The translocation inhibitor tuberactinomycin binds to nucleic acids and blocks the in vitro assembly of 50S subunits

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ABSTRACT

Binding studies were performed with a \( ^{14} \text{C} \)-labelled derivative of viomycin, tuberactinomycin 0 (TUM 0). TUM 0 bound to 30S and 50S subunits. The binding component was the RNA, since ribosomal proteins did not bind the drug. Other RNAs such as tRNA, phage RNA (MS2), and homopolynucleotides also bound the drug. Striking differences in the binding capacity of the various homopolynucleotides were found. Poly(U) bound strongly, poly(G) and poly(C) bound intermediately, whereas poly(A) showed a very low binding. DNA also bound TUM 0, although with native DNA the binding was only weak.

Finally the effects of viomycin on the assembly in vitro of the 50S subunit from E. coli were tested. A very strong inhibition was found: when the reconstitution was performed at 0.5 \( \times 10^{-6} \) M viomycin the particles formed sedimented at about 50S, but showed a residual activity of less than 10%. The inhibitory power of viomycin with respect to the in vitro assembly is more pronounced than that found in in vitro systems for protein synthesis.

INTRODUCTION

Viomycin inhibits the translational apparatus in intact bacteria of Mycobacterium avium and Rhizobium meliloti (1,2) as well as in in vitro systems derived from E. coli (3) and M. smegmatis (4). Detailed analyses have revealed that viomycin stabilizes 70S couples (5) and inhibits the translocation reaction (6,7). A unique feature of this antibiotic is its specific high activity against mycobacteria in the culture. Accordingly, viomycin resistant mutants with altered ribosomes were isolated from M. smegmatis. Mutants with a high level of resistance could be isolated only by a multistep selection. The altered ribosomes were cross-resistant to streptomycin and kanamycin. These stu-
dies suggested that the expression of high level resistance to viomycin can be generated only by a combination of several alterations of the ribosomal constituents (8-10).

Analysis of various mutants demonstrated that some of them contained altered 50S subunits, whereas others had altered 30S subunits (4). Genetic and biochemical studies have confirmed that there are two loci for viomycin resistance on the chromosome of M. smegmatis. One locus (vicA) was related to an alteration of the 50S and the other (vicB) to an alteration of the 30S subunit (11-13). In both cases the RNA moiety conferred the resistance property (14).

In this paper we describe binding experiments with a viomycin derivative (tuberactinomycin O). Multiple binding sites on 30S and 50S subunits were found. The ribosomal RNA was identified as the binding component. The antibiotic inhibited ribosomal assembly in reconstitution systems.

MATERIALS AND METHODS

Antibiotics and reagents.

$[^{14}C]$-tuberactinomycin O (TUM O, Fig. 1) was prepared by incubating the antibiotic with $[^{14}C]$-urea in 3N HCl for 40 days at room temperature, followed by purification on a Sephadex G-10

![Chemical structure of various tuberactinomycins. The asterisk represents radioactive carbon. VM means viomycin.](image)

Fig. 1: Chemical structure of various tuberactinomycins. The asterisk represents radioactive carbon. VM means viomycin.
The specific activity of $^{14}$C-TUM O was 20 Ci/mol. ($^{14}$C-urea was purchased from the Radiochemical Centre, Amersham, England.) The purity of the labelled antibiotic was checked by paper chromatography, using a solvent system containing acetone, 10% ammonium acetate, and 10% ammonia in a ratio of 30 : 9 : 1. Viomycin was a product of Pfitzer Ltd. (Japan). Streptomycin, chloramphenicol, aurintricarboxylic acid, calf thymus DNA, poly(U), poly(A), poly(C), poly(G), MS2 RNA, and E. coli tRNA were purchased from Boehringer Mannheim, Germany.

**Strains and culture media.**

E. coli strain A19 and M. smegmatis strain Rabinowitchi (R) were used. The parental strain of M. smegmatis used in this study was R-15 (argA-6, met-5). The viomycin resistant mutants employed in this study were R-31 (argA-6, met-5, vicA-1, str-15) which has altered 50S subunits, and R-33 (argA-6, met-5, vicB-3) which has altered 30S subunits. The other characteristics of these mutants have been described previously (11,12). The culture media used for E. coli and M. smegmatis were as described previously (4).

**Buffers:** TMXNYSH (10 mM Tris-HCl, pH 7.8, X mM Mg-acetate, Y mM $\text{NH}_4\text{Cl}$, 6 mM 2-mercaptoethanol) were used. E.g., TM10N100SH contains 10 mM of Mg-acetate and 100 mM of $\text{NH}_4\text{Cl}$.

**Preparation of ribosomes, proteins and RNAs.**

Isolation of ribosomes, ribosomal subunits, ribosomal RNA and total proteins from 50S subunits (TP50) and from 30S subunits (TP30) were as described (15).

**Reconstitution and poly(U) assay.**

Reconstitution of 50S subunits from TP50 and RNAs and the subsequent poly(U) assay followed the procedure of ref. 15.

**Equilibrium dialysis.**

The equilibrium cells have been described previously (16). One chamber contained 100 μl of ribosomes, ribosomal subunits, core particles, RNAs, DNAs, or protein in TMXNYSH buffer. The amounts of the various components in the chamber for the appropriate experiments are given in the legends to the Figures. Con-
trols with sample buffer only were run in parallel. The other chamber was filled with 100 μl of sample buffer containing the indicated amounts of [14C]-tubercactinomycin. Dialysis reached equilibrium after a minimum of 19 hrs. The cells were shaken at 4°C for 19-24 hrs, then two 40 μl aliquots were withdrawn with a syringe from each chamber and counted in a scintillation counter. The mean values of the two aliquots were calculated. Sampling error was within 1%. From the difference between the two chambers, the number of moles of antibiotic bound to the samples was calculated.

RESULTS AND DISCUSSION

The dependence of binding on ionic strength.

The binding of [14C]-TUM O to ribosomes was measured by means of equilibrium dialysis as described in Materials and Methods. Fig. 2A illustrates the dependence on magnesium of the binding to 70S ribosomes in the presence of 320 mmol NH₄⁺; and the NH₄⁺ dependence is shown in Fig. 2B in the presence of 9 mmol Mg. The binding increases with decreasing concentrations of either cation. Below either 8 mM Mg or 150 mM NH₄⁺, more than one drug molecule was bound per 70S ribosome, indicating multiple binding sites. Accordingly, for the following experiment, a binding buffer was chosen which contained 8 mM Mg and 100 mM ammonium (TM8-N100SH). Under these conditions about 1.9 antibiotic molecules per 70S ribosomes were bound.

The binding of [14C]-TUM O to ribosomal particles and components.

70S ribosomes and their subunits bound about 50 to 80 pmol per A₂₆₀ unit, which is equivalent to about 1.2 to 1.9 molecules of antibiotic per ribosomes (see Table 1). Total protein fractions extracted from the subunits did not bind the drug, in contrast to the ribosomal RNAs. (23S+5S)RNA bound the drug slightly less than 16S RNA (74 and 102 pmol per A₂₆₀ unit, respectively; see Table 1). In addition, tRNA from E. coli bound similar amounts (90 pmol per A₂₆₀ unit). Core particles with a decreasing protein content were produced from both subunits by incubation with increasing concentrations of LiCl. The 50S derived cores showed a slight increase of binding after washing off the first proteins.
Fig. 2: The effect of Mg\textsuperscript{+} and NH\textsubscript{4}\textsuperscript{+} on the binding of \([^{14}\text{C}]-\text{TUM O}\) to 70S ribosomes. A, equilibrium dialysis was carried out with 70S ribosomes from E. coli in TMXN320SH buffer containing various concentrations of Mg acetate. One compartment was filled with 100 \(\mu\)l of 430 pmoles of ribosomes, the other one with 2000 pmoles of \([^{14}\text{C}]-\text{TUM O}\). B, the effect of NH\textsubscript{4}Cl on the binding of \([^{14}\text{C}]-\text{TUM O}\) to 70S ribosomes derived from E. coli. Equilibrium dialysis was performed as described under A except that TM9NXSH buffer was used with various concentrations of NH\textsubscript{4}Cl.

(compare 50S subunits and 1.3c cores in Fig. 3A), whereas the 30S derived cores showed about the same binding capacity (Fig. 3B). Clearly, the influence of the protein on the conformation of the ribosomal RNA does not contribute to the RNA-dependent binding of the drug. Furthermore, a surprisingly large portion of the RNA within the ribosomal subunit is still capable of binding the drug.

The drug resistance against viomycin can be conferred by either the 30S and the 50S subunit. In both cases the RNA moieties are the altered components. 50S subunits were isolated from the parental strain R-15 and the drug resistant strain R-31, which contains an altered 50S subunit conferring the resistance. Fig. 4 demonstrates that both resistant and sensitive 50S subunits bind about the same amount of antibiotic at various Mg\textsuperscript{++} concentrations. Equivalent results were obtained at various NH\textsubscript{4}\textsuperscript{+} concentrations.
Table 1: Comparison of TUM O binding to ribosomal constituents and related macromolecules.

<table>
<thead>
<tr>
<th>Components used</th>
<th>Bound TUM O (pmol) per A\textsubscript{260} unit</th>
<th>Number of TUM O molecules bound per component</th>
</tr>
</thead>
<tbody>
<tr>
<td>70S ribosomes</td>
<td>46</td>
<td>1.9</td>
</tr>
<tr>
<td>50S particles</td>
<td>54</td>
<td>1.5</td>
</tr>
<tr>
<td>30S particles</td>
<td>80</td>
<td>1.2</td>
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<tr>
<td>TP50</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>TP30</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>23S RNA (+ 5S RNA)</td>
<td>74</td>
<td>2.0</td>
</tr>
<tr>
<td>16S RNA</td>
<td>102</td>
<td>1.6</td>
</tr>
<tr>
<td>tRNA</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>poly(A)</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>poly(U)</td>
<td>216</td>
<td></td>
</tr>
<tr>
<td>poly(G)</td>
<td>142</td>
<td></td>
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<tr>
<td>poly(C)</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>poly(AUG)</td>
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</tr>
<tr>
<td>MS2 RNA</td>
<td>108</td>
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</tr>
<tr>
<td>E. coli DNA</td>
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<td></td>
</tr>
<tr>
<td>calf thymus DNA</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>denatured calf thymus DNA</td>
<td>50</td>
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</tr>
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</table>

Equilibrium dialysis was carried out in TM8N100SH buffer as described in the text. The number of TUM O molecules bound per ribosome was calculated by assuming that 1 A\textsubscript{260} unit of 30S, 50S and 70S ribosomes corresponds to 64 pmol, 36 pmol, and 24 pmol, respectively. The same assumption was made for the isolated RNA moiety from each ribosomal particle. A\textsubscript{260} equivalent unit of TP50 and TP30 was calculated as described in ref. 15.

concentrations and with various amounts of drug (data not shown). Similarly, resistant and sensitive 30S subunits did not show any significant difference with respect to binding of the antibiotic (data not shown). It should be noted that it is important to perform a complete experimental series with one and the same ribosome preparation. In our hands, the extent of binding to ribo-
Fig. 3: Binding of TUM O to LiCl core particles derived from E. coli 50S (A) and 30S (B) subunits. A, one chamber was filled with 180 pmoles of 50S subunits, or 50S derived cores, or 23S RNA. The other one contained 2200 pmoles of [14C]-TUM O. The buffer was TM11N160SH. B, one chamber contained 320 pmoles of 30S, 0.5 c, 1.0 c, 3.0 c, 4.0 c, or 16S RNA, and the other one 2200 pmoles of [14C]-TUM O. TM7N70SH was used.

somes within a single preparation was highly reproducible. However, different ribosomal preparations could vary considerably in their binding capacity. The reason for this variation is not clear, but one possibility is that the various preparations represent slightly different ribosomal conformers which expose their ribosomal RNA to various degrees.

Our finding that binding is observed equally well with sensitive and resistant ribosomes is in disagreement with a previous publication (ref.17) which claimed that resistant ribosomes were unable to bind the drug.

The binding to various polynucleotides.

Surprisingly, striking difference in binding capacity of the drug were found with various homopolyribonucleotides. The binding to poly(A) was about 6-fold weaker than that to poly(U) (35 and 216 pmol antibiotic per A260 unit, respectively, see Table 1). An intermediate level of binding was found with poly(G) and poly(C) as well as with poly(AUG). Native DNA from calf
Fig. 4: Comparison of TUM O binding to parental R-15 (••••) and drug-resistant R-31 (○○○○) 50S subunits of M. smegmatis at increasing concentration of Mg acetate. The buffer was TMXN100SH. One chamber contained 110 pmoles of 50S subunits, and the other one 2460 pmoles of [14C]-TUM O.

thymus bound very small amounts of the drug, but the binding capacity could be significantly increased by denaturing the DNA (10 and 50 pmol per A260 units, see Table 1).

Viomycin affects the in vitro assembly of the 50S subunits.

Since as shown above viomycin binds to RNA, it is conceivable that the drug would influence assembly of the ribosomal subunits. To test a possible interference of viomycin with the reconstitution of the 50S subunits, the following experiment was performed: 50S subunits from E. coli were reconstituted in the presence of various amounts of viomycin, and, as a control, native 50S subunits were subjected to the reconstitution procedure, also in the presence of various concentrations of viomycin. After the two step incubation, the samples were dialysed against TM20W400SH buffer in order to remove the excess of the drug. After adding native 30S subunits, the poly(U) directed poly(Phe) synthesis was measured. Fig. 5A demonstrates that even at very
Fig. 5: Activities of 50S subunits reconstituted in presence of viomycin (A) and some other drugs (B). A, poly(U)-directed polyphenylalanine synthesis of 50S particles reconstituted in presence of increasing amounts of viomycin (TUM B, o--o). As a control, native 50S particles were subjected to the reconstitution procedure in presence of the same amounts of the drug (e--e). After the reconstitution incubations the samples were dialyzed against 3L of TM20N400SH buffer for 2 hrs in order to remove the drug and then assayed for poly(U)-directed polyphenylalanine synthesis. B, effects of streptomycin (e--e), chloramphenicol (o--o), and aurintricarboxylic acid (o--o) on the reconstitution of 50S subunits. The experimental conditions were as described under A.

low concentrations of viomycin the assembly of the E. coli ribosomes was severely affected. The activity of native 50S subunits in the control was hardly affected, indicating that the dramatic reduction of activity seen with the reconstituted particles is not due to a transfer of drug from the incubation system to the poly(U) system, but is due to a strong interference with the assembly of the 50S subunit. The 50S particles obtained in the presence of the drug show a similar sedimentation coefficient to that of native 50S subunits in sucrose gradients (data not shown).

A further control was performed in a parallel experiment, which was designed to test chloramphenicol, aurintricarboxylic acid, and streptomycin for their inhibitory effect on reconstitution (see Fig. 5B). Chloramphenicol and aurintricarboxylic
acid did not affect the reconstitution procedure at all, whereas streptomycin (which also binds to RNA, ref. 18) influences the in vitro assembly of the large subunit (solid circles in Fig. 5B). However, streptomycin affects the in vitro assembly of the 50S subunit much less than viomycin. At a concentration of streptomycin of 1 μM, the reconstituted particles show about 60% activity, whereas at this concentration of viomycin only background activity was found.

The in vitro assembly of the E. coli 50S subunit is even more strongly inhibited than protein synthesis systems with E. coli ribosomes (poly(U) system as well as natural mRNA system, compare Fig. 1 in ref. 5 with Fig. 5A). It is therefore likely that viomycin also affects assembly in vivo. However, it is difficult to understand how a resistant mutant can counteract the interference of the drug with the assembly process. Even more puzzling is the fact that resistant mutants exist, where the resistance is conferred by the RNA of either the small or large subunit. It should be noted that resistance is not generated by spontaneous mutation, but is rather the result of a multistep mutational process (ref. 10). Possibly, the assembly of both ribosomal subunits occurs in a coordinated manner, and an appropriate alteration of either ribosomal RNA (16S or 23S RNA) can counteract the effect of the drug on the assembly process. Evidence for a coordinate assembly of both ribosomal subunits have been reported (19).

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