# STUDIES ON TRANSLOCATION OF F-MET-tRNA AND PEPTIDYL-tRNA WITH ANTIBIOTICS.

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Summary: The translocation of fMet-tRNA is inhibited by bottromycin $A_2$ but not by fusidic acid. The G factor-catalysed release of deacylated tRNA is inhibited by fusidic acid but not by bottromycin $A_2$. The ribosome-catalysed translocation of peptidyl-tRNA and mRNA, which occurs as the donor site is vacated, is inhibited by bottromycin $A_2$ but not by fusidic acid. The results indicate that G factor primarily participates in the release of deacylated tRNA and the translocation per se is catalysed by the 50S ribosomal subunit.

It has been demonstrated that fusidic acid, a steroidal antibiotic, and bottromycin $A_2$, a peptide antibiotic, interfere with translocation of peptidyl-tRNA and mRNA on ribosomes (1-11). Both antibiotics do not significantly affect the puromycin reaction in the absence of G factor and GTP, but inhibit the puromycin reaction enhanced by G factor and GTP (1,6,9). Fusidic acid interacts with G factor (2,9) and bottromycin $A_2$ with the 50S ribosomal subunit (10). The ribosome-dependent GTPase activity of G factor is inhibited by the former but not by the latter (1,5). The mechanism of translocation of peptidyl-tRNA and mRNA has been studied, using these antibiotics as tools, and the results are presented in this communication.

The preparation of E. coli washed ribosomes and initiation factor, f2 phage RNA, and f-$^3$H-Met-tRNA followed the methods previously described (15). The ribosomes charged with $^{14}$C-polylysyl-$^3$H-tRNA were prepared as described previously (10), using $^3$H-tRNA (7,800 cpm/μg RNA) isolated from an uracil-less mutant of E. coli

K-12 grown in the presence of $^{3}$H-uracil. The ribosomes were extensively washed to remove G factor (10).

It was observed by the method previously described (15) that bottromycin $A_2$ and fusidic acid slightly inhibited the formation of f2 RNA-ribosome-fMet-tRNA complex (Table 1). It indicates that the antibiotics might affect the formation of initiation complex, although the grade of inhibition is far less than that of protein synthesis. Therefore the inhibition of initiation complex formation seems to be insignificant (17).

Table 1. Effects of antibiotics on binding of fMet-tRNA to ribosomes.

| Additions | | fMet-tRNA bound (pmoles/tube) | %inhibition |
|---|---|---|---|
| None | | 4.54 | |
| Bottromycin $A_2$ | $2 \times 10^{-4}$ M | 3.47 | 22 |
| | $2 \times 10^{-5}$ | 4.31 | 3 |
| | $2 \times 10^{-6}$ | 4.67 | - |
| Fusidic acid | $2 \times 10^{-4}$ M | 4.05 | 9 |
| | $2 \times 10^{-5}$ | 4.58 | - |

The reaction mixture contained: 50 mM Tris-HCl, pH 7.5, 60 mM $NH_4Cl$, 6 mM $Mg(AcO)_2$, 10 mM 2-mercaptoethanol, 2 mg/ml washed 70S ribosomes, 0.6 mg/ml initiation factor, 150 µg/ml f-$^{14}$C-Met-tRNA (300,000 cpm/mg), 1 mg/ml f2-RNA, 0.2 mM GTP, 0.1 ml in each tube. It was incubated at 37°C for 15 min. The radioactivity, collected on Millipore filter, was assayed with corrections for values without messenger.

The reaction of puromycin with fMet-tRNA on 70S ribosomes with f2-RNA was inhibited by bottromycin $A_2$. Approximately 90 % inhibition was observed at the concentration of $10^{-4}$ M, 32 % inhibition at $10^{-5}$ M, and no inhibition at $10^{-6}$ M (Exp. I in Table 2).

Kolakofsky et al. reported that fMet-tRNA binds to the acceptor site and then translocates to the donor site (16). Kasugamycin was observed to inhibit the binding of fMet-tRNA more significantly than the binding of Phe-tRNA (15). It suggests that the binding site of fMet-tRNA is closely related to, but not identical with the acceptor site. Here it is tentatively called the "entry" site.

Table 2. Inhibition by bottromycin $A_2$ of fMet-puromycin reactions.

| Additions | | 70S initiation complex | | |
|---|---|---|---|---|
| | | not preformed (Exp. I) | preformed with GTP (Exp. II) | preformed with GDPCP (Exp. III) |
| None | | 100 | 100 | 100 |
| Bottromycin $A_2$ | $10^{-4}$ M | 6 | 61 | 0 |
| | $10^{-5}$ | 68 | 108 | 28 |
| | $10^{-6}$ | 102 | | 80 |
| Fusidic acid | $10^{-4}$ | 104 | 102 | 99 |

100 % was 13,220 cpm in Exp. I, 1,000 cpm in Exp. II, and 1,096 cpm in Exp. III.
The reaction mixture in 0.25 ml of the standard buffer (0.1 M $NH_4Cl$, 0.05 M Tris-acetate, pH 7.2, 0.01 M Mg acetate and 0.01 M 2-mercaptoethanol) contained:
in Exp. I, 200 µg ribosomes, 25 µg f2-RNA, 44 µg f-$^3$H-Met-tRNA (15,000 cpm), 150 µg initiation factor, 0.025 µmoles GTP and 0.05 µmoles puromycin; in Exp. II, 300 µg initiation complex (1,1000 cpm) prepared with GTP and 0.1 µmoles puromycin; and in Exp. III, 300 µg initiation complex (1,240 cpm) prepared with GDPCP, 0.025 µmoles GTP and 0.05 µmoles puromycin. The reaction mixture was incubated at 37°C for 20 min. and the radioactivity, extracted with ethyl acetate at pH 5, was determined in a gas flow counter.
Preparation of initiation complex with GTP or with GDPCP;The reaction mixture in 1 ml of the standard buffer contained: 4 mg ribosomes, 200 µg f2-RNA, 50 µg f-$^3$H-Met-tRNA (16,500 cpm), 0.2 µmoles GDPCP or GTP, and 600 µg initiation factor. It was incubated at 37°C for 20 min. The mixture in 2.5 ml was overlayered on 2.5 ml of 10 % sucrose in the standard buffer, and centrifuged at 150,000 x g for 120 min. The pellet was rinsed and suspended in the stantard buffer.

The effects of bottromycin $A_2$ on the puromycin reaction was further studied with fMet-tRNA bound to the "entry" site and with that bound to the donor site (Fig. 1). The GTP-stimulated puromycin reaction on the initiation complex, prepared in the presence of GDPCP, was more significantly inhibited by bottromycin $A_2$: complete inhibition at $10^{-4}$ M, 72 % inhibition at $10^{-5}$ M, and 20 % inhibition at $10^{-6}$ M (Exp. III in Table 2). The grade of inhibition was significantly less with the initiation complex, produced in the presence of GTP: 39 % inhibition at $10^{-4}$ M and no inhibition at $10^{-5}$ M (Exp. II in Table 2). More fMet-tRNA may bind to the "entry" site and translocate to the donor site by addition of GTP in Exp. III; while more fMet-tRNA may bind to the donor site in Exp. II. The grade of

inhibition by bottromycin $A_2$ of the puromycin reaction was more significant in the former than in the latter. The results indicate that the antibiotic may primarily inhibit ribosome-catalysed translocation of fMet-tRNA from the "entry" site to the donor site on the ribosome. It may also interfere with the peptidyl transferase reaction. However, the grade of inhibition is less and it seems to be the secondary effect. Fusidic acid did not significantly affect the fMet-puromycin reaction nor the ribosome-catalysed translocation of fMet-tRNA (Table 2).

The effects of bottromycin $A_2$ and fusidic acid on peptide chain elongation was examined in a polylysyl-tRNA system, and the results are summarized in Table 3. Most of polylysyl-tRNA was reactive with puromycin, indicating binding to the donor site. The puromycin reaction did not result in the release of tRNA (line 2 in Table 3). Bottromycin $A_2$ and fusidic acid did not significantly affect the

Table 3. Effects of bottromycin $A_2$ and fusidic acid on the release of deacylated tRNA and polylysine from the ribosomes during translocation.

| Additions | Relative activity remaining on ribosomes | |
|---|---|---|
| | $^3$H-tRNA | $^{14}$C-polylysine |
| 1. None | 100 | 100 |
| 2. PM | 98 | 42 |
| 3. PM + BM | 100 | 40 |
| 4. PM + FA | 103 | 42 |
| 5. G + GTP | 97 | 97 |
| 6. PM + G + GTP | 50 | 24 |
| 7. PM + BM + G + GTP | 52 | 40 |
| 8. PM + FA + G + GTP | 101 | 41 |

PM: puromycin 2 x $10^{-4}$ M, BM: bottromycin $A_2$ 2 x $10^{-4}$M, FA: fusidic acid 4 x $10^{-4}$ M, G: G factor 10 µg/ml, GTP: guanosine triphosphate $10^{-4}$ M. The radioactivity 100 was 11,070 cpm for $^3$H-tRNA and 4,560 cpm for $^{14}$C-polylysine.
The control reaction mixture contained: 400 µg ribosomes charged with $^{14}$C-polylysyl-$^3$H-tRNA, 15 µg poly A, 50 µmoles Tris-HCl, pH 7.2, 100 µmoles $NH_4Cl$, 12 µmoles Mg acetate, and 10 µmoles 2-mercaptoethanol, in a total volume of 1 ml. It was incubated at 35°C for 10 min. without GTP and further incubated for 10 min. with GTP. After addition of 200 µg *E. coli* tRNA as a carrier, the Millipore assay was used to determine the level of $^3$H-tRNA and $^{14}$C-polylysine that remained bound.

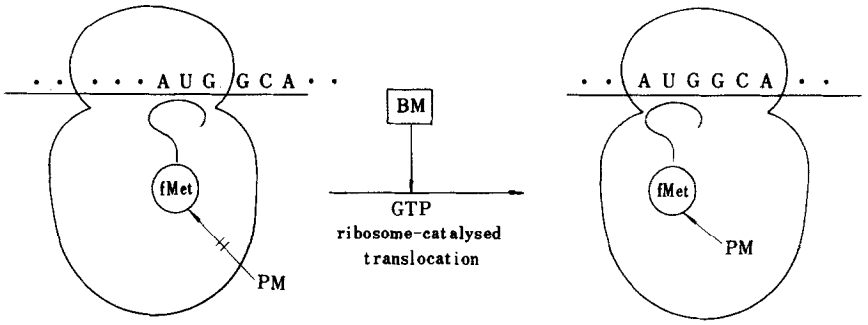


Fig. 1. Diagrammatic representation of translocation of fMet-tRNA and action of bottromycin $A_2$. BM: bottromycin $A_2$, PM: puromycin.

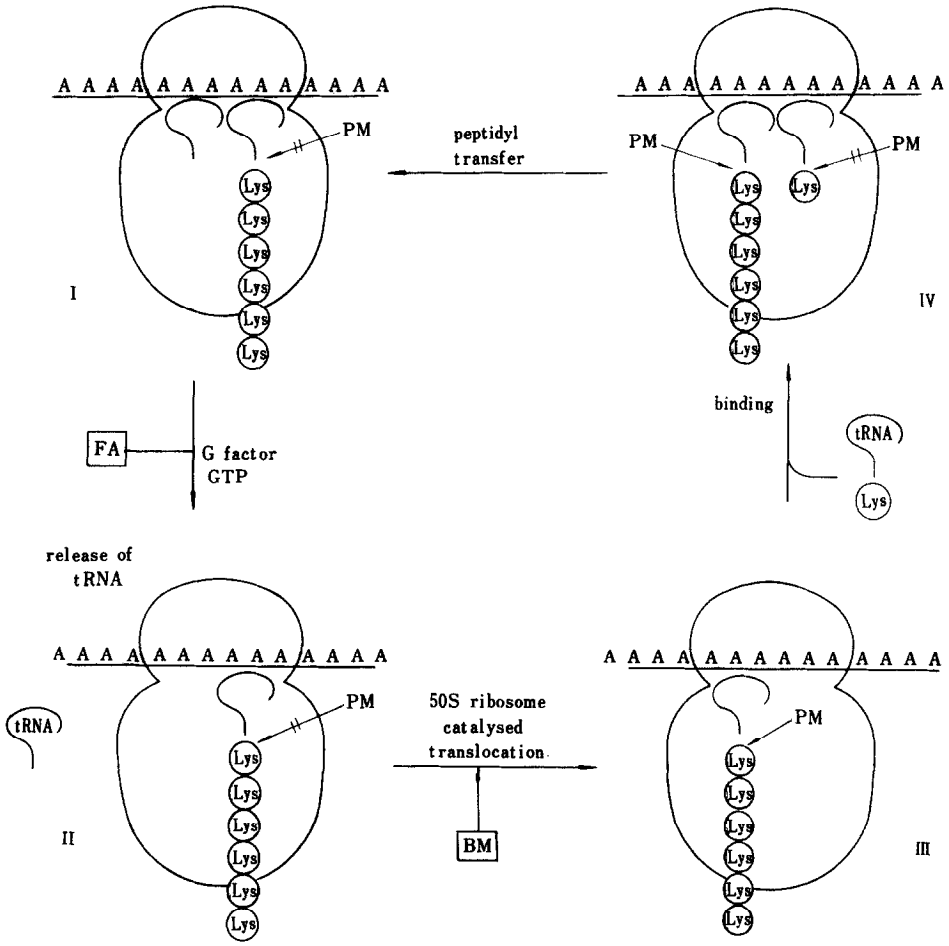


Fig. 2. Diagrammatic representation of translocation of poly-lysyl-tRNA and sites of action of antibiotics. FA: fusidic acid, BM: bottromycin $A_2$, PM: puromycin.

puromycin reaction (peptidyl transfer) and the attachment of tRNA to the ribosomes (lines 3 and 4 in Table 3). Addition of G factor and GTP to this system increased the amount of puromycin-reactive polylysyl-tRNA, indicating that the translocation of polylysyl-tRNA occurred (1,5), and the deacylation by puromycin resulted in the release of tRNA (lines 5 and 6 in Table 3). Bottromycin $A_2$ and fusidic acid were observed to inhibit G factor- and GTP-stimulated puromycin reaction, indicating that they interfere with translocation of peptidyl-tRNA and mRNA. Fusidic acid inhibited the release of tRNA, but bottromycin $A_2$ did not significantly affect it (lines 7 and 8 in Table 3).

The reaction of puromycin with Ac-$^{14}$C-Leu-oligonucleotide (RNase $T_1$-digested) on the 50S ribosomal subunit in the presence of methanol was inhibited by bottromycin $A_2$. However, the grade of inhibition was less than that of translocation. The results indicate that bottromycin $A_2$ primarily inhibits translocation and the inhibition of peptidyl transferase may be the secondary effect.

Table 4. Effects of antibiotics on the fragment reaction.

| Additions | | Ac-$^{14}$C-Leu-puromycin formed |
|---|---|---|
| None | | 100 |
| Bottromycin $A_2$ | $2 \times 10^{-4}$ M | 82 |
| | $2 \times 10^{-5}$ | 92 |
| Blasticidin S | $2 \times 10^{-5}$ M | 17 |
| | $2 \times 10^{-6}$ | 43 |
| Mikamycin A | $3 \times 10^{-6}$ M | 3 |

100 = 1,432 cpm.
The reaction mixture, in 0.1 ml, contained: E. coli 50S ribosomes 130 µg, Ac-$^{14}$C-Leu-oligonucleotide (digested with RNase $T_1$) 8 µM (2,400 cpm), puromycin 1 mM, Tris-HCl, pH 7.4, 50 mM, KCl 400 mM, $Mg(AcO)_2$ 20 mM and DTT 2 mM. It was kept at 0°C for 30 min. and the radioactivity extracted with ethylacetate was determined.

The sites of action of bottromycin $A_2$ and fusidic acid, which are concluded from the results, are illustrated in Fig. 1 and 2. The

present experiment shows that the release of donor tRNA and translocation can be uncoupled by using antibiotics: bottromycin $A_2$ and fusidic acid. Since fusidic acid interacts with G factor (2,9) and bottromycin $A_2$ with the 50S ribosomal subunit (11), it is concluded from the resuts that the release of deacylated tRNA is the function of G factor and translocation of peptidyl-tRNA and mRNA to the vacated donor site is catalysed by the 50S ribosomal subunit. The assumption is in accordance with that of Roufa et al. (14). However, it opposes the proposal by Kuriki and Kaji (12) and that by Lucas-Lenard and Haenni (13). They indicated that the G factor-catalysed release of deacylated tRNA requires the presence of an adjacent tRNA or peptidyl-tRNA and is coupled with translocation.

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