EFFECT OF SIOMYCIN ON THE ACCEPTOR SITE OF Escherichia coli RIBOSOMES

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SUMMARY

The binding of N-acetyl-^{14}C-phenylalanyl-tRNA to ribosomes pretreated with polyuridylic acid and deacylated tRNA was almost completely inhibited by tetracycline and partly by siomycin.

The N-acetyl-^{14}C-phenylalanyl-tRNA bound to ribosomes preincubated with deacylated tRNA was found to be unreactive to puromycin. By incubating the ternary complex with G factor and GTP, however, the greater part of the bound N-acetyl-^{14}C-phenylalanyl-tRNA became reactive to puromycin, this amount being equivalent to that amount of N-acetyl-^{14}C-phenylalanyl-tRNA whose binding to deacylated tRNA-treated ribosomes is inhibited by siomycin.

We have previously suggested that siomycin interacts with 50S ribosomal subunits and inhibits the translocation of peptidyl-tRNA from the acceptor site to the donor site of ribosomes, since this antibiotic inhibits the G factor and GTP dependent increment of the synthesis of N-acetyl-^{14}C-phenylalanyl-puromycin and the G factor dependent hydrolysis of GTP by Escherichia coli ribosomes (1,2).

According to the current ideas on protein biosynthesis, which postulate two tRNA binding sites — the acceptor site and the donor site — on ribosomes (3), the acceptor site of the ribosomes should be occupied by peptidyl-tRNA and the donor site by deacylated tRNA just after the peptidyl transfer reaction (pretranslocation stage).

In this present work we have found that by pretreating E. coli ribosomes with deacylated tRNA, the binding of N-acetyl-^{14}C-phenylalanyl-tRNA is inhibited almost completely by tetracycline and that the N-acetyl-^{14}C-phenylalanyl-tRNA bound to such pretreated ribosomes is unreactive unless the formed ribosomal ternary complex is incubated with G factor and GTP.

We have also studied the effect of siomycin on this binding of N-acetyl-^{14}C-phenylalanyl-tRNA to the ribosomes.

MATERIALS AND METHODS

Ribosomes and G factor were prepared from Escherichia coli Q13 cells as previously described (2). Deacylated tRNA was prepared from E. coli Q13 cells as described in the previous paper (4). N-Acetyl-^{14}C-phenylalanyl-tRNA was obtained by the method of Lapidot, De Groot and Fry-Shafrir (5). Siomycin (mono-thiomalic acid-siomycin A) was kindly provided by Dr. Ebata and his colaborators in this laboratory.

Assays for the binding of N-acetyl-^{14}C-phenylalanyl-tRNA to ribosomes and the synthesis of N-acetyl-^{14}C-phenylalanyl-puromycin were carried out by combination of the following incubations.

Preincubation of ribosomes with deacylated tRNA ----- A mixture (50 µl) containing 3.78 A_{260} units of ribosomes, 1.50 A_{260} units of deacylated tRNA, 12.5 µg of polyuridylic acid, 2.5 µmoles of Tris-HCl (pH 7.8), 0.5 µmole of magnessium acetate, 8.0 µmoles of NH_4Cl and 0.25 µmole of dithiothreitol was incubated at 37°C for 10 minutes.

Incubation for the binding of N-acetyl-^{14}C-phenylalanyl-tRNA ----- To the incubation mixture described above, an equal volume of a mixture containing 2.5 µmoles Tris-HCl (pH 7.8), 1.0 µmole magnessium acetate, 8.0 µmoles NH_4Cl, 0.25 µmole dithiothreitol, and N-acetyl-^{14}C-phenylalanyl-tRNA (0.16 A_{260} unit, 5.3 p moles of ^{14}C-phenylalanine, 455 µCi/µmole) was added and the mixture was incubated at 37°C as stated in the legend to the Figure and the Tables. To estimate the N-acetyl-^{14}C-phenylalanyl-tRNA bound to the ribosomes at the end of the incubation period, the reaction mixture was diluted to 2 ml with ice cold buffer containing 10 mM Tris-HCl (pH 7.8), 15 mM magnessium acetate, and 160 mM NH_4Cl, and filtered

through nitrocellulose membrane (millipore HAWP). The membrane was washed three times with the same buffer, and after drying, the radioactivity retained on the membrane was measured by liquid scintillation spectrometer using conventional toluene scintillation mixture.

Incubation for translocation ----- To the incubation mixture as used for the binding of N-acetyl-^{14}C-phenylalanyl-tRNA was added 25 µl of a solution containing 1.25 µmoles Tris-HCl (pH 7.8), 0.375 µmole magnessium acetate, 4.0 µmoles NH_4Cl, 0.125 µmole dithiothreitol, 3.63 µg G factor, 10 mµmoles GTP, 0.1 µmole phosphoenolpyruvate and 1.0 µg pyruvate kinase, and the mixture was incubated at 37°C for 10 minutes.

Incubation for puromycin reaction ----- To the incubation mixture described above, puromycin 0.1 µmole (10 µl) was added, and the mixture further incubated at 0°C for 30 minutes. The formation of N-acetyl-^{14}C-phenylalanyl-puromycin was assayed according to the method of Leder and Bursztyn (6).

RESULTS AND DISCUSSION

As shown in Table I, the binding of N-acetyl-^{14}C-phenylalanyl-tRNA to ribosomes preincubated with deacylated tRNA and polyuridylic acid was almost completely inhibited by tetracycline, which has been shown to interfere with the binding of aminoacyl-tRNA to the acceptor site of ribosomes (7-9); however, the binding to the ribosomes preincubated only with polyuridylic acid under these particular conditions was hardly affected by this antibiotic. The rate of binding of N-acetyl-^{14}C-phenylalanyl-tRNA to ribosomes preincubated with deacylated tRNA and polyuridylic acid was slower than that to ribosomes preincubated without deacylated tRNA, although the maximum amounts of N-acetyl-^{14}C-phenylalanyl-tRNA bound to ribosomes were almost the same under both of these conditions (Fig. 1).

As shown in Table II, the N-acetyl-^{14}C-phenylalanyl-tRNA bound to the ribo-

Table I

Effects of tetracyline and siomycin on the binding of N-acetyl-^{14}C-phenylalanyl-tRNA to ribosomes preincubated with poly U in the presence or absence of deacylated tRNA

Preincubation of ribosomes	Incubation period for binding	Antibiotics added*	N-acetyl-^{14}C-Phe-tRNA bound (p moles)	(%)
Without deacylated tRNA	10 min.	None	3.47	100
		Tetracycline	3.28	94.5
		Siomycin	2.81	81.0
		Tetracycline and siomycin	3.09	89.2
With deacylated tRNA	10 min.	None	1.88	100
		Tetracycline	0.16	8.60
		Siomycin	0.49	25.7
		Tetracycline and siomycin	0.15	7.96
	60 min.	None	3.17	100
		Tetracycline	0.27	8.40
		Siomycin	1.22	38.6
		Tetracycline and siomycin	0.19	5.92

* The amounts of antibiotics added in the incubation for binding were as follows; 50 mμmoles tetracycline and 5.0 mμmoles siomycin.

somes preincubated with deacylated tRNA was found to be unreactive to puromycin, while that bound to the ribosomes preincubated without deacylated tRNA was almost completely converted to N-acetyl-^{14}C-phenylalanyl-puromycin by the addition of puromycin in the absence of G factor and GTP.

The greater part of the N-acetyl-^{14}C-phenylalanyl-tRNA bound to ribosomes pretreated with deacylated tRNA became reactive to puromycin by incubating with G factor and GTP. It was interesting to find that the amount of N-acetyl-^{14}C-phenyl-

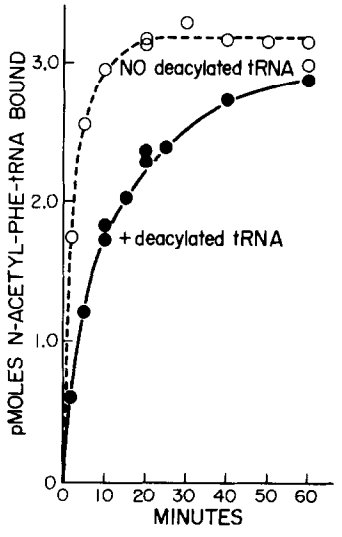

Fig. 1. The binding of N-acetyl-^{14}C-phenylalanyl-tRNA to ribosomes preincubated with poly U in the presence or absence of deacylated tRNA.
Preincubation of ribosomes was carried out in the presence or absence of deacylated tRNA. Incubation periods for the binding of N-acetyl-^{14}C-Phe-tRNA are indicated in the Figure. Experimental conditions in detail are described in Materials and Methods.

Table II

Requirement of G factor and GTP on the synthesis of N-acetyl-^{14}C-phenylalanyl-puromycin by ribosomes preincubated with poly U in the presence or absence of deacylated tRNA

Preincubation of ribosomes	Incubation period for binding	Additions*	N-acetyl-^{14}C-Phe-puromycin formed (p moles)	(%)
Without deacylated tRNA	10 min.	Complete	3.11	100
		- G factor and GTP	2.65	85.0
With deacylated tRNA	10 min.	Complete	1.56	100
		- G factor and GTP	0.17	11.1
	60 min.	Complete	1.73	100
		- G factor and GTP	0.14	8.18

* The ommission of G factor and GTP in the incubation for translocation includes that of PEP and pyruvate kinase.

alanyl-tRNA converted to N-acetyl-^{14}C-phenylalanyl-puromycin by the addition of puromycin after the incubation with G factor and GTP was almost equivalent to that amount of N-acetyl-^{14}C-phenylalanyl-tRNA whose binding was inhibited by siomycin.

In this model of the pretranslocation state of ribosomes, N-acetyl-^{14}C-phenylalanyl-tRNA at the acceptor site should attach to a 70S ribosome at at least two sites, namely; at the decoding site of the 30S ribosomal subunit, which recognizes N-acetyl-^{14}C-phenylalanyl-tRNA possibly by the decoding mechanism under the participation of polyuridylic acid; and at the acceptor site of peptide elongation center of the 50S subunit, which recognizes N-acetyl-^{14}C-phenylalanyl-tRNA possibly by the presence of N-acetyl-^{14}C-phenylalanyl residue at the CCA end of $tRNA^{Phe}$. From the previous findings on the binding sites of tetracycline (8, 10) and siomycin (1, 2, 11), it may safely be considered that the inhibitory effect of tetracycline is mainly due to the interference of the binding of N-acetyl-^{14}C-phenylalanyl-tRNA at the site on the 30S ribosomal subunit and that siomycin affects the affinity of N-acetyl-^{14}C-phenylalanyl-tRNA at the site of the 50S ribosomal subunit.

Thus if we assume that N-acetyl-^{14}C-phenylalanyl-tRNA correctly bound to 70S ribosomes at the acceptor site is successively translocated in the presence of G factor and GTP, then the data obtained in this study suggest that for such binding, N-acetyl-^{14}C-phenylalanyl-tRNA must attach at the 30S ribosomal site which is sensitive to tetracycline and at the 50S ribosomal site which is sensitive to siomycin, and that the attachment at these two sites is a basic necessity for the correct (functional) binding at the acceptor site of 70S ribosomes.

It was also confirmed that when siomycin was added after the binding of N-acetyl-^{14}C-phenylalanyl-tRNA to the ribosomes, the antibiotic does not stimulate the release of bound N-acetyl-^{14}C-phenylalanyl-tRNA from the ribosomes, but does inhibit the translocation of the N-acetyl-^{14}C-phenylalanyl-tRNA by the addition of G factor and

GTP. This may indicate that the binding site of siomycin on the 50S ribosomal subunit does not completely overlap with the acceptor site on the 50S ribosomal subunit.

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