ACTION OF STREPTOTHRICIN F ON RIBOSOMAL FUNCTIONS

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The effect of streptothricin F on elongation factor-dependent and on elongation factor-free translation systems was studied. Streptothricin F inhibits factor-dependent as well as factor-free polypeptide synthesis. The results suggest that streptothricin F inhibits polypeptide synthesis *via* interaction with the ribosome. In partial reactions streptothricin F impairs EF-G-dependent translocation and to a lesser extent EF-T_u-dependent binding of aa-RNA to the ribosome, while it does not affect peptide bond formation significantly.

We recently examined the effect of streptothricin F (ST-F) on cell-free protein synthesis[1]. ST-F has been shown to inhibit protein synthesis specifically in intact bacterial cells and in cell-free systems of *Escherichia coli*, while the antibiotic did not inhibit cell-free protein synthesis in rat liver extracts. Furthermore ST-F induced misreading of synthetic homopolynucleotides in *E. coli* cell-free systems.

In the present paper the action of ST-F on the individual reactions of polyphenylalanine formation on *E. coli* ribosomes is described.

Materials and Methods

Materials

[^{14}C] Phe-tRNA(182 mCi/m Mol) and ac[^{14}C]Phe-tRNA (182 mCi/m Mol) were prepared as described by ČERNÁ *et al.*[2]. Ac[^{14}C]Leu-pentanucleotide (CACCA-acLeu, 164 mCi/m Mol) was prepared as described by MONRO *et al.*[3]. Radioactivity was determined in a Packard-Tricarb scintillation spectrometer (counting efficiency for ^{14}C was 53%) and a methane flow counter (Frieseke-Hoepfner, counting efficiency for ^{14}C was 41%).

The antibiotics ST-F, neomycin, turimycin-H_5 and streptomycin were dissolved in water while oxytetracycline was dissolved in 0.01 N HCl. Antibiotic solutions were prepared immediately before use.

Preparation of ribosomes

Ribosomes were prepared from *E. coli* B by ammonium sulfate precipitation according to GAVRILOVA and SPIRIN[5] and GAVRILOVA *et al.*[6]. The bacteria were broken by grinding with aluminium oxide powder and the ribosomes were pelleted by centrifugation at 105,000 *g* in a buffer containing 10 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 5 mM NH_4Cl and 1 mM β-mercaptoethanol. The ribosomal pellets were washed 4 times with 1 M NH_4Cl in 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ and 1 mM β-mercaptoethanol and were precipitated at 0°~4°C by ammonium sulfate adding 49 g of the dry salt per 100 ml of the ribosome suspension containing 2.5 mg ribosomes per ml. The ribosomal precipitate was stored under ammonium sulfate at 4°C. Immediately before use 2 ml of the ribosome suspension were centrifuged for 40 minutes at 15,000 rpm. The pellet was suspended in 10 mM Tris-HCl (pH 7.8), 100 mM KCl and 20 mM $MgCl_2$ and dialysed against the same buffer to complete removal of the ammonium sulfate. Dialysis was continued against a buffer with the $MgCl_2$ concentration required for the experiment.

Preparation of elongation factor EF-T_u·GDP and elongation factor EF-G

Crystalline EF-T_u·GDP from *E. coli* B was prepared according to ARAI *et al.*[7]. The fraction with EF-G activity obtained after DEAE-Sephadex column chromatography of the postribosomal S–100 supernatant from *E. coli* cells served for the preparation of elongation factor EF-G[7]. The fraction was first precipitated by solid ammonium sulphate. The precipitate formed between 38% and 59% saturation was dissolved in 0.02 M Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol and 0.25 M sucrose, frozen and stored in dry ice. Just before use an aliquot of the preparation was diluted with one volume of distilled water, heated for 4 minutes at 55°C, the precipitate so formed was removed by centrifugation and the clear supernatant diluted again with one volume of distilled water. The preparation was completely free of EF-T_u activity when tested in a cell-free poly(U)-dependent polyphenylalanine formation system.

Factor-free and factor-dependent translation systems

The elongation factor-free and the elongation factor-dependent translation systems were prepared according to SPIRIN *et al.*[8]. The reaction mixture for factor-free translation contained in 50 μl: 10 mM Tris-HCl (pH 7.1), 100 mM KCl, 13 mM $MgCl_2$, 0.1 mM *p*-chloromercuribenzoate, 10 μg poly(U), 20 μg ribosomes, 44.7 pMol [^{14}C]Phe-tRNA. After incubation for 3 hours at 25°C the reaction was stopped with 3 ml of 5% trichloroacetic acid. The samples were hydrolysed for 15 minutes at 90°C, cooled and filtered through Synpor 6 filters (Chemapol, ČSSR)[9]. Radioactivity was measured in a Packard-Tricarb scintillation spectrometer. The reaction mixture for the factor-dependent translation contained in 50 μl: 10 mM Tris-HCl (pH 7.1), 100 mM KCl, 13 mM $MgCl_2$, 2 mM dithiothreitol, 10 μg poly(U), 20 μg ribosomes, 44.7 pMol [^{14}C]Phe-tRNA, 0.4 mM GTP and either 3 μg EF-T_u and 2.3 μg EF-G in the complete factor-dependent system or 3 μg EF-T_u or 2.3 μg EF-G in the one factor-dependent systems. The complete factor-dependent system was incubated for 20 minutes at 25°C while the EF-T_u- and EF-G-dependent systems were incubated for 3 hours at 25°C. Treatment of the samples was as in the factor-free translation system.

EF-T_u-dependent binding of Phe-tRNA to ribosomes

The composition of the final reaction mixture (100 μl) was the following: 50 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 150 mM NH_4Cl, 10 mM β-mercaptoethanol, 10 μg poly(U), 100 μg tRNA, 50 μg ribosomes, 44.7 pMol [^{14}C]Phe-tRNA, 4 mM GTP and 2 μg EF-T_u·GDP. The reaction mixture (50 μl) without [^{14}C]Phe-tRNA, GTP and EF-T_u·GDP was first preincubated for 10 minutes at 30°C. Then [^{14}C]Phe-tRNA, GTP and EF-T_u·GDP in 50 μl of the reaction buffer were added and the incubation was continued for further 10 minutes at 30°C. Subsequently 2 ml of cold incubation buffer were added and the ribosome-bound[^{14}C]Phe-tRNA was measured by Millipore-filtration technique[10].

Peptidyltransferase reaction

The transfer of the acPhe-residue from ac[^{14}C]Phe-tRNA to puromycin was measured according to RYCHLÍK *et al.*[11] whilst the transfer of the acLeu-residue from ac[^{14}C]Leu-pentanucleotide (CACCA-ac[^{14}C]Leu) to puromycin was measured according to MONRO *et al.*[3]. The reaction mixtures with acPhe-tRNA contained in 100 μl: 100 mM Tris-acetate (pH 7.2), 100 mM ammonium acetate, 10 mM magnesium acetate, 84 pMol ac[^{14}C]Phe-tRNA, 250 μg ribosomes and 10 μg poly(U). After preincubation for 20 minutes at 35°C, puromycin to a final concentration of 100 μM was added and the incubation continued for further 20 minutes at 35°C. Incubation was terminated by adding 100 μl of 200 mM sodium acetate (pH 5.5) saturated with $MgSO_4$. The acPhe-puromycin formed was extracted with ethyl acetate (1.5 ml) and the radioactivity was measured on planchets in a methane flow counter. The reaction mixtures with CACCA-acLeu contained (before adding methanol) in 100 μl: 60 mM Tris-HCl (pH 7.4), 40 mM KCl, 20 mM magnesium acetate, 150 μg ribosomes, 12 pMol ac[^{14}C]Leu-pentanucleotide and 500 μM puromycin. The reaction was started with 50 μl methanol. Incubation at 0°C was terminated after 30 minutes and product extracted as described in the experiment with acPhe-tRNA.

Translocation of acPhe-tRNA

Translocation was followed by the puromycin reaction in the presence of EF-G according to LIOU and TANAKA[12]. The reaction mixture contained in 100 μl: 50 mM Tris-HCl (pH 7.8), 10 mM

magnesium acetate, 60 mM NH_4Cl, 2 mM dithiothreitol, 50 μg poly(U), 84 pMol ac[^{14}C]Phe-tRNA, 100 μg ribosomes, 0.5 mM puromycin, 0.2 mM GTP and 1 μg EF-G. The mixture (70 μl) of all reaction ingredients with the exception of puromycin, GTP and EF-G was incubated for 10 minutes at 30°C. After addition of the remaining compounds (30 μl) in the same buffer, incubation was continued for further 10 minutes at 30°C. Incubation was terminated by adding 100 μl of 200 mM sodium acetate (pH 5.5) saturated with $MgSO_4$. The acPhe-puromycin formed was extracted with ethyl acetate (1.5 ml) and the radioactivity determined in a methane flow counter.

Results

1. Effect of ST-F on Factor-free and Factor-dependent Synthesis of Polyphenylalanine in Cell-free Systems of *E. coli*

It has been reported by PESTKA[18] and by SPIRIN and GAVRILOVA[8] that ribosomes programmed with poly(U) can synthesize polyphenylalanine in the complete absence of elongation factors EF-T_u and EF-G. This indicates that the addition elongation factors does not activate any new principle mechanism in protein synthesis but stimulates mechanisms already intrinsic to the ribosome[6]. Factor-free polyphenylalanine synthesis is stimulated by the sulfhydryl-group reagent *p*-chloromercuribenzoate[5].

Fig. 1 shows the effect of ST-F on factor-free and factor-dependent translation. It is seen that the antibiotic very effectively suppresses factor-free translation as well as the complete factor-dependent translation. However, factor-free translation is more sensitive to ST-F than the factor-dependent translation. This indicates that

Fig. 1. Effect of ST-F on poly(U) directed factor-free, EF-T_u-dependent, EF-G-dependent and complete factor-dependent translation systems.

Experimental conditions as described in Materials and Methods. The data are expressed as percent of the control without ST-F. The incorporation of phenylalanine was 10.7 pMol in the factor-free, 10.7 pMol in the EF-T_u-dependent, 12.5 pMol in the EF-G-dependent and 9.8 pMol in the complete factor-dependent translation system.

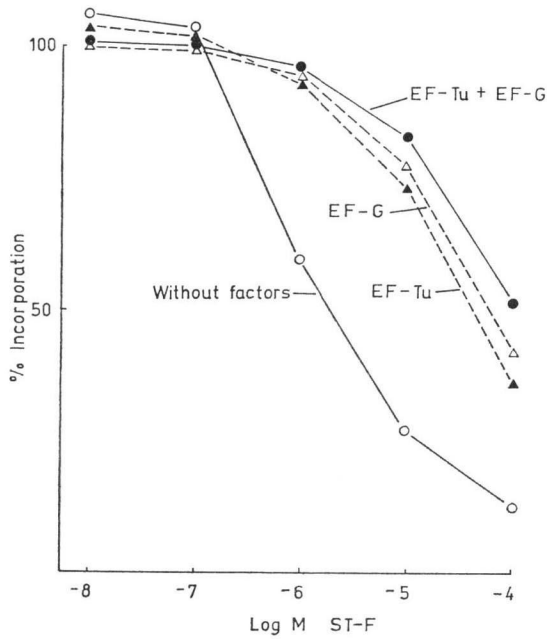
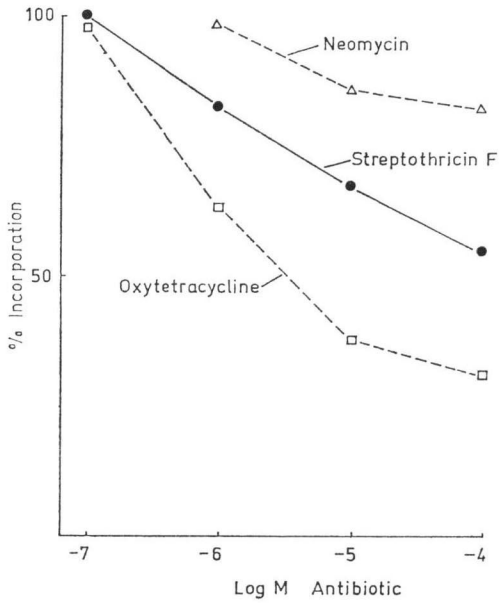


Fig. 2. Effect of antibiotics on EF-T_u-dependent binding of Phe-tRNA to ribosomes.

Experimental conditions as described in Materials and Methods. The data are expressed as percent of the control without antibiotics. 11.7 pMol Phe-tRNA were bound in the control.



elongation factors can, to some degree, overcome ST-F inhibition of ribosomal functions.

In order to clarify which of the elongation factors antagonizes ST-F inhibition, the effect of the antibiotic on the one factor-dependent system such as EF-T_u- or the EF-G-dependent system[6] was investigated. As is shown in Fig. 1, EF-T_u as well as EF-G can overcome ST-F inhibition partially. These results suggest that the antibiotic might interfere with the functioning of either elongation factor. To examine this possibility, the effect of ST-F on single steps of elongation was determined in separate experiments.

2. Effect of ST-F on Peptide Chain Elongation on the Ribosome

The process of peptide chain elongation involves EF-T_u-dependent binding of aminoacyl-tRNA to the ribosome, ribosome catalysed peptide bond formation and EF-G-dependent translocation of peptidyl-tRNA from the acceptor site to the donor site of the ribosome. The effect of ST-F on each reaction was studied.

Fig. 2 shows the effect of ST-F on the EF-T_u-dependent binding of Phe-tRNA to the ribosome in comparison to the effect of oxytetracycline, a typical inhibitor of this reaction, and neomycin which should not inhibit this process.[14] The results indicate that enzymatic binding of Phe-tRNA is not significantly influenced by neomycin, is markedly inhibited by oxytetracycline and moderately inhibited by ST-F.

The reaction of acetylphenylalanyl-tRNA or CACCA-acLeu with puromycin on the 70 S ribosome in the absence of EF-G and GTP was employed as a model of the peptidyltransferase reaction. The results presented in Table 1 show that the puromycin reaction with acetylphenylalanyl-tRNA or with the CACCA-acLeu fragment was at most only slightly inhibited by 1 mM ST-F. The reaction was profoundly blocked by the macrolide antibiotic turimycin-H_5, which is a typical inhibitor of peptidyltransferase[4]. The results indicate that ST-F does not inhibit peptide bond formation.

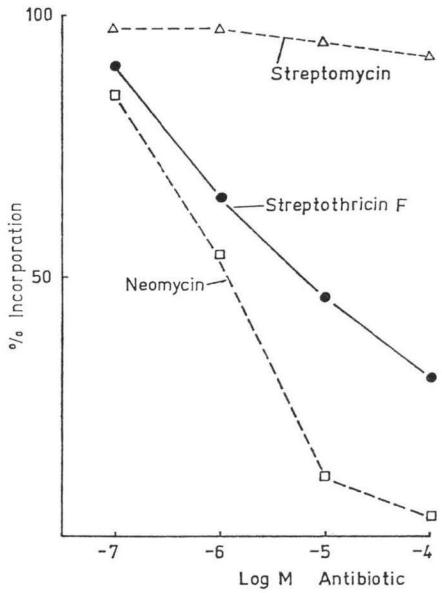
Table 1. The effect of antibiotics on the puromycin reaction with ac[^{14}C]Phe-tRNA and CACCA-ac[^{14}C]Leu as donor substrates

Antibiotic	Conc. (μM)	AcPhe-puromycin formed		AcLeu-puromycin formed	
		pMol	%	pMol	%
None		8.9	100	5.5	100
Streptothricin F	1	8.4	94	5.4	98
	10	8.2	92	5.4	98
	100	8.0	90	5.3	96
Turimycin-H_5	1	6.1	68	2.3	41
	10	2.9	33	0.6	11
	100	2.1	24	0.3	5

Experiments were performed as described under Materials and Methods.

Fig. 3. Effect of antibiotics on EF-G-dependent translocation of acPhe-tRNA.

Experimental conditions as described in Materials and Methods. The data are expressed as percent of the control without antibiotics. 3.2 pMol acPhe-puromycin were formed in the absence of EF-G and GTP and 7.5 pMol in the presence of EF-G and GTP. Values without EF-G and GTP were subtracted.



The EF-G+GTP dependent reaction of puromycin with acetylphenylalanyl-tRNA bound to the ribosomal A-site was used as a model of the translocation process. Our experiments indicate that ST-F inhibits the translocation reaction (Fig. 3). The sensitivity of translocation was tested with other antibiotics for comparison (Fig. 3) and was profoundly blocked by the well known translocation inhibitor neomycin[14] while the reaction was not inhibited by streptomycin, which is consistent with published data[15]. Inhibition of translocation by ST-F was studied by using limiting concentrations of EF-G. The effect of ST-F can be effectively reversed by the addition of increasing amounts of EF-G close to saturation (data not shown). The sensitivity of the translocation reaction is greater than that of the complete polyphenylalanine synthesis system.

Discussion

In our previous paper ST-F has been shown to inhibit polypeptide synthesis and to induce misreading in cell-free systems of *E. coli* directed by synthetic homopolynucleotides[1].

Further characterization of the action of ST-F has used the factor-free and factor-dependent translation systems[8,13,16]. Our results suggest that ST-F inhibits factor-free as well as factor-dependent polyphenylalanine synthesis. From these results it can be concluded that the antibiotic inhibits polypeptide synthesis *via* interaction with the ribosome and not by a direct interaction with the elongation factors. The results with the complete factor-dependent system further suggest that elongation factors can partially overcome ST-F inhibition of factor-free translation system. This indicates that the site of the action of ST-F involve certain ribosomal components involved in the interaction of both EF-T_u and EF-G.

From studies with one factor-dependent translation systems we suggest that ST-F affects the reactions promoted by both elongation factors, EF-T_u and EF-G. This assumption was confirmed by our studies of EF-T_u-dependent binding of aa-tRNA to the ribosome and EF-G dependent translocation. ST-F significantly inhibits translocation and to a somewhat lesser degree binding of aa-tRNA while it does not inhibit peptide bond formation.

The dual action of ST-F on EF-T_u-dependent binding of aa-tRNA and on EF-G-dependent translocation reaction may be explained by the presence of common or overlapping binding sites for both elongation factors on the ribosome which are affected by the antibiotic[17]. The reversion of the inhibition of EF-G-dependent translocation reaction by increasing EF-G concentrations further suggests that the EF-G and ST-F binding sites may be located in close proximity on the ribosome or may overlap.

With respect to inhibition of translocation and induction of misreading, ST-F resembles aminoglycosides, such as neomycin, paromomycin, kanamycin, gentamicin and hygromycin B[14,15,18] though it differs in its chemical structure from this group of antibiotics which are characterized by a deoxystreptamine moiety. The question arises whether induction of misreading and inhibition of translocation are associated phenomena which can result from action of the antibiotic at the same site or at different sites on the ribosome. Such a relationship has been discussed by CABAÑAS *et al.*[18] for aminoglycosides and would be reasonable for ST-F, too. However, the ability to induce misreading does not necessarily lead to simultaneous inhibition of translocation since the misreading inducing antibiotics streptomycin and negamycin[19,20] do not inhibit the translocation reaction effectively[15,21]. In the case of streptomycin a moderate inhibition of translocation was described by CABAÑAS *et al.*[14]. On the other hand the aminoglycoside antibiotics, kasugamycin and spectinomycin which do not induce misreading[22,23] fail to inhibit translocation[12,24,25].

In addition to the action of ST-F on translocation, aa-tRNA binding and induction of misreading it is still possible that ST-F might interfere with other steps of polypeptide synthesis.

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