DUAL ACTIONS OF VIOMYCIN ON THE RIBOSOMAL FUNCTIONS

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Summary: Viomycin was observed to inhibit poly[U]- or f2 RNA-directed protein synthesis in an E. coli cell-free system. The former was more profoundly affected than the latter. Both initiation complex formation on the 30S ribosomal subunit and on 70S ribosomes were prevented by the antibiotic. In the peptide chain elongation process, viomycin did not significantly affect aminoacyl-tRNA binding to ribosomes and the peptidyl transferase reaction, but markedly inhibit translocation of peptidyl-tRNA from the acceptor site to the donor site. The mechanism of action of the drug appeared to be unique.

Viomycin is a basic peptide antibiotic, active primarily against Mycobacteria, and less against other bacteria. Although E. coli is rather resistant to the antibiotic, it inhibits protein synthesis in an E. coli cell-free system. Viomycin does not cause codon misreading, in spite of biological activity similar to that of aminoglycoside antibiotics (1). Yamada et al.(2) have investigated viomycin-resistant mutants of M. smegmatis, and observed that some of the resistance mutations are attributable to alteration of the 30S subunit of ribosomes and the others to alteration of the 50S subunit. Their results have stimulated us to perform further experiments concerning the mechanism of action of viomycin. Some of the effects of the antibiotic on ribosomal functions are reported in this communication.

The growth-inhibitory activity of viomycin is low with E. coli. However, since the cell-free protein-synthesizing system of E. coli is well established and is blocked by the antibiotic, this system has been employed in this study. It has been demonstrated that viomycin exhibits a dual action on the ribosomal functions.

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The antibiotic significantly affects both initiation of protein synthesis and peptide chain elongation: i.e. translocation of peptidyl-tRNA.

MATERIALS AND METHODS

[^{14}C]Phenylalanine (522 mCi/mM), and [^{14}C]leucine (283 mCi/mM) were obtained from Daiichi Pure Chemical Co., Tokyo. [^{35}S]Methionine (436.6 Ci/mM) was purchased from New England Nuclear, Boston, Mass. Viomycin was a product of Pfizer, Ltd. (Japan). Kasugamycin was generously given by Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo, and thiopeptin by Fujisawa Pharmaceutical Co., Osaka. Phosphoenolpyruvate, GTP, E. coli tRNA, and pyruvate kinase were obtained from Boehringer Mannheim (Germany). ApUpG and poly-[U] were purchased from Miles Lab. (U.S.A.). The preparation of S-30 and S-100 fractions, washed ribosomes, ribosomal subunits, and initiation factors of E. coli W1895 followed the method as described by Ohta et al.(3). [^{35}S]fMet-tRNA was synthesized by the procedure of Hershey and Thach (4), and N-acetyl-[^{14}C]Phe-tRNA by that of Haenni and Chapeville (5). Elongation factors EF-T and EF-G were prepared by the method of Nishizuka et al.(6), and f2 phage RNA according to Nathans et al.(7).

RESULTS

The effects of viomycin on poly[U]- or f2 phage RNA-directed protein synthesis in an E. coli cell-free system.

Viomycin was found to block both natural and synthetic mRNA-dependent protein syntheses. The degree of inhibition was about 10 times greater with f2 RNA-directed protein synthesis than with polyphenylalanine synthesis. Approximately 50% inhibition was demonstrated at concentrations of 10^{-5} to 10^{-6} M of the antibiotic in the f2 RNA system (Table 1). In a simultaneous experiment, kasugamycin, an inhibitor of initiation of protein synthesis (8), was observed to interfere with f2 RNA-dependent protein synthesis, but not significantly with polyphenylalanine formation. Thiopeptin, an inhibitor of peptide chain elongation (9), prevented both protein syntheses to the same extent.

The specific initiation mechanism is involved in natural mRNA-directed protein synthesis, but not in synthetic messenger-dependent polypeptide formation. Since viomycin blocks the former

Table 1. Inhibition by antibiotics of poly[U]- or f2 RNA-directed protein synthesis in an E. coli cell-free system.

Antibiotics		Incorporation of	
		Leu with f2 RNA	Phe with poly[U]
None		100	100
Viomycin	10^{-6} M	72	91
	10^{-5}	37	60
	10^{-4}	17	41
Thiopeptin	10^{-5}	19	18
Kasugamycin	10^{-4}	16	98

The numbers represent per cent of control.
The reaction mixture in 0.1 ml contained: 50 mM Tris-HCl,pH 7.6, 10 mM Mg acetate, 160 mM NH_4Cl, 2 mM dithiothreitol, 200 µg E. coli S-30 fraction, 50 µg f2 phage RNA or poly[U], 20 µg E. coli tRNA, 0.02 uCi [^{14}C]leucine or phenylalanine, 2 mM ATP, 5 mM phosphoenolpyruvate, 2 µg pyruvate kinase, 0.1 mM GTP, 2 µM 19 kinds of amino acids, and the antibiotic. It was incubated at 37°C for 20 minutes. The hot CCl_3COOH-insoluble radioactivity was determined with correction for the values obtained in parallel mixtures without messenger. The incorporation of leucine was 9,884 cpm and that of phenylalanine 7,006 cpm in the control samples.

more markedly than the latter, the results suggest that the antibiotic may affect the initiation step of protein synthesis.

Inhibition by viomycin of initiation complex formation on the 30S ribosomal subunit or on the 70S ribosome.

In the initiation step in protein synthesis, fMet-$tRNA_F$, an initiator, binds to the initiation codon ApUpG or GpUpG of template RNA on the 30S ribosomal subunit. For the purpose of studying the effect of viomycin on initiation complex formation, the binding of [^{35}S]fMet-tRNA was investigated. The antibiotic was found to inhibit formation of the initiation complex on the 30S ribosomal subunit with f2 RNA or ApUpG as well as on the 70S ribosome (Table 2). Approximately 50% inhibition was observed at the concentration of 10^{-5} M of viomycin, which corresponded to or a little less than the degree of inhibition of f2 RNA-directed protein synthesis. In a parallel experiment, the fMet-tRNA binding was pronouncedly

blocked by kasugamycin, an inhibitor of initiation (8), but slightly by thiopeptin, inhibiting primarily the elongation process (9).

Effects of viomycin on peptide chain elongation on the ribosome.

The process of peptide chain elongation involves aminoacyl-tRNA

Table 2. Effects of antibiotics on initiation complex formation.

Antibiotics		fMet-tRNA bound to ribosomes with mRNA			
		70S ApUpG	70S f2 RNA	30S ApUpG	30S f2 RNA
None		100	100	100	100
Viomycin	10^{-6} M	89	96	81	84
	10^{-5}	63	78	55	60
	10^{-4}	13	30	26	35
Thiopeptin	10^{-5}	81	75	86	93
Kasugamycin	10^{-4}	6	24	20	27

The numbers represent per cent of control.
The reaction mixture in 0.1 ml contained: 50mM Tris-HCl,pH 7.5, 60 mM NH_4Cl, 6 mM Mg acetate, 10 mM 2-mercaptoethanol, 0.2 mg washed 70S ribosomes or 0.1 mg 30S ribosomal subunit, 0.06 mg initiation factors, 10 μg (4 x 10^5 cpm) [^{35}S]fMet-tRNA, 10 μg ApUpG or 50 μg f2 RNA, 0.2 mM GTP, and the antibiotic. It was incubated at 37°C for 15 minutes. The radioactivity, collected and washed on Millipore filters, was determined with correction for the values obtained in parallel mixtures without messenger. The radioactivities in control tubes were 21,176 cpm, 85,630 cpm, 4,890 cpm, and 4,637 cpm respectively.

Table 3. Effects of antibiotics on EF-Tu-dependent binding of Phe-tRNA to the ribosomes with poly[U].

Antibiotics		[^{14}C]Phe-tRNA bound
None		100
Viomycin	10^{-6} M	95
	10^{-5}	85
	10^{-4}	81
Thiopeptin	10^{-5}	25
Kasugamycin	10^{-4}	97

The numbers represent per cent of control.
The reaction mixture in 0.1 ml contained: 50 mM Tris-HCl,pH 7.5, 60 mM NH_4Cl, 7 mM Mg acetate, 2 mM dithiothreitol, 0.2 mg washed ribosomes, 20 μg EF-T, 40 μg poly[U], 20 μg [^{14}C]Phe-tRNA, 0.2 mM GTP and antibiotics. It was incubated at 37°C for 10 minutes. The radioactivity, collected and washed on Millipore filters, was determined with correction for the values obtained in parallel mixtures without messenger. The radioactivity incorporated in controls was 8,182 cpm.

binding, peptidyl transferase reaction, and translocation of peptidyl-tRNA with mRNA on the ribosome. The effects of viomycin on each reaction were investigated.

The EF-T-dependent binding of [^{14}C]Phe-tRNA to the ribosome with poly[U] was scarcely blocked by viomycin or kasugamycin, but pronouncedly by thiopeptin (table 3). The results with the latter two antibiotics are consistent with the previous observations(8,9).

N-Acetylphenylalanyl-puromycin synthesis with N-acetyl-[^{14}C]Phe-tRNA and puromycin on the ribosome in the absence of EF-G and GTP was employed as a model of peptidyl transferase reaction. The puromycin reaction was not significantly or only slightly affected by viomycin as well as by thiopeptin (9), fusidic acid (12), and kasugamycin (8). It was profoundly blocked by chloramphenicol, an inhibitor of peptidyl transferase (Table 4).

Table 4. Effects of antibiotics on N-acetylphenylalanyl-puromycin formation in the absence or presence of EF-G and GTP.

Antibiotics		N-Ac-[^{14}C]Phe-puromycin formed	
		without EF-G	enhanced by EF-G
None		100	100
Viomycin	10^{-6} M	95	24
	10^{-5}	85	10
	10^{-4}	78	2
Thiopeptin	10^{-5}	97	11
Fusidic acid	10^{-4}	97	13
Kasugamycin	10^{-4}	96	98
Chloramphenicol	10^{-5}	48	8

The numbers represent per cent of control.
The reaction mixture in 0.1 ml contained: 50mM Tris-HCl, pH 7.5, 10 mM Mg acetate, 60 mM NH_4Cl, 2 mM dithiothreitol, 50 µg poly-[U], 30 µg N-acetyl-[^{14}C]Phe-tRNA, and 0.2 mg washed ribosomes. After it was incubated at 30°C for 10 minutes and cooled in an ice-bath, 0.2 mM puromycin and the antibiotic with or without 50 µg/ml EF-G and 0.1 mM GTP were added to the mixture. It was further incubated at 30°C for 10 minutes, and was extracted with 1.5 ml of ethyl acetate after addition of 0.5 ml of 0.2 M potassium acetate, pH 5. The radioactivity of the solvent layer was assayed after evaporation. The control radioactivity was 746 cpm in the absence of EF-G and GTP, and that enhanced by EF-G and GTP was 1,384 cpm.

The enhanced puromycin reaction observed in the presence of EF-G and GTP was definitely inhibited by viomycin, thiopeptin, and fusidic acid; but not by kasugamycin (Table 4). The results indicate that viomycin, like thiopeptin and fusidic acid, prevents translocation of peptidyl-tRNA from the acceptor site to the donor site on the ribosome with simultaneous movement of mRNA (9,12).

DISCUSSION

The results with f2 RNA and poly[U] suggest that viomycin affects the initiation process of protein synthesis. This was confirmed by the study of fMet-tRNA binding to the ribosome with initiation factors. Kasugamycin (8), edeine (10), and aurintricarboxylic acid (11) were reported to prevent the 30S initiation complex formation. Kasugamycin primarily blocks the initiation step, but the latter two affect both chain initiation and elongation. Viomycin is a new member of initiation inhibitors. However, it was suggested that viomycin may also interfere with peptide chain elongation, because it inhibits polyphenylalanine synthesis with poly[U] in which the initiation mechanism is not primarily involved. In measurements of peptide chain elongation, the antibiotic was observed to affect significantly neither EF-T-dependent aminoacyl-tRNA binding to the ribosome with messenger nor the peptidyl transferase reaction; but to prevent translocation of peptidyl-tRNA from the acceptor site to the donor site on the ribosome.

Viomycin seems to possess two sites of action on the ribosome. This is in accord with the reports that two kinds of viomycin-resistant mutants have been obtained: one is due to alteration of the 30S ribosomal subunit, and the other to that of the 50S subunit (2). An alternative explanation is that the antibiotic may have a single site of action on the ribosome which may lead to the inhibition of initiation and translocation. If this were the case,

the two reaction may have some as yet unknown relationship.

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