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Inhibition by Siomycin and Thiostrepton of Both Aminoacyl-tRNA and Factor G Binding to Ribosomes

(Millipore filter/T_u/tetracycline/streptogramin/puromycin)

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ABSTRACT **Siomycin, a peptide antibiotic that interacts with the 50S ribosomal subunit and inhibits binding of factor G, is shown also to inhibit binding of aminoacyl-tRNA; however, it does not impair binding of fMet-tRNA and completion of the initiation complex. Moreover, unlike other inhibitors of aminoacyl-tRNA binding (tetracycline, sparsomycin, and streptogramin A), siomycin completely abolishes the GTPase activity associated with the binding of aminoacyl-tRNA catalyzed by factor T_u. A single-site interaction of siomycin appears to be responsible for its effect on both the binding of the aminoacyl-tRNA–T_u–GTP complex and that of factor G.**

Factor G, one of the supernatant proteins necessary for ribosomal activity in *Escherichia coli*, is involved in the coupling of GTP hydrolysis to polypeptide elongation. This hydrolysis is thought to provide the energy necessary for the rearrangements of tRNA, mRNA, and ribosomal subunits, in each round of peptide bond formation, that result in translocation of mRNA and peptidyl-tRNA (reviewed in ref. 1). GTP hydrolysis can also occur in the absence of mRNA and peptidyl-tRNA, the reaction requiring only factor G and ribosomes (2, 3). In recent work this reaction has been used to show that there is a binding site for factor G (the G-site) on the 50S subunit (4, 5). Our studies have further shown that a peptide antibiotic, siomycin, inactivates this site, preventing the interaction of this site with factor G. Moreover, the G-site is evidently distinct from the peptidyl transferase center on the 50S subunit (5).

We have now shown that siomycin-treated ribosomes can carry out the initiation step in protein synthesis, with either viral messenger or triplet AUG, and the bound fMet-tRNA can reach the puromycin-reactive position. However, no peptide bond can be formed because the ribosomes do not bind aminoacyl-tRNA in the recognition site (the A-site). Moreover, the siomycin-treated ribosomes can complement neither the GTPase activity associated with factor T nor that associated with factor G. These effects appear to be the consequence of a single action of siomycin on the 50S subunit, thus suggesting a relationship between the binding site of factor G and that of the aminoacyl-tRNA–T_u–GTP complex.

MATERIALS AND METHODS

Ribosomes washed with ammonium chloride (1 M) and crude initiation factors from *E. coli* MRE600 were prepared as described (6, 7). Homogeneous initiation factor F_1 and partially purified factor F_2 were a gift of Dr. S. Ochoa. Elongation factors G and T (T_s plus T_u) and [γ-^{32}P]GTP were prepared as described (2, 8). Commercial deacylated tRNA was charged with labeled methionine (under formylating conditions), phenylalanine, or alanine in the absence of other unlabeled amino acids. Specific activities were 3900 cpm/pmol f-[^{3}H]Met-tRNA, 740 cpm/pmol [^{14}C]Phe-tRNA and 180 cpm/pmol [^{14}C]Ala-tRNA.

Binding of aminoacyl-tRNA to ribosomes was assayed by filtration (9). Reaction mixtures for the binding of fMet-tRNA contained: 80 mM NH_4Cl, 50 mM Tris·HCl (pH 7.7), 5 mM Mg(acetate)$_2$, 6 mM mercaptoethanol, 0.4 mM GTP, 0.3 mg/ml of crude initiation factors, 4.7 pmol f-[^{3}H]Met-tRNA per A_{260} unit ribosomes, 1.2 mg/ml of R17-RNA, 35 A_{260} units/ml of ribosomes, and other additions as specified. Unless otherwise indicated, incubation was at 34°C for 10 min and was followed by filtration and analysis.

Ala-tRNA was bound to preformed fMet-tRNA–70S ribosome–R17 RNA complex. An incubated reaction mixture for the binding of fMet-tRNA was cooled to 0°C, and supplemented with 7.8 pmol of [^{14}C]Ala-tRNA per A_{260} unit of ribosomes and 0.08 mg/ml of T-factor. Binding was measured after incubation at 18°C for 15 min. In all experiments, binding was corrected for the values obtained in parallel mixtures without messenger.

T-factor-dependent binding of Phe-tRNA to ribosomes and its associated GTPase activity were measured as follows: ribosomes (68 A_{260} units/ml) were first incubated at 30°C for 12 min in a mixture containing 150 mM NH_4Cl, 16 mM Mg(acetate)$_2$, 85 mM Tris·HCl (pH 7.7), 1.6 mM dithiothreitol and 90 μg/ml of poly(U). This treatment was found to decrease nonenzymic binding of Phe-tRNA without affecting the T-factor-dependent binding. After cooling at 0°C, 40-μl portions were supplemented with antibiotics and other components for the binding reaction (final volume, 70 μl). Final concentrations were: 90 mM NH_4Cl, 24 mM $(NH_4)_2SO_4$ (carried over with the T-factor preparation), 50 mM Tris·HCl (pH 7.7), 9 mM Mg (acetate)$_2$, 1 mM dithiothreitol, 0.43 μM [γ-^{32}P] GTP, 800–1200 cpm/pmol, 50 μg/ml of poly(U), 8 pmol of [^{14}C] Phe-tRNA per A_{260} unit ribosomes, 27 μg/ml of T-factor, and 38 A_{260} units/ml of ribosomes. After incubation at 30°C for 10 min, the reaction mixtures were chilled and divided into two portions: one was analyzed for [^{14}C]Phe-tRNA bound to ribosomes (9) and the other for ^{32}P-labeled inorganic phosphate (8). Binding of Phe-tRNA

Abbreviations: $(Me)_2SO$, dimethylsulphoxide; Complex II, GTP–factor T_u–aminoacyl-tRNA complex; Sio, siomycin.

TABLE 1. *Binding of fMet-tRNA to 70S ribosomes and reaction with puromycin, insensitivity to siomycin*

Expt.	Messenger	Addition	Incubation time (min)	fMet-tRNA bound (pmol/A unit) − Sio	+ Sio
1	R17-RNA	—	10	0.26	0.17
		GTP	10	1.00	1.11
2	AUG	F_2	15	2.6	3.9
		$F_2 + F_1$	15	4.6	5.5
3	R17-RNA	GTP	10	—	0.97
		GTP	12	—	0.97
		GTP; at 10 min puromycin	12	—	0.22

Reaction mixtures for Expts. 1 and 3 were as described in *Methods*, except that GTP was present only when indicated. In Expt. 3, 0.1 mM puromycin was added to one portion of the reaction mixture and the incubation was continued for two more minutes before the analysis. In Expt. 2, the reaction mixture contained 0.06 mM AUG, 0.2 mM GTP, 1 mM dithiothreitol, 0.069 units/ml of factor F_2 (see ref. 10) with or without 60 μg/ml of factor F_1, 19 A_{260} units/ml of 70S ribosomes and 13.6 pmol of f-[^{3}H]Met-tRNA per A_{260} unit of ribosomes. Incubation was at 25°C. All mixtures contained 2.5% Me_2SO. Siomycin was 11 μM. Subtracted values from control reactions without messenger were between 0.13 and 0.48 pmol.

and hydrolysis of GTP were corrected for the values obtained in parallel mixtures without T-factor or without [^{14}C]Phe-tRNA, respectively.

Siomycin and thiostrepton were dissolved in dimethylsulfoxide $(Me)_2SO$. Consequently, between 2 and 4% Me_2SO was present in reaction mixtures with the antibiotics. Unless otherwise indicated, an equal amount of Me_2SO was added to other reaction mixtures in the experiment.

AUG was obtained from Miles Laboratories. Phage R17 RNA was prepared as described (6). Poly(U) was from Schwartz. [^{3}H]Methylmethionine (11.1 Ci/mmol), [^{14}C]-phenylalanine (0.477 Ci/mmol) and [^{14}C]alanine (0.152 Ci/mmol) were from The Radiochemical Center, Amersham.

RESULTS

Lack of effect of siomycin on initiation

Siomycin did not inhibit, but slightly stimulated, the binding of f-[^{3}H]Met-tRNA to NH_4Cl-washed ribosomes, either with crude initiation factors and R17-RNA (Table 1, Expt. 1) or with purified factors and triplet AUG (Expt. 2). Moreover, with ribosomes washed according to a different procedure (5), this small effect was not detected. The release by puromycin (which acts on 70S but not on 30S particles) was not impaired (Table 1, Expt. 3), showing that the 50S subunit could join the initiation complex and fMet-tRNA could reach the ribosomal P site. Evidently siomycin does not interfere with formation or completion of the initiation complex.

Binding of aminoacyl-tRNA to initiation complex: inhibition by siomycin and thiostrepton

With R17-RNA as messenger, most ribosomes initiate at the coat protein cistron (11), which codes for alanine after formylmethionine (12). We therefore studied the binding of Ala-tRNA by initiation complexes to start the process of elongation. Although we used crude initiation factors to form the initiation complex (which could result in substantial contamination with elongation factors, acylated-tRNA, and other undefined components), subsequent binding of [^{14}C]-Ala-tRNA showed a good requirement for T factor, fMet-tRNA, and R17-RNA (Table 2). Moreover, binding of Ala-tRNA to ribosomes was not the result of extensive polypeptide formation: when the ^{14}C-labeled product retained by the Millipore filter was released by 1 M NH_4OH and hydrolyzed with 0.5 M KOH at 37°C for 30 min (7), 75% of the radioactivity was found to have the same electrophoretic mobility as chemically synthesized formylmethionyl-alanine (data not shown). Thus, under the conditions employed, most of the Ala-tRNA retained by ribosomes did bind to the recognition site (the A site) of initiation complexes and accepted the transfer of formylmethionine.

TABLE 2. *Requirements for Ala-tRNA binding to 70S ribosomes*

	pmol bound/A unit			
	f-[^{3}H]Met-tRNA		[^{14}C]Ala-tRNA	
	−T	+T	−T	+T
Complete	2.02	2.02	0.24	1.78
-fMet-tRNA	—	—	0.11	0.21
-R17-RNA	0.52	0.53	0.19	0.33

[^{14}C]Ala-tRNA was bound to ribosomes carrying prebound f-[^{3}H]Met-tRNA.

With this system it could be shown (Table 3) that siomycin, and the closely related antibiotic thiostrepton (13), strongly inhibited the binding of Ala-tRNA. Moreover, in agreement with studies in other systems (14–17) tetracycline,

TABLE 3. *Effect of antibiotics on the binding of Ala-tRNA to preformed fMet-tRNA–70S ribosome–R17-RNA complex*

Expt.	Additions	pmol bound/A unit f-[^{3}H]Met-tRNA	[^{14}C]Ala-tRNA
1	None	1.32	1.36
	Fusidic acid (1.2 mM)	1.30	1.35
	Tetracycline (0.1 mM)	1.46	0.21
	Me_2SO (2.5%)	1.61	1.86
	Siomycin (0.011 mM) + Me_2SO (2.5%)	1.69	0.16
	Thiostrepton (0.012 mM) + Me_2SO (2.5%)	1.74	0.14
2	None	1.45	1.59
	Ethanol (2.5%)	1.47	1.89
	Streptogramin A (0.05 mM) + ethanol (2.5%)	1.39	0.23
	Sparsomycin (0.05 mM) + ethanol (2.5%)	1.73	0.14

Portions (20 μl) of an incubated reaction mixture containing initiation complex (1.34 and 1.50 pmol of f-[^{3}H]Met-tRNA bound per A_{260} unit of ribosomes in Expt. 1 and 2, respectively) were mixed with [^{14}C]Ala-tRNA, T factor, and specified additions. Ethanol was present in reaction mixtures of Expt. 2 because the streptogramin A and sparsomycin stock solutions were made up in 50% ethanol.

TABLE 4. *Effect of antibiotics on T-dependent Phe-tRNA binding to 70S ribosomes and associated GTP hydrolysis*

Expt.	Antibiotic	pmol/*A* unit [^{14}C]Phe-tRNA bound	pmol/*A* unit [^{32}P]GTP hydrolyzed
1	None	1.42	1.36
	Siomycin (0.013 mM)	0.13	−0.03
2	None	1.18	1.15
	Thiostrepton (0.014 mM)	0.06	−0.26
	Tetracycline (0.1 mM)	0.32	1.01
3	None	1.33	1.44
	Fusidic acid (1 mM)	1.04	1.14
	Streptogramin A (0.029 mM)	0.47	1.22
	Tetracycline (0.1 mM)	0.34	0.92

Binding of Phe-tRNA and hydrolysis of GTP were corrected with the values obtained from control reaction mixtures without T factor (between 0.48 and 0.97 pmol) or without Phe-tRNA (between 0.42 and 1.34 pmol), respectively. Stimulation of GTP hydrolysis by Phe-tRNA was not observed in the absence of T factor.

streptogramin A, and sparsomycin were also good inhibitors, though they did not unstabilize initiation complexes, as measured by the retention of fMet-tRNA. In contrast, fusidic acid, an inhibitor of translocation (18), was without effect on either binding.

It has previously been shown that siomycin inhibits the binding of factor G by interacting with ribosomes (5). The same interaction with the antibiotic appears to be responsible for the inhibition of Ala-tRNA binding, since this reaction and the G-factor-dependent GTPase were inhibited in parallel by increasing concentrations of siomycin (Fig. 1), 1–2 molecules per ribosome being sufficient in each case to cause over 90% inhibition.

Inhibition by siomycin and thiostrepton of enzymic binding of Phe-tRNA and associated GTP hydrolysis

The GTP hydrolysis associated with the T-dependent binding of aminoacyl-tRNA (19–22) is easily masked by more active GTPases present in partially purified systems. We therefore used a highly purified system for poly(U)-directed binding of Phe-tRNA to ribosomes. Table 4 shows that siomycin and thiostrepton markedly inhibited the T-stimulated binding and abolished accompanying GTPase activity*. Moreover (results not shown), like the G-dependent GTPase and the Ala-tRNA binding, the T-dependent GTPase activity and the Phe-tRNA binding were also completely inhibited by less than 2 molecules of siomycin per ribosome. Fusidic acid, which inhibits the G-dependent GTPase (18), was added as a control: it had little effect on either binding or GTPase (Table 4). In agreement with other studies (20, 21), tetracycline was found to uncouple GTPase from binding, inhibiting binding to a much larger extent than GTPase (Table 4, Expts. 2 and 3). Streptogramin A appeared to uncouple the activities even more clearly (Table 4).

* Control experiments showed that about 80% of the ^{14}C bound in uninhibited reactions was in the form of Phe-tRNA (see legend to Table 5). Siomycin inhibited its binding, as well as the formation of a small amount of $(Phe)_2$-tRNA.

TABLE 5. *Effect of siomycin on nonenzymic binding of Phe-tRNA to ribosomes*

Mg^{++} conc. (mM)	Sio	Phe-tRNA bound: − poly-(U)	+ poly-(U)	Stimulated by poly(U)	Product recovered from ribosomes: Phe	Phe_2	$Phe_{\geq 3}$
		(pmol/A_{260} unit)					
8	−	0.16	2.6	2.4	—	—	—
	+	0.15	1.9	1.8	—	—	—
20	−	1.1	5.1	4.0	3.4	0.45	<0.1
	+	1.0	2.7	1.7	2.0	0.24	<0.1

Reaction mixtures (25 μl) contained: 120 mM NH_4Cl, 60 mM Tris·HCl (pH 7.7), 1.3 mM dithiothreitol, 6.7 pmol of [^{14}C]Phe-tRNA per A_{260} unit of ribosomes, 41 A_{260} units/ml of 70S ribosomes, 2% Me_2SO; 80 μg/ml of poly(U) 9 μM siomycin, and Mg $(acetate)_2$ as indicated. Contrary to the experiments in Table 4, ribosomes were not preincubated in order to preserve their nonenzymic binding activity. Incubation at 30°C for 10 min was followed by filtration analysis. Radioactivity retained by the Millipore filters from 75-μl reaction mixtures was released by treatment with 1 M NH_4OH (21) in the presence of unlabeled phenylalanine (Phe), diphenylalanine (Phe_2), and triphenylalanine (Phe_3). The product was hydrolyzed in 0.5M KOH at 37°C for 40 min, neutralized with $HClO_4$, and analyzed by paper chromatography in *n*-butanol saturated with 3% NH_4OH. The paper strips were cut and radioactivity was determined by liquid scintillation. Spots were identified by comparison with unlabeled standards. More than 80% of the radioactivity retained by the Millipore filters was recovered from the chromatograms.

Inhibition of nonenzymic binding of Phe-tRNA at high Mg^{++} concentration

Poly(U)-directed binding of Phe-tRNA to ribosomes can take place also in the absence of T factor and GTP, the binding being most efficient at high Mg^{++} concentrations (9). Table 5 shows that siomycin inhibited much of the binding observed at 20 mM Mg^{++}, while the lesser binding observed at 8 mM Mg^{++}, which takes place mainly in the P site (23), was less affected. Moreover, the same amount of siomycin was required to inhibit G-dependent GTPase (unpublished observations). To eliminate the possibility that siomycin might be inhibiting oligophenylalanine synthesis rather than Phe-tRNA binding, the bound product was characterized. Table 5 shows that it consisted mainly of Phe-tRNA. Siomycin inhibited its binding, as well as a small synthesis of $(Phe)_2$-tRNA. Inhibition of nonenzymic binding of Phe-tRNA at high Mg^{++} concentration was also observed with thiostrepton.

DISCUSSION

Siomycin has recently been shown to inhibit the binding of factor G to the ribosomes and its associated GTPase activity by interacting with the 50S subunit and blocking a site that binds this factor (5). Its site of action appears to be distinct from the so-called peptidyl transferase center (5, 24). We have now found that the antibiotic also inhibits the T-factor-dependent binding of aminoacyl-tRNA to the A site, including both Ala-tRNA on the initiation complex formed with R17-RNA and Phe-tRNA on the complex with poly(U). Since the GTPase activity associated with the enzymic binding of

Phe-tRNA is also inhibited, siomycin presumably prevents detectable interaction between the GTP-T_u-aminoacyl tRNA complex (called Complex II, refs. 19, 22) and the ribosome. Thiostrepton, which seems to have the same mechanism of action as siomycin (5, 24–28), also has the same effect on the interaction of Complex II with the ribosome (Tables 3 and 4). In contrast, tetracycline, which binds to the 30S subunit (14), and sparsomycin and streptogramin A, which act at the peptidyl transferase center on the 50S subunit (14–16), evidently allow interaction of Complex II with the ribosome; though they inhibit aminoacyl-tRNA binding, they do not block the T-dependent GTPase (Table 4 and refs. 17, 20, 21).

Siomycin binds to the 50S subunit, but not to the 30S subunit (24). The binding of G and that of Complex II seem to be inhibited by the same single-site interaction of the antibiotic with the 50S subunit: the G-dependent GTPase and the binding of Ala-tRNA to initiation complexes are inhibited in parallel by increasing concentrations of siomycin, and less than 2 molecules per ribosome are sufficient to cause over 90% inhibition in both reactions (Fig. 1).† The possibility that inhibition of aminoacyl-tRNA binding might be a secondary effect of the action of siomycin on another ribosomal function is made unlikely by several observations: (*a*) siomycin does not interfere with attachment of mRNA to ribosomes or with the formation of or stability of the complete initiation complex (Tables 1 and 3); (*b*) siomycin most likely does not act directly on Complex II since if the antibiotic is prebound to ribosomes Phe-tRNA binding is inhibited (unpublished observation).

If siomycin, as suggested, interacts with only one ribosomal site, the simplest explanation of the results is that the antibiotic competes directly with G and with Complex II for binding to the same, or partially overlapping, sites on the 50S subunit. Alternatively, siomycin, factor G, and Complex II might bind at different, but allosterically interacting, sites. The results thus raise the possibility that the binding site of factor G (the G site) and that of Complex II (which includes the A site) are near each other on the 50S subunit, or may even overlap. Such proximity or overlapping might make obligatory the release of factor G after translocation, before binding of Complex II (29), and the release of factor T_u before binding of G. Since 50S subunits appear to be able to complement both the GTPase of G (4, 5) and that of T_u (23), it is tempting to suggest that a common, siomycin-sensitive site on this subunit might activate the GTPase of both factors. Accordingly, this site could be visualized as a region controlling both entry of acylated-tRNA into the A site mediated by T_u, and exit, mediated by G.

In the absence of T, binding of Phe-tRNA at lower than 10 mM Mg^{++} is inhibited only slightly by siomycin, but the stimulation of binding caused by increasing the Mg^{++} concentration to 20 mM is quite sensitive (Table 5). This reinforces the view that nonenzymic bindings at high and low Mg^{++} concentration are qualitatively different, only the first taking place mainly in the ribosomal A-site (23). The inhibition of nonenzymic binding further suggests that the sites for interaction of T_u and G with the 50S subunit

† No attempts have been made to correlate the amount of antibiotic bound with the number of ribosomes active in each reaction.

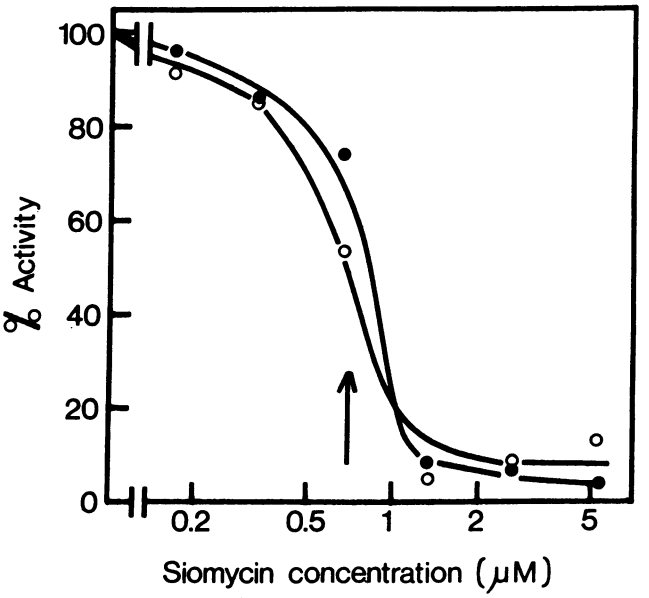

FIG. 1. Effect of siomycin concentration on Ala-tRNA binding to ribosomes (O——O) and on G-dependent GTPase (●——●). Binding of [^{14}C]Ala-tRNA to f-[^{3}H]Met-tRNA–70S ribosome–R17-RNA complex in the presence of increasing concentrations of siomycin was conducted as described in *Methods* and the legend to Table 3. In the absence of the antibiotic, binding was 1.22 and 1.40 pmol per A_{260} unit of ribosomes of f-[^{3}H]Met-tRNA and [^{14}C]Ala-tRNA, respectively. G-dependent GTPase was measured in reaction mixtures (20 μl) containing: 55 mM NH_4Cl, 39 mM NH_4-$(SO_4)_2$, 50 mM Tris·HCl(pH 7.7), 5 mM Mg(acetate)$_2$, 6 mM mercaptoethanol, 0.16 mM[γ-^{32}P]GTP (11,500 cpm/nmol), 33 A_{260} units/ml of 70S ribosomes, 40 μg/ml of factor G, and siomycin as specified. After 20 min incubation at 18°C, the mixtures were analyzed for [^{32}P]P_i as described (8). Uninhibited reactions hydrolyzed about 50% of the GTP present. Controls without G or without ribosomes showed undetectable GTPase. *Arrow* shows the concentration of ribosomes in the reaction mixtures.

are near to or overlap with the A site. The moiety of the tRNA molecule that interacts with the siomycin-sensitive site is presumably not the CCA terminus [which interacts with the peptidyl transferase center (15)] or the anticodon loop (which interacts with the mRNA on the 30S subunit), but might be the GTφCG loop.

The lack of effect of siomycin on initiation (Tables 1 and 3) suggests that the G site and the portion of the A site on the 50S subunit are not involved in the binding of fMet-tRNA, including the GTPase activity catalyzed by initiation factor F_2 (ref. 30). Our findings thus support the view that during initiation fMet-tRNA probably does not go through the ribosomal A-site (31).

NOTE ADDED IN PROOF

After the completion of this work we learned that Dr. N. Tanaka [*Molecular Mechanisms of Antibiotic Action on Protein Biosynthesis and Membranes* (Proceedings of a Symposium held in Granada, Spain, June 1971), (Springer-Verlag, Berlin, 1971), in the press] has also observed that siomycin inhibits the T factor-dependent binding of Phe-tRNA to ribosomes and the GTPase activity associated with this reaction.

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