STREPTOMYCIN INDUCED RELEASE OF fMET-tRNA FROM THE RIBOSOMAL INITIATION COMPLEX

J.C. Lelong, M.A. Cousin, D. Gros, M. Grunberg-Manago and F. Gros

Institut de Biologie Moléculaire
Faculté des Sciences - Paris 5e, France

Received December 23, 1970

SUMMARY

The streptomycin effect on the initial steps of mRNA translation in vitro was studied. The drug does not inhibit the primary assembling step during which initiator tRNA is positioned on a 30S-ribosome-mRNA complex but causes release of bound fMet-tRNA from the preformed initiation complex. The observed requirement for both subunits and GTP hydrolysis indicated that fMet-tRNA could not be released until bound to the puromycin reactive site on sensitive ribosomes. Other aminoglycoside antibiotics which cause misreading do not induce fMet-tRNA release showing that streptomycin specifically induces distortion of the P site.

The effect of streptomycin on translation mechanisms has been largely documented over the last five years (1-3). The identification of one of the ribosomal proteins P_{10}, as the antibiotic target site (4) has provided a new basis to study its mode of action, but the nature of modifications induced on sensitive ribosomes appears complex and is still debated.

Luzzato et al (5) have shown that in streptomycin treated cells, polysomes disappear while 70S messenger RNA complexes accumulate. This has led to the proposal that streptomycin selectively inhibits message reading at the initiation step. This interpretation was put in question by Modolell and Davis (6); their results indicate that streptomycin in bacteriostatic amounts causes in vitro almost instantaneous arrest in polypeptide chain extension. Furthermore, they have described an antibiotic induced breakdown of the messenger ribosome peptidyl tRNA complex, and have proposed that streptomycin creates some distortion in the A site which labilizes the ribosome peptidyl-tRNA association during peptidyl-transfer to that site.

We have reinvestigated the effect of streptomycin on initiation. According to our results streptomycin does not inhibit the primary assembling step during which fMet-tRNA is positioned on a 30S ribosome-messenger RNA complex (7-9), but causes a release of fMet-tRNA from a preformed initiation complex. No such release occurs when GMPPCP is used in the place of GTP clearly indicat-

ing that initiator tRNA is released from a peptidyl site. Streptomycin induced fMet-tRNA release is partly inhibited by tetracyclin suggesting that distortion of the P site by streptomycin could cause artificial transfer of this tRNA to the A site before effective release.

Inhibition of fMet-tRNA binding to sensitive ribosomes

Table I shows the effects of streptomycin on fMet-tRNA binding to sensitive MRE 600 70S ribosomes in the presence of an AUG triplet, poly AUG or T_4 mRNA. It is clear that regardless of the messenger added to the system, a severe inhibition is observable. That streptomycin inhibition can be attributed to a drug ribosome interaction rather than some artefactual effect is supported by the observed lack of inhibition when ribosomes from a Sm-resistant strain (MRE 600 Sm^r) are used.

Since streptomycin selectively binds to the 30S subunit (10) we have

Table

Effects of streptomycin and aminoglycoside antibiotics on fMet-tRNA binding to ribosomes

Expt	Messenger	Ribosomes	^{3}H fMet-tRNA bound (pmoles/A 260 unit)				
			control	Sm	Pm	Neo B.	Genta
1	ApUpG	70S (Sm^s)	2.0	0.2 (86)*	-	-	-
2	poly AUG	70S (Sm^s)	2.1	0.7 (65)	1.5 (30)*	2.2 (0)*	2.1 (0)*
3	T_4 RNA	70S (Sm^s)	2.0	0.6 (70)	2.0 (0)	1.8 (10)	2.2 (0)
		70S Q13 (Sm^s)	1.7	0.5 (67)	-	-	-
		70S (Sm^r)	1.3	1.0 (22)	-	-	-
4	poly AUG	30S (Sm^s)	1.0	1.1 (0)	-	-	-
5	ApUpG	30S (Sm^s)	0.7	0.8 (0)	-	-	-

Reaction mixtures : (0.05 ml) Tris HCl, pH 7.5, 50 mM, NH_4 Cl 80 mM, Mg acetate 5 mM (expt 1-2) 8 mM (expt 3) 10 mM (expt 4-5) mercaptoethanol 7 mM, GTP 1 mM, purified ribosomes 1 A 260 unit, crude initiation factors (13) in saturating amount. ^{3}H fMet-tRNA 8 pmoles - 3200 cpm - ApUpG, 0.15, A 260 units or poly AUG 0.25 , A 260 units or T_4RNA prepared according to Salser et al (14) 70 µg. Streptomycin 4 µg/ml; other antibiotics 8 µg/ml. Incubation 15 min at 25°C (ApUpG or poly AUG) or 37°C (T_4 RNA). ^{3}H fMet-tRNA bound to ribosomes was measured by the filtration technique of Nirenberg et al (15). Backgrounds without messenger were deduced (AUG or poly AUG : 15 % - T_4 RNA : 38 %).

* % of inhibition - Abbreviations : Sm - streptomycin ; Pm - paromomycin ; Neo B. - neomycin B ; genta - gentamycin.

repeated the previous experiments using 30S rather than 70S ribosomes. Surprisingly no inhibition was observed. This suggests that formation of a ribosomal dimer on the messenger is a prerequisite to the streptomycin effect on fMet-tRNA binding.

Streptomycin induced release of fMet-tRNA from initiator complex

Addition of streptomycin after fMet-tRNA has become attached to the 70S ribosome-messenger complex causes a gradual reduction in the amount of bound initiator tRNA. The decay is relatively slow with a half-life comprised between 90 and 120 seconds (Figure 1) and its extent usually varies between 50 to 70% depending upon the messenger. It can thus be concluded that the antibiotic induces fMet-tRNA release from the initiation complex which is responsible for

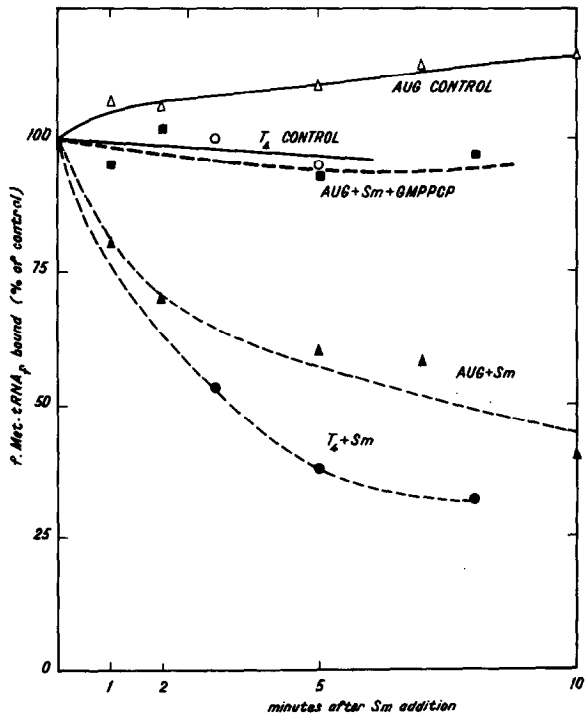

Fig. 1 - Streptomycin induced release of fMet-^{3}H tRNA bound to the ribosomes. Sm (8 µg/ml) was added to the preformed complex after 15 minutes as in Table I. GMPPCP (from Miles) 1 mM - 100% values (cpm) were : 600 (T_4 RNA) 1850 (AUG in the presence of GTP) 910 (AUG in the presence of GMPPCP). Backgrounds without messenger were deduced.

the afore-mentioned effect on fMet-tRNA binding. Accordingly, the level of bound initiator tRNA reached in the presence of streptomycin is independent of the time at which the antibiotic is added, in the course of complex formation (Figure 2).

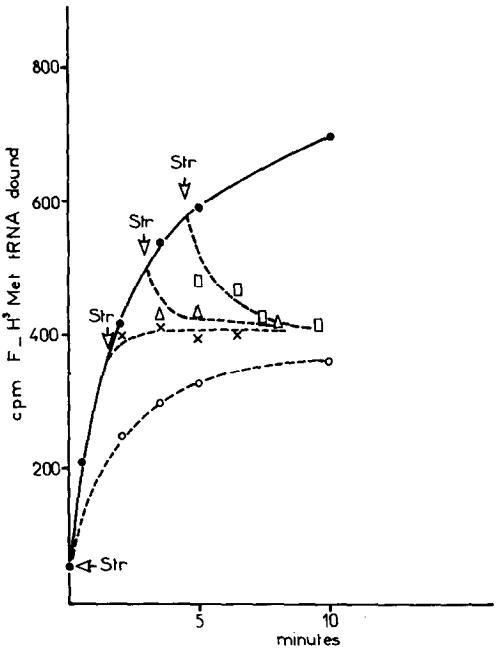

Fig. 2 - Effect of streptomycin added in the course of complex formation. Sm (4 μg/ml) was added in identical samples at different intervals indicated by arrows and fMet-^{3}H tRNA bound was measured as in Table I. •——• control without Sm.

The streptomycin-induced loss of bound fMet-tRNA could be interpreted to mean that streptomycin causes dissociation of the total initiation complex or that it releases fMet-tRNA only, leaving the 70S ribosome-messenger complex intact. To explore this question experiments were repeated using either radioactive T_4 mRNA or a radioactive AUG triplet. Radioactive T_4 mRNA binds to 70S *E. coli* ribosomes only if fMet-tRNA plus initiation factors are present (Figure 3A). Yet if streptomycin is added to this complex once formed and the system is further analyzed on sucrose gradient, the amount of bound T_4 mRNA appears unchanged although 60% of fMet-tRNA has been lost. When AUG is used as a messenger the situation is somewhat different since a definite streptomycin induced release of the radioactive triplet can be shown to occur (Figure 3B). Nevertheless this release is clearly slower than tRNA release. Thus breakdown of the initiation complex whenever it takes place is probably the consequence of tRNA release from that complex.

Ribosomal site involved in streptomycin effect

If streptomycin is added at intervals following the onset of fMet-tRNA

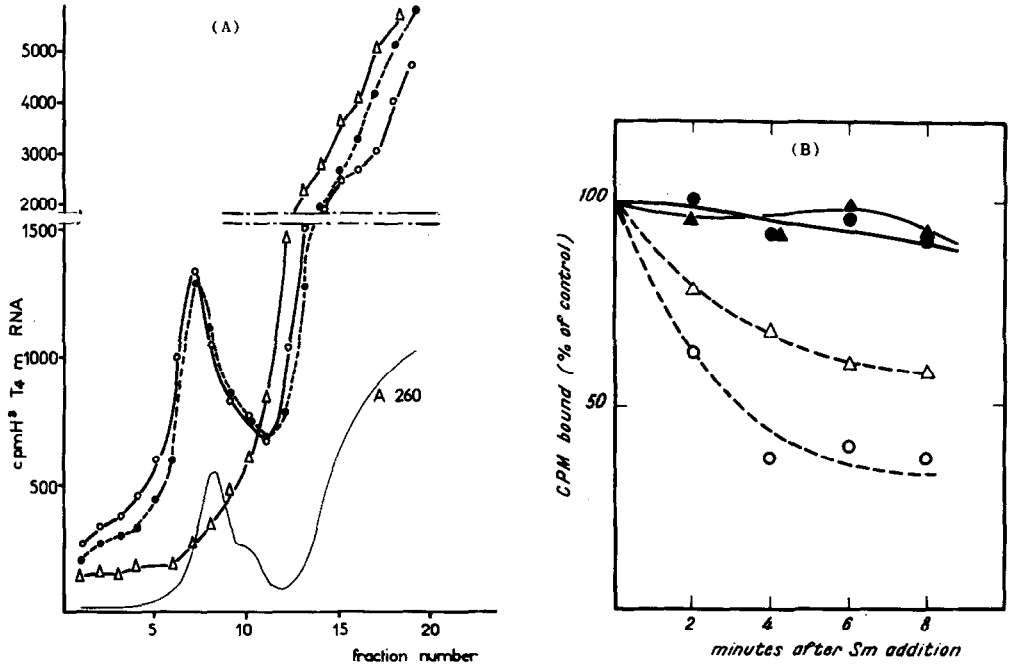

Fig. 3 - Stability of the messenger ribosome association after streptomycin action

A - Sucrose gradient analysis of the T_4 RNA messenger-70S ribosome complex 5 min after Sm addition. Conditions were the same as in Figure 1, except that ^3H T_4 RNA (in vivo late messenger) 0.5 A 260 units (50 000 cpm) and cold fMet-tRNA 0.7 A 260 units were used. o——o complete system without Sm; •——• Sm added after 5 min; Δ——Δ omit fMet-tRNA. Incubation time 10 min at 37°C. Samples were layered on 5-20% sucrose gradients in binding buffer, and centrifuged 60 min at 60 000 rpm in a SW 65 K Spinco rotor. After recording the UV absorption fractions were directly collected in scintillation vials and counted in Bray's fluid.

B - Comparative kinetics of fMet-tRNA and AUG release during streptomycin action. Sames conditions as in Figure 1 except for : GTP : 1 mM - ribosomes : 0.9 A 260 units - ^3H fMet-tRNA (400 cpm/plole) 20 pmoles. ApUpG (^{14}C) 70 cpm/pmole, 250 pmoles. Streptomycin 8 µg/ml or H_2O was added after 12 min at 25°C. Control 100% values were 2.3 pmoles ^3H fMet-tRNA and 2.1 pmoles AUG. Background without AUG (1.1 pmole fMet-tRNA) was deduced. No AUG was bound in the absence of fMet-tRNA. ^3H fMet-tRNA •——• control o——o plus Sm [^{14}C]AUG ▲——▲control Δ——Δ plus Sm.

---

binding on ribosome, it is observed that during the first minute - when binding is only 20% of the saturation level - the antibiotic causes no release. As fMet-tRNA binding proceeds, the streptomycin dissociating effect appears (Figure 4). One interpretation of these results could be that fMet-tRNA cannot be released until it is translocated into a particular, presumably peptidyl-site. This interpretation is well supported by the lack of streptomycin effect when the initiation complex is formed, on 70S ribosomes, in the presence of a

non-splittable GTP analog, GMPPCP, as is shown on Figure 1. It is known that, under such conditions, initiator tRNA cannot reach a puromycin reactive site.

The effects of simultaneous addition of puromycin and streptomycin to the preformed initiation complex (in the presence of GTP) were also investigated. With ApUpG and poly AUG templates, fMet puromycin formation is only slightly inhibited (37% and 13% respectively). In contrast, when T_4 RNA is used, inhibition can reach as much as 76%. The relative insensitivity of the fMet transfer reaction to puromycin with synthetic messengers is probably related with the fact that this reaction is much faster than the streptomycin induced release of initiator tRNA.

Comparison with other aminoglycosidic antibiotics

We have tested other antibiotics of the aminoglycoside series - which like streptomycin cause some misreading - for their effect on the initiation complex. At doses which completely prevent (T_4 RNA directed) protein synthesis, paromomycin, neomycin B and gentamycin hardly inhibit binding of radioactive fMet-tRNA in the presence of T_4 mRNA. A small inhibition is observed with paromomycin when poly AUG is used as a messenger (Table). Thus, in spite of great similarities in their mechanisms of action, these various antibiotics do not seem to alter the fMet-tRNA decoding site by the same way as does streptomycin.

DISCUSSION

The present results clearly indicate that streptomycin does not impair the first step in initiation of polypeptide synthesis since it does not prevent binding of fMet-tRNA to the 30S-messenger RNA complex in the presence of GTP plus initiation factors. Reformation of a 70S dimer on the previous complex also seems to occur normally since streptomycin does not inhibit fMet-tRNA binding to 70S in the presence of a non-splittable GTP analog, GMPPCP. In contrast, when initiator tRNA is engaged into the peptidyl site - a reaction probably concomitant with GTP hydrolysis - streptomycin causes a very marked tRNA release.

It is interesting to notice that, in spite of this release, a natural messenger such as T_4 mRNA remains bound to 70S ribosomes. This might possibly explain the accumulation of inactive monosomes by streptomycin treated cells, as reported by Luzzato et al (5).

These findings have also several implications both for the mechanism of streptomycin action and for the study of fMet-tRNA decoding sites.

First, the present data can be best explained if one assumes a drug induced distortion of the peptidyl site; this would also account for the slow release of peptidyl-tRNA which occurs when polysomes are mixed with streptomycin

in vitro (6). A possibility would be that after fMet-tRNA has become attached to the P site, streptomycin alters ribosomal conformation in such a way as to cause initiator tRNA to pass artificially into the A site from which it would be excluded. This is supported by the fact that tetracyclin which inhibits occupancy of the A site by (internal) aminoacyl-tRNA (11) does not cause any change in the amount of fMet-tRNA bound to an AUG ribosome complex (12), while counteracting streptomycin effect on this binding (inhibition in the presence of streptomycin alone was 40%, and 17% only in the presence of both streptomycin and tetracyclin).

As shown on Figure 4, there is a certain latency during fMet-tRNA binding before streptomycin can cause release of this tRNA from the ribosomal complex. Moreover the final fMet-tRNA level reached after the streptomycin induced chasing is the same regardless of the time at which the drug is added.

In view of the afore-mentioned streptomycin sensitivity of the peptidyl site, it can thus be proposed that, in the course of a normal binding reaction, initiator tRNA is not directly (or immediately) engaged into the P site but enters a different site, or region, on the ribosome which is not alterable by streptomycin. That this "entry site" is a typical "A" site appears excluded however, in view of its insensitivity to tetracyclin (12).

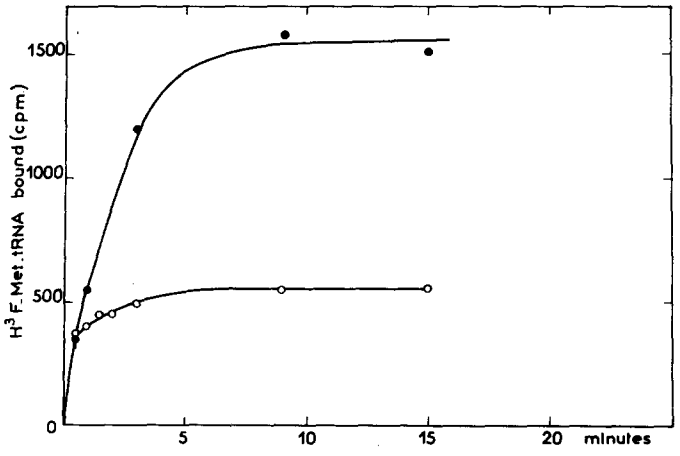

Fig. 4 - Kinetics of the Sm induced release of fMet-^{3}H tRNA from the ribosomes Reaction mixtures as in Table I : poly AUG as messenger. ●——● fMet-tRNA bound in the absence of Sm at the time indicated. o——o fMet-tRNA bound in the presence of Sm added at the time indicated on abscissa, but measured 10 min after Sm addition.

ACKNOWLEDGEMENTS

This work was supported by grants from the Centre National de la Recherche Scientifique, the Fonds de Développement de la Recherche Scientifique et

Technique, the Commissariat à l'Energie Atomique, the Ligue Nationale Française contre le Cancer, and the Fondation pour la Recherche Médicale Française.

REFERENCES

1. Davies, J., Gilbert, W., and Gorini, L., Proc. Natl. Acad. Sci. U.S., 51, 883 (1964).
2. Gorini, L., and Kataja, E., Proc. Natl. Acad. Sci. U.S., 51, 487 (1964).
3. Weisblum, B., and Davies, J. Bacteriol. Rev., 32, 493 (1968).
4. Ozaki, M., Mizushima, S., and Nomura, M., Nature, 222, 333 (1969).
5. Luzzato, L., Apirion, D., and Schlessinger, D., Proc. Natl. Acad. Sci. U.S., 60, 873 (1968).
6. Modolell, J., and Davis, D.B., Nature, 224, 345 (1969).
7. Nomura, M., and C.V. Lowry, Proc. Natl. Acad. Sci., U.S., 58, 946 (1967).
8. Hille, M.B., Miller, M.J., Iwasaki, K., and Wahba, A.J., Proc. Natl. Acad. Sci. U.S., 58, 1652 (1967).
9. Ghosh, H.P., and Khorana, H.G., Proc. Natl. Acad. Sci. U.S., 58, 2455(1967).
10. Kaji, H., and Tanaka, Y., J. Mol. Biol., 32, 221 (1968).
11. Sarkar, S., and Thach, R.E., Proc. Natl. Acad. Sci. U.S., 60, 1479 (1968).
12. Zagorska, L., Lelong, J.C., Gros, F., and Grunberg-Manago, M., in preparation.
13. Revel, M., Brawerman, G., Lelong, J.C., and Gros, F., Nature, 219, 1016 (1968).
14. Salser, W., Gesteland, W., and Bolle, M.A., Nature, 215, 588 (1967).
15. Nirenberg, M., and Leder, P., Science, 145, 1399 (1964).