Effect of streptomycin on the stoichiometry of GTP hydrolysis in a poly(U)-dependent cell-free translation system

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The technique of Sepharose-bound template translation has been used to estimate the stoichiometry of GTP hydrolysis during peptide elongation in the presence of streptomycin. The presence of streptomycin has been shown to have no great effect on the elongation rate and the stoichiometry of GTP hydrolysis during codon-specific peptide elongation in the poly(U)-directed translation system: the molar ratio of hydrolysed GTP to incorporated phenylalanine was about 2. At the same time streptomycin exerted a significant effect during misreading when a ribosome-bound peptide in the poly(U)-programmed system was elongated by leucine or isoleucine residues: the miselongation was stimulated and hence the ratio of hydrolysed GTP per peptide bond was strongly reduced, as compared with the excessive GTP hydrolysis which is characteristic of the misreading system in the absence of streptomycin [(1984) FEBS Lett. 178, 283–287]. The conclusion has been made that streptomycin blocks the stage of correction ('proof-reading') following GTP hydrolysis during EF-Tu-dependent aminoacyl-tRNA binding.

Misreading *Proof-reading* *Sepharose-bound poly(U) translation technique*

1. INTRODUCTION

It has long been known that streptomycin stimulates misreading of the template during translation [1]. This implies that streptomycin results in a decrease in the selectivity of codon-dependent aminoacyl-tRNA binding to the ribosome. According to the theory first proposed by Hopfield [2], the selectivity is the product of 2 successive discrimination steps in aminoacyl-tRNA binding: the initial codon-anticodon interaction of the aminoacyl-tRNA·EF-Tu·GTP complex with the template on the ribosome, and the retention/rejection of the codon-bound aminoacyl-tRNA on the ribosome after GTP hydrolysis; the latter is called the correction, or proof-reading step. Using poly(U)-programmed ribosomes with pre-bound AcPhe-tRNA it has been demonstrated that EF-Tu·GTP-promoted binding and incorporation into dipeptide of non-cognate aminoacyl-tRNAs, such as Leu-tRNA or Ile-tRNA, are accompanied by a significant increase in the amount of GTP hydrolysed per peptide bond, as compared with cognate aminoacyl-tRNA binding, and that streptomycin decreases the GTP/peptide bond ratio [3,4]. Hence the correction step of aminoacyl-tRNA binding has been suggested to be inhibited by streptomycin. This conclusion, however, has been based on the results of binding and dipeptide formation in the absence of real translation (elongation) (see [5] for criticism). More recently, experiments with a poly(U)-programmed total translation system have also indicated that stimulating misreading streptomycin lowers the GTP/peptide ratio and thus seems to inhibit the second discrimination (correction) step [6]. In this case, however, the difficulty of direct measurement of peptide bond-coupled GTP hydrolysis in a background of uncoupled EF-Tu and EF-G-catalysed GTP hydrolysis on the non-

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translating ribosomes was inevitable, so that the authors used an indirect method [5,7,8]. Now a direct estimation of the GTP hydrolysis stoichiometry during peptide elongation on translating ribosomes has become possible due to application of the Sepharose-bound poly(U) translation technique [9–11].

Here we present the results of the study of the streptomycin effect on GTP stoichiometry in this system during codon-specific elongation and misreading.

2. MATERIALS AND METHODS

Escherichia coli MRE-600 ribosomes were purified from GTPase activity by washing 4 times in 1 M NH_4Cl [12,13]. EF-Tu and EF-G were isolated and checked as described in [14]. GTPase activity in the preparations of ribosomes, EF-Tu and EF-G was no higher than in [9,11].

E. coli tRNA (Serva) was acylated with [^{14}C]phenylalanine (spec. act. 496 Ci/mol) or [^{14}C]leucine (339 Ci/mol), or [^{14}C]isoleucine (354 Ci/mol); all the radioactive amino acids were from Amersham. Aminoacyl-tRNA was precipitated with cetavlon [15] and finally deproteinized with phenol which fully removed GTPase activity [16].

Poly(U) with $s^{o}_{20,w}$ = 3.4–6.0 S was oxidized with periodate and then covalently coupled to Sepharose 2B or 6B hydrazide [17].

Elongation of poly(Phe), poly(Leu) or poly(Ile) was performed starting with the complex of oligo(Phe)-tRNA · ribosome · poly(U)-Sepharose [9–11], in the presence or absence of 5×10^{-6} M streptomycin. All components of the translation system except the ribosomal complex were present in saturating amounts. Each 0.2 ml aliquot contained 2.0–2.5 A_{260} units of the oligo(Phe)-tRNA · ribosome · poly(U)-Sepharose complex, 8 μg EF-T, 6 μg EF-G, 0.05 mM [γ-^{32}P]GTP (170–250 Ci/mol, Amersham), 200–300 μg total *E. coli* tRNA, acylated with either [^{14}C]phenylalanine, or [^{14}C]leucine, or [^{14}C]isoleucine, 1 μg pyruvate kinase and 0.5 mM phospho*enol*pyruvate in buffer consisting of 10 mM Tris-HCl, pH 7.3, at 37°C, 50 mM KCl, 50 mM NH_4Cl, 1 mM dithiothreitol, 0.5% glycerol and 3–20 mM $MgCl_2$. Incubation was done at 37°C. Peptide elongation was recorded by measuring the ^{14}C label increase in the hot 5% trichloroacetic acid precipitate [9]. Hydrolysis of [γ-^{32}P]GTP to GDP and [^{32}P]orthophosphate was measured as in [18,19].

3. RESULTS AND DISCUSSION

Fig.1 demonstrates the dependences on Mg^{2+} concentration of the rates of elongation of poly(Phe), poly(Leu) and poly(Ile) using poly(U)-Sepharose as a template. It is seen that the elongation rate of each polypeptide has a fairly narrow optimum. It is noteworthy that the Mg^{2+} optima for the elongation rates of the 3 polypeptides synthesized are very different; the lowest, about 6 mM Mg^{2+}, characterizes codon-specific elongation, whereas maximal rates of mistranslation require higher Mg^{2+} concentrations. The difference in Mg^{2+} optima between poly(Leu) and poly(Ile) elongation may be consistent with the assumption that the lower the affinity of anticodon to codon the more Mg^{2+} is required for aminoacyl-tRNA uptake by the ribosome.

In special experiments it was shown that the presence of streptomycin at 10^{-4} M does not affect the Mg^{2+} dependences of both the codon-specific elongation and the miselongation (not shown). In the presence of streptomycin the elongation rates were found to be the same within the range

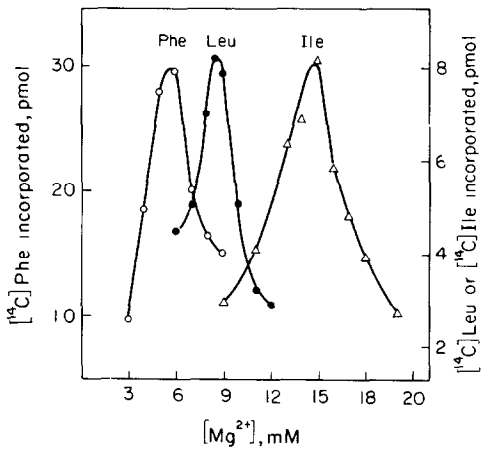

Fig.1. Mg^{2+} dependence of Phe, Leu or Ile incorporation in peptides elongating in the oligo(Phe)-tRNA · ribosome · poly(U)-Sepharose system at 37°C. Incubation: Phe, 1.5 min; Leu or Ile, 10 min.

10^{-6}–10^{-4} M streptomycin. A concentration of streptomycin of 5×10^{-6} M was chosen for further experiments.

The influence of streptomycin on the GTP hydrolysis and peptide synthesis rates during codon-specific elongation and misreading was studied at 3 different concentrations for each polypeptide synthesized: at the Mg^{2+} optimum for a given polypeptide and at lower and higher Mg^{2+} concentration points where the elongation rate was half that in the optimum. In each experiment the kinetics of peptide elongation and elongation-coupled GTP hydrolysis as well as background GTP hydrolysis in the mixture without ribosomes

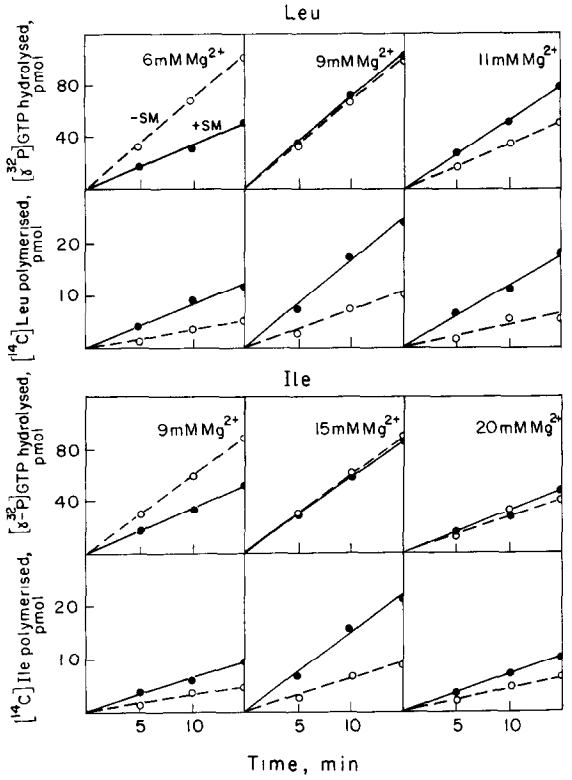

Fig.2. Kinetics of peptide elongation and GTP hydrolysis during misreading in the oligo(Phe)-tRNA·ribosome·poly(U)-Sepharose system at 37°C. Miselongation for poly(Leu) at 6, 9 and 11 mM $MgCl_2$; for poly(Ile) at 9, 15 and 20 mM $MgCl_2$. Without streptomycin (○---○); with 5×10^{-6} M streptomycin (●——●). Experimental series with Leu or Ile were done simultaneously using the same sample of the oligo(Phe)-tRNA·ribosomes·poly(U)-Sepharose complex.

were recorded. Examples of the kinetic curves for misreading are given in fig.2. The results of all experiments are summarized in table 1.

First of all, in the case of codon-specific elongation the presence of streptomycin (5×10^{-6} M) had little effect on both the elongation and GTP hydrolysis rates. Just as under normal conditions (in the absence of streptomycin, see [9,11]), the molar ratio of GTP cleaved per peptide bond was around 2 in the presence of streptomycin.

In the cases of misreading, however, the effect of streptomycin was obvious. The rates of elongation of both poly(Leu) and poly(Ile) on poly(U) were stimulated 2–4-fold, an increase in synthesis being observed in the presence of streptomycin at all Mg^{2+} concentrations. At the same time, the rate of GTP hydrolysis was not affected significantly by streptomycin at the Mg^{2+} optima for elongation (9 and 15 mM for Leu and Ile, respectively) and at higher Mg^{2+} concentrations, or it was even decreased at Mg^{2+} concentrations below the optimum. Hence the augmentation of misreading in the presence of streptomycin was not due to a correspondingly increased consumption of non-cognate ternary aa-tRNA·EF-Tu·GTP complexes, but to an increased economy of their use, i.e. higher efficiency of the final binding and incorporation of non-cognate aminoacyl-tRNAs. During misreading in the absence of streptomycin the number of GTP molecules cleaved per peptide bond was about 20 below the Mg^{2+} optimum for elongation, about 10 at the Mg^{2+} optimum, and about 6 above the Mg^{2+} optimum (see table 1); this means that only one of, respectively, 20, 10 or 6 non-cognate aminoacyl-tRNA molecules entering the ribosome as ternary complexes (with GTP) is incorporated into the peptide bond, whereas the rest are rejected after GTP cleavage. In the presence of streptomycin the level of rejection is strongly reduced; as a result the incorporation of non-cognate amino acid residues increases, and the ratio of the GTP cleaved per peptide bond reaches 2–4, thus approaching that in the case of codon-specific elongation. In other words, it is the correction (proof-reading) step that seems to be affected by streptomycin. Thus, our data on the direct determination of the stoichiometry of GTP cleavage during elongation in a complete cell-free system fully confirm earlier observations and conclusions made with less direct techniques [3,4,6].

Table 1

Stoichiometry of GTP hydrolysis during peptide elongation in the oligo(Phe)-tRNA·ribosome·poly(U)-Sepharose system in the absence and presence of streptomycin (−SM and +SM) at 37°C (average values)

^{14}C-labelled amino acid	[Mg^{2+}] (mM)	Amino acid, polymerized (pmol/min)		[γ-^{32}P]GTP, hydrolysed (pmol/min)		GTP cleaved per peptide bond	
		−SM	+SM	−SM	+SM	−SM	+SM
Phe	3	8.5	7.7	18.8	16.2	2.2	2.1
	6 (opt)		22.5		51.7		2.3
	9	7.0	6.3	15.8	13.3	2.2	2.1
Leu	6	0.4	0.8	7.2	3.3	20.4	4.0
	9 (opt)	0.8	2.8	7.3	8.0	9.1	2.9
	11	0.6	1.3	4.0	5.1	6.4	4.0
Ile	9	0.5	1.5	8.5	4.8	18.9	3.3
	15 (opt)	0.8	2.0	5.7	5.7	7.3	2.8
	20	0.6	1.1	3.2	3.9	5.9	3.5

GTP hydrolysis background without ribosomes was 0.63 ± 0.39 pmol GTP hydrolyzed per min in 54 experiments summarized. Data are without background subtraction

We previously demonstrated that the ribosomal proof-reading mechanism is manifested mainly at Mg^{2+} concentrations below the Mg^{2+} optimum for elongation [10,20,21]. At higher Mg^{2+} the proof-reading becomes less effective. Here it has been shown that this is determined not by the absolute Mg^{2+} concentration, but rather by the relative position of the Mg^{2+} point on the Mg^{2+} dependence curve of a given elongation system: the same proof-reading is attained at 9 mM Mg^{2+} in the case of poly(Ile) elongation and at 6 mM Mg^{2+} in the case of poly(Leu) elongation, both points being on the left slope of their Mg^{2+} dependence curves (see fig.1). The left slope of the curve seems to provide the conditions where aminoacyl-tRNA binding is the rate-limiting step of the elongation cycle (whereas the Mg^{2+} range above the Mg^{2+} optima makes translocation a rate-limiting step) [14]. It is likely that the ratio of the kinetic parameters of the elongation cycle, rather than absolute Mg^{2+} concentration, are of decisive importance for proof-reading efficiency. Naturally, the maximum effect of streptomycin is exerted under conditions where the proof-reading is manifested the most.

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REFERENCES

[1] Davies, J., Gilbert, W. and Gorini, L. (1964) Proc. Natl. Acad. Sci. USA 51, 883–890.
[2] Hopfield, J.J. (1974) Proc. Natl. Acad. Sci. USA 71, 4135–4139.
[3] Yates, J.L. (1979) J. Biol. Chem. 254, 11550–11554.
[4] Thompson, R.C., Dix, D.B., Gerson, R.B. and Karim, A.M. (1981) J. Biol. Chem. 256, 6676–6681.
[5] Ruusala, T., Ehrenberg, M. and Kurland, C.G. (1982) EMBO J. 1, 741–745.
[6] Ruusala, T. and Kurland, C.G. (1984) Mol. Gen. Genet. 198, 100–104.
[7] Andersson, D.I. and Kurland, C.G. (1983) Mol. Gen. Genet. 191, 378–381.

[8] Ruusala, T., Andersson, D.I., Ehrenberg, M. and Kurland, C.G. (1984) EMBO J. 3, 2575–2580.
[9] Kakhniashvili, D.G., Smailov, S.K., Gogia, I.N. and Gavrilova, L.P. (1983) Biokhimiya 48, 959–969.
[10] Smailov, S.K., Kakhniashvili, D.G. and Gavrilova, L.P. (1984) Biokhimiya 49, 1868–1873.
[11] Gavrilova, L.P., Kakhniashvili, D.G. and Smailov, S.K. (1984) FEBS Lett. 178, 283–287.
[12] Gavrilova, L.P. and Smolyaninov, V.V. (1971) Mol. Biol. (USSR) 5, 883–891.
[13] Kakhniashvili, D.G., Smailov, S.K. and Gavrilova, L.P. (1980) Biokhimiya 45, 1999–2012.
[14] Gavrilova, L.P., Kostiashkina, O.E., Koteliansky, V.E., Rutkevitch, N.M. and Spirin, A.S. (1976) J. Mol. Biol. 101, 537–552.
[15] Jones, A.S. (1953) Biochim. Biophys. Acta 10, 607–612.
[16] Smailov, S.K., Kakhniashvili, D.G. and Gavrilova, L.P. (1982) Biokhimiya 47, 1747–1751.
[17] Ustav, M.B., Remme, Ya.L., Lind, A.Ya. and Villems, R.L.E. (1979) Bioorg. Khim. 5, 365–369.
[18] Nishizuka, Y. and Lipmann, F. (1966) Arch. Biochem. Biophys. 116, 344–351.
[19] Kaziro, Y., Inoue-Yokosawa, N. and Kawakita, M. (1972) J. Biochem. (Tokyo) 72, 853–863.
[20] Gavrilova, L.P., Perminova, I.N. and Spirin, A.S. (1981) J. Mol. Biol. 149, 69–78.
[21] Gavrilova, L.P. and Perminova, I.N. (1981) Proc. 4th Int. Symp., Metabolism and Enzymology of Nucleic Acids, Smolenice, June 8–11, 1981, Publ. House of the Slovak Acad. Sci., Bratislava, 1982.