tRNA binding to programmed ribosomes increases the ribosomal affinity for tuberactinomycin O

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The binding of ^{14}C-labelled tuberactinomycin O was analysed in equilibrium dialysis cells. The ionic conditions and the concentration of the labelled drug used in the binding assays allowed the binding of just one drug molecule per non-programmed ribosome. Under these conditions, the occupation of the ribosomal P-site by deacylated tRNA^{Phe} in the presence of poly(U) increased the amount of [^{14}C]tuberactinomycin O bound by a factor of two. Kanamycin, gentamicin and neomycin reduced the binding of tuberactinomycin O, whereas chloramphenicol, tetracycline, streptomycin and puromycin had no effect. A stimulation of the binding of tuberactinomycin O was found upon addition of erythromycin.

Tuberactinomycin O binding *Protein synthesis inhibitor* *Equilibrium dialysis* *Ribosomal tRNA binding* *Antibiotic*

1. INTRODUCTION

Viomycin (= tuberactinomycin B) is a peptide antibiotic which inhibits polypeptide synthesis in cultures of *Mycobacterium avium* [1], and in the cell-free systems of both *Escherichia coli* [2] and *Mycobacterium smegmatis* [3]. The drug blocks the translocation reaction [4,5], reduces tRNA binding to the ribosomal A-site and severely impairs the accuracy of tRNA selection at the A-site [6].

It is likely that the effect on ribosomal function involves a concerted action of both subunits, since it was found that resistance against viomycin could be conferred by an altered rRNA either in the 30 S or in the 50 S subunit [3,7]. This view is supported by the finding that viomycin favours the association and inhibits the dissociation of the subunits [8].

Here we demonstrate that the binding of [^{14}C]tuberactinomycin O to ribosomes is significantly enhanced by tRNA binding to the ribosomal P-site. Furthermore, binding of the drug was reduced by kanamycin, gentamicin and neomycin, whereas streptomycin, chloramphenicol and tetracycline had no effects.

2. MATERIALS AND METHODS

2.1. *Antibiotics and reagents*

Crude tuberactinomycin O was a gift from Toyo Jozo Co. It was purified by the procedure in [9]. Gentamicin (a mixture of C1a and C2) was a gift from Dr A. Böck (Universität München). Neomycin and puromycin were purchased from Sigma, Heidelberg, and kanamycin, poly(U), and tRNA^{Phe} were from Boehringer, Mannheim. [^{14}C]Urea (55 mCi/mmol, 887 μCi/mg) was obtained from the Radiochemical Centre, Amersham, England.

2.2. *Strain, culture media and ribosomes*

E. coli K12, strain A19, was cultured in 1% bactotryptone (Difco), 0.5% yeast extract (Difco), 0.09 M NaCl, 0.01 N NaOH and 0.2% glucose. Ribosomes (tight couples) were prepared as in [10].

2.3. *Preparation of [^{14}C]tuberactinomycin O*

[^{14}C]Tuberactinomycin O was prepared by incubating 3.75 mg tuberactinomycin O and 1 mCi [^{14}C]urea in 75 μl of 3 N HCl at room temperature for 3 days, followed by purification on a Sephadex

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G-10 column. The purity of the labelled antibiotic was checked by thin-layer chromatography on silica gel, using a solvent system consisting of acetone, 10% ammonium acetate, and ammonia in the ratio 10:9:1. The [^{14}C]tuberactinomycin O thus obtained was used in concentrations of 1.9 nmol/μl and 14 cpm/pmol.

2.4. *Equilibrium dialysis*

Equilibrium dialysis was carried out with cells containing two chambers each with a volume of 200 μl as described [11]. The dialysis was performed in the presence of 50 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 160 mM NH_4Cl, 6.7 mM mercaptoethanol. One chamber contained 144–180 pmol ribosomes in 100 μl, the other chamber 1700 pmol [^{14}C]tuberactinomycin O in the same volume. The equilibrium cells were gently shaken at 4°C for 19–24 h, then 2 × 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. From the difference in the values found for the two chambers the amount of [^{14}C]tuberactinomycin O bound to ribosomes was calculated. Where indicated, the ribosome chamber contained 75 μg (1.5 A_{260} units) of poly(U) and $tRNA^{Phe}$ as given in the tables.

3. RESULTS AND DISCUSSION

The binding of [^{14}C]tuberactinomycin O to ribosomes was measured by equilibrium dialysis under conditions which yield the binding of one drug molecule per ribosome (see table 1, first line, 182 pmol tuberactinomycin bound per 180 pmol ribosomes). The binding was stimulated by adding increasing amounts of deacylated $tRNA^{Phe}$ in the presence of the message poly(U). At a two-molar excess of tRNA the stimulation was more than two-fold. When poly(U) was omitted the addition of deacylated tRNA did not induce a significant effect.

In the absence of ribosomes poly(U) alone showed some low binding of the antibiotic whereas with $tRNA^{Phe}$ no binding was found (see controls in table 1). The relatively low binding of tuberactinomycin to nucleic acids observed here is due to the higher ionic strength we applied in these equilibrium dialysis experiments in contrast to the previous data [12]. Increasing the ionic strength

Table 1

Binding of [^{14}C]tuberactinomycin O to ribosomes in the presence of various amounts of $tRNA^{Phe}$

Ribosomes (180 pmol)	Poly(U)	Added $tRNA^{Phe}$ (pmol)	Bound tuberactinomycin O (pmol)	Relative binding	
				%	(drug/70 S)
+	+	0	182	100	(1.00)
+	+	150	319	175	(1.75)
+	+	375	404	222	(2.22)
+	+	750	428	235	(2.35)
+	−	0	193	106	(1.06)
+	−	150	205	113	(1.13)
+	−	375	247	136	(1.36)
+	−	750	234	129	(1.29)
Controls					
−	+	0	127	69	
−	−	0	0	0	
−	−	150	0	0	
−	−	375	0	0	
−	−	750	0	0	

dramatically reduces the binding of tuberactinomycin to nucleic acids [12].

Maximal stimulation of the binding of tuberactinomycin to programmed ribosomes was achieved by the addition of a two-molar excess of $tRNA^{Phe}$ (375 pmol $tRNA^{Phe}$, see table 1). At this molar ratio $tRNA^{Phe}$ binds exclusively to the ribosomal P-site [13]. The addition of more tRNA does not further increase the drug binding (table 1, cf. binding of 2.22 and 2.35 drug molecules per ribosome at the addition of 375 and 750 pmol $tRNA^{Phe}$, respectively).

The strikingly enhanced drug binding in the presence of tRNA suggests that tRNA binding to the P-site of programmed ribosomes may alter the ribosomal conformation or, alternatively, the presence of tRNA at the P-site stabilizes a defined conformational state which now represents an enlarged fraction of the ribosome population. Our finding is in accord with the observation of an increased ribosomal affinity for chloramphenicol and anisomycin, if the ribosomes bear a peptidyl tRNA at the P-site [14,15].

In the next experiments we compared the binding of tuberactinomycin O with an excess of various other antibiotics. Table 2 demonstrates that kanamycin, gentamicin and neomycin were able to compete with the binding of tuberactinomycin O when $tRNA^{Phe}$ was bound to the P-site of programmed ribosomes. Erythromycin stimulated the binding of tuberactinomycin O even further, whereas the other antibiotics tested (chloramphenicol, tetracycline, streptomycin, and puromycin) had no significant effect. The competition of the first 3 drugs was less pronounced when poly(U) and $tRNA^{Phe}$ were omitted from the assays (see table 2), and the stimulating effect of erythromycin was even reversed, leading to a slight depression. It is clear that A-site specific antibiotics such as tetracycline, streptomycin, chloramphenicol and puromycin had no effect on the binding of tuberactinomycin O. Even the filling of the A-site with *N*-acetyl-$tRNA^{Phe}$ did not influence the binding of tuberactinomycin O (not shown). Thus, tRNA present at the ribosomal P-site stimulates the binding of tuberactinomycin O but, on the other hand, ligands of the ribosomal A site do not interfere with the binding of tuberactinomycin. It is therefore evident that the binding site of tuberactinomycin O is located neither at the ribosomal P- nor at the ribosomal A-site, but is allosterically linked to the ribosomal P-site.

Table 2

Effect of various antibiotics on the binding of [^{14}C]tuberactinomycin O to ribosomes

Antibiotic added	(μM)	Tuberactinomycin O bound to programmed 70 S			Tuberactinomycin O bound to non-programmed 70 S		
		pmol	Relative binding		pmol	Relative binding	
			%	(drug/70 S)		%	(drug/70 S)
None	–	330	100	(2.3)	200	100	(1.4)
Kanamycin A	165	204	62	(1.4)	186	93	(1.3)
Gentamicin C	170	202	61	(1.4)	161	81	(1.1)
Neomycin B	130	187	57	(1.3)	178	89	(1.2)
Chloramphenicol	250	353	107	(2.5)	197	99	(1.4)
Tetracycline	180	309	94	(2.2)	190	95	(1.3)
Streptomycin	140	351	106	(2.4)	211	106	(1.5)
Puromycin	170	363	113	(2.5)	197	99	(1.4)
Erythromycin	110	451	137	(3.1)	153	77	(1.1)

The [^{14}C]tuberactinomycin O concentration was 8.5 μM (1700 pmol per assay); 'programmed 70 S' means that 144 pmol of 70 S ribosomes were preincubated with 75 μg poly(U) (1.5 A_{260} units) and 300 pmol $tRNA^{Phe}$ (per assay); 'non-programmed 70 S' means that 144 pmol 70 S ribosomes were present per assay without poly(U) or $tRNA^{Phe}$

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