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. 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. 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 thioestrepton and siomycin are included. Thiostrepton and siomycin have been reported to act on 50S ribosomal subunits resulting in inhibition of their elongation factor EF G-dependent activity. 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. 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.

Isolation and studies of mutants resistant to an antibiotic are generally undertaken as an effective approach to understanding of the mechanism of its action. 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 NH₄Cl. 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 ultracentrifuge) 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 14C-phenylalanine (80,000 cpm/mg RNA). Poly U was the product of Boehringer Co., Ltd. 14C-Phenylalanine (342 mCi/m mole) was purchased from Daiichi Pure Chemical Co., Ltd., and 3H-GDP (620 mCi/m mole) from the Radiochemical Centre.

The assay for EF T-dependent binding of 14C-phe-tRNA to ribosomes was carried out by the Millipore filtration method11, and EF G-dependent binding of 3H-GDP to ribosomes in the presence of fusidic acid was examined similarly2). Since thiopeptin is sparingly soluble in water, the antibiotic 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 5×10⁻⁷~5×10⁻⁶ 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 siomycin7). 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 I 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.
Inhibition by thiopeptin 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 NH₄Cl, 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 ¹⁴C-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. For the hydrolysis of GTP to occur interaction with 50S ribosomal subunits is required. EF G exhibits another
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.

<table>
<thead>
<tr>
<th>Series</th>
<th>pmoles phe-tRNA bound to ribosomes from sensitive strain</th>
<th>pmoles phe-tRNA bound to ribosomes from resistant strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.5 (100)</td>
<td>6.6 (100)</td>
</tr>
<tr>
<td>− EF T</td>
<td>1.3 (17)</td>
<td>1.1 (17)</td>
</tr>
<tr>
<td>+ $5 \times 10^{-7} M$</td>
<td>5.1 (64)</td>
<td>5.8 (88)</td>
</tr>
<tr>
<td>$5 \times 10^{-6} M$</td>
<td>1.7 (23)</td>
<td>4.2 (61)</td>
</tr>
</tbody>
</table>

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$_4$Cl 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.

<table>
<thead>
<tr>
<th>Series</th>
<th>pmoles GDP bound to ribosomes from parent strain</th>
<th>pmoles GDP bound to ribosomes from resistant strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.2 (100)</td>
<td>18.4 (100)</td>
</tr>
<tr>
<td>− ribosomes</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>− EF G</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>+ $5 \times 10^{-7} M$</td>
<td>12.1 (80)</td>
<td>16.1 (89)</td>
</tr>
<tr>
<td>$5 \times 10^{-6} M$</td>
<td>4.1 (25)</td>
<td>14.2 (76)</td>
</tr>
</tbody>
</table>

Reaction mixtures, 0.1 ml, contained: 50 mM Tris-HCl, pH 7.4, 10 mM Mg acetate, 60 mM NH$_4$Cl, 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$_4$Cl 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 percent 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$^{19,20,23,5}$. In the presence of fusidic acid the GDP-EF G-ribosome complex accumulates$^{20}$, 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 inhibitory 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 is 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.

Acknowledgement

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References

aminoacyl transfer ribonucleic acid to ribosomes. J. Biol. Chem. 244: 4555–4564, 1969