EFFECT OF SPECTINOMYCIN ON PEPTIDE CHAIN INITIATION

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Spectinomycin inhibits the formation and causes dissociation of preformed specific cell-free initiation complexes prepared with the trinucleotide A-U-G or R_{17} phage RNA, fmet-RNA_F and either 30S ribosomal subunits or 70S ribosomes. Both the initiation factor and the Mg^{2+}-induced processes are subject to inhibition by spectinomycin. The only exception is the Mg^{2+}-induced 70S initiation system formed with A-U-G which is stimulated by spectinomycin.

Previous studies on the mode of action of spectinomycin suggested that this antibiotic inhibits protein synthesis and more specifically that it might impair ribosomal functions associated with the translocation step. This conclusion was largely reached by a process of elimination in which the antibiotic failed to inhibit other ribosomal functions in cell-free systems and the observation that protoplasts treated with spectinomycin maintain high levels of polysomes[1,2]. DAHLBERG *et al.*[3] on the other hand found a rapid change in the electrophoretic mobility of the polysomal patterns in cell extracts from spectinomycin-treated *Escherichia coli* cells: Within 1~2 minutes after addition of the antibiotic a transition to shorter polysomes and predominantly 70S particles occurred which was followed by a further change to intermediate size polysomes and the disappearance of large size polysomes. These results would indicate that spectinomycin inhibits the process of chain initiation. More recently WALLACE *et al.*[4] suggested that spectinomycin inhibits ribosomal functions occurring immediately after initiation.

This report presents evidence that spectinomycin inhibits the process of peptide chain initiation *per se* when studied in specific cell-free initiation systems.

Materials and Methods

Salt-washed ribosomes and 30S and 50S ribosomal subunits were prepared as described previously[5]. *E. coli* $tRNA_F^{met}$ was obtained from the Oak Ridge National Laboratories. Synthetase-transformylase enzyme required for the synthesis of fmet-$tRNA_F$ was isolated by the procedure of DUBNOFF and MAITRA[6]. [^{3}H] fmet-$tRNA_F$ was prepared as described by CASKEY *et al.*[7] and had a specific activity of approximately 900 cpm/pmole. Initiation factors were isolated from the first ribosomal salt wash fluid[6]. The trinucleotide A–U–G was obtained from Collaborative Research, Inc., Waltham, Mass. Binding of fmet-$tRNA_F$ to 70S ribosomes and 30S ribosomal subunits was assessed by the nitrocellulose filtration technique.

The reaction mixtures contained in a total volume of 0.15 ml: 50 mM Tris·HCl, pH 7.8; 100 mM NH_4Cl; 4 mM or 20 mM Mg acetate; 4 mM mercaptoethanol; 2 mM GTP; 0.04 OD_{260} units (optical density at 260 nm) of A–U–G or 0.5 OD_{260} units of phage RNA; 4.5 OD_{260} units of 70S ribosomes or 1.5 OD_{260} units of 30S subunits; 0.11 OD_{260} units of [^{3}H] fmet-$tRNA_F$ containing ~30,000 cpm; and 25 μg of initiation factors where applicable. Spectinomycin concentration =0.05 mM unless indicated otherwise.

Results

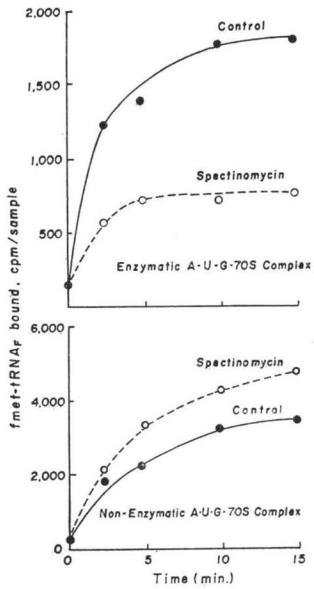
Binding of fmet-tRNA$_F$

Control experiments showed that no fmet-tRNA$_F$ was bound to salt-washed ribosomes in the presence of mRNA but in the absence of initiation factors if the Mg^{2+} concentration in the reaction mixtures amounted to 2 ~ 4 mM. Hence, our system was strictly dependent on the participation of initiation factors to form an initiation complex in the presence of low Mg^{2+} concentrations. If the Mg^{2+} concentration in the test system was raised to 15 or 20 mM, the participation of initiation factors was no longer required to form initiation complexes. Binding carried out at low Mg^{2+} concentrations (2 ~ 4 mM) with the participation of initiation factors is referred to as enzymatic initiation and initiation carried out in the presence of high Mg^{2+} concentrations (10 ~ 20 mM) not requiring initiation factors is referred to as nonenzymatic or Mg^{2+}-induced initiation. We recently demonstrated that the antibiotic rubradirin specifically prevents the formation of enzymatic initiation complexes but does not affect the formation of nonenzymatic ones.[8] Hence, these two types of initiation complexes must differ in some significant aspect.

Initiation Complexes Formed with the Codon A–U–G

Fig. 1. Effects of spectinomycin on formation of enzymatic (top) and Mg^{2+}-induced (bottom) 70S initiation complexes with A-U-G.

The reaction mixtures contained in a total volume of 0.15 ml; 50 mM Tris-(hydroxymethyl)-aminomethane-hydrochloride, pH 7.8; 100 mM NH_4Cl; Mg·acetate 4 mM or 20 mM; 4 mM mercaptoethanol; 2 mM guanosine 5′-triphosphate; 0.04 OD_{260} units (optical density at 260 nm) of A-U-G; 4.8 OD_{260} units of 70S ribosomes, 0.11 OD_{260} units of [^{3}H]fmet-tRNA$_F$ containing ~30,000 counts/min; and 25 μg of initiation factors where applicable. The reaction mixtures were incubated at 37°C. Spectinomycin concentration = 0.05 mM.



Spectinomycin was found to inhibit the enzymatic formation of 70S initiation complexes formed with A–U–G, fmet-tRNA$_F$, initiation factors and salt-washed 70S ribosomes in the presence of 4 mM Mg^{2+} (Fig. 1). Conversely, non-enzymatic formation of 70S initiation complexes induced in the presence of 20 mM Mg^{2+} was stimulated by the antibiotic (Fig. 1). Spectinomycin thus inhibits the enzymatic formation of the 70S initiation complexes but stimulates the Mg^{2+}-induced ones.

It was also of interest to assess the stability of enzymatic 70S initiation complexes. Initiation factor induced 70S complexes were thus pre-

Fig. 2. Effect of spectinomycin on stability of enzymatic 70S complex with A-U-G. Reaction mixtures were preincubated for 10 minutes prior to addition of spectinomycin.

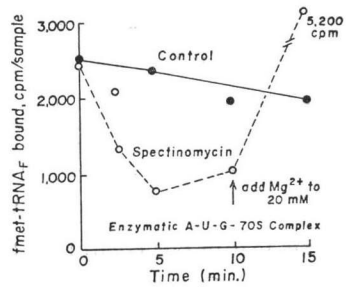


Fig. 3. Effects of spectinomycin on formation of 30S initiation complexes with A-U-G; top= enzymatic complex; bottom=Mg^{2+}-induced complex.

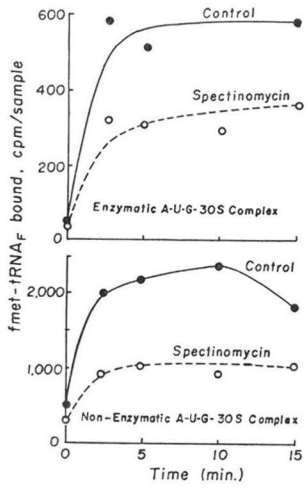


Fig. 4. Effect of addition of 50S subunits to Mg^{2+}-induced 30S complex with A-U-G.
50S subunits=2.8 OD_{260} units/sample.

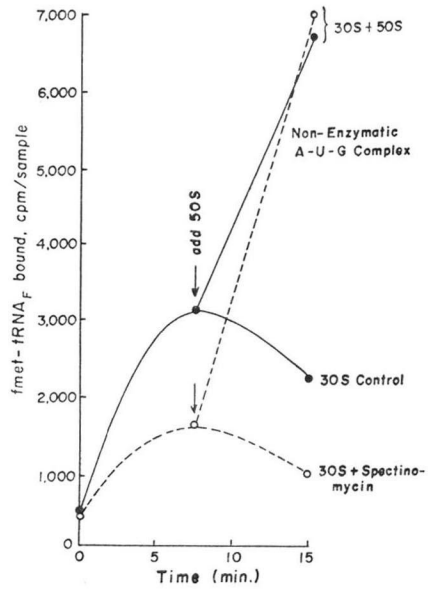
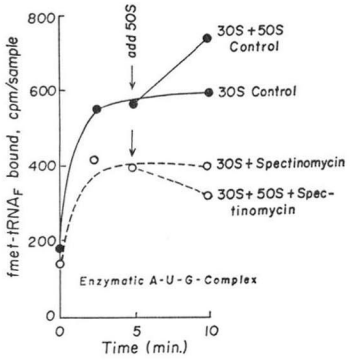


Fig. 5. Effect of addition of 50S subunits to enzymatic 30S complex with A-U-G. 50S subunit conc. see Fig. 4.



formed in the absence of spectinomycin and then exposed to the antibiotic. Under these conditions dissociation of the complex started immediately after addition of the drug (Fig. 2). Readjustment of the Mg^{2+} concentration to 20 mM in the reaction mixtures after dissociation had occurred resulted in an immediate reassociation of the complex. This suggests that ribosomal bound fmet-tRNA$_F$ was released as such and no concomitant deacylation took place.

The enzymatic formation of 30S initiation complexes which functionally preceeds the formation of the 70S complex was also subject to spectinomycin inhibition (Fig. 3). Unexpectedly, non-enzymatic initiation complex formation was also inhibited by spectinomycin (Fig. 3). As shown above the corresponding 70S initiation complex formation process was stimulated by spectinomycin. We thus formed non-enzymatic initiation complexes in the presence or absence of spectinomycin and then added 50S subunits to such reaction mixtures. The 50S preparation contained only trace amounts of 30S subunits as evidenced by electrophoresis on composite polyacrylamide-agarose gels prepared as described by HERMOSO and SZER.[9] Addition of 50S subunits to the 30S initiation complex resulted in a drastic increase of the amount of fmet-tRNA$_F$ bound in both the control samples and the ones containing spectinomycin (Fig. 4). As only one molecule of fmet-tRNA$_F$ will bind per 30S subunit this suggests that in our control mixtures a portion of the 30S subunits was unable to combine efficiently with A–U–G and fmet-tRNA$_F$ but was rendered reactive after the addition of 50S subunits.

Fig. 6. Effects of spectinomycin on stability of enzymatic (top) and Mg^{2+}-induced (bottom) 30S initiation complexes formed with A-U-G. The complexes were preformed in the absence of spectinomycin by incubating the reaction mixtures for 10 minutes (enzymatic system) or 15 minutes (Mg^{2+}-induced system) prior to addition of the antibiotic.

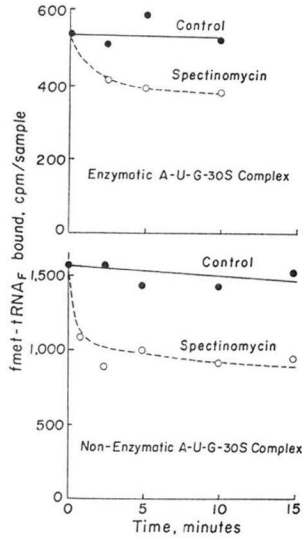


Fig. 8. Effects of spectinomycin on the formation of non-enzymatic (top) and enzymatic (bottom) phage RNA-induced 30S initiation complexes.

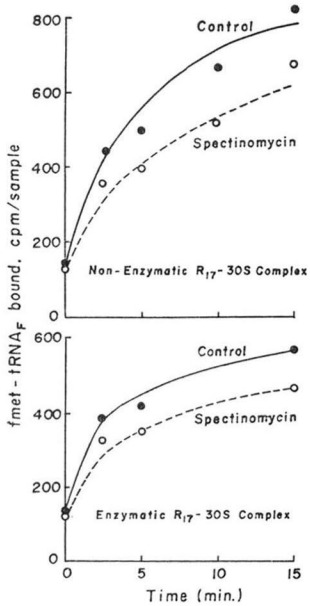


Fig. 7. Effects of spectinomycin on the formation of non-enzymatic (top) and enzymatic (bottom) phage RNA-induced 70S initiation complexes.

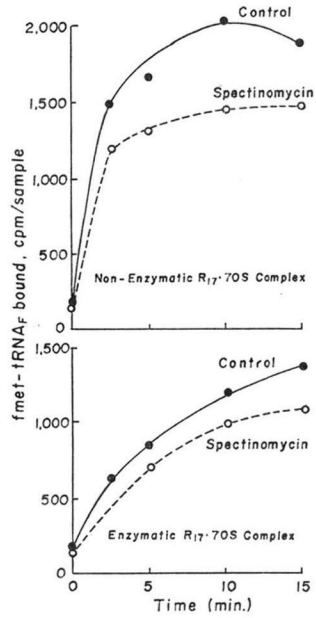
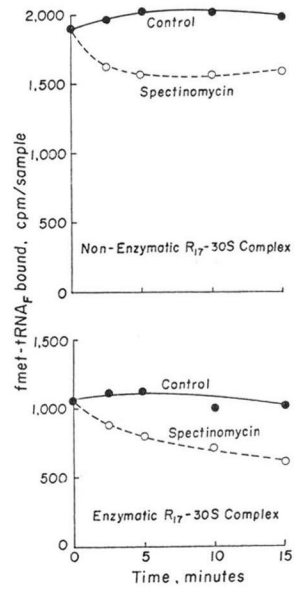


Fig. 9. Effects of spectinomycin on stability of preformed non-enzymatic (top) and enzymatic (bottom) 30S initiation complexes induced with phage RNA. The non-enzymatic complexes were preformed for 15 minutes, the enzymatic ones for 10 minutes prior to addition of spectinomycin.



The 50S subunits may thus exert some catalytic effect upon the 30S subunit in such a manner that the latter one can now participate in the initiation process. Spectinomycin did inhibit the formation of 30S initiation complexes in the absence of 50S subunits as expected but following the addition of 50S subunits inhibition was reversed and minor stimulation of fmet-$tRNA_F$ binding was observed. Hence spectinomycin apparently suppresses the formation of Mg^{2+} induced 30S initiation complexes but this inhibition is completely reversed upon addition of 50S subunits.

Enzymatic 30S initiation complexes were also formed, followed by the addition of 50S subunits. In this case spectinomycin-induced inhibition persisted after the addition of 50S subunits and was thus not reversed by 50S subunits as observed with the corresponding Mg^{2+}-induced system (Fig. 5). Furthermore, it was apparent that the addition of 50S subunits to 30S complexes in the control samples did not significantly enhance the extent of fmet-$tRNA_F$ binding as we had observed in the Mg^{2+}-induced system.

If the enzymatic 30S initiation complex was preformed and then exposed to spectinomycin, dissociation of the complex occurred (Fig. 6). Similarly, preformed Mg^{2+}-induced 30S initiation complexes underwent dissociation after exposure to spectinomycin (Fig. 6). Hence enzymatic as well as non-enzymatic preformed 30S initiation complexes are rendered unstable by spectinomycin.

Initiation Complexes Formed with R_{17} Phage RNA

Spectinomycin was found to inhibit the enzymatic as well as the non-enzymatic formation of 70S complexes consisting of R_{17} phage RNA, fmet-$tRNA_F$ and salt-washed 70S ribosomes (Fig. 7). It was somewhat surprising to find that non-enzymatic initiation in the phage-induced system was also subject to spectinomycin inhibition as we had found previously that this process is stimulated by the antibiotic if the initiator triplet A–U–G was used.

Spectinomycin was also found to inhibit both the enzymatic and non-enzymatic assembly of 30S initiation complexes induced with phage RNA (Fig. 8). The formation of the 30S complex preceeds the formation of the 70S complex. It thus follows that spectinomycin inhibits initiation by interacting with an event or events occurring during the formation of the 30S complex.

Preformed enzymatic as well as Mg^{2+}-induced 30S initiation complexes started to dissociate immediately after the addition of spectinomycin (Fig. 9). The antibiotic thus also renders phage RNA induced complexes unstable.

Discussion

The results presented show that spectinomycin inhibits the formation of both enzymatically and Mg^{2+}-induced 30S initiation complexes formed with A–U–G, fmet-$tRNA_F$ and 30S ribosomal subunits. The antibiotic also induces dissociation of preformed 30S initiation complexes regardless whether they are formed enzymatically or non-enzymatically which indicates that such complexes are rendered unstable upon exposure to spectinomycin. On the other hand, only the enzymatic formation or stability of 70S initiation complexes is impaired by spectinomycin. The non-enzymatic initiation process is actually stimulated by spectinomycin. Addition of 50S subunits to Mg^{2+}-induced 30S initiation complexes reverses the inhibitory activity of spectinomycin on the formation of the 30S complex. Spectinomycin also inhibits the enzymatic and non-enzymatic initiation processes studied in 30S and 70S phage RNA induced systems. As the 30S complex formation preceeds the 70S complex formation, it follows that spectinomycin interacts with one or several initiation functions associated with the assembly of the 30S complex. This conclusion is further substantiated by the observation that preformed 30S complexes are rendered unstable when exposed to the antibiotic.

As both the enzymatic (initiation factor-induced) and non-enzymatic (Mg^{2+}-induced) initiation processes are subject to spectinomycin inhibition, this also indicates that, in all likelihood, initiation factor associated functions are not the primary target for spectinomycin inhibition. Hence spectinomycin appears to impair some function (s) associated with the 30S ribosomal subunit *per se*. It is of interest to note that whole cells treated with spectinomycin show a transition of their polysomal pattern from large-sized units to tri, di and predominantly monosomes as shown by DAHLBERG *et al.*[3] and also evident from the data of WALLACE *et al.*[4] although these authors interpreted their results different from ours. This transition from large polysomes to oligo and particularly to monosomes in whole cells is compatible with our observation that spectinomycin inhibits the initiation process as already initiated ribosomes engaged in peptide chain elongation will continue to synthesize protein in the presence of spectinomycin until they reach the end of the translational message. At this point they will release from the mRNA and as they are unable to reinitiate, they will accumulate as monosomes.

Within the time span the reactions were run, initiation in these specific cell-free systems was never completely suppressed by the antibiotic, even in the presence of high antibiotic concentrations. The inhibition kinetics also show that the inhibitory action of spectinomycin exerted on the initiation processes studied is slow as significant inhibition takes effect only several minutes after the start of the reaction. However, this slow rate of expression does not argue against the conclusion that spectinomycin inhibits protein synthesis by virtue of inhibiting chain initiation as the final effect (meaning cessation of protein synthesis) is the same regardless of the slow kinetics.

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