PROTECTION OF *E. COLI* RIBOSOMES AGAINST COLICIN E3-INDUCED INACTIVATION BY BOUND AMINOACYL-tRNA

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1. Introduction

Colicin E3 inactivates bacterial ribosomes by introducing a single endonucleolytic break in the 16 S ribosomal RNA [1, 2]. The cleavage is observed only when colicin acts on 70 S ribosomes. Isolated 30 S subunits or purified 16 S RNA are not substrates for the reaction [3, 4]. It has recently been reported that streptomycin, tetracycline and gentamycin, antibiotics known to interact specifically with the 30 S ribosomal subunit, counteract the effect of colicin both in vivo and in vitro [5].

In the experiments described in this communication we tested the ability of molecules that normally bind to ribosomes during polypeptide synthesis to provide protection against colicin E3-induced inactivation. It has been found that ribosomes complexed with Phe-tRNA* and poly U are resistant to the inactivation by colicin. The protection was observed irrespective of the mode of binding; nonenzymatically and enzymatically bound aminoacyl-tRNA's were similarly effective.

2. Experimental

Cells of *E. coli* MRE-600 were the source of ribosomes, S-100 fraction and partially purified factors.

70 S ribosomes, isolated by sucrose density centrifugation [6], were layered on a cushion of 30% sucrose in 1 M NH_4Cl, 0.01 M $Mg(OAc)_2$, 0.05 M Tris–HCl (pH 7.5) and 0.01 M mercaptoethanol and were pelleted by centrifugation. The ribosomes were then suspended in and dialyzed against buffer A containing 0.1 M NH_4Cl, 0.01 M $Mg(OAc)_2$, 0.05 M Tris–HCl (pH 7.5) and 0.01 M mercaptoethanol. EF-T (Tu+Ts) was prepared according to Lucas-Lenard and Lipmann [7].

Polyphenylalanine synthesis was assayed in 50 μl or 150 μl reaction mixtures containing 0.1 M NH_4Cl, 0.01 M $Mg(OAc)_2$, 0.02 M Tris–HCl (pH 7.5), 0.006 M DTT, 0.001 M GTP, saturating amounts of S-100 fraction, limiting amounts of ribosomes and 10 μg of poly U and 4×10^4 cpm of [^{14}C]Phe-tRNA ($6–9 \times 10^5$ cpm/mg, 455 mCi/mmole, 750 cpm/pmole) per 50 μl of reaction mixture. Incubation was carried out at 25°C for 5 or 10 min, a period during which polymerization progressed linearly. Hot trichloroacetic acid-insoluble material was collected on glass fiber filters (Whatman GF/C).

Purified colicin E3 was a gift of Dr. D.R. Helinski to Dr. S. Sarid. The colicin was dissolved in TMAI buffer containing 0.03 M NH_4Cl, 0.01 M $Mg(OAc)_2$, 0.01 M Tris–HCl (pH 7.5) and 0.006 M mercaptoethanol.

3. Results

The reaction of colicin E3 with ribosomes was assessed in the present experiments by its inactivating effect on the ribosomes. To test the effect of bound aminoacyl tRNA on colicin E3-induced inactivation, ribosomes were first allowed to form binding complexes with aminoacyl-tRNA which were then incubated with colicin. Following the incubation, the reaction mixtures were diluted 30-fold with cold buffer or, they were layered on and centrifuged through a sucrose solution containing 1 M NH_4Cl. The

* *Abbreviations*: Phe-tRNA, phenylalanyl-transfer RNA; fMet-tRNA, formylmethionyl-transfer RNA; poly U, polyuridylic acid; DTT, dithiothreitol; OAc, acetate; GMPPCP, 5'guanylyl methylendiphosphate.

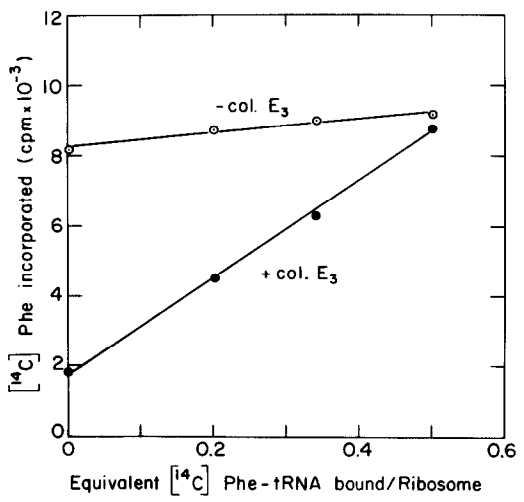

Fig. 1. The effect of non-enzymatically bound Phe-tRNA on colicin-induced inactivation of ribosomes binding of Phe-tRNA: The binding mixtures in 25 μl contained 0.1 M NH_4Cl, 0.02 M $Mg(OAc)_2$, 0.026 M Tris–HCl (pH 7.5), 0.006 DTT, 10 μg poly U, 310 μg (115 pmoles) 70 S ribosomes and 20, 40 or 133 pmoles of [^{14}C]Phe-tRNA. Incubation of the binding mixtures as well as control ribosomes without Phe-tRNA and poly U, was for 15 min at 37°C. The mixtures were chilled and 2 μl samples were removed to determine the amount of Phe-tRNA bound. Colicin treatment and incorporation assay: To a 10 μl aliquot of the binding mixture 2 μl of colicin solution was added to give a final colicin concentration of 0.25 mg/ml. To another 10 μl aliquot, 2 μl of TMAI buffer was added. The samples were incubated for 30 min at 37°C. Aliquots of 3 μl containing 30 μg of 70 S ribosomes were removed and added to 100 μl of cold buffer containing 0.15 M NH_4Cl, 0.015 M $Mg(OAc)_2$ and 0.03 M Tris–HCl (pH 7.5). The mixtures were supplemented with all the necessary components for phenylalanine polymerization (as indicated in the experimental section) to give a final volume of 150 μl. Polymerization was performed for 5 min.

dilution practically stopped the reaction with colicin, without causing a significant dissociation of the bound Phe-tRNA. The centrifugation method not only freed the ribosomes from the colicin, and components of the binding reaction but also caused dissociation of the bound Phe-tRNA.

As is shown in fig. 1, the non-enzymatic poly U-dependent binding of Phe-tRNA protects the ribosomes against inactivation by colicin. The degree of protection is directly proportional to the amount of Phe-tRNA bound, i.e. to the number of complexed ribosomes in the mixture. Nearly complete protection is observed when 0.5 equivalents of Phe-tRNA are bound per ribosome. This would correspond to 50% of the ribosomes being in a complexed state assuming each ribosome binds a single molecule of Phe-tRNA. This extent of binding represents the maximal binding ability of the ribosome preparation employed. The results indicate that only the ribosomes capable of non-enzymatic binding are functional in polyphenylalanine synthesis.

The protective ability of non-enzymatically bound Phe-tRNA was compared with that of enzymatically bound aminoacyl-tRNA in the experiment summarized in table 1. The enzymatic and non-enzymatic reactions were performed at 8 mM Mg^{2+} and 20 mM Mg^{2+} respectively and resulted in a similar level of bound Phe-tRNA. Following the colicin treatment, reaction mixtures were centrifuged through a sucrose solution to avoid effect of components of the binding mixtures on the subsequent polymerization assay. It should be noted that the lower inactivation observed in this experiment as compared with the experiment described in fig. 1 is due to the lower concentration of colicin used. The results show that enzymatically formed complexes were protected from colicin attack similarly to complexes formed non-enzymatically. Binding with GMPPCP appears to provide, for the amount of Phe-tRNA bound, even a better protection than the binding with GTP. Analysis of the bound material revealed, however, that in the presence of GTP, but not with GMPPCP, a significant amount of the bound material was in the form of di-Phe-tRNA and therefore the amount of Phe-tRNA bound in this case exceeded the amount of complexed ribosomes. In similar experiments bound poly U alone decreased the level of colicin-induced inactivation by 5–10% whereas no other single component of the binding mixture had any protective effect. This indicates that the whole aminoacyl-tRNA binding complex is required for the effective protection of the ribosome.

Other binding complexes were also tested (experiments not shown). It was found that the ribosomal binding complexes with *N*-acetyl-Phe-tRNA directed by poly U and with fMet-tRNA directed by poly AUG, both formed in the presence of initiation factors, were also largely protected against colicin E3 inactivation. The degree of protection appeared however to be somewhat lower than that provided by Phe-tRNA.

Table 1
The effect of enzymatically bound Phe-tRNA on colicin-induced inactivation of ribosomes.

	Preincubation			Colicin treatment	Activity	
	Phe-tRNA poly U	Additions	Phe-tRNA bound (Equivalents/ribosome)		[^{14}C]Phe incorporation cpm	Relative activity
1)	–	–	–	–	4125	100
				+	2185	53
2)	+	EF-T, GTP	0.67	4090	4630	100
				+	–	89
3)	+	EF-T, GMPPCP	0.57	–	4147	100
				+	3910	95
4)	+	–	0.57	–	3880	100
				+	3830	99

Preincubation: The 90-μl preincubation mixtures each in duplicate, contained 90 pmoles of 70 S ribosomes in 0.1 M NH_4Cl, 0.02 M Tris–HCl (pH 7.5) and 0.005 M DTT. Concentration of $Mg(OAc)_2$ was 0.008 M in mixtures 1, 2 and 3, and 0.02 M in mixture 4. When indicated 30 μg of poly U and 106 pmoles of [^{14}C]Phe-tRNA were added. EF-T (Tu+Ts) was present in saturating amounts and GTP and GMPPCP were added at 0.001 M and 0.006 M respectively. After incubation for 15 min at 37°C, 2-μl samples were removed to determine the amount of Phe-tRNA bound. *Colicin treatment and activity assay*: 3 μl of the colicin solution containing 5 μg were added and the mixtures were incubated for 30 min at 37°C. Controls were incubated after adding 3 μl of TMAI buffer. Following the incubation, the mixtures were immediately layered on 1.5 ml of 30% sucrose in 1 M NH_4Cl, 0.01 M $Mg(OAc)_2$, 0.05 M Tris–HCl (pH 7.5) and 0.01 M mercaptoethanol in 2-ml centrifuge tubes. The ribosomes were pelleted by centrifugation for 7 hr at 50 000 rpm and were suspended in an dialyzed against buffer A. Phenylalanine polymerization was performed in 50 μl reaction mixtures with 6 μg ribosomes for 10 min.

4. Discussion

In the present experiments colicin E3 activity was followed by the inactivation of ribosomes in polyphenylalanine synthesis. The protection of ribosomes complexed with aminoacyl-tRNA against inactivation is likely to be due to prevention of the colicin-induced endonucleolytic cleavage. The possibility that cleavage did occur but the ribosomes maintained their active structure due to the bound aminoacyl-tRNA was ruled out by the finding that the treated ribosomes retained their activity even when Phe-tRNA was dissociated from the ribosomes before the polymarization assay (cf. table 1).

Protection of ribosomes against colicin E3 inactivation does not require a specific mode of binding since it was observed with aminoacyl-tRNA's bound under different conditions and presumably at different sites. A plausible interpretation of these results is that the protection is not due to a direct interference of bound aminoacyl-tRNA with sites of colicin attachment or action. Rather, it appears that the formation of any of the binding complexes affects the overall conformation of the ribosome and renders it resistant to the action of colicin. A similar interpretation was offered by Dahlberg et al. [5] for the protective effect of antibiotics. Still, the possibility of some direct interference cannot be excluded.

The finding that ribosomes complexed with mRNA and aminoacyl-tRNA are resistant to colicin action raised some questions as to the mechanism of colicin-induced inactivation in vivo. According to the present experiments it appeared that ribosomes would be most susceptible to colicin inactivation when in a non-complexed state. If such ribosomes fail to initiate peptide synthesis (as has been demonstrated in experiments not shown) then the primary effect of colicin would be to block the initiation step. However, from the study of extracts of colicin-treated bacteria Senior et al. [8] concluded that colicin affected the

elongation rather than the initiation step. This raises the possibility that there exists a stage in the elongation cycle when ribosomes even in a complexed form become susceptible to colicin. This aspect is currently under study.

Acknowledgements

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