DOES TRANSLOCASE (G-FACTOR) REQUIRE THE PRESENCE OF UNESTERIFIED tRNA ON THE DONOR SITE FOR ITS ACTION? - THE EFFECT OF FUSIDIC ACID.*

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Summary: For the action of G-factor and its inhibition by fusidic acid, the presence of unesterified tRNA at the donor site is not necessary.

The peptide chain elongation consists of at least three steps, namely, the binding of aminoacyl-tRNA to the acceptor site of the ribosome, peptide bond formation, and translocation of peptidyl-tRNA from the acceptor site to the donor site (1,2). In the normal situation, the substrate for peptidyl-tRNA translocation by G-factor (3) would be a ribosomal complex having unesterified tRNA on the donor site and peptidyl-tRNA on the acceptor site. In this communication, we present evidence that G-factor can act on an abnormal substrate which contains aminoacyl-tRNA on the acceptor site but no tRNA on the donor site of the ribosome. It was further determined whether fusidic acid could inhibit such an abnormal translocation even in the absence of tRNA at the donor site. The result indicated that fusidic acid could inhibit such translocation.

Materials and methods: E.coli extract and other materials; Preparations of ribosomes from E.coli Q-13 and aminoacyl-tRNA have been described in the previous communication (4). The ribosomes were washed three times with a buffer containing 0.1M Tris-HCl (pH 7.8), 0.01M magnesium acetate, 0.06M KCl, 0.006M β-mercaptoethanol and 0.5M NH_4Cl and were practically free from elongation and initiation factors. Preparation of T and G factor

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was described previously (3,5). Counting efficiency of ^{14}C was 10^6 cpm/μc. Specific activity of ^{14}C-phenylalanine was 375 C/mole.

Separation of phenylalanyl-puromycin and diphenylalanyl-puromycin by Sephadex G-15 chromatography; Puromycin derivatives of phenylalanine formed were analyzed by Sephadex G-15 chromatography (4). Puromycin derivative in 0.5ml of 0.5M acetic acid was applied to a Sephadex G-15 column (0.8x150cm) which had been equilibrated with 0.5M acetic acid. The elution was carried out by 0.5M acetic acid with a flow rate of 12 - 15min/1ml. Each fraction (1ml) was mixed with 10ml of Bray's solution (6) and radioactivity of puromycin derivatives of ^{14}C-phenylalanine was measured. The material first eluted was identified as phenylalanyl-puromycin and the material which followed the first peak was identified as diphenylalanyl-puromycin (4).

Results: In the experiment illustrated in Figure 1, the complexes of ribosome, poly U and ^{14}C-phenylalanyl-tRNA were prepared in the presence of 5mM Mg^{++} and T-factor (4). To this isolated complex, puromycin was added and resulting radioactive puromycin derivatives of ^{14}C-phenylalanine were analyzed by Sephadex G-15 chromatography. It is noted in this figure that in the absence of G-factor, phenylalanyl-puromycin as well as diphenylalanyl-puromycin were formed. The formation of diphenylalanyl-puromycin is perhaps due to the nonenzymatic translocation of diphenylalanyl-tRNA from the acceptor site to the donor site (7). On the other hand, when G-factor was added to the puromycin reaction, a marked stimulation of phenylalanyl-puromycin and diphenylalanyl-puromycin formation was observed. The fact that the formation of phenylalanyl-puromycin was significantly stimulated by G-factor strongly indicated that the acceptor site-bound phenylalanyl-tRNA can be translocated even in the absence of unesterified tRNA at the donor site. This is because, under the condition for the formation of the ribosomal complex, peptide bond formation between the donor site and the acceptor site-bound phenylalanyl-tRNA takes place, resulting in formation of diphenylalanyl-tRNA bound at the acceptor site. If the peptide bond formation does not take place because of partial inactivation of peptidyl transferase on the ribosomes,

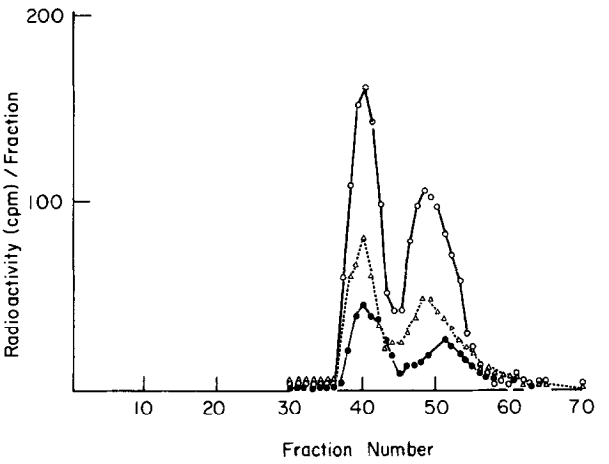

Fig.1 Sephadex G-15 column chromatography of puromycin derivatives of (^{14}C)-phenylalanine formed from (^{14}C)-phenylalanyl-tRNA bound to ribosomes in the presence of T-factor.

For the isolation of the complex, the reaction mixture (0.5ml) contained 50mM Tris-HCl (pH 7.2), 40mM NH_4Cl, 85µg of T-factor, 6mM magnesium acetate, 0.2mM GTP, 2mM dithiothreitol (DTT), 400ug of poly U, 5.8 mg of ribosomes and 5.2 mg of tRNA containing 8.5×10^5cpm of ^{14}C-phenylalanyl-tRNA (4). The complete reaction mixture (0.5ml) for the formation of puromycin derivatives contained 50mM Tris-HCl (pH 7.2), 80mM NH_4Cl, 6mM magnesium acetate, 12µg of G-factor, 0.2mM GTP and 0.3ml of the ribosomal complex containing 11,256 cpm of (^{14}C)-phenylalanyl-tRNA prepared as above. The mixture was incubated for 60 min. at 22° C. During this incubation period, 1,990 cpm and 6,400 cpm of the puromycin derivatives of (^{14}C)-phenylalanine were formed in the absence and the presence of G-factor, respectively. When fusidic acid (5×10^{-4}M) was added to the mixture with G-factor, 3,420 cpm of the puromycin derivatives of (^{14}C)-phenylalanine were formed.

o–o 2,100 cpm of the puromycin derivatives formed with G-factor were placed on the Sephadex column
Δ–Δ 1,140 cpm of that formed with G-factor and fusidic acid were analyzed
●–● 850 cpm of that formed in the absence of G-factor were placed on the Sephadex column as described in the text

these complexes will not be subjected to the action of G-factor because the G-factor can not release aminoacyl tRNA from the donor sites (8). Thus, the G-factor dependent phenylalanyl-puromycin formation most likely came from the ribosomal complex which had only phenylalanyl-tRNA at the acceptor site and no tRNA at the donor site. We therefore tentatively conclude that this abnormal complex, having aminoacyl tRNA only at the acceptor site, can be a substrate for G-factor.

Having answered the question whether G-factor requires the presence of tRNA at the donor site for its action, we proceeded to answer the question whether fusidic acid can still exert its inhibitory action even if the donor site is empty. As shown in this figure, the G-factor dependent phenylalanyl-puromycin formation was as strongly inhibited by fusidic acid as the G-factor dependent diphenylalanyl-puromycin formation.

The possibility existed in the preceding experiment that $tRNA^{phe}$ (tRNA specific for phenylalanine) may have been present in some of the ribosomal complexes because of the possibility that ^{14}C-phenylalanyl-tRNA used may contain some unesterified $tRNA^{phe}$. If such contamination of $tRNA^{phe}$ exists, one might have a situation where some ribosomes contain $tRNA^{phe}$ at the donor site and phenylalanyl-tRNA at the acceptor site. The increase of phenylalanyl-puromycin formation due to G-factor may have been observed because of the existence of such a complex. In order to rule out this possibility, in the experiment indicated in Table 1, $tRNA^{phe}$ at the donor site was measured by assaying the amount of $tRNA^{phe}$ released by G-factor. It has been shown that $tRNA^{phe}$ at the donor site is released during translocation (8,9). In this experiment, two step reactions were carried out. To avoid the complication due to the formation of a peptide bond, a complex of N-acetyl-^{14}C-phenylalanyl-tRNA, poly U and the ribosome was used. This complex was prepared in the presence of 13mM Mg^{++} where both acceptor and donor sites were occupied (4,10) by N-acetyl-^{14}C-phenylalanyl-tRNA. To this complex, G-factor was added and $tRNA^{phe}$ released by the addition of G-factor was measured. The next step was carried out by the addition of puromycin to this complex. During the second step, namely peptide bond formation with puromycin, fusidic acid was added to stop translocation completely. A control experiment was carried out similarly except for no addition of G-factor. The amount of the formation of N-acetyl-phenylalanyl-puromycin due to the presence of G-factor in the first step was compared to the amount of $tRNA^{phe}$ released in this step. It is clear from this table that the G-factor dependent N-acetyl-phenylalanyl-puromycin formation was far greater than the G-factor dependent release of $tRNA^{phe}$. A large amount of N-acetyl-phenylalanyl-puromycin was formed in the absence of G-factor. This is expected because

Table 1. Comparison of G-factor dependent release of $tRNA^{phe}$ and formation of N-acetyl-(^{14}C)-phenylalanyl-puromycin

Fusidic acid	N-acetyl-(^{14}C)-phenylalanyl-puromycin formed (cpm)	$tRNA^{phe}$ released (expressed as acceptor capacity for (^{14}C)-phenylalanine, cpm)
none	2830	235
10^{-4} M	1195	43

The reaction mixture (1 ml) for the formation of the ribosomal complex with N-acetyl-(^{14}C)-phenylalanyl-tRNA contained 50mM Tris-HCl (pH 7.2), 40mM NH_4Cl, 13mM magnesium acetate, 3.5mg of ribosomes, 100µg of poly U and tRNA mixture containing 6.4×10^5cpm of N-acetyl-(^{14}C)-phenylalanyl-tRNA. The complex was isolated as described previously (4). The reaction mixture (0.5ml) for the translocation reaction of acceptor site-bound N-acetyl-(^{14}C)-phenylalanyl-tRNA contained 40mM Tris-HCl (pH 7.2), 13mM magnesium acetate, 40mM NH_4Cl, 5mM DTT, 10µg of G-factor, 0.4mM GTP and 290µg of ribosomal complex containing 2×10^4cpm of N-acetyl-(^{14}C)-phenylalanyl-tRNA. Fusidic acid was added where indicated. The mixture was incubated for 6 min. at 26° C. In the control reaction mixture, G-factor and GTP were omitted. The release of $tRNA^{phe}$ with 0.4ml of the above reaction mixture was measured as described previously (8). In the absence of G-factor and GTP, 1075 cpm of $tRNA^{phe}$ was released. The reaction mixture (0.25ml) for N-acetyl-(^{14}C)-phenylalanyl-puromycin formation contained 0.1ml of the reaction mixture for translocation described above, 40mM Tris-HCl (pH 7.2), 13mM magnesium acetate, 40mM NH_4Cl, 5×10^{-4}M fusidic acid and 0.4mM puromycin. The mixtures were incubated for 6 min. at 26° C and N-acetyl-(^{14}C)-phenylalanyl-puromycin was measured by counting ethylacetate extractable radioactivity. In the absence of G-factor, 10,205 cpm of N-acetyl-(^{14}C)-phenylalanyl-puromycin was formed.

all N-acetyl-phenylalanyl-tRNA bound at the donor site would react with puromycin (4). Those complexes having N-acetyl-phenylalanyl-tRNA on both acceptor and donor site would not be influenced during the first step because of the inability of G-factor to eject aminoacyl or N-acetyl-aminoacyl-tRNA from the donor site (8). The small but significant amount of G-factor dependent release of $tRNA^{phe}$ observed probably originated from the contaminated $tRNA^{phe}$ in the N-acetyl-^{14}C-phenylalanyl-tRNA preparation which was used for the formation of the ribosomal complex. If translocation of N-acetyl-phenylalanyl-tRNA from acceptor site to donor site was dependent on this trace amount of $tRNA^{phe}$ at the donor site, one would expect that the amount of $tRNA^{phe}$ released from the donor site would be equal to the G-factor dependent formation of N-acetyl-phenylalanyl-puromycin. The fact

that the latter was far greater than the former indicates that translocation of N-acetyl-phenylalanyl-tRNA from acceptor to donor site does not indeed require the donor site bound $tRNA^{phe}$. In order to confirm the results obtained in Figure 1 that fusidic acid can inhibit the translocation of phenylalanyl-tRNA from acceptor site to empty donor site, fusidic acid was added during the first step as well as the second. As shown in the second line in this table, addition of fusidic acid, prior to the puromycin reaction, during the translocation step, inhibited the release of $tRNA^{phe}$ and the G-factor dependent N-acetyl-^{14}C-phenyl-alanyl-puromycin formation.

Discussion. The natural substrate for the translocase (G-factor) would be the ribosomal complex having unesterified tRNA at the donor site and peptidyl-tRNA at the acceptor site. It was conceivable that the presence of unesterified tRNA on the donor site may be necessary for G-factor to act on. However, the present results that phenylalanyl-puromycin formation was greatly stimulated by G-factor indicated such a configurational requirement did not exist for the action of G-factor. Thus, we can conclude that the action of G-factor is to move the peptidyl-tRNA from acceptor site to donor site regardless of the presence of the unesterified tRNA at the donor site. If, however, unesterified tRNA is present, simultaneous release of this tRNA takes place as the peptidyl tRNA is translocated (8,9). The well-known inhibitor of translocation, fusidic acid, can act in two conceivable fashions. The first possibility is that fusidic acid fixes unesterified tRNA on the donor site and thus prevents the movement of aminoacyl- or peptidyl- tRNA from the acceptor site to the donor site. The second possible mechanism is that it stops the movement of aminoacyl- or peptidyl-tRNA from the acceptor site to the donor site even if the donor site is empty. Present results eliminated the first possibility.

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