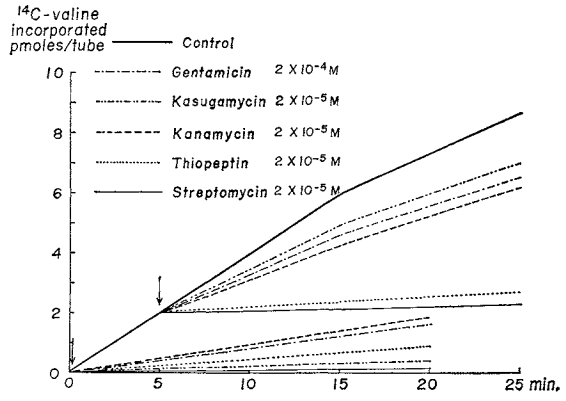
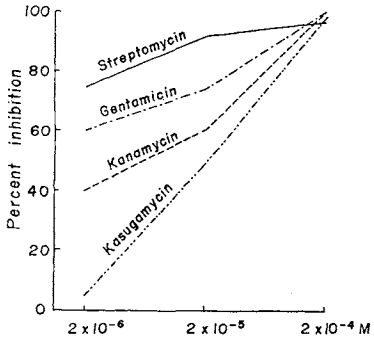


Fig. 3. Effect of aminoglycosides on binding of fMet-tRNA to 70S ribosomes in the presence of f2 RNA.

The reaction mixture contained: 50 mM Tris-HCl, pH 7.5, 60 mM NH_4Cl, 6 mM $Mg(AcO)_2$, 10 mM 2-mercaptoethanol, 2 mg/ml washed 70S ribosomes, 0.6 mg/ml initiation factor, 150 μg/ml f-^{14}C-Met-tRNA (300,000 cpm/mg), 1 mg/ml f2 RNA, 0.2 mM GTP; 0.1 ml in each tube. It was incubated at 37°C for 15 minutes. The radioactivity, collected on Millipore filter, was assayed with corrections for values without messenger.



The reaction mixture was allowed to translate viral RNA for 5 minutes and then the antibiotics were added. Further protein synthesis was completely inhibited by streptomycin. But the grade of inhibiton by kasugamycin, kanamycin and gentamicin was significantly less than that observed when they were addded at the beginning of protein synthesis (Fig. 2). The concentration of kasugamycin employed was 10 times that for other aminoglycosides since it is much less effective in inhibiting bacterial growth and protein synthesis[12]. Thiopeptin, a peptide antibiotic, has been demonstrated to block chain elongation[13]. It was observed to exhibit the same tendency of inhibition as streptomycin (Fig. 2). From these results we suggest that kasugamycin, kanamycin, and gentamicin inhibit a process at or near initiation more markedly than they affect chain elongation. Streptomycin may interfere with chain elongation or with both chain initiation and elongation.

Table 1. Effect of streptomycin and kasugamycin on binding of fMet-tRNA to unwashed 70S ribosomes in the presence of f2 phage RNA

fMet-tRNA	Antibiotics	fMet-tRNA bound (pmoles)	% Inhibition
Unfractionated (^{14}C-labelled)	None	8.32	
	Streptomycin 2×10^{-5} M	5.16	38
	Kasugamycin 2×10^{-4}	1.50	82
Fractionated (^{3}H-labelled)	None	7.11	
	Streptomycin 2×10^{-5} M	5.97	16
	Kasugamycin 2×10^{-4}	1.56	78

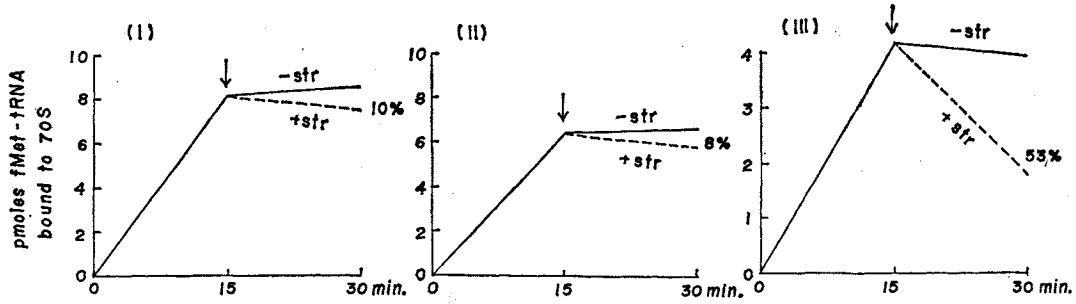
The binding of fMet-tRNA was performed in the reaction mixture, as described in the lengend of Fig. 3, in which washed 70S ribosomes and initiation factors were replaced by 2 mg/ml unwashed 70S ribosomes. In the case fractionated $tRNA_F^{Met}$, 30 μg/ml f-^{3}H-Met-$tRNA_F$ (1,024 μCi/μmole) was used.

Fig. 4. Release by streptomycin of fMet-tRNA from the 70S initiation complex.

str: streptomycin, %: % breakdown of the 70S initiation complex.

The 70S initiation complex was formed as described in the legend of Fig. 3. At 15 minutes, streptomycin was added at the concentration of 2×10^{-5} M and further incubated for 15 minutes.

(I) Unwashed ribosomes (70S) and f-^{14}C-Met-tRNA (unfractionated) were used.
(II) Unwashed 70S ribosomes and f-^{3}H-Met-$tRNA_F$ (fractionated) were used.
(III) Washed 70S ribosomes, initiation factors and f-^{14}C-Met-tRNA (unfractionated) were used.



The binding of fMet-tRNA to washed 70S ribosomes in the presence of f2 RNA and initiation factors was significantly affected by streptomycin, kanamycin, genta-

micin, and kasugamycin. The extents of inhibition were comparable with those of protein synthesis (Figs. 1 and 3).

In the experiments, in which washed ribosomes and initiation factors were replaced by unwashed ribosomes, the inhibition by streptomycin of fMet-tRNA binding was significantly reduced. However, virtually the same order of inhibition was observed with kasugamycin (Table 1). No significant difference was found with fractionated fMet-$tRNA_F$ and with unfractionated fMet-tRNA.

Streptomycin, kanamycin, and gentamicin were observed to induce release of up to 60% of fMet-tRNA bound to the 70S initiation complex (Fig. 4 and Table 3). The grade of induced breakdown of the 70S initiation complex was less than that of inhibition of the 70S initiation complex formation. It indicated that the apparent inhibition by these antibiotics of initiation complex formation may be caused not only by breakdown of the complex but also by the inhibition of ribosomal dissociation[14].

The fMet-tRNA release was much less induced by the antibiotics, when the initiation complex was formed with unwashed ribosomes; up to 20% release was observed (Fig. 4 and Table 3). Most of fMet-tRNA was puromycin-reactive in the initiation complex formed with unwashed 70S ribosomes as well as with washed 70S ribosomes and initiation factors. Ala-tRNA or deacylated $tRNA^{Ala}$ was observed not

Table 2. The reactivity of $fMet\text{-}tRNA_F$ in the 70S initiation complex to puromycin

System	fMet-puromycin formed (pmoles)
Complete	4.64
− f2 RNA	1.01
− puromycin	0.48
+ tRNA 200 μg/ml	3.06
+ Ala-$tRNA^{Ala}$ 50 μg/ml	3.39
+ $tRNA^{Ala}$ 30 μg/ml	3.62

The 70S initiation complex was formed with unwashed 70S ribosomes as described in Table 1. At 15 minutes, puromycin was added at the concentration of 2×10^{-4} M, and the reaction mixture was further incubated for 15 minutes. The reaction was terminated by addition of 1 ml of 0.1 M sodium acetate, pH 5.5, and fMet-puromycin was extracted with ethyl acetate.

Table 3. Effect of antibiotics on release of fMet-tRNA from 70S initiation complex and on binding of Ala-tRNA to it

	Antibiotics	f-^{14}C-Met-tRNA		^{3}H-Ala-tRNA	
		Bound (pmoles)	Release (%)	Bound (pmoles)	Inhibition of binding (%)
I. Initiation complex formed with washed 70S and initiation factors.	None	1.26		1.03	
	Streptomycin 2×10^{-5} M	0.63	50	0.36	65
	Kanamycin 2×10^{-5}	0.64	49	0.52	50
	Gentamicin 2×10^{-5}	0.73	42	0.50	51
	Kasugamycin 2×10^{-4}	1.17	7	0.98	5
	Thiopeptin 2×10^{-5}	1.22	3	0.04	96
II. Initiation complex formed with unwashed 70S ribosomes.	None	7.54		5.82	
	Streptomycin 2×10^{-5} M	6.11	19	2.33	60
	Kanamycin 2×10^{-5}	6.11	19	5.35	8
	Gentamicin 2×10^{-5}	6.03	20	4.83	17
	Kasugamycin 2×10^{-4}	7.40	2	5.83	0
	Thiopeptin 2×10^{-5}	6.94	8	0.00	100

Exp. I. The reaction mixture contained: 50 mM Tris-HCl, pH 7.5, 60 mM NH_4Cl, 6 mM $Mg(AcO)_2$, 10 mM 2-mercaptoethanol, 2 mg/ml 70S initiation complex, 10 μg/ml T factor, 0.2 mM GTP, ^{3}H-Ala-tRNA (3,000 μCi/μmole), and antibiotics; 0.1 ml in each tube. It was incubated at 37℃ for 10 minutes. The radioactivity of ^{3}H-Ala-tRNA was determined with correction for values without T factor. The 70S initiation complex was formed as described in Fig. 3 and was pelletted and washed by ultracentrifugation.

Exp. II. The 70S initiation complex was formed as described in Table 1. At 15 minutes, the tubes were cooled to 0℃ and 10 μg/ml T factor, 0.2 mM GTP, ^{3}H-Ala-tRNA and antibiotics were added in 0.2 ml final volume. It was incubated further for 10 minutes at 37℃. The radioactivity of ^{14}C and ^{3}H was determined with correction for values without messenger.

to stimulate the fMet-puromycin reaction. It indicated that most of fMet-tRNA was bound to the donor stie in the 70S initiation complex (Table 2). The results also suggested that the ribosomal proteins washed out in 1 M NH_4Cl may protect the target sites of the aminoglycoside antibiotics, or may exhibit a certain unknown functions for the activity of the antibiotics. The activity of kasugamycin on the initiation complex formation was virtually the same both with unwashed ribosomes and with washed ones (Fig. 3 and Table 1).

Table 4. Effects of aminoglycoside antibiotics on fMet-puromycin reaction

Antibiotics	fMet-puromycin formed (pmoles)	% Inhibition
None	8.67	
Kasugamycin 2×10^{-4} M	8.70	0
Kanamycin 2×10^{-5}	8.43	3
Gentamicin 2×10^{-5}	8.26	5
Streptomycin 2×10^{-5}	7.91	9

The assay procedure was the same as described in Table 2. The aminoglycosides were added simultaneously at 15 minutes.

Kasugamycin did not induce a significant release of fMet-tRNA from the 70S initiation complex, while it blocked the initiation complex formation (Table 3 and Fig. 3).

The T factor- and messenger-dependent binding of Ala-tRNA to 70S ribosomes was inhibited by streptomycin but was not significantly affected by kasugamycin (Table 3). The results were in accordance with the assumption that streptomycin interferes with chain elongation as well as chain initiation, while kasugamycin selectively inhibits chain elongation.

The binding of Ala-tRNA was reduced by the presence of kanamycin and gentamicin with the washed ribosomes, from which fMet-tRNA was significantly released. But the binding was less affected with the unwashed ribosomes, from which less fMet-tRNA was released (Table 3). Most of fMet-tRNA was attached to the donor site in both ribosomes (Table 2). If mRNA was released by kanamycin and gentamicin concomitantly with fMet-tRNA from the ribosomes[11], then mRNA-dependent Ala-tRNA binding did not occur, regardless of the antibiotic effects. On the contrary, if mRNA was still attached to the ribosomes[7], the results indicated that kanamycin and gentamicin may inhibit Ala-tRNA binding to the initiation complex formed with washed ribosomes but may not significantly affect the binding to the ones formed with unwashed ribosomes. The discrepancy may be due to the different sensitivity of the two forms of ribosomes to the antibiotics. Formylmethionyl-puromycin formation was not significantly affected by kasugamycin, kanamycin, gentamicin, and streptomycin (Table 4).

Discussion

The results presented in this publication and the previous one[12] indicate that kasugamycin is a selective inhibitor of initiation of protein synthesis. It inhibits the formation of 30S initiation complex and subsequently the 70S initiation complex formation, while the other aminoglycoside antibiotics do not significantly affect the 30S initiation complex formation. Contrary to streptomycin, kasugamycin does not significantly induce release of fMet-tRNA from the initiation complex nor inhibit the binding of Ala-tRNA to the ribosomes which is dependent on T factor and messenger (f2 RNA).

Streptomycin induces release of fMet-tRNA from the 70S initiation complex. The results are in accordance with those of LELONG *et al.*[9,10] and those of MODOLLEL and

DAVIS[11]. It also inhibits the binding of Ala-tRNA to the ribosomes with f2 RNA in the presence of T factor and GTP. The results indicate that streptomycin affects chain elongation as well as chain initiation.

The release by streptomycin of fMet-tRNA from the 70S initiation complex is much reduced when unwashed ribosomes are employed instead of washed ribosomes and initiation factors. The results may be in accordance with those of LENNETTE and APIRION[8], who have observed that fMet-tRNA is attached to streptomycin-monosomes formed *in vivo*. Streptomycin, kanamycin, and gentamicin are observed to exhibit more pronounced effects with washed ribosomes than with unwashed ribosomes. The results seem to be in accordance with the view that the site of action of the aminoglycosides has to be uncovered first *in vivo*[15,16]. The binding of ^{3}H-streptomycin to ribosomes is markedly enhanced by the washing procedure with 1 M NH_4Cl (unpublished data).

Kanamycin and gentamicin also induce release of fMet-tRNA from the 70S initiation complex. The results are different from those of LELONG *et al.*[9] The discrepancy remains to be elucidated. However, since the sensitivity of washed ribosomes to the antibiotics is significantly different from that of unwashed ribosomes, the discrepancy may be due to the state of ribosomes employed.

The effects of kanamycin and gentamicin on chain elongation, particularly on Ala-tRNA binding, have been not well established by the methods employed. However, it was demonstrated by the kinetic study that kanamycin and gentamicin affect chain initiation more markedly than chain elongation.

Streptomycin, kanamycin, neomycin and spectinomycin have been reported to inhibit dissociation of 70S ribosomes by initiation factor F_3[14]. The inhibition by streptomycin and kanamycin of the 70S initiation complex formation may be the sequence of the inhibition of ribosomal dissociation. The grade of inhibition of the 70S initiation complex formation is higher than the grade of induced release of fMet-tRNA from the complex. Therefore it is plausible that the inhibition by the aminoglycoside antibiotics of ribosomal dissociation and the induced brakdown of 70S initiation complex result in the apparent inhibition of 70S initiation complex formation.

Thiopeptin, a peptide antibiotic, has been observed to interact with 50S subunit of ribosomes and interfere with both T and G factor-associated functions[13]. It does not significantly affect chain initiation nor induce breakdown of the initiation complex. But it markedly inhibits T factor-dependent binding of Ala-tRNA to the ribosomes with f2 RNA. The results are in accordance with the previous paper[13].

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