THE EFFECTS OF STREPTOMYCIN AND TETRACYCLINE ON CODON–ANTICODON INTERACTIONS

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1. Introduction

The antibiotics streptomycin and tetracycline affect the attachment of aminoacyl-tRNA's to the ribosome. A common feature of both drugs is that they inactivate the ribosome by binding to the 30 S subunit [1–3]. The attachment of streptomycin to an area around the ribosomal protein P 10 is well documented [4]. No such singular binding site for tetracycline has been found, even the number of drug molecules per ribosome is still uncertain [5, 6]. The mode of attachment, however, is partially known as tetracycline has been found to bind to ribosomal components by magnesium bridges [7]. The exact nature of the disturbances originating from both bound streptomycin and tetracycline is as yet unknown. Since mRNA binding and codon recognition are believed to occur on the 30 S ribosomal particle, we attempted to reveal the molecular details of the mode of action of both antibiotics by studying their effect on ribosome free codon–anticodon interactions. As described previously, codon–anticodon interactions can be observed even in the absence of ribosomes [8] and the strength of these interactions may be estimated by equilibrium dialysis techniques [9, 10]. Determinations of ribosome free codon–anticodon interactions in the presence of streptomycin or tetracycline showed that the former had no effect while the latter strongly interfered with this reaction. Based on these results, a model for the mode of action of tetracycline is proposed.

2. Materials and methods

Tetracycline hydrochloride and pyrrolidinomethyl-tetracycline (Rolitetracycline®) were obtained from Pfizer Corp. and from Farbwerke Hoechst, respectively. Streptomycin sulfate was from Biochemie Ges.m.b.H., Vienna. [^{3}H] Tetracycline HCl was purchased from the Radiochemical Centre, Amersham. The sources for all other materials are listed in previous communications [9, 11].

2.1. *tRNA$_f^{Met}$ (E. coli) preparations*

The procedure described earlier [11] was followed. Magnesium was removed from the tRNA either by additional chromatography on benzoylated DEAE cellulose using a gradient from 0.45 M NaCl (Solution A) to 1.5 M NaCl (Solution B), both solutions containing 0.001 M EDTA, 0.005 M mercaptoethanol and 0.005 M Na-formate, pH 4, or by extensive dialysis of the tRNA against solution A.

2.2. *Estimation of the magnesium content of tRNA*

24 mg commercial crude *E. coli* tRNA was dissolved in 10 ml of 0.45 M NaCl and subsequently precipitated with ethanol, washed and dried. This and an untreated sample were dissolved in glass distilled water and the magnesium content of each was determined with a Perkin-Elmer Model 303 atomic absorption spectrophotometer. The untreated sample contained 75.5 μg Mg^{2+} per A_{260} unit while the EDTA treated tRNA contained 1.91 μg Mg^{2+} per A_{260} unit.

2.3. *Equilibrium dialysis experiments*

Micro dialysis cells [12] were used throughout. The experimental procedure and the introduced modifications have previously been described [9, 11]. In all cases it was established that within the time allowed for each equilibrium dialysis experiment, an

even distribution of freely diffusible substances was possible. All experiments were conducted at 2°.

3. Results and discussion

Tetracycline, when applied at concentrations lower than 0.1 mM, inhibits mRNA dependent attachment of aminoacyl-tRNA to the ribosomal A-site both in the nonenzymatic [13, 14] and in the T factor-stimulated [15] binding reaction. The chemical causes for this behaviour have thus far been unresolved. In an attempt to elucidate the molecular details of the drug's mode of action we studied its influence upon codon–anticodon interactions since this parameter, in addition to interactions between tRNA and the ribosomal surface, provides the energy for the attachment of tRNA to the ribosome. The possible interference by tetracycline with codon–anticodon interactions was assayed using an equilibrium dialysis system which permits the quantitative estimation of triplet binding to tRNA [9, 11]. The $tRNA_f^{Met}$–ApUpG association was chosen as a model reaction since it offers a number of experimental conveniences in addition to the advantage that the details of this complex formation have been well established [9, 11]. The selection of this model, however, was not related to the fact that $tRNA_f^{Met}$ functions as an initiator tRNA.

The complex formation of tetracycline with divalent metal ions precluded employment of previously used buffers as they contain magnesium ions. The presence of noticeable quantities of such chelates could make an interpretation of the dialysis data impossible. However, before magnesium could be omitted from the dialysis system, its effect on the binding of ApUpG to $tRNA_f^{Met}$ had to be investigated. In the absence of magnesium the attachment of triplets to the complementary anticodon of tRNA was found to be reduced but still strong enough to be accurately measured. Another point of concern was the possible removal of a tightly bound magnesium ion from $tRNA_f^{Met}$ by tetracycline. We could not a priori dismiss a mechanism that such a metal ion is required for the anticodon loop to retain its correct conformation and its elimination, therefore, prevents the establishment of a codon–anticodon interaction. To exclude this possibility the tRNA preparations were treated with an EDTA containing buffer, a procedure which was shown to reduce the magnesium content of tRNA to less than 0.05 atoms per tRNA molecule.

The results from equilibrium dialysis experiments performed in the presence of a highly water soluble derivative, pyrrolidino-methyl-tetracycline (Rolitetracycline®), are depicted in fig. 1. It is apparent from this graph that codon–anticodon interactions become unstable at tetracycline concentrations above 5×10^{-4} M. This inhibition may reflect either a competition for the anticodon bases or the intercalation of the planar part of the drug molecule into the double helical regions of tRNA. Binding of tetracycline to polynucleotides has been observed earlier [5, 6]. We measured the binding of tetracycline to tRNA by equilibrium dialysis using the labeled antibiotic. Two types of binding were established from a Scatchard plot (fig.2), the stronger one, K_{ass} 27,000 M^{-1}, involves only about 0.1 binding site and probably arises from contaminating metal ions adhering tightly to the tRNA molecule. The weaker binding appears to be responsible for the inhibitory effect of tetracycline. The results indicate approximately three sites on the $tRNA_f^{Met}$ molecule for this attachment. The

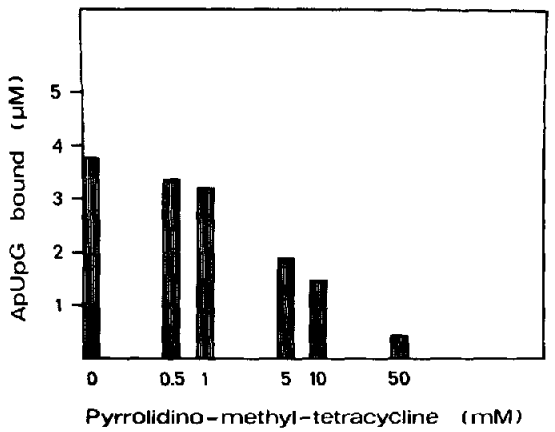

Fig. 1. Effect of pyrrolidino-methyl-tetracycline on codon–anticodon interactions. A solution of $tRNA_f^{Met}$ in 0.2 M NaCl, 0.01 M HEPES, pH 6, at a concentration of 1.012×10^{-4} M was placed on one side of the dialysis membrane and a mixture of equal volumes of pyrrolidino-methyl-tetracycline and [^{3}H] ApUpG dissolved in the same buffer, on the other side. The initial triplet concentration was 8.14×10^{-5} M. The plotted tetracycline values are the final concentrations. The dialysis experiments lasted 6 hr.

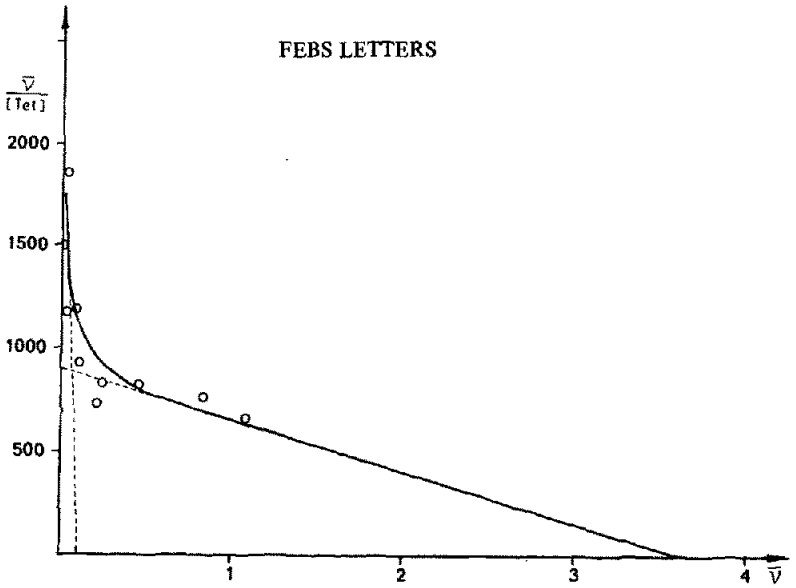

Fig. 2. Scatchard plot of tetracycline-HCl binding to $tRNA_f^{Met}$. $tRNA_f^{Met}$ (1.36×10^{-4} M) in 0.4 M NaCl, 0.02 M HEPES, pH 6, was dialyzed to equilibrium against aqueous solutions of [^{3}H] tetracycline-HCl. The poor solubility of tetracycline prevented measurements at concentrations greater than 1.7 mM.

Table 1
Measured and calculated association constants of the $tRNA_f^{Met}$–ApUpG complex in the presence of various tetracycline concentrations.

Pyrrolidino-methyl-tetracycline (M)	K_{app} experimental (M^{-1})	K_{app} calculated (M^{-1})
0	2083	–
5×10^{-4}	1739	1835
1×10^{-3}	1692	1640
5×10^{-3}	987	886
1×10^{-2}	757	563
5×10^{-2}	470	144

association constant of 270 M^{-1} has been estimated for these complexes.

The apparent association constants of the ApUpG–$tRNA_f^{Met}$ binding in the presence of varying tetracycline concentrations listed in table 1 were calculated using the data of fig. 1. This table also includes the apparent association constants which were calculated using formula 1, the mathematical treatment for competitive binding of two different molecules to the same binding site on a third molecular species.

$$K_{app} = \frac{K_o}{1 + K_i[Tet]}. \quad (1)$$

In this equation K_o represents the binding constant of the uninhibited association, K_i the association constant of the $tRNA_f^{Met}$–tetracycline complex, and [Tet] the tetracycline concentration. The good correlation of observed *vs.* calculated binding constants strongly suggests that competitive binding of tetracycline to tRNA inhibits codon–anticodon interactions rather than intercalating drug molecules. Supporting this theory is the difference in strength and in the number of binding sites seen between tetracycline–tRNA and ethidium bromide–tRNA interactions [16]. Ethidium bromide is known to be intercalated into helical regions of tRNA. According to our exploratory study with Dreiding atomic models, the hydroxy and keto groups on C-10, C-11 and C-12 of tetracycline may be perfectly paired with the nucleotide-bases to give hydrogen bonded complexes. In a single helical polynucleotide chain each tetracycline molecule would thus occlude the space of a triplet along the helical axis. Although it was not rigorously proven, the idea of one of the three tetracycline molecules adhering to the anticodon and thereby preventing the attachment of the codon in a competitive way appears to be the most plausible explanation for our results.

The unspecific, i.e. messenger independent, binding of tRNA to ribosomes is nearly unchanged by tetracycline, an observation first made by Takeda et al. [17]. Their experiments and similar results from this laboratory [18] obtained from EF-T factor dependent binding of several aminoacyl-tRNA's to ribosomes support our idea that impairment of codon–anticodon interactions is the drug's mode of action.

Ribosomes are known to attach tetracycline through magnesium ions [7, 19]. The number of drug molecules on the ribosomal surface, however, is still a matter of controversy. On the basis of our experiments we propose that one of the ribosome-bound tetracycline molecules adheres close to the area of codon-anticodon interaction on the A-site and thus increases the local concentration of the drug in that region. This favourably positioned tetracycline molecule causes the disruption of the codon-anticodon binding while the tRNA-ribosome interactions, which are chemically still undefined, remain unaffected but are too weak to permit the tRNA molecule from fully attaching to the ribosome.

Most of the known data on tetracycline activity in bacterial protein synthesis are consistent with our model. For example, the inhibition of f-Met-tRNA binding to the P-site [20] by drug concentrations of the same magnitude as those effective in the ribosome-free system, suggests that freely diffusible or less specifically bound tetracycline molecules prevent the aminoacyl-tRNA attachment to the P-site.

Streptomycin, after binding to the 30 S ribosomal subunit, causes either miscoding [21] or detachment of tRNA's from ribosomes [22, 23] and, therefore, possibly disturbs codon–anticodon interactions. However, even at a concentration of 12.5 mM the antibiotic showed no inhibitory effect on ribosome-free attachment of AUG to $tRNA_f^{Met}$ when tested in the system described in fig. 2. On the basis of this result another explanation is required for the mode of action of streptomycin, such as the concept of a distorted binding site as proposed by Modolell and Davis [23].

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