EFFECT OF A SOLUBLE TRANSFER FACTOR ON THE REACTION OF AMINOACYL-tRNA WITH PUROMYCIN

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Lucas-Lenard and Lipmann (1966) have demonstrated that three soluble protein factors (referred to as Ts, Tu, and G) are required for polypeptide synthesis in bacterial systems. The exact role of these factors in protein synthesis is still unclear. Recent studies have shown that GTP reacts with Ts and Tu, together, to form a protein GTP complex (Ertel et al., 1968) and that this complex may be involved in the messenger dependent binding of aminoacyl-tRNA to the ribosome (Ravel et al., 1967; Gordon, 1968). Factor G, which initially was separated from a T fraction (Ts and Tu) by Allende et al. (1964) catalyzes the hydrolysis of GTP in the presence of ribosomes. The extent of this hydrolysis has been related to the number of peptide bonds formed during the polymerization reaction (Nishizuka and Lipmann, 1966a). More specifically, Nishizuka and Lipmann (1966b) have suggested that G may function in the process of translocation.

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Several reports (Traut and Monro, 1964; Hultin, 1966; Zamir et al., 1966; Gottesman, 1967) have appeared showing that the reaction of puromycin with acylated-tRNA requires ribosomes and messenger and is stimulated by the addition of a soluble cell extract. These data have been consistent with the view that a soluble factor is needed for the movement (translocation) of the acylated-tRNA from a puromycin unreactive site on the ribosome to one which is subsequently puromycin reactive. However, the effect of the individual factors (Ts, Tu, and G) on the puromycin reaction have not been reported.

We have recently separated the three soluble transfer factors from extracts of Escherichia coli (Ertel et al., 1968) and have tested their ability to stimulate the reaction of puromycin with ^{14}C-phenylalanyl-tRNA (phe-tRNA). Under the conditions employed, Factor G and GTP are specifically required for the formation of a puromycin peptide. While these studies were in progress, Tanaka et al. (1968) have shown that in the presence of Factor G, GTP, and puromycin, there is a decrease in the amount of phe-tRNA bound to the ribosome.

Experimental

E. coli B cells were obtained from the Grain Processing Corporation and the transfer factors Tu, Ts, and G were purified as previously described (Ertel et al., 1968). Tu and Ts were free of G, and G had no Tu or Ts activity.

The preparation of ^{14}C-phe-tRNA was done according to

the procedure of Conway (1964). Ammonium chloride washed ribosomes were prepared by a modification (Ertel et al., 1968) of the procedure of Hershey and Thach (1967). At least four washes with 1 M NH_4Cl were needed in order to remove virtually all of the Factor G activity from the ribosomes. γ-^{32}P-GTP (sp. act. 1.66 C/mmole) was purchased from International Chemical and Nuclear Corporation, ^{14}C-phenylalanine (sp. act. 315 μc/μmole) from Schwarz BioResearch, Inc., 5'-guanylylmethylene-diphosphonate (GMP-PCP), and the triplet, UpUpC (UUC), were obtained from Dr. Marshall Nirenberg.

Puromycin Reaction - Three separate incubations were used to study the formation of a puromycin reacting material. The puromycin product was then assayed by a modification of the extraction procedure described by Leder and Bursztyn (1966). The first incubation (binding reaction) was for five 5 minutes at 23° and contained in a final volume of 85 μl, Tris-HCl buffer pH 7.4, 50 mM, 2-mercaptoethanol, 12 mM; NH_4Cl, 160 mM; $MgCl_2$, 20 mM; Poly U, 50 μg; ribosomes, 1.1 A_{260}; and ^{14}C-phe-tRNA, 10 $\mu\mu$moles. At the end of this incubation, Factor G and GTP were added in a volume of 5 μl and the second incubation carried out for 5 minutes at 37° (Factor G and GTP reaction). All other additions to the incubation mixtures, as noted in Table 1, were always made prior to the second incubation. The incubation tubes were then placed into an ice-water mixture and 10 μl of a 0.01 M neutralized solution of puromycin were added. The final incubation (puromycin reaction) was done at 0°. At the end of 30 minutes, 0.9 ml of 0.1 M

potassium phosphate buffer pH 8.0 was added and the aqueous solution containing the puromycin peptide was extracted with 3 ml of ethyl acetate. Two ml of the organic phase were removed and assayed for radioactivity in a Packard Tri-Carb Spectrometer using a counting fluid as described by Bray (1960).

The binding of phe-tRNA to ribosomes was performed essentially as described by Nirenberg and Leder (1964).

Results and Discussion

Table 1 shows that in the presence of GTP and Factor G there is a marked stimulation of the reaction of ribosomal bound phe-tRNA with puromycin. Other guanosine nucleotides, including GMP-PCP, and ATP could not substitute for GTP, and purified fractions of Ts and Tu (Ertel et al., 1968) were not able to replace Factor G. The formation of a puromycin product was dependent upon the presence of poly U and ribosomes in the binding reaction (first incubation), and under the conditions used, between 50-75% of the phe-tRNA present in the incubation was bound to the ribosomes at the time puromycin was added.

In other experiments, no effect of G and GTP was obtained when the trinucleotide, UUC, (0.06 A_{260}) was substituted for poly U during the binding reaction. With this triplet, about 1.5 μμmoles of phe-tRNA were bound to the ribosomes under the conditions used, but less than 0.1 μμmole of puromycin product was formed whether or not Factor G and GTP were present.

Table 1

Requirements for the Formation of a Puromycin Product

Omissions from 1st Incubation 5' 23°	Omissions from 2nd Incubation 5' 37°	Puromycin-Product μμmoles
None	None	2.1
None	- G	0.18
None	- GTP	0.15
None	- G,-GTP	0.12
None	- GTP + GMP	0.15
None	- GTP + GDP	0.25
None	- GTP + GMP-PCP	0.20
None	- GTP + ATP	0.18
None	- G + Tu, Ts	0.15
None	+ Tu, Ts	2.4
None	None (0°)	0.12
- Poly U	None	0.09
- Ribosome	None	0

The reaction mixture and incubation conditions are described in the text and the values were corrected for extractable radioactivity (0.4 μμmoles) observed in the absence of puromycin. All additions were made prior to the second incubation. The incubation mixtures contained, where indicated, G factor; 0.24 μg of protein, Tu; 6.2 μg of protein and Ts; 3.6 μg of protein; and 10 mμmoles of the nucleotides.

The purified G factor used in this study catalyzed the ribosomal dependent hydrolysis of 9 μmoles of GTP per mg of protein in 10 minutes at 37° as assayed by the procedure described by Conway and Lipmann (1964). The incubations for the assay of Factor G contained in a total

volume of 0.2 ml: Tris-HCl buffer pH 7.4, 50 mM; Mg acetate, 10 mM; NH_4 acetate, 100 mM; GTP-γ-^{32}P, 25 mμmoles (specific activity 2000 cpm/mμmole), and ammonium chloride washed ribosomes, A_{260} 1.5.

The Ts and Tu used in this study bound 18 $\mu\mu$moles of GTP by the millipore technique described by Ertel et al. (1968).

The maximum amount of puromycin product was obtained when the second and third incubations were for 5 and 30 minutes, respectively, and 1×10^{-4} M GTP was present.

The formation of a puromycin peptide described in the present experiments probably involves at least two partial reactions; a movement of the aminoacyl-tRNA on the ribosome and the subsequent reaction of this translocated aminoacyl-tRNA with puromycin to form the puromycin peptide. There have been several reports which show that the latter reaction, i.e., the formation of the peptide bond, is catalyzed by an enzyme bound to the ribosome, and does not require a soluble factor or GTP (Zamir et al., 1966; Gottesman, 1967; Monro and Marcker, 1967; Pestka, 1968). Thus, the stimulation of the puromycin reaction, by Factor G and GTP, reported here, support the original idea put forth by Nishizuka and Lipmann (1966a) that Factor G and GTP are needed for the translocation process.

It is interesting to note that in the present experiments, UUC could not replace poly U, and GMP-PCP did not substitute for GTP. These results indicate that although messenger dependent binding is required for the stimulation of the reaction of phe-tRNA with puromycin, the size of the messenger is important and that hydrolysis of GTP may occur during this reaction.

The data are also consistent with the view that puromycin reacts only with peptidyl or acylated-tRNA which is bound to a specific site on the ribosome, presumably the "peptidyl-tRNA site", while the acylated-tRNA occupying the "aminoacyl-tRNA site" is unreactive toward the antibiotic. The amount of puromycin product obtained in the absence of Factor G and GTP (Table 1) is probably due to aminoacyl-tRNA bound to the peptidyl site during the incubation. The marked stimulation of the puromycin reaction by G and GTP, varying between 6 and 10 fold in the present experiments, appears to be related to at least 3 parameters. Thus, only 2-3 fold stimulations were observed when; 1) the first incubation was carried out at 37° or for longer times at 23°; 2) a larger quantity of ribosomes was used; and 3) the ribosomes were not completely free of Factor G.

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