STUDIES ON THE RIBOSOMES OF A THIOPEPTIN-RESISTANT MUTANT OF *ESCHERICHIA COLI*

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A thiopeptin-resistant mutant of *E. coli* has been obtained. Resistance was shown to be associated with the 50S ribosomal subunit of the mutant. It was also demonstrated that both elongation factor EF T-dependent binding of aminoacyl-tRNA to the mutant ribosomes and the formation of the elongation factor EF G-GDP-mutant ribosome complex were resistant to thiopeptin.

A number of antibiotics are known to inhibit protein synthesis by acting on ribosomes.[1] The variety of specificities in their mode of action is a reflection of the complexity of ribosomal structure and function. Many laboratories are investigating the physico-chemical properties of individual ribosomal RNAs and proteins, their separate functions in the overall process of translation of messenger RNA, and the location of each of these components in the translation complex[2~4]. In these investigations antibiotics possessing unique and specific modes of action are very helpful.

Thiopeptin is one of a group of sulfur-containing peptide antibiotics in which thiostrepton and siomycin are included.[5] Thiostrepton and siomycin have been reported to act on 50S ribosomal subunits resulting in inhibition of their elongation factor EF G-dependent activity[6~10]. We observed that thiopeptin, as well as siomycin, also inhibits the elongation factor EF T-dependent function of ribosomes, *i. e.* EF T-dependent binding of aminoacyl-tRNA to ribosomes and accompanying GTP hydrolysis[11]. This led to the suggestion that on 50S ribosomal subunits there is a common site that participates in interactions both with EF T and EF G. MODOLELL *et al.* reported a similar observation with thiostrepton and siomycin[12].

Isolation and studies of mutants resistant to an antibiotic are generally undertaken as an effective approach to understanding of the mechanism of itsa ction. We have isolated an *Escherichia coli* mutant resistant to thiopeptin in which resistance was shown to reside in the ribosomes. In this paper a partial characterization of the ribosomes is described. With the ribosomes from the mutant both the EF T-dependent binding of aminoacyl-tRNA and the EF G-dependent binding of GDP in the presence of fusidic acid were resistant to thiopeptin. Thus resistance was attributable to an alteration of the 50S ribosomal subunits.

Materials and Methods

Thiopeptin-resistant mutants were obtained from *E. coli* Q 13 by treatment with N-methyl-N′-nitro-N-nitrosoguanidine. The cells harvested from 10 ml of overnight culture in Bacto Penassay Broth (PAB (Difco)) were suspended in 1 ml of physiological saline containing 1 mg of the mutagen and incubated at 37°C for 10 minutes. The mutagenized cells were collected by centrifugation, washed with physiological saline and resuspended in 20 ml of PAB. After subsequent overnight

incubation at 37°C, 0.1 ml of the culture was inoculated into 200 separate tubes each containing 0.9 ml of PAB with 250 μM EDTA and 12.5 μg/ml thiopeptin. These tubes were incubated at 37°C for 2 days and then each of the cultures from 40 tubes, which showed most pronounced growth, was subjected to two successive single colony isolations. Forty clones derived from these 40 cultures were scored for thiopeptin resistance by a dilution method in PAB. Twenty thiopeptin-resistant mutants were thus obtained. Selection of the resistant mutants on agar medium and the agar dilution method to score sensitivity were not used because of the low antibacterial activity of the antibiotic on agar medium.

Cell-free extracts (S-30) were prepared from each of the 20 mutants and tested for sensitivity to thiopeptin of polyphenylalanine synthesis directed by poly U. One strain was found to show resistance to thiopeptin (Fig. 1).

Ribosomes from the mutant strain and the parent strain were isolated and washed four times with 0.5 M NH_4Cl. 30S and 50S ribosomal subunits were obtained by dialysis of washed ribosomes against 0.5 mM Mg, followed by centrifugation on a 10~30% sucrose gradient in SW 25 rotor (Beckman ultracetrifuge) for 17 hours. Elongation factors EF T and EF G were prepared from the parent strain by the method of NISHIZUKA *et al.*[13] The EF T fraction was purified up to the step of hydroxyapatite column chromatography and used without further separation into EF Tu and EF Ts. *E. coli* tRNA was purchased from Boehringer Co., Ltd. and labeled with ^{14}C-phenylalanine (80,000 cpm/mg RNA). Poly U was the product of Boehringer Co., Ltd. ^{14}C-Phenylalanine (342 mCi/mmole) was purchased from Daiichi Pure Chemical Co., Ltd., and ^{3}H-GDP (620 mCi/mmole) from the Radiochemical Centre.

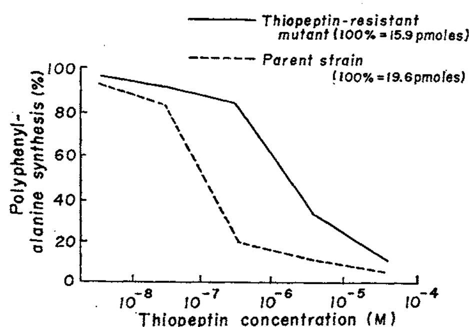
The assay for EF T-dependent binding of ^{14}C-phe-tRNA to ribosomes was carried out by the Millipore filtration method[11], and EF G-dependent binding of ^{3}H-GDP to ribosomes in the presence of fusidic acid was examined similarly[21,22]. Since thiopeptin is sparingly soluble in water, the antibioitc was first dissolved in dimethylformamide and diluted with water. The final concentration of dimethylformamide in the reaction mixtures was less than 0.5%, which was without effect on polyphenylalanine synthesis and other reactions described in this paper.

Results

A mutant strain of *E. coli* was obtained in which protein synthesis was resistant to thiopeptin, as proved by *in vitro* assays. Fig. 1 illustrates inhibition of poly U-directed synthesis of polyphenylalanine by the antibiotic in unfractionated extracts (S-30) of the mutant cells and of the cells of the parent strain. It can be seen that in the mutant extract the concentration of the antibiotic necessary for 50% inhibition is higher by about an order of magnitude than in the extract of the parent sensitive strain; at an antibiotic concentration in the range of $5\times10^{-7}\sim5\times10^{-6}$ M inhibition is markedly different between the two systems. The degree of inhibition by the antibiotic varies according to the concentration of ribosomes in the reaction mixture, as is the case with siomycin[7]. The amounts of S-30 were adjusted in respect to protein concentration in this experiment, so the observed difference in the inhibition could not be accounted for by a difference, if any, of ribosome concentrations.

Ribosomes of both strains were separated and equal amounts were combined with the supernatant (S-100) of the sensitive and resistant strains; inhibition of poly U-directed polyphenylalanine synthesis by thiopeptin was examined in these mixed systems. The results presented in Table 1 show that resistance to the antibiotic is a property of the ribosomes of the mutant. It is possible that the mutant ribosomes have acquired the ability to destroy thiopeptin. This was, however, ruled out on observing that preincubation of the antibiotic with the mutant ribosomes did not affect its inhibitory activity against sensitive ribosomes of the parent strain.

Fig. 1. Inhibition by thiopeption of poly U-directed polyphenylalanine synthesis of S-30 from the thiopeptin-resistant mutant and the parent strain



Reaction mixtures, in 0.1 ml, contained: 50 mM Tris-HCl, pH 7.4, 10 mM Mg acetate, 160 mM NH_4Cl, 2 mM dithiothreitol, 0.2 μmoles ATP, 0.5 μmoles phosphoenolpyruvate, 5 μg pyruvate kinase, 0.01 μmole GTP, 4 μg poly U, 20 μg tRNA, 0.02 μCi ^{14}C-phenylalanine, 200 μg protein of S-30 and the antibiotic. S-30 was preincubated to remove endogenous mRNA activity and dialyzed. The reaction mixtures were incubated at 30°C for 20 minutes. The hot TCA-insoluble fraction was collected on glass fiber filters and the radioactivity was measured by a liquid scintillation counter with PPO (5 g/liter) and dimethyl POPOP (0.3 g/liter) in toluene. The data are expressed as percent of the control obtained without the antibiotic.

To determine which subunit of the mutant ribosome is responsible for resistance to thiopeptin, ribosomes of the sensitive and resistant strains were separated into subunits and reconstituted in different 30S and 50S subunit combinations. The effect of the antibiotic on polyphenylalanine synthesis using these ribosomes was investigated with the results shown in Table 2. The inhibition was different depending on whether 50S subunits were derived from the mutant or the parent strain, while it did not differ substantially with the origin of 30S subunits. This indicates that resistance to thiopeptin is due to an alteration of the 50S ribosomal subunits induced by the mutation.

EF T is involved in codon-directed binding of aminoacyl-tRNA to ribosomes. This factor interacts in the form of an aminoacyl-tRNA-EF T-GTP complex with ribosomes, whereby the aminoacyl-tRNA is transferred to ribosomes and the GTP is hydrolyzed[14~16] For the hydrolysis of GTP to occur interaction with 50S ribosomal subunits is required[17,18]. EF G exhibits another

Table 1. Inhibition by thiopeptin of poly U-directed polyphenyl-alanine synthesis in different combinations of ribosomes and S-100 from the mutant and the parent strain

Source		Thiopeptin	pmoles Phe polymerized
Ribosomes	S-100	M×10⁶	(% inhibition)
S	S	–	12.5
S	S	1	2.6 (79)
S	R	–	12.4
S	R	1	3.9 (69)
S	R	2	2.1 (83)
R	R	–	10.4
R	R	1	9.2 (12)
R	S	–	10.6
R	S	1	8.1 (24)
R	S	2	5.2 (52)

The composition of the reaction mixture (0.1 ml) was the same as in the experiment in Fig. 1 except that 200 μg washed ribosomes and 200 μg protein of dialyzed S-100 were used in place of S-30. Incubation was 30°C for 20 minutes.

S: from the parent strain, R: from the mutant.

Table 2. Thiopeptin sensitivity of ribosomes reconstituted from 30S and 50S subunits isolated from sensitive and resistant strains

Source		Thiopeptin	pmoles Phe polymerized
30S	50S	M×10⁶	(% inhibition)
S	S	–	16.4
S	S	1	3.5 (80)
S	R	–	12.6
S	R	1	8.6 (31)
S	R	2	6.2 (50)
R	R	–	14.2
R	R	1	11.4 (20)
R	S	–	15.4
R	S	1	3.6 (76)
R	S	2	2.1 (86)

Reaction mixtures, in 0.1 ml, contained: 100 μg 30S subunits, 200 μg 50S subunits, 250 μg protein of S-100 prepared from the parent strain, and other components as in the experiment in Fig. 1. Incubation was at 30°C for 20 minutes. The amounts of both subunits were estimated by absorbance at 260 mμ, assuming that 14.4 A_{260} units are eqivalent to 1 mg/ml.

Table 3. Effect of thiopeptin on EF T-dependent binding of phe-tRNA to ribosomes from the thiopeptin-resistant mutant and the thiopeptin sensitive parent strain.

Series	pmoles phe-tRNA bound to ribosomes from	
	sensitive strain	resistant strain
Control	7.5 (100)	6.6 (100)
− EF T	1.3 (17)	1.1 (17)
+ thiopeptin		
5×10^{-7}M	5.1 (64)	5.8 (88)
5×10^{-6}M	1.7 (23)	4.2 (61)

Reaction mixtures, in 0.1 ml, contained: 2 µg EF T, 200 µg ribosomes, 4 µg poly U and 50 µg ^{14}C-phe-tRNA in a medium consisting of 50 mM Tris-HCl, pH 7.4, 7 mM Mg acetate, 60 mM NH_4Cl and 2 mM dithiothreitol. After incubation at 30°C for 10 minutes, 2 ml of cold buffer with identical constitution as the reaction medium was added. ^{14}C-phe-tRNA bound to ribosomes was collected on Millipore filters, washed with the same buffer, and counted. The numbers in the brackets are percent of the control.

Table 4. Effect of thiopeptin on EF G-dependent binding of GDP to ribosomes in the presence of fusidic acid.

Series	pmoles GDP bound to ribosomes from	
	parent strain	resistant strain
Control	16.2 (100)	18.4 (100)
− ribosomes	0.6	
− EF G	1.2	1.5
+ thiopeptin		
5×10^{-7}M	12.1 (80)	16.1 (89)
5×10^{-6}M	4.1 (25)	14.2 (76)

Reaction mixtures, 0.1 ml, contained: 50 mM Tris-HCl, pH 7.4, 10 mM Mg acetate, 60 mM NH_4Cl, 2 mM dithiothreitol, 200 µg ribosomes, 6 µg EF G, 18 nmoles ^{3}H-GDP and 250 µM fusidic acid. After incubation at 18°C for 10 minutes, 2 ml of ice-cold buffer (10 mM Tris-HCl, pH 7.4, 10 mM Mg acetate, 60 mM NH_4Cl and 2 mM dithiothreitol) containing 200 µM fusidic acid was added, and the GDP bound to ribosomes was collected on Millipore filters, washed with fusidic acid-containing buffer, and counted. The numbers in the brackets are per cent of the control.

GTPase activity in association with ribosomes which is presumed to be involved in translocation of peptidyl-tRNA on ribosomes. In this GTPase reaction also the factor interacts with 50S ribosomal subunits, forming GTP-EF G-ribosome complex[19,20,23]. In the presence of fusidic acid the GDP-EF G-ribosome complex accumulates[21], which makes complex formation easily detectable. GDP is effective in place of GTP in the formation of the ternary complex. Thiopeptin has been reported to block both the interactions of EF T and EF G with ribosomes[11]. The effects of the antibiotic on EF T-dependent binding of phe-tRNA and EF G-dependent binding of GDP to the mutant ribosomes were investigated. The data given in the Tables 3 and 4 show that the inhbitory effects are markedly weaker on both reactions with the mutant ribosomes than with the ribosomes from the parent strain. From these and the above results it was concluded that in this mutant not only EF T-dependent but also EF G-dependent ribosomal functions have become resistant to thiopeptin due to an alteration of 50S ribosomal subunits.

Discussion

The nature of the ribosomal involvement in GTP hydrolysis associated with EF T and EF G and mechanisms linking these reactions with the binding of aminoacyl-tRNA and translocation remain to be elucidated. The present study is concerned with these problems.

It was not possible to isolate thiopeptin-resistant mutants on agar plates because of low solubility of the antibiotic. Instead, the selection was performed in a liquid medium, as described in Materials and Methods, on the basis of a finding that the antibiotic exhibits much greater activity against *E. coli* Q 13 in liquid medium containing EDTA.

From the results obtained in this study with a thiopeptin-resistant mutant it can be concluded that a single action of thiopeptin on 50S ribosomal subunits brings about dual effects, namely, inhibition of the EF T-dependent reaction and the EF G-dependent reaction. This can be simply explained by the view, as suggested previously[11,12], that there si a common site on 50S ribosomal

subunits that participates in interactions with EF T and EF G. In this connection it was observed that EF T-dependent binding of aminoacyl-tRNA to ribosomes is prevented when EF G remains bound to ribosomes[24~27]. As more direct evidence for the common site, a ribosomal protein that is involved in GTP hydrolysis dependent both on EF T and on EF G was recognized[28,29] and identified as 50S ribosomal protein L 12 and its acetylated form, L 7[30]. It will be interesting to know if this protein is altered in the thiopeptin-resistant mutant we have isolated. However, it was recently reported that 50S ribosomal subunits retain the capability to bind thiostrepton after treatment with CsCl that removes L 7 and L 12[31]. This indicates that the site for the binding of the antibiotic is probably different from the site shared by EF T and EF G.

The conclusion stated above is based on the assumption that the mutant we have isolated is a single-step mutant, which has not been proved. The liquid selection technique used may have provided multiple mutants more frequently than ordinary agar selection. However, the conclusion is reasonable in consideration of the results of the previous study[11] and several other reports[12,24~29] supporting the view of a common site on 50S ribosomal subunits shared by EF T and EF G.

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References

1) PESTKA, S.: Inhibitors of ribosomal functions. Ann. Rev. Microbiol. 25: 478~562, 1971
2) KURLAND, C. G.: Structure and function of bacterial ribosome. Ann. Rev. Biochem. 41: 377~408, 1972
3) WITTMAN, H. G. & G. STÖFFLER: Structure and function of bacterial ribosomal proteins. In "The mechanism of protein synthesis and its regulation", ed. by L. BOSCH; North-Holland Publ. Co., Amsterdam, pp. 285~351, 1972
4) NOMURA, M.: Assembly of bacterial ribosomes. Science 179: 864~873, 1973
5) MIYAIRI, M.; T. MIYOSHI, H. AOKI, M. KOHSAKA, H. IKUSHIMA, K. KUNIGATA, H. SAKAI & H. IMANAKA: Studies on thiopeptin antibiotics. I. Characteristics of thiopeptin B. J. Antibiotics 23: 113~119, 1970
6) WEISBLUM, B. & V. DEMOHN: Thiostrepton, an inhibitor of 50S ribosomal subunits function. J. Bact. 101: 1073~1075, 1970
7) TANAKA, K.; S. WATANABE, H. TERAOKA & K. TAMAKI: Effect of siomycin on protein synthesizing activity of *E. coli* ribosomes. Biochem. Biophys. Res. Comm. 39: 1189~1193, 1970
8) PESTKA, S.: Thiostrepton: A ribosomal inhibitor of translocation. Biochem. Biophys. Res. Comm. 40: 667~674, 1971
9) MODOLELL, J.; D. VAZQUEZ & R. E. MONRO: Ribosomes, G factor and siomycin. Nature New Biol. 230: 109~112, 1971
10) BODLEY, J. W.; L. LIN & J. H. HIGHLAND: Studies on translocation. V. Thiostrepton prevents the formation of ribosomes-G factor-guanosine nucleotide complex. Biochem. Biophys. Res Comm. 41: 1406~1411, 1970
11) KINOSHITA, T.; L. LIOU & N. TANAKA: Inhibition by thiopeptin of ribosomal functions associated with T and G factors. Biochem. Biophy. Res. Comm. 44: 895~863, 1971
12) MODOLELL, J.; B. CABRER, A. PARMEGGIANI & D. VAZQUEZ: Inhibition by siomycin and thiostrepton of both aminoacyl-tRNA and factor G binding to ribosomes. Proc. Natl. Acad. Sci. U.S.A. 68: 1796~1800, 1971
13) NISHIZUKA, Y.; F. LIPMANN & J. LUCAS-LENARD: Isolation and properties of the *E. coli* amino acid polymerizing enzymes. Methods in Enzymology, Vol. 12 B, ed. by L. GROSSMAN & K. MOLDAVE, Academic Press, pp. 708~721, 1968
14) ONO, Y.; A. SKOULTCH, J. WATERSON & P. LENGYEL: Stoichiometry of aminoacyl-tRNA binding and GTP cleavage during chain elongation and translocation. Nature 223: 679~701, 1969
15) SHOREY, L. H.; J. M. RAVEL, C. W. GORNER & W. SHIEVE: Formation and properties of aminoacyl transfer ribonucleic acid-guanosine triphosphate-protein complex. An intermediate in the binding of

aminoacyl transfer ribonucleic acid to ribosomes. J. Biol. Chem. 244: 4555~4564, 1969
16) GORDON, J.: Hydrolysis of guanosine 5'-triphosphate associated with binding of aminoacyl transfer ribonucleic acid to ribosomes. J. Biol. Chem. 244: 5680~5686, 1969
17) BROT, N.; B. REDFIELD & H. WEISSBACH: Studies on the reaction of the aminoacyl-tRNA-Tu-GTP complex with ribosomal subunits. Biochem. Biophys. Res. Comm. 41: 1388~1395, 1970
18) WEISSBACH, H.; B. RFDFIELD, E. YAMAZAKI & N. BROT: Interaction of a phe-tRNA-Tu-GTP complex with ribosomal subunits. Arch. Biochem. Biophys. 149: 560~562, 1972
19) BROT, N.; C. SPEARS & H. WEISSBACH: The formation of a complex containing ribosomes transfer factor G and guanosine nucleotide. Biochem. Biophys. Res. Comm. 34: 843~847, 1969
20) PARMEGGIANI, A. & E.M. GOTTSCHALK: Propertes of the crystalline amino acid polymerization factors from *E. coli*: Binding of G to ribosomes. Biochem. Biophys. Res. Comm. 35: 861~867, 1969
21) BROT, N.; C. SPEARS & L. LIN: Interaction of transfer factor G, ribosomes and guanosine nucleotides in the presence of fusidic acid. Arch. Biochem. Biophys. 143: 286~296, 1970
22) BODLEY, J. W.; F. J. ZIEVE, L. LIN & S. T. ZIEVE: Studies on translocation. III. Conditons necessary for the formation and detection of a stable ribosome-G-guanosine diphosphate complex in the presence of fusidic acid. J. Biol. Chem. 245: 5656~5661, 1970
23) BODLEY, J. W. & L. LIN: Interaction of *E. coli* G factor with 50s ribosomal subunit. Nature 227; 60~62, 1970
24) RICHMAN, N. & J. W. BODLEY: Ribosomes cannot interact simultaneously with elongation factors EF Tu and EF G. Proc. Natl. Acad. Sci. U. S. A. 69: 686~689, 1972
25) CABRER, B.; D. VAZQUEZ & J. MODOLELL: Inhibition by elongation factor EF G of aminoacyl-tRNA binding to ribosomes. Proc. Natl. Acad. Sci. U. S. A. 69: 733~736, 1972
26) MILLER, D. L.: Elongation factors EF Tu and EF G interact at related sites on ribosomes. Proc. Natl. Acad. Sci. U. S. A. 69: 752~755, 1972
27) RICHTER, D.: Inability of *E. coli* ribosomes to interact simultaneously with the bacterial elongation factors EF Tu and EF G. Biochem. Biophys. Res. Comm. 46: 1850~1856, 1972
28) HAMEL, E. & T. NAKAMOTO: Requirement of an *E. coli* 50S ribosomal protein for effective interaction of the ribosome with T and G factors and guanosine triphosphate. J. Biol. Chem. 247: 805~814, 1972
29) WEISSBACH, H.; B. REDFIELD, E. YAMAZAKI, R. C. DAVIS, Jr., S. PESTKA & N. BROT: Studies on the ribosomal sites involved in factors Tu and G-dependent reactions. Arch. Biochem. Biophys. 149: 110~117, 1972
30) KASCHA, K.; W. MÖLLER & G. STÖFFLER: Reconstitution of a GTPase activity by a 50S ribosomal protein. Nature 233: 62~63, 1971
31) MOHAM, L. & P. LENGEL: Components of the 50S ribosomal subunits involved in GTP cleavage. Biochem. Biophys. Res. Comm. 46: 238~244, 1972