THE LEVEL OF Fmet-tRNA ON RIBOSOMES FROM STREPTOMYCIN TREATED CELLS

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SUMMARY

Ribosomes of a sensitive strain of Escherichia coli K12 treated with streptomycin contain about twice as much formylmet-tRNA as before. Considering this and previous findings on the effect of streptomycin in whole cells we suggest that streptomycin exerts its main effect on translocation but only when protein chains are being initiated.

Treatment of sensitive Escherichia coli strainswith streptomycin causes the accumulation of up to 60% of the ribosomes in monosomes (1-3). These streptomycin (str) monosomes are attached to relatively long mRNA molecules, but are inactive in protein synthesis (2). These observations suggested that str-monosomes are blocked near or at the initiation of new protein chains (1-3). If the str-monosomes are blocked at initiation then each of them should contain an Fmet-tRNA molecule.

In order to distinguish between these two possibilities we decided to assess the abundance of Fmet-tRNA on ribosomes before and after treatment with streptomycin and to determine the relative amount of Fmet-tRNA to ala-tRNA and leu-tRNA, as alanine appears frequently in the beginning of E. coli proteins, and leucine is a frequent internal amino acid in many proteins (4-6)

EXPERIMENTAL PROCEDURES AND RESULTS

tRNA was isolated from ribosomes in two ways. Firstly, total RNA was extracted from ribosomes by phenol. The second method involved a low Mg^{2+} concentration (10^{-4}M) wash to release the attached tRNA from the ribosomes. The freed tRNA was then recovered from the wash by phenol extraction. The

amount of a specific tRNA in a given RNA fraction was determined by the amount of radioactive aminoacyl-tRNA that could be obtained after chemical discharge and enzymatic acylation. According to Cannon, Krug and Gilbert (7), the second procedure released about one mole of tRNA per mole of ribosome. The tRNA recovered in our experiments varied from 0.5 - 1.0* molecule per ribosome (as estimated by absorption at 260 mμ). In general, the amino acid acceptor ability of the recovered material equalled or exceeded that of the commercially obtained tRNA from E. coli K12. This was found to be the case for all the three amino acids tested, L-alanine, L-leucine and L-methionine.

The release of the tRNA bound to ribosomes is facilitated by the dissociation of the subunits at the low Mg^{2+} concentration. In order to insure that tRNA bound to the streptomycin-monosomes was not discriminated against by the low Mg^{2+} wash, we compared the stability of the str-monosomes to that of untreated ribosomes. The extracts of strain AB301, streptomycin treated and untreated, were dialyzed against a tris buffer (0.01 M) containing various concentrations of Mg^{2+} and centrifuged in sucrose gradients containing the relevant Mg^{2+} concentrations. Ten different concentrations of Mg^{2+} were tested, ranging from 10^{-4} to 5×10^{-3}M. Ribosomes from the streptomycin treated cells dissociated to the same extent as those from the untreated cells The same results were observed whether or not streptomycin was present in the dialysis buffer and in the sucrose gradients.

Since both methods used for isolating tRNA from ribosomes led to similar results, we shall report here data obtained with tRNA released from ribosomes at a low Mg^{2+} concentration. tRNA isolated from ribosomes from treated and untreated cells from strain AB301 (Str sensitive) and N21 (Str resistant) treated cells were deacylated chemically and charged enzymatically (for details, see Table 1). The capacity of the tRNA to be charged with methionine, alanine and leucine was measured. While the efficiency of the

*A certain amount of the RNA recovered should be mRNA and therefore this is an upper limit value.

Table 1: Relative Charging Capacity of tRNA Isolated from Ribosome before and after Treatment with Streptomycin.

tRNA Isolated from Strain	Presence of Streptomycin	Charged tRNA percent* (mean and standard deviation) Met	Ala	Leu
AB301	No	21.6 ± 1.7	33.3 ± 2.3	45.1 ± 4.0
AB301	Yes	27.7 ± 1.3	32.2 ± 1.0	40.1 ± 1.1
K12†	No	24.7	26.0	49.3

* 100% being the sum of methionyl, alanyl and leucyl tRNA
† Commercial stripped tRNA of E. coli K12

Streptomycin (75 μg/ml) was added to exponentially growing cultures in nutrient broth of AB301 sensitive and N21 (a resistant derivative) strains. One hour later, the cells were rapidly chilled and harvested. Cells were ground with alumina and their ribosomes were extracted by standard procedures (15). After the ribosomes were washed in a high Mg^{2+} buffer (0.01M) they were spundown and resuspended in tris buffer (0.01M) and Mg^{2+} 1mM, and dialyzed against the same buffer. The dialyzed ribosomes were layered on 30% sucrose and centrifuged for 5 hours at 150,000 g to sediment the ribosomal subunits. The top 1/3 of the tube provided the source of tRNA. It was recovered and phenol extracted as described by Cannon, Krug and Gilbert (7). The tRNAs thus isolated were stripped chemically according to Nathan and Lipmann (16), and tested for their capacity to accept radioactive amino acids. The reaction mixture, modified from Berg et al, (17), for charging tRNA with ^{14}C-amino acids contained in μmoles per ml: Tris-HCl pH 7.2, 100; $MgCl_2$ 10; KCl 10; ATP, (neutralized with KOH) 2.5; GSH, 4; a mixture of 19 cold amino acids, 0.4 of each; ^{14}C-amino acid to be tested 0.1, (specific activities per mmole L-methionine 46 μC, L-alanine 151, L-leucine 25); and 200 μl of E. coli supernatant of an 150,000 g centrifugation of a crude extract; and 0.1-0.2 mg of the tRNA to be tested. 50 μl aliquots of the reaction mixture were incubated at 30°C for 20 minutes and the acid precipitable material was filtered through glass fiber filters. The filters were dried and counted in a low background gas glow counter with counting efficiency of 30%. The data are from three separate experiments using three different preparations of tRNA from strain AB301. The results obtained for the charging of 40 μμmoles aliquots of RNA (as determined by A260) isolated from ribosomes of untreated cells were as follows in μμmoles; met-tRNA 1.04, ala tRNA 1.62 and leu-tRNA 2.72; for treated cells 1.2, 1.46 and 1.92 respectively. Backgrounds without RNA, of up to 20%, were subtracted.

charging reactions varied from experiment to experiment, the relative acceptor activity for each of the amino acids tested remained unchanged. The results from three experiments for the streptomycin sensitive strain using three different tRNA preparations are presented in Table 1. It is evident that

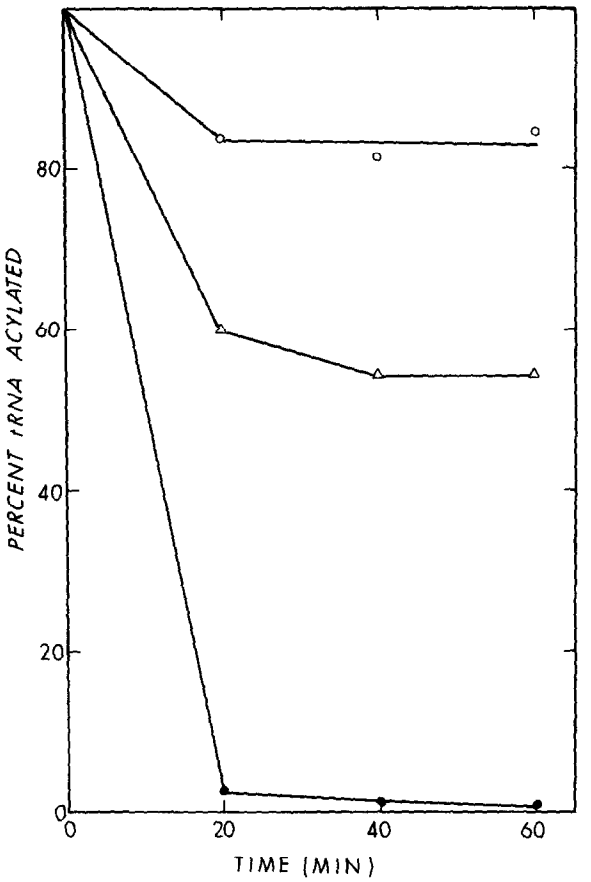

Figure 1. Discharging of amino acyl tRNA by the pyrophosphate exchange reaction.

tRNA isolated from ribosomes by dissociation in low Mg^{2+} (see legend to Table 1) was deacylated chemically (16) and charged with ^{14}C-met in the presence of a formyl donor. The reaction mixture was identical to the one described in Table 1, with the addition per ml of 30 µg of calcium salt of formyl tetrahydropteroylglutamic acid (calcium Leucovorin, Lederle Laboratories Division). For control the tRNA was charged with ^{14}C-ala; identical results were obtained whether ^{14}C-ala-tRNA were formed in the presence or absence of Leucovorin. 0.250 ml of the charging mixtures was incubated for 20 minutes at 30°C. 0.025 ml aliquots in duplicates were rapidly withdrawn and precipitated with 3 ml of 5% cold TCA. 0.8 ml of discharging mixture modified from Marcker (9) was added to the remaining charging reaction mixture. The discharging mixture contained in µmoles per ml: $MgCl_2$, 30; cacodylate buffer (pH 7.2) 50; AMP (sodium salt) 5; sodium pyrophosphate, 5; and 300 µl of E. coli S-100. At the indicated time interval, 0.125 ml samples in duplicates were removed from the incubating reaction mixture precipitated with cold 5% TCA filtered and counted (see Legends to Table 1). The samples withdrawn prior to the addition of the discharging mixture were taken as 100%. ●—●, ^{14}C-ala-tRNA using commercial tRNA from E. coli K12; O—O, ^{14}C-met-tRNA from streptomycin treated cultures of AB301; Δ—Δ, ^{14}C-met-tRNA from cultures of AB301 not treated with streptomycin.

that amount of met-tRNA, as compred to leucyl- and alanyl-tRNA, increased slightly on ribosomes from streptomycin treated cells. The relative amount of met-tRNA on ribosomes from the streptomycin resistant strain was somewhat lower than that on ribosomes from the streptomycin sensitive strain.

In order to assess what portion of the met-tRNA was Fmet-tRNA, the charging reactions were carried out in the presence of a formyl donor and were followed by an enzymatic deacylation. The latter reaction is only effective on the non-formylated met-tRNA, whereas the formylated met-tRNA is left intact (8,9). The results presented in Fig. 1 showed that while 85% of the met-tRNA isolated from ribosomes of the streptomycin treated cells could be formaylated, only 55% of the met-tRNA isolated from untreated cells could accept a formyl group. A similar percentage (60) of met-tRNA isolated from the ribosomes of the streptomycin resistant strain could be formylated. From the combined data obtained from the charging and deacylating experiments (Table 1 and Fig. 1), it was possible to determine the amount of Fmet-tRNA on ribosomes, and to evaluate the ratio of Fmet-tRNA to the other tRNAs in streptomycin treated and untreated cells. The ratio comparison, shown in Table 2, suggested that after treating streptomycin sensitive cells with

Table 2: Ratio of Fmet and ala-tRNA to leu-tRNA before and after Treatment with Streptomycin

Strain	Presence of Streptomycin	Fmet - tRNA* / leu - tRNA		ala - tRNA / leu - tRNA	
AB301	No	0.26	1.0	0.74	1.0
AB301	Yes	0.58	2.0	0.81	1.1

* Experiments were conducted as described in Legend of Fig. 1 and Table 1. The relative amount of Fmet-tRNA is calculated from data obtained from Table 1 and Fig. 1, i.e., 85% of the met-tRNA from treated culture of AB301 and 55% of the met-tRNA from untreated AB301 could be formylated. The number presented in the left columns are molar ratios.

streptomycin the ribosomes contained twice as much Fmet-tRNA as compared to leu-tRNA.

DISCUSSION

Studies by Luzzatto, Apirion and Schlessinger (1,2) showed that the distribution of ribosomal subunits, monosomes and polyribosomes in streptomycin treated and untreated cells was as follows: untreated cells, 65.5% polyribosomes, 9.1% monosomes, 24.1% subunits; treated cells, 29.6% polyribosomes, 53.3% monosomes, 15.9% subunits. These observations are probably applicable to our studies since the streptomycin treatment in both cases was similar.

If str-monosomes represent initiation complexes arrested by the drug, (the initiation complex defined here as the complex formed before the first peptide bond is catalyzed) then every str-monosome is expected to carry an Fmet-tRNA molecule. This being the case there should be an increase of ribosomal associated Fmet-tRNA by a factor of six in streptomycin treated cells. In the above calculations we assumed that no tRNA is bound to the subunits and that the polyribosomes in both kinds of cells contain about the same amount of Fmet-tRNA. We consider the number of polyribosomes in treated and untreated cells to be similar, due to the fact that polyribosomes from str treated cells are smaller than those from untreated cells. This expected six fold relative increase in Fmet-tRNA in streptomycin treated cells is probably an underestimate. Firstly, if the number of Fmet-tRNA molecules is increasing then the number of leu-tRNA molecules should decrease. Secondly, while in streptomycin treated cells every str-ribosome whether it is in a monosome or in the beginning of a polyribosome should contain an Fmet-tRNA, not every monosome and polyribosome in an untreated cell should contain an Fmet-tRNA.*

The value obtained for Fmet-tRNA could be an underestimate if str-monosomes

*If we assume the following: that the number of ribosomes in treated and untreated cells are those given in Table 5, ref. 2; that Fmet-tRNA is present in the streptomycin treated cells on every monosome and on every second polyribosome, and in the treated cells on every second monosome or polyribosome; that leu-tRNA is present in both cases only on polyribosomes in the amount of 0.075 per ribosome (see Table 1, untreated cells), then the ratio of Fmet-tRNA to leu-tRNA after streptomycin treatment should be twenty times the ratio before treatment.

were stable in low Mg^{2+}. However, as was discussed, the dissociation of the ribosomes at lower Mg^{2+} concentrations was identical whether the ribosomes were from streptomycin treated or untreated cells. Therefore, while we accept the two-fold difference observed in the ratio of Fmet to leu-tRNA in treated versus untreated cells as significant, the fact that it is not larger suggests to us that not every str monosome contains an Fmet-tRNA molecule.

We conclude that initiation per se is not inhibited by streptomycin *in vivo* and suggest that translocation of the ribosome during formation of the initial peptide bond is.

Movement of ribosomes along the mRNA is possible in the presence of antibiotics like, Chloramphenicol, spectinomycin and tetracycline (10). That is, polyribosomes continue to form in the drug-treated cells in the absence of protein synthesis. Unlike the situation with these three antibiotics, where the flow of ribosome between polyribosomes and ribosome subunits is only slightly affected, streptomycin causes an extensive accumulation of monosomes plus a small build up of disomes and trisomes, at the expense of larger polyribosomes (1-3). This and the fact that mRNA does join str ribosomes (1,2) suggests that perhaps in the presence of streptomycin there is a limited movement of ribosomes along the messenger. In the process of this limited ribosome movement, the Fmet-tRNA is probably replaced by tRNA corresponding to subsequent codons, although not to the same extent as in the absence of streptomycin.

This view of streptomycin action *in vivo* is in agreement with the concept of White and White (11) that the site of action of streptomycin has to be uncovered first. The uncovering is probably the completion and release of the polypeptide chain. Therefore we suggest that while elongation of a polypeptide chain *is not inhibited* by streptomycin when a peptidyl-tRNA is present on the ribosome *it is* when peptidyl-tRNA is absent. It is quite likely that this is only a problem of accessibility of streptomycin

to its site of action, since the studies of Modelell and Davis (12,13) and Luzzatto, Apirion and Schlessinger (14) showed that *in vitro* this site was accessible to streptomycin even when a peptidyl-tRNA was present on the ribosome.

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Erratum

Vol. 41, No. 1 (1970), in the Communication, "A DNA-Dependent ATPase from E. coli. Infected with Bacteriophage T4," by Nancy Debreceni, Margaret T. Behme, and K. Ebisuzaki, pp. 115-121, the following sentence was omitted from the Acknowledgments:

"This work was supported by the National Cancer Institute of Canada and Medical Research Council (Canada)."