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MECHANISM OF INHIBITION OF TRANSLOCATION BY KANAMYCIN AND VIOMYCIN: A COMPARATIVE STUDY WITH FUSIDIC ACID

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SUMMARY

Kanamycin and viomycin were found to block a single cycle of translocation on the poly[U]-ribosome, carrying N-acetyl-diPhe-tRNA on the acceptor site and deacylated tRNA at the donor site. The inhibition of translocation was demonstrated by enhanced puromycin-reactivity of N-acetyl-diPhe-tRNA and by release of deacylated tRNA. The GTPase reaction, catalyzed by EF-G and ribosomes, was not significantly affected by the antibiotics. The results with kanamycin and viomycin differed from those obtained with fusidic acid, indicating that the mechanism of translocation inhibition may be different. Kanamycin and viomycin seemed to interfere with the translocation by fixing N-acetyl-Phe-tRNA to the acceptor site, but not to the donor site.

Kanamycin, viomycin, and related antibiotics have been recently observed to interact with both large and small subunits of ribosomes, and to inhibit translocation of peptidyl-tRNA from the acceptor site to the donor site (1-6). The results of a more detailed study of the mechanism of translocation inhibition are presented in this communication.

Since fusidic acid is known to block the overall translocation without affecting the first cycle of translocation (7-9)[cf. a review (20)], we have investigated the effects of kanamycin and viomycin on a single cycle of translocation on the ribosome, carrying N-acetyl-diPhe-tRNA on the acceptor site and deacylated tRNA on the donor site. In addition, the effects of the antibiotics on the interaction of EF-G, GTP and ribosomes with or without fusidic acid, and stabilization of peptidyl-tRNA at the acceptor and donor sites have been examined.

MATERIALS AND METHODS

[^{14}C]Phenylalanine (513 mCi/mmole) and [5,6-^{3}H]uracil (43.4 Ci/mmole) were purchased from New England Nuclear, Boston, Mass., and [^{14}C]GTP (495 mCi/mmole) from the Radiochemical Centre, Amersham, England. Puromycin, poly[U], *E. coli* tRNA, GTP and GMPP(NH)P were products of Boehringer-Mannheim, Germany.

EF-G and 1 M NH_4Cl-washed ribosomes were prepared from *E. coli* Q13, as described previously (3). N-Acetyl-[^{14}C]phenylalanyl-tRNA was synthesized by the method of Haenni and Chapeville (10). The preparation of [^{3}H]tRNA and the assay for the release of [^{3}H]

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tRNA during translocation followed the procedure of Lucas-Lenard and Haenni (11). The release of $tRNA^{Phe}$ was assayed by the method of Ishitsuka et al. (14). Puromycin reaction was carried out at 0°C for 30 min. by the technique of Leder and Bursztyn (12).

RESULTS

Effects of antibiotics on a single cycle of translocation of N-acetyl-diPhe-tRNA:

A single cycle of translocation was observed by GMPP(NH)P- and EF-G-dependent N-acetyl-diphenylalanyl-puromycin synthesis on the ribosome, possessing N-acetyl-diPhe-tRNA at the acceptor site and deacylated $tRNA^{Phe}$ at the donor site. The translocation was promoted by the presence of a stoichiometric amount of EF-G, when GMPP(NH)P was substituted for GTP. The single cycle of translocation was markedly inhibited by kanamycin and viomycin, but not by fusidic acid, when a substrate amount of EF-G was employed (Fig. 1). In a simultaneous experiment, the translocation with GTP and a catalytic amount of EF-G was prevented by all the three antibiotics (data are not shown). The results were in accord with the previous reports (1,3,5,7-9).

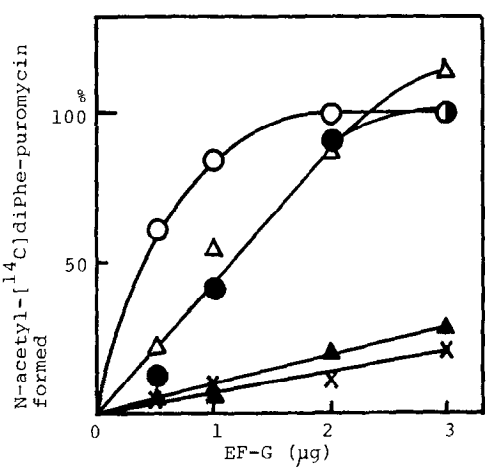

Fig. 1. The effects of antibiotics on the translocation of N-acetyl-[^{14}C]diPhe-tRNA in the presence of GMPP(NH)P: The dependency upon EF-G concentration.

The assay for the translocation was performed by the procedure of Inoue-Yokosawa et al.(13). The reaction mixture, in 0.05 ml, contained: 50 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 160 mM NH_4Cl, 10 mM 2-mercaptoethanol, *E. coli* ribosomes (70 μg) carrying 5.5 pmoles of N-acetyl-[^{14}C]diPhe-tRNA, 0.1 mM GTP or GMPP(NH)P, and EF-G. The 100 % value obtained with GTP and 2.0 μg of EF-G was 4.8 pmoles, and that with GMPP(NH)P and 3.0 μg of EF-G was 3.6 pmoles.
(○) GTP, (●) GMPP(NH)P, (✕) GMPP(NH)P + 0.1 mM kanamycin, (▲) GMPP(NH)P + 0.1 mM viomycin, and (△) GMPP(NH)P + 1 mM fusidic acid.

Table 1. The effects of antibiotics on the release of deacylated $tRNA^{Phe}$ and translocation of N-acetyl-diPhe-tRNA on the ribosome with N-acetyl-diPhe-tRNA at the acceptor site and deacylated $tRNA^{Phe}$ at the donor site.

Additions	Exp. I	Exp. II	
	$tRNA^{Phe}$ released	[^{3}H]tRNA bound	N-Ac-[^{14}C]diPhe-puromycin formed
None	0.7	9.4	0.8
EF-G	1.1	9.3	0.4
EF-G, GTP	9.3	4.6	9.7
EF-G, GTP, fusidic acid 1 mM	10.4	4.4	9.6
EF-G, GTP, viomycin 0.1 mM	2.2	9.0	1.7
EF-G, GTP, kanamycin 0.1 mM	2.7	8.6	2.0
EF-G, GMPP(NH)P	7.1	5.2	7.1
EF-G, GMPP(NH)P, fusidic acid 1 mM	7.6	4.3	7.8
EF-G, GMPP(NH)P, viomycin 0.1 mM	2.4	8.3	1.4
EF-G, GMPP(NH)P, kanamycin 0.1 mM	2.0	8.0	1.6

The number represents pmoles per 0.1 ml of the reaction mixture.
Exp. I: The assay was performed by the method of Inoue-Yokosawa et al.(13). The reaction mixture, in 0.1 ml, contained: 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, 140 μg *E. coli* ribosomes with poly[U] and 11 pmoles of N-acetyl-[^{14}C]diPhe-tRNA and $tRNA^{Phe}$; and 0.1 mM GTP or GMPP(NH)P, and 8 μg EF-G where indicated.
Exp. II: The reaction mixture was the same as above, except that the ribosome with 12 pmoles of N-acetyl-[^{14}C]diPhe-tRNA and [^{3}H]tRNA were used.

Effects of antibiotics on the release of deacylated $tRNA^{Phe}$ from the donor site during translocation:

The exit of deacylated $tRNA^{Phe}$ from the ribosomal donor site with concomitant translocation of N-acetyl-diPhe-tRNA from the acceptor site to the donor site was assayed by the following two procedures. In the first method, the release of deacylated $tRNA^{Phe}$ was determined by aminoacylation with [^{14}C]phenylalanine by Phe-tRNA synthetase. In the second one, the amount of [^{3}H]tRNA bound to ribosomes was measured by a nitrocellulose (Millipore) filter technique. The results are presented in Table 1. In Exp. I, using the first assay, the exit of deacylated $tRNA^{Phe}$ from the donor site occurred in the presence of a substrate amount of EF-G with GTP or GMPP(NH)P. It was profoundly blocked by kanamycin and viomycin, but not by fusidic acid. In Exp. II, using the second procedure, the release of [^{3}H]tRNA and simultaneous translocation of N-acetyl-[^{14}C]diPhe-tRNA, induced by a substrate amount of EF-G with GTP or GMPP(NH)P, were also inhibited by kanamycin and viomycin, but not significantly by fusidic acid.

Effects of antibiotics on the release of N-acetyl-Phe-tRNA bound to the acceptor or donor site:

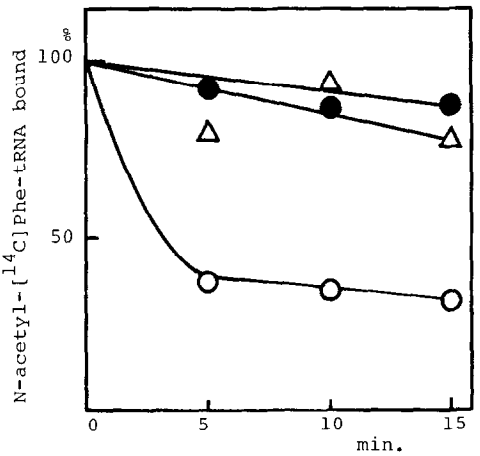

Fig. 2. Kinetics of protection by antibiotics against the release of N-acetyl-[^{14}C]Phe-tRNA from the ribosomal acceptor site, induced by NH_4^+ depletion.

The non-enzymic binding of N-acetyl-[^{14}C]Phe-tRNA to the acceptor site followed the procedure of Watanabe (15). The reaction mixture, in 50 µl, contained: 50 mM Tris-HCl, pH 7.5, 15 mM magnesium acetate, 40 mM NH_4Cl, 140 µg ribosomes, and 1.8 µM N-acetyl-[^{14}C]Phe-tRNA. The binding was performed at 37°C for 20 min. Then NH_4^+ was diluted to 8 mM, and the antibiotic was introduced to the mixture. The amount of bound acetyl-[^{14}C]Phe-tRNA was determined by a Millipore filter method. (○) No antibiotic, (●) 0.1 mM kanamycin, and (△) 0.1 mM viomycin.

Since N-acetyl-Phe-tRNA bound to the acceptor site is released by decreasing NH_4^+ concentration (15), the effects of antibiotics on the N-acetyl-Phe-tRNA exit were examined by this procedure. As illustrated in Fig. 2, kanamycin and viomycin were observed to block the release of N-acetyl-Phe-tRNA, indicating that the antibiotics fixed N-acetyl-Phe-tRNA to the acceptor site. The results with viomycin were in accord with those reported by Modolell and Vazquez (5).

N-Acetyl-Phe-tRNA bound to the donor site was released by increasing Mg^{2+} concentration or by digestion with ribonuclease T_1 (18). It was revealed by these methods that kanamycin and viomycin did not significantly affect the release of N-acetyl-Phe-tRNA from the donor site (Fig. 3). The results showed that the antibiotics do not fix N-acetyl-Phe-tRNA to the donor site.

Effects of antibiotics on the GTPase reaction, catalyzed by EF-G and ribosomes:

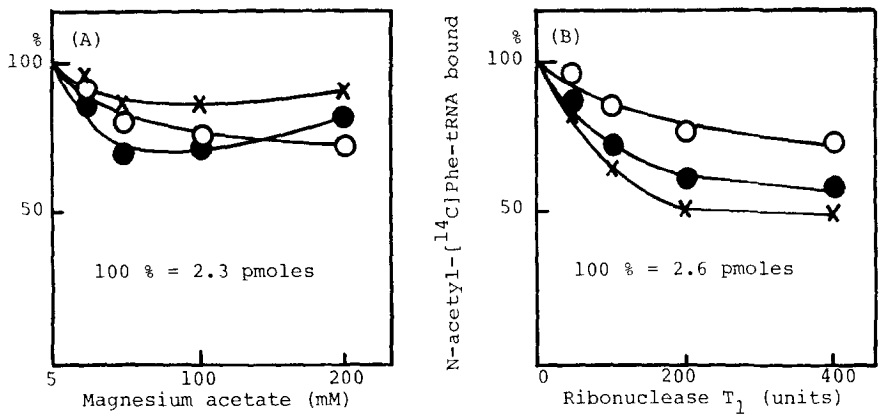

Fig. 3. The effects of antibiotics on the release of N-acetyl-[^{14}C]-Phe-tRNA from the ribosomal donor site, caused by increasing Mg^{2+} concentration (A), and by digestion with ribonuclease T_1 (B).

The binding of N-acetyl-[^{14}C]Phe-tRNA to the ribosomal donor site was carried out at 30°C for 20 min. in a reaction mixture containing: 50 mM Tris-HCl, pH 7.5, 160 mM NH_4Cl, 6 mM magnesium acetate, 6 mM 2-mercaptoethanol, 0.2 mg/ml poly[U], 2.8 mg/ml ribosomes, and 2.3 μM N-acetyl-[^{14}C]Phe-tRNA. The mixture was sedimented at 45,000 rpm for 90 min., and the pellet was resuspended in the above solution without poly[U], N-acetyl-[^{14}C]Phe-tRNA and ribosomes. The ribosomes (140 μg), carrying N-acetyl-[^{14}C]Phe-tRNA on the donor site, were then incubated with or without the antibiotic at 30°C for 15 min. in 50 μl of a buffer: 50 mM Tris-HCl, pH 7.5, 6 mM magnesium acetate, 160 mM NH_4Cl, and 6 mM 2-mercaptoethanol.

(A): The Mg^{2+} concentration was raised as indicated in Fig. (A), and the mixture was further incubated at 30°C for 20 min.

(B): The mixture was further incubated at 30°C for 20 min. with ribonuclease T_1 at concentrations, indicated in Fig. (B).

The amount of N-acetyl-[^{14}C]Phe-tRNA, bound to the ribosome, was determined by a Millipore filter method.

(○) No antibiotic, (●) kanamycin 0.1 mM, and (×) viomycin 0.1 mM.

The hydrolysis of GTP to GDP by EF-G and ribosomes was not significantly affected by kanamycin or viomycin, although a slight inhibition was observed at high antibiotic concentrations of 0.2 mM (Table 2). In a simultaneous experiment, fusidic acid blocked the GTPase reaction. The formation of EF-G-[^{14}C]GDP-ribosome complex with or without fusidic acid was not significantly affected by kanamycin or viomycin (data are not shown). The results indicated that kanamycin and viomycin do not interfere with the interaction of EF-G, GTP, and ribosomes.

DISCUSSION

Kanamycin, viomycin, and related antibiotics have been previously demonstrated to inhibit the translocation of peptidyl-tRNA in a recycling system (1-6). The current experiments show that a single

Table 2. The effects of antibiotics on ribosome-dependent GTPase reaction of EF-G.

Additions		[^{14}C]GTP hydrolyzed
None		100 % (21 pmoles)
Viomycin	0.02 mM	94
	0.2	77
Kanamycin	0.02 mM	91
	0.2	72
Fusidic acid	0.1 mM	16

The production of [^{14}C]GDP from [^{14}C]GTP was assayed by thin layer chromatography on polyethylene-imine impregated cellulose (17). The reaction mixture, in 50 µl, contained: 50 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.56 mg *E. coli* ribosomes, 0.5 µg EF-G, and 0.1 mM [^{14}C]GTP in the presence or absence of antibiotics. The mixture was incubated at 30°C for 15 min.

turnover of the translocation, resistant to fusidic acid, is also blocked by kanamycin and viomycin. The results suggest that kanamycin and viomycin are direct inhibitors of the translocation, but fusidic acid is an indirect inhibitor. GTP hydrolysis, catalyzed by EF-G and ribosomes, has been found in the present experiments to be resistant to kanamycin and viomycin. On the contrary, the GTPase reaction is known to be highly sensitive to fusidic acid and the thiostrepton group of antibiotics [cf. reviews by Tanaka (20), and by Pestka and Bodley (21)]. Therefore, the current results indicate that the mechanism of translocation inhibition by kanamycin and viomycin may be different from that by fusidic acid and thiostrepton. It is in accord with the previous reports that fusidic acid binds to EF-G (9,19), whereas kanamycin and viomycin bind to the ribosome (2,3).

In addition, the present experiments show that the inhibition of translocation by kanamycin and viomycin may be caused by fixing peptidyl-tRNA to the acceptor site but not to the donor site. The results with viomycin agree to those reported by Modolell and Vazquez (5). Both antibiotics interact with the large, as well as small, subunits of ribosomes (2,3). It remains to be determined which ribosomal subunit, large or small, possesses the acceptor site blocked by the drugs. Alternatively, both subunits may participate in preventing the exit of peptidyl-tRNA from the acceptor site. Concerning the mechanism of translocation inhibition, no substantial difference has been found between kanamycin and viomycin in the current experiments.

The mechanism of translocation of peptidyl-tRNA and release of deacylated tRNA is not well established. Inoue-Yokosawa *et al.*

(13) have suggested that a single cycle of translocation occurs without GTP hydrolysis, which is necessary to remove EF-G from the ribosome. This hypothesis seems to be supported by the current results that kanamycin and viomycin inhibit the translocation without affecting the GTPase reaction. Concerning the release of deacylated tRNA from the donor site, there appear to be two possibilities. The tRNA release may be caused by the shift of peptidyl-tRNA from the acceptor site to the donor site. Alternatively, it may occur prior to the translocation of peptidyl-tRNA (11, 14,16). The former assumption seems to be supported by the present results: i.e. kanamycin and viomycin inhibit both release of tRNA and translocation of peptidyl-tRNA.

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REFERENCES

1) Liou, Y.-F., and Tanaka, N. (1976) Biochem. Biophys. Res. Commun. 71, 477-483.
2) Misumi, M., Tanaka, N., and Shiba, T. (1978) Biochem. Biophys. Res. Commun. 82, 971-976.
3) Misumi, M., Nishimura, T., Komai, T., and Tanaka, N. (1978) Biochem. Biophys. Res. Commun. 84, 358-365.
4) Choi, E.C., Misumi, M., Nishimura, T., Tanaka, N., Nomoto, S., Teshima, T., and Shiba, T. (1979) Biochem. Biophys. Res. Commun. 87, 904-910.
5) Modolell, J., and Vazquez, D. (1977) Eur. J. Biochem. 81, 491-497.
6) Campuzano, S., Vazquez, D., and Modolell, J. (1979) Biochem. Biophys. Res. Commun. 87, 960-966.
7) Tanaka, N., Kinoshita, T., and Masukawa, H. (1968) Biochem. Biophys. Res. Commun. 30, 278-283.
8) Bodley, J.W., Zieve, F.J., Lin, L., and Zieve, S.T. (1969) Biochem. Biophys. Res. Commun. 37, 437-447.
9) Okura, A., Kinoshita, T., and Tanaka, N. (1971) J. Antibiotics 24, 655-661.
10) Haenni, A.L., and Chapeville, F. (1966) Biochim. Biophys. Acta 114, 135-138.
11) Lucas-Lenard, J., and Haenni, A.L. (1969) Proc. Natl. Acad. Sci. U.S.A. 63, 93-97.
12) Leder, P., and Bursztyn, H. (1966) Biochem. Biophys. Res. Commun. 25, 233-238.
13) Inoue-Yokosawa, N., Ishikawa, C., and Kaziro, Y. (1974) J. Biol. Chem. 249, 4321-4323.
14) Ishitsuka, H., Kuriki, Y., and Kaji, A. (1970) J. Biol. Chem. 245, 3346-3351.
15) Watanabe, S. (1972) J. Mol. Biol. 67, 443-457.
16) Roufa, D.J., Skogerson, L.E., and Leder, P. (1970) Nature 227, 567-570.

17) Kornberg, A., Scott, J.E., nad Bertsch, L.L. (1978) J. Biol. Chem. 253, 3298-3304.
18) Černá, J., Rychlík, I., and Jonák, J. (1973) Eur. J. Biochem. 34, 551-556.
19) Okura, A., Kinoshita, T., and Tanaka, N. (1970) Biochem. Biophys. Res. Commun. 41, 1545-1550.
20) Tanaka, N. (1974) Antibiotics III Mechanism of Action of Antimicrobial and Antitumor Agents pp. 436-447, Springer-Verlag, Berlin-Heidelberg-New York.
21) Pestka, S., and Bodley, J.W. (1974) ibid. pp. 551-576.