Vol. 87, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 13, 1979 Pages 904-910

VIOMYCIN RESISTANCE: ALTERATIONS OF EITHER RIBOSOMAL SUBUNIT AFFECT THE BINDING OF THE ANTIBIOTIC TO THE PAIR SUBUNIT AND THE ENTIRE RIBOSOME BECOMES RESISTANT TO THE DRUG

Eung Chil Choi, Masarou Misumi, Toshio Nishimura, and Nobuo Tanaka*
Institute of Applied Microbiology, University of Tokyo, Tokyo

Shinya Nomoto, Tadashi Teshima, and Tetsuo Shiba
Department of Chemistry, Faculty of Science, Osaka University, Osaka, Japan

Received March 1,1979

SUMMARY

Viomycin inhibited polypeptide synthesis and translocation of peptidyl-tRNA on ribosomes derived from a sensitive strain of M. smegmatis (R-15), but not significantly on ribosomes from viomycin-resistant mutants (R-31 and R-43). The binding of [^{14}C]tuberactinomycin O, a viomycin analog, to ribosomal particles was studied by Millipore filter method. The sensitive ribosome exhibited higher affinity for the antibiotic than the resistant ribosomes. The resistance was localized on the large ribosomal subunit in a mutant (R-31), and on the small subunit in another mutant (R-43). The binding of the drug to the sensitive ribosomal subunit was markedly reduced by combination with the resistant pair subunit, and the entire ribosome became resistant to the antibiotic.

Viomycin has been observed to inhibit protein synthesis on bacterial ribosomes at two steps: initiation and elongation. The antibiotic blocks 30S initiation complex formation and translocation of peptidyl-tRNA from the acceptor site to the donor site (1,2). It has been demonstrated that [^{14}C]tuberactinomycin O, a drug closely related to viomycin, binds to E. coli ribosomes. The ribosome has two major binding sites: one on the large ribosomal subunit and another on the small subunit. Since the binding of tuberactinomycin O is reversed by viomycin, the two antibiotics seem to possess the same binding sites on the ribosome (3).

Yamada et al. (4,5) have reported that the resistance to viomycin is due to alteration of the small ribosomal subunit in some of resistant mutants of Mycobacterium smegmatis , and to that of the large subunit in other mutants.

We have prepared ribosomes and ribosomal subunits from viomycin-sensitive and -resistant strains of M. smegmatis; and studied effects of viomycin on polypeptide synthesis and translocation of peptidyl-tRNA on hybrid ribosomes, and the interaction of [^{14}C]tuberactinomycin O with ribosomes and ribosomal subunits. It has been observed that the resistant subunit affects the binding of the antibiotic to the sensitive pair subunit. The results are presented in this publication.

* To whom reprint requests should be addressed.

0006-291X/79/070904-07$01.00/0

MATERIALS AND METHODS

Mycobacterium smegmatis R-15, R-31 and R-43 were generously given by Prof. Yasuo Mizuguchi, University of Occupational and Environmental Health, Kita-Kyushu City, Japan. R-15 was sensitive to viomycin (MIC 5 μg/ml). R-31 (vicA) was resistant to the drug (MIC 300 μg/ml) and had altered large ribosomal subunit. R-43 (vic B) was also resistant (MIC 100 μg/ml), possessing altered small subunit (5).

[^{14}C]Phenylalanine (522 mCi/mM) and [^{14}C]leucine (354 mCi/mM) were purchased from the Radiochemical Centre, Amersham, England. Phosphoenolpyruvate, GTP, ATP, pyruvate kinase, poly[U] and E. coli tRNA were products of Boehringer Mannheim, Germany.

The preparation of S-30 fraction, ribosomes and ribosomal subunits of M. smegamatis followed the method described by Ohta et al. (6). For the purpose of removing RNase, the ribosomes were washed with 0.5 M LiCl-containing buffer (10 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$, 6 mM 2-mercaptoethanol and 10 μg/ml polyvinylsulfate) (7). N-acetyl-[^{14}C]phenylalanyl-tRNA was synthesized by the procedure of Haenni and Chapeville (8), and elongation factors were prepared by the method of Nishizuka et al. (9). The preparation and characteristics of [^{14}C]tuberactinomycin O (15.0 Ci/mole) were described in previous papers (3,10).

The incorporation of [^{14}C]leucine with endogenous mRNA and polyphenylalanine synthesis were carried out as described by Nirenberg (11) with a few minor modifications. The binding of [^{14}C]tuberactinomycin O to ribosomal particles was assayed by Millipore filter method.

RESULTS

The effect of viomycin on polypeptide synthesis in cell-free systems from the drug-sensitive and -resistant strains of Mycobacterium smegmatis.

Viomycin was observed to block both endogenous mRNA- and poly[U]-directed polypeptide synthesis in a ribosomal system obtained from M. smegmatis R-15, a strain sensitive to viomycin. Approximately 50 % inhibition was found at antibiotic concentrations of 0.2 to 0.4 μM. Much less effect of the antibiotic were demonstrated in cell-free systems derived from M. smegmatis R-31 and R-43, viomycin-resistant mutants, at antibiotic concentration range of 0.15 to 1.5 μM. It was further confirmed by experiments, using reconstituted hybrid ribosomes, that the resistance of R-31 was localized on the large ribosomal subunit and that of R-43 on the small subunit (Data are not shown). The results were in accord with previous reports (4,5).

Effects of viomycin on peptidyl transferase reaction and translocation of peptidyl-tRNA on sensitive and resistant ribosomes.

N-Acetylphenylalanyl-puromycin synthesis on ribosomes in the absence of EF-G and GTP was employed as a model system of peptidyl transferase reaction. The puromycin reaction on ribosomes of R-15 as well as on those of R-31 and R-43 was not significantly affected by viomycin (Table 1). In a simultaneous experiment, it was profoundly blocked by blasticidin S, an inhibitor of peptidyl transferase (12).

Table 1. Effects of viomycin on peptidyl transfer and translocation of peptidyl-tRNA on ribosomes, derived from viomycin-sensitive and -resistant strains of Mycobacterium smegmatis.

Mycobacterium smegmatis strains	Antibiotics	Ac-Phe-puromycin formation − EF-G, GTP cpm	%	+ EF-G, GTP cpm	Translocation of peptidyl-tRNA cpm	%
R-15 (sensitive to viomycin)	None	960	100	1,943	983	100
	Viomycin 0.15 μM	880	92	1,631	751	76
	1.5	756	79	882	126	13
	15.	779	81	815	36	4
	Fusidic acid 0.19 mM	892	93	1,167	275	28
	Blasticidin S 0.24 mM	212	22			
R-31 (viomycin-resistant alteration on the large ribosomal subunit)	None	667	100	1,450	783	100
	Viomycin 0.15 μM	702	105	1,468	766	98
	1.5	636	95	1,348	712	91
	15.	583	87	1,408	825	105
	Fusidic acid 0.19 mM	662	99	937	275	35
	Blasticidin S 0.24 mM	172	27			
R-43 (viomycin-resistant alteration on the small ribosomal subunit)	None	686	100	1,354	668	100
	Viomycin 0.15 μM	704	102	1,322	618	93
	1.5	689	100	1,346	657	98
	15.	556	81	1,185	629	94
	Fusidic acid 0.19 mM	680	99	780	100	15
	Blasticidin S 0.24 mM	171	25			

The reaction mixture in 0.1 ml contained: 50 mM Tris-HCl, pH 7.8, 15 mM $Mg(OAc)_2$, 150 mM NH_4Cl, 1 mM dithiothreitol, 10 μg poly[U], 44 μg N-acetyl-[^{14}C]Phe-tRNA, and 2 A_{260} units washed ribosomes. It was incubated for an hour, and then the antibiotic with or without 200 μg EF-G and 0.2 mM GTP were added to the mixture. The incubation was carried out at 37°C for 10 minutes; and then 0.45 mM puromycin was introduced to the mixture, which was further incubated at 0°C for 30 minutes. The reaction was terminated by addition of one ml of 0.1 M sodium acetate, pH 5.5, and extracted with 1.5 ml of ethyl acetate. The radioactivity of the solvent layer was determined in Bray's scintillator.

The effect of viomycin on translocation of peptidyl-tRNA from the acceptor site to the donor site on ribosomes was studied with the puromycin reaction enhanced by addition of EF-G and GTP (13). The stimulated reaction was sensitive to fusidic acid on R-15, R-31 and R-43 ribosomes. The translocation was markedly inhibited by the drug on R-15 ribosomes (ca. 50 % inhibition at 0.4 μM), but not significantly on R-31 and R-43 ribosomes. The results indicated that translocation of peptidyl-tRNA is resistant to viomycin not only on R-31 ribosomes (the large subunit resistance) but also on R-43 ribosomes (the small subunit resistance) (Table 1).

Table 2. The effect of viomycin on translocation of peptidyl-tRNA on hybrid ribosomes.

Source of ribosomal subunits		Viomycin	Ac-Phe-puromycin synthesis enhanced by EF-G and GTP		Sensitivity to viomycin
30S	50S	μM	cpm	%	
R-15	R-15	0	1,065	100	
		0.15	503	47	sensitive
		1.5	137	13	
R-31	R-31	0	2,457	100	
		0.15	2,498	102	resistant
		1.5	2,342	95	
R-31	R-15	0	1,372	100	
		0.15	773	56	sensitive
		1.5	69	5	
R-15	R-31	0	2,096	100	
		0.15	2,130	102	resistant
		1.5	1,957	93	
R-43	R-43	0	1,235	100	
		0.15	1,198	97	resistant
		1.5	1,106	90	
R-43	R-15	0	752	100	
		0.15	736	98	resistant
		1.5	602	80	
R-15	R-43	0	1,737	100	
		0.15	1,175	68	sensitive
		1.5	512	30	

The experiments were performed by the procedure described in the legend of Table 1, in which washed ribosomes were replaced by 1.4 A_{260} units of the large ribosomal subunit and 0.7 A_{260} units of the small subunit.

The effect of viomycin on translocation of peptidyl-tRNA on hybrid ribosomes.

The puromycin reaction, enhanced by EF-G and GTP, on reconstituted ribosomes consisting of the 50S and 30S subunits from R-15, on those of 50S from R-43 and 30S from R-15, and those of 30S from R-31 and 50S from R-15 were markedly inhibited by viomycin, showing that all these ribosomal subunits are sensitive to the drug. On the contrary, the reaction was not signficantly affected by the antibiotic on hybrid ribosomes, containing the 50S subunit from R-31 or the 30S subunit from R-43, indicating that these subunits are resistant to viomycin (Table 2). The results suggested that the resistance of peptidyl-tRNA translocation is localized in the large subunit on R-31 ribosomes and in the small subunit on R-43 ribosomes; and hybrid ribosomes, consisting of the sensitive subunit and resistant one, become resistant to the antibiotic, regardless of which ribosomal subunit is derived from the resistant or sensitive strain.

Binding of [^{14}C]tuberactinomycin O to sensitive and resistant ribosomes and ribosomal subunits.

The interaction of [^{14}C]tuberactinomycin O, an analog of viomycin, with ribosomes and ribosomal subunits, obtained from viomycin-sensitive and -resist-

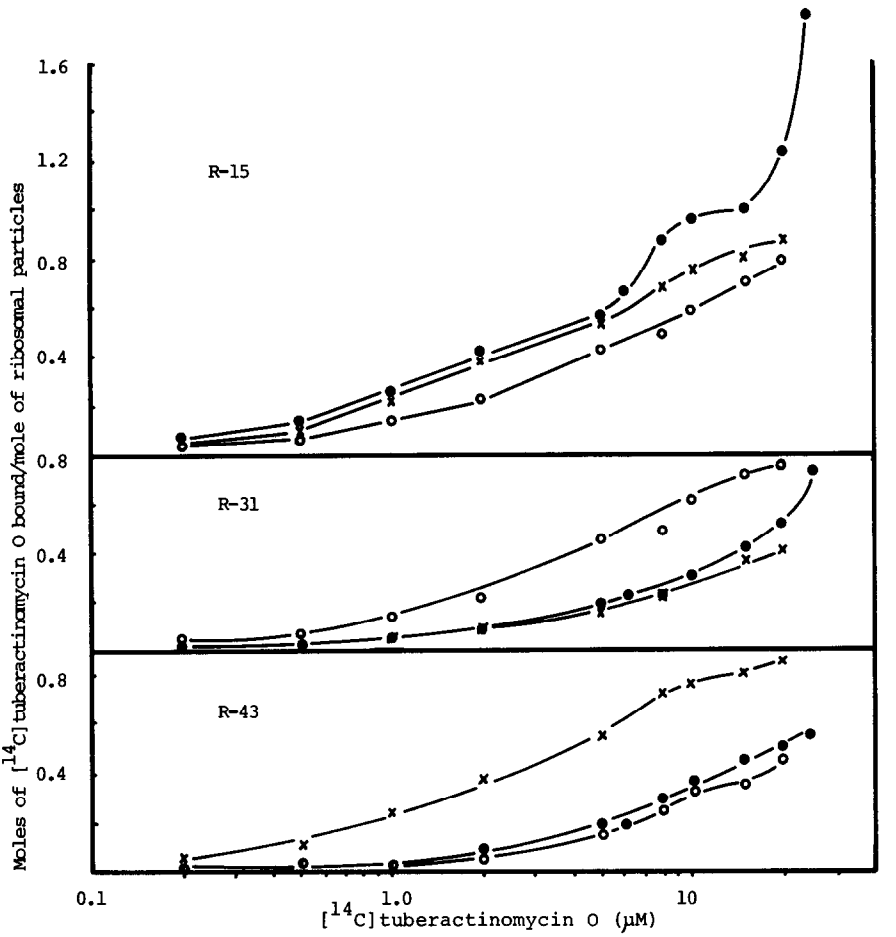

Fig. 1. The binding of [^{14}C]tuberactinomycin O to ribosomal particles, derived from viomycin-sensitive and -resistant strains of Mycobacterium smegmatis.
●——● ribosomes, ×——× the large ribosomal subunit, o——o the small ribosomal subunit.
The assay was carried out as described in the legend of Table 3.

ant strains of M. smegmatis, were studied by Millipore filter method at antibiotic concentration range of 0.2 to 25 μM. In order to determine the association constants and the numbers of binding sites, the data were plotted according to the Scatchard equation for equilibrium binding, $r/A = Kn - Kr$, where r, A, K and n represent moles of bound [^{14}C]antibiotic per mole of ribosomes, molar concentration of free [^{14}C]antibiotic, association constant, and number of binding sites on a ribosome, respectively.

The dependency of the antibiotic concentration for its binding to ribosomal particles is illustrated in Fig. 1. For instance, approximately 0.23 moles of

Table 3. Association constants (Ka) obtained from the Scatchard plots of data for binding of [^{14}C]tuberactinomycin O to the ribosome or ribosomal subunits.

Ribosomal particles	Mycobacterium smegmatis strains		
	R-15	R-31	R-43
Ribosome	4.3×10^5 M^{-1}	4.4×10^4	5.0×10^4
Large subunit	3.4×10^5	4.6×10^4	3.5×10^5
Small subunit	1.9×10^5	1.8×10^5	5.1×10^4

The binding of [^{14}C]tuberactinomycin O to ribosomal particles of Mycobacterium smegmatis was assayed by Millipore filter method. The reaction mixture in 0.1 ml contained: 10 mM Tris-HCl, pH 7.8, 10 mM $Mg(OAc)_2$, 30 mM NH_4Cl, 6 mM 2-mercaptoethanol, 1 μg polyvinylsulfate, 1 μM ribosomes or ribosomal subunits, and 0.2 - 25 μM [^{14}C]tuberactinomycin O. It was incubated at30°C for 5 min. and immediately cooled in an ice-bath; and then one ml of cold washing buffer was added to the mixture. The radioactivity, collected on Millipore filters, was determined with correction for the values in the parallel mixture without ribosomal particles.

[^{14}C]tuberactinomycin O bound to one mole of R-15 ribosomes at antibiotic concentration of one μM, when the ribosomes were used at one μM; and about 0.9 moles of the drug interacted with one mole of ribosomes at antibiotic concentration of 8 μM. The Scatchard plot showed that R-15 ribosome appears to possess one binding site with an association constant (Ka) of approximately 4.3×10^5 M^{-1}, and at least one more site with lower affinity (Table 3). Less amounts of [^{14}C]tuberactinomycin O bound to resistant ribosomes (R-31 and R-43): i.e. 0.22-0.3 moles of the drug bound to one mole of ribosomes at antibiotic concentration of 8 μM. R-31 and R-43 ribosomes seemed to possess one major binding site with Ka 4.4×10^4 and 5.0×10^4 M^{-1}, respectively, which were about one-tenth of Ka of R-15 ribosomes. The results showed that the resistant ribosomes (R-31 and R-43) interact with the antibiotic more weakly than the sensitive ribosome (R-15).

The R-31 large ribosomal subunit showed lower affinity for the drug (Ka 4.6×10^4 M^{-1}) than the R-15 and R-43 large subunits (Ka 3.4-3.5 $\times 10^5$ M^{-1}), indicating that the former one is resistant to and the latter two are sensitive to the antibiotic. The R-15 and R-31 small subunits exhibited higher affinity (Ka 1.8-1.9 $\times 10^5$ M^{-1}) than the R-43 small subunit (Ka 5.1×10^4), showing that the former two are sensitive and the latter one is resistant. The results indicated that the drug resistance is attributed to poor affinity of the ribosomal subunit for the antibiotic, and the resistant subunit markedly affects the binding ability of the sensitive pair subunit.

DISCUSSION

The mechanism of viomycin resistance in R-31 and R-43 mutants appears to be due to alterations of either large or small ribosomal subunit. The binding of

the drug to the sensitive large or small sibosomal subunit is drastically influenced by resistant alterations of the pair ribosomal subunit. The cause of mutual interference of both ribosomal subunits remains to be determined. However, the decrease of affinity of the ribosome for the drug may occur either by changing the conformation of the whole ribosome or by masking the binding site or both. Similar phenomena have been recently reported (14,15). Saltzman and Apirion (14) have observed that the binding of erythromycin to the large ribosomal subunit is affected by alterations in the small subunit. Sutton et al. (15) have reported that cycloheximide resistance of Tetrahymena thermophilia can be mediated through either ribosomal subunit.

ACKNOWLEDGEMENTS

This work is partly supported by a grant-in-aid from Ministry of Education, Science and Culture, Japan. The authors express their deep thanks to Hamao Umezawa, Institute of Microbial Chemistry, Tokyo for his kind advice and cooperation in the current experiments.

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