BINDING OF [^{14}C]TUBERACTINOMYCIN O TO RIBOSOMES FROM *MYCOBACTERIUM SMEGMATIS*

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The peptide antibiotics, tuberactinomycin O (Tum O) and viomycin (=tuberactinomycin B, VM) are closely related in chemical structure; both are more active against mycobacteria than other bacterial species[1]. The mode of action of VM has been studied in detail; VM inhibits protein synthesis in *Mycobacterium avium*[2] and in cell-free systems derived from *Escherichia coli*[3] and *Mycobacterium smegmatis*[4]. It is a ribosomal inhibitor because resistance to VM in mutants of *M. smegmatis* is due to ribosome alterations[4,5]. Some of the mutants contained altered 50S subunits, whereas others had altered 30S subunits[4]. Genetic and biochemical studies have confirmed that there are two loci for VM resistance on the chromosome of *M. smegmatis*[6,7]. The antibiotic inhibits the dissociation of, and favours association of, ribosomal subunits[8]. Inhibition of translocation by VM has been also reported[9,10]. Equilibrium dialysis has demonstrated the binding of [^{14}C]Tum O to 30S and 50S subunits of *E. coli* ribosomes[11,12]. In addition, ribosomes were prepared from the drug-sensitive strain R-15 and resistant mutants of *M. smegmatis* and their drug binding affinity was compared. Binding was equal on both sensitive and resistant ribosomes when measured by equilibrium dialysis[12], however a significant difference was found when the Millipore filter method[13] was used.

In this paper, we have compared the binding affinity of the drug to ribosomes derived from *M. smegmatis*, a VM-resistant mutant of *M. smegmatis*, and *E. coli* by means of equilibrium dialysis and dialysis against a large volume of buffer. The following conclusions were obtained: 1) [^{14}C]Tuberactinomycin O binds more strongly to 70S ribosomes from *M. smegmatis* than those from *E. coli* and a drug-resistant mutant of *M. smegmatis*. 2) The agent had a higher affinity for 70S ribosomes than either ribosomal subunit derived from *M. smegmatis*. *M. smegmatis* 70S ribosomes seem to carry the binding sites of the antibiotic important for inhibition of polypeptide synthesis.

VM was a product of Pfizer Taito Co., Ltd. (Japan). Tum O was a gift from Toyo Jozo Co., Ltd. (Japan). [^{14}C]Tum O was prepared according to the procedures described previously[12]. Sources of reagents for *in vitro* protein synthesis were as reported previously[4]. *M. smegmatis* strain R-15, VM-resistant *M. smegmatis* strains R-33 and R-31, and *E. coli* HAK10 were used in this study[1]. *M. bovis* BCG was obtained from ATCC. The culture medium for *M. smegmatis* and *E. coli* was as described previously[4]. *M. bovis* BCG was grown in Kirchner medium[1]. TMXNYSH (10 mM Tris-HCl, pH 7.8, X mM Mg-acetate, Y mM NH_4Cl, 6 mM 2-mercaptoethanol) were used. TMNSH: T10M10N60SH buffer. The preparation of ribosomes and the cell-free system for poly U-directed polyphenylalanine synthesis were as described previously with a few modifications[4]. The standard reaction mixture (0.1 ml) contained the following: 95 mM Tris-HCl, pH 7.8; 5.8 mM 2-mercaptoethanol; 7.5 mM phosphoenolpyruvate trisodium salt; 0.9 mM adenosine triphosphate; 0.028 mM guanosine triphosphate; 0.1 mM [^{14}C]phenylalanine (specific activity, 100 μCi/5.5 μmol); 48 mM NH_4Cl; 1.5 μg of phosphoenolpyruvate kinase, 10 μl of supernatant fluid (absorbance at 280 nm, 20); 3 A_{260} units of ribosomes, 100 μg of polyU; 50 μg of *E. coli* transfer RNA; and 10 mM magnesium acetate. The binding of [^{14}C]Tum O to ribosomes was assayed by equilibrium dialysis[12] and dialysis against excess amounts of buffer. In the latter assay, the reaction mixture contained 400 pmol of ribosomes and 1,500 pmol of [^{14}C]Tum O in 100 μl of TMNSH buffer and was incubated at 37°C for 30 minutes, followed by dialysis against 10 liters of T20M20N400SH buffer for 18 hours at 4°C. Ribosomes were then centrifuged (rotor 30, Beckman) in 30% sucrose in the same buffer at 30,000 rpm for 18 hours. The resulting pellets were dissolved in 100 μl of TMNSH buffer. A_{260} and radioactivity of the solutions

Table 1. Binding of [^{14}C]Tum O[a].

Bacterial strain	Ribosome	pmol of [^{14}C]Tum O per pmol of ribosomes	
		A[b]	B[c]
M. smegmatis R-15	70S	0.22~0.48	2.6
	50S	<0.045	
	30S	<0.05	
M. bovis BCG	70S	ND[d]	2.8
VM-resistant *M. smegmatis* R-33	70S	0.1~0.06	3.0
E. coli	70S	0.07~0.08	2.2

[a] Experimental procedures are described in the text.

[b] The binding was examined by means of dialysis against excess amounts of buffer. The values show the variation of at least 3 separate estimates.

[c] For equilibrium dialysis, one chamber contained 180 pmol of 70S and the other 2 nmol of [^{14}C]Tum O in 100 μl of TMNSH buffer. When equilibrium was reached after 20 hours, aliquots were removed and counted in a scintillation counter.

[d] Not done.

were determined. Amount (pmol) of ribosomes were calculated assuming that one A_{260} unit of 70S, 50S and 30S ribosomes corresponds to 24, 36 and 64 pmol respectively.

The results obtained by equilibrium dialysis suggest that the drug binds to ribosomes derived from *M. smegmatis*, a VM-resistant mutant of *M. smegmatis*, *M. bovis* BCG and *E. coli* (Table 1). No significant differences in the binding affinities were observed in these four cases. This observation is consistent with our previous report[12].

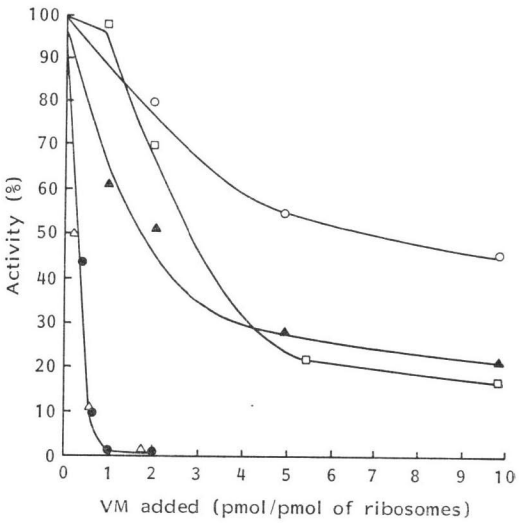
On the contrary, binding studies by means of extensive dialysis indicated that the Tum O bound to ribosomes from *E. coli* and the VM-resistant mutant of *M. smegmatis* can be easily dissociated, whereas it binds strongly to ribosomes of drug-sensitive *M. smegmatis* ribosomes.

Whether the difference seen between the two dialysis methods represents simply differences in affinities, or a different type of interaction for the sensitive ribosomes is not clear.

The pattern of inhibition of poly U-directed polyphenylalanine synthesis by various concentrations of VM is presented in Fig. 1. As expected, VM has a greater effect on ribosomes from *M. smegmatis* and *M. bovis* BCG than on those from *E. coli* and the drug-resistant mutant of *M. smegmatis*.

Fig. 1. Effects of VM on poly U-directed polyphenylalanine synthesis on the ribosomes from *M. bovis* BCG (△), *M. smegmatis* R-15 (●), VM-resistant strains R-33 (▲) and R-31 (○) of *M. smegmatis*, and *E. coli* (□).

The experimental conditions are as described in text.



As shown in Table 1, the drug bound to either 50S or 30S subunits from *M. smegmatis* can be easily removed. This observation suggests that the antibiotic has strong affinity to 70S ribosomes very weak affinity to isolated ribosomal subunits.

This was confirmed by the following experiments. Excess amounts of either 50S or 30S subunits were added to a poly U-directed polyphenylalanine synthesizing system containing 70S ribosomes from *M. smegmatis* R-15, and the inhibitory effects of VM at various concentrations were examined. If the added subunits were capable of binding the drug, they should reduce the antibiotic-induced inhibition of polypeptide synthesis. The experimental results are presented in Figs. 2 and 3. As expected, neither subunit had any effect in restoring polypeptide synthesis in the presence of a variety of concentrations of VM. The addition of more 70S ribosomes reduced the extent of the inhibition.

Thus, we suggest that 70S ribosomes possess unique binding site(s) for the inhibitory effects of VM. Detailed analyses of the binding of Tum O to mycobacterial 70S ribosomes rather

Fig. 2. Inhibitory effect of VM on cell-free polypeptide synthesis.

The cell-free system contained a 4-fold excess of 30S subunits to 70S ribosomes (○). The pattern of the inhibition of poly U-directed polyphenylalanine synthesis by VM on 70S ribosomes alone is shown as control (●). Experimental conditions are described in text. The incorporation of [^{14}C]phenyl alanine into acid-insoluble material in the respective system in the absence of VM represents 100% activity.

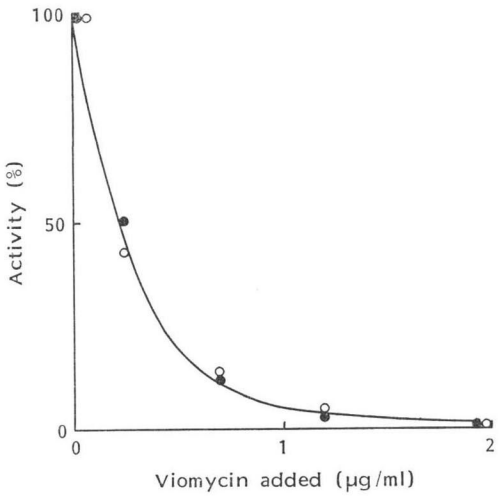
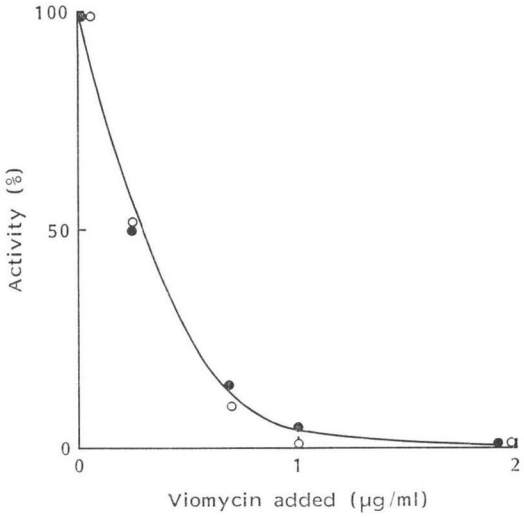


Fig. 3. Inhibitory effects of VM on cell-free polyphenylalanine synthesis.

The system containing a 4-fold excess 50S subunits to 70S ribosomes (○). The pattern of inhibition by VM on 70S alone is shown as control (●). The other conditions are as described in Fig. 2.



than to ribosomal subunits might be an important approach to an understanding of both the mode of action of VM and the complexity of ribosomal structure and function.

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