The excess GTP hydrolyzed during mistranslation is expended at the stage of EF-Tu-promoted binding of non-cognate aminoacyl-tRNA

D.G. Kakhniashvili, S.K. Smailov and L.P. Gavrilova*

Institute of Protein Research, Academy of Sciences of the USSR, 142292 Pushchino, Moscow Region, USSR

Received 6 November 1985

The system of translation of Sepharose-bound poly(U) in which all ribosomes are active in peptide elongation was used to determine the stoichiometry of GTP hydrolysis at the stage of EF-Tu-promoted aminoacyl-tRNA binding. The ratio of GTP hydrolyzed at this stage per peptide bond was assayed during codon-specific elongation (polyphenylalanine synthesis) and misreading (polyleucine sythesis). It was demonstrated directly that the excess GTP hydrolyzed during misreading [(1984) FEBS Letters 178, 283–287] is expended at the stage of Ef-Tu-promoted binding of non-cognate aminoacyl-tRNA.

Misreading *Proof-reading* *Poly(U)-Sepharose translation system* *Elongation factor Tu function*

1. INTRODUCTION

Using a technique of Sepharose-bound template translation in which all ribosomes are active in peptide elongation, the stoichiometry of GTP hydrolysis was directly determined during both codon-specific elongation and misreading [1–4]. The expenditure of GTP during misreading, e.g. elongation of poly(Leu) or poly(Ile) on a poly(U) template was found to be much higher than that in the process of codon-specific elongation of poly(Phe). Under certain conditions (at Mg^{2+} concentrations where ribosomal proof-reading is clearly displayed [5]), the ratio of GTP hydrolyzed per peptide bond during misreading (GTP/Leu or GTP/Ile) was 10-times higher than that during codon-specific elongation (GTP/Phe) [3,4]. However, the experiments performed did not allow determination of which of the GTP-dependent stages of the elongation cycle, i.e. either EF-Tu-promoted aminoacyl-tRNA binding or EF-G-promoted translocation, is responsible for the excess of GTP expenditure.

Here we report on experiments where the expenditure of GTP at the stage of EF-Tu-promoted aminoacyl-tRNA binding was determined selectively during elongation, by using preformed tertiary complexes with labeled GTP (Aa-tRNA·EF-Tu·[γ-^{32}P]GTP) in a Sepharose-bound poly(U) translation system. Poly(Leu) elongation of oligo(Phe) presynthesized on the Sepharose·poly(U)·ribosome complex [6] was exploited as a model for misreading. The results show that virtually all the excess GTP hydrolyzed during poly(U) misreading is expended at the stage of EF-Tu-promoted binding of non-cognate aminoacyl-tRNA.

2. MATERIALS AND METHODS

Ribosomes from *Escherichia coli* MRE-600, total *E. coli* tRNA acylated with [^{14}C]phenylalanine or [^{14}C]leucine, and Sepharose-poly(U)·ribosome·oligo(Phe)-tRNA complexes were prepared as described in [1] (see also [3–5]). 1 mg *E. coli* tRNA (Serva) acylated with [^{14}C]Phe or [^{14}C]Leu (Amersham, 496 and 330 Ci/mol,

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

respectively) contained 800 pmol [^{14}C]Phe-tRNA or 1500 pmol [^{14}C]Leu-tRNA.

The EF-Tu·GTP complex was prepared from EF-Tu·GDP (1 : 1 molar ratio) by incubation with a 4-fold molar excess of [γ-^{32}P]GTP (Amersham, 300–500 Ci/mol) or [^{14}C]GTP (Amersham, 400 Ci/mol) for 20 min at 37°C in buffer A [10 mM Tris-HCl, pH 7.3 (at 37°C), 50 mM KCl, 50 mM NH_4Cl, 6 mM $MgCl_2$, 3 or 5 mM 2-mercaptoethanol, 0.5% glycerol, 1 mM phospho*enol*pyruvate and 0.001–0.002% pyruvate kinase]. The EF-Tu-labeled GTP complex formed was measured by adsorption on nitrocellulose filters [7]. For this purpose, 25–40-μl aliquots of the incubation mixture were diluted with 3 ml of a buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NH_4Cl and 10 mM $MgCl_2$, and the mixture was passed through a nitrocellulose filter; the filter was washed with the same buffer, dried, and the radioactivity counted.

Titration of EF-Tu·[^{14}C]GTP complex by amino[^{14}C]acyl-tRNA was done in a mixture containing 10 μM [^{14}C]GTP, 2.5 μM EF-Tu·[^{14}C]GTP and increasing amounts, from 0 to 2.5 μM, of [^{14}C]Phe-tRNA or [^{14}C]Leu-tRNA, in buffer A at 37°C; incubation was for 5 min. The mixture was then passed through a nitrocellulose filter. The amount of ternary complex formed (amino[^{14}C]acyl-tRNA·EF-Tu·[^{14}C]GTP) was determined by subtraction of the amount of the non-reacted binary complex EF-Tu·[^{14}C]GTP (adsorbed on the nitrocellulose filter) from the total amount of the binary complex EF-Tu·[^{14}C]GTP introduced into the mixture. The titration curve is presented in fig.1. An EF-Tu/Aa-tRNA molar ratio of 2.5 was chosen in all subsequent experiments with amino[^{14}C]acyl-tRNA and EF-Tu·[γ-^{32}P]GTP.

The rate of exchange of GTP in the binary complex for exogenous GTP was measured in a mix-

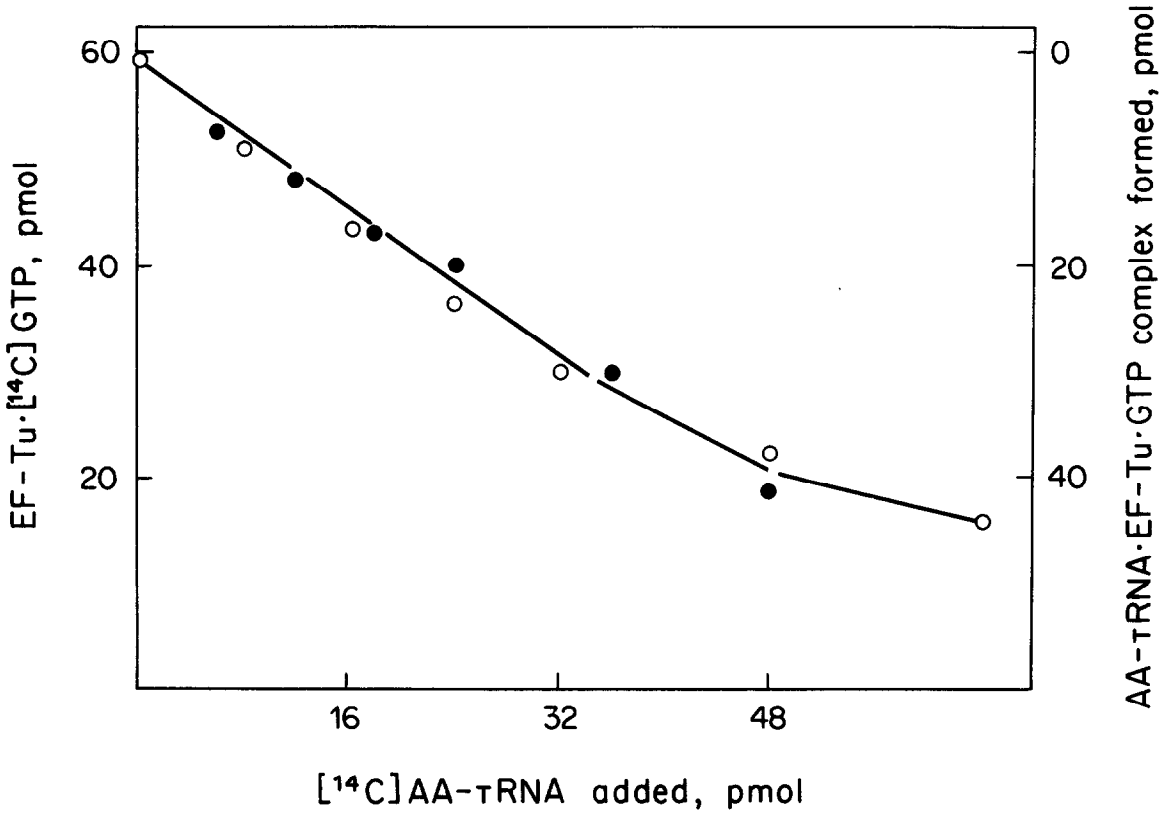

Fig.1. Titration curves of the EF-Tu·[^{14}C]GTP complex by [^{14}C]Phe-tRNA (—●—) or [^{14}C]Leu-tRNA (—○—) at 37°C. Experimental conditions are given in section 2.

ture containing 2.5 μM EF-Tu·[^{14}C]GTP, 10 μM [^{14}C]GTP and 400 μM GTP; incubation was carried out in buffer A at 37°C. The amount of EF-Tu·[^{14}C]GTP during incubation was determined by the nitrocellulose filter technique [7].

The rate of exchange of GTP in the ternary complex for exogenous GTP was assayed as follows: a mixture containing 2.5 μM EF-Tu·[^{14}C]GTP, 10 μM [^{14}C]GTP and 4 mg/ml total *E. coli* tRNA acylated with phenylalanine was preincubated for 5 min at 37°C in buffer A, then 400 μM unlabeled GTP was added and the incubation continued at 37°C. The ternary complex was precipitated from the incubation mixture by a cold saturated solution of $(NH_4)_2SO_4$, the precipitate collected on GF/F filter (Whatman), washed with a cold saturated solution of $(NH_4)_2SO_4$ and counted for radioactivity.

Elongation of poly(Phe) and poly(Leu) on the Sepharose-poly(U)·ribosome·oligo(Phe)-tRNA complex was performed at 6 mM $MgCl_2$ and at 37°C; all components of the translation system were present in excess over the ribosomal complex [1]. 1 ml incubation mixture in buffer A contained 10 A_{260} units of poly(U) of the Sepharose-poly(U)·ribosome·oligo(Phe)-tRNA complex, 10 μM [γ-^{32}P]GTP, 2.5 μM EF-Tu·[γ-^{32}P]GTP complex, 1 μM [^{14}C]Phe-tRNA or [^{14}C]Leu-tRNA, 0.5 μM EF-G and 400 μM GTP. In some experiments 400 μM GTP was replaced by 50 μM [γ-^{32}P]GTP. 5×10^{-6} M streptomycin was present where indicated. Before the elongation was started, [γ-^{32}P]GTP, EF-Tu·[γ-^{32}P]GTP complex and amino[^{14}C]acyl-tRNA were incubated for 5 min at 37°C to form the ternary complex. Then the Sepharose-poly(U)·ribosome·oligo (Phe)-tRNA complex was added to the mixture. The elongation was initiated by addition of EF-G and GTP. An aliquot for measuring the rate of [γ-^{32}P]GTP hydrolysis was taken 1 min after initiation of elongation. Polyphenylalanine and polyleucine synthesis rates were measured within 1 min and 5 min periods after the start, respectively (i.e. within initial linear parts of the kinetic curves for elongation of the corresponding peptides). Elongation was determined by the increase in hot 5% trichloroacetic acid-insoluble ^{14}C label ([^{14}C]Phe or [^{14}C]Leu) during incubation. GTP hydrolysis was measured from ^{32}P counting, as in [8,9].

3. RESULTS

Fig.2 shows the rate of exchange of labeled GTP contained in the binary complex EF-Tu·[^{14}C]GTP and in the ternary complex Phe-tRNA·EF-Tu·[^{14}C]GTP with exogenous unlabeled GTP. It can be seen that while the exchange rate of GTP in the binary complex is relatively high (more than 50% GTP is exchanged within 15 s at 37°C), the ternary complex proves to be slowly exchangeable so that only about 15% of its GTP is replaced by exogenous GTP over 1 min at 37°C. These results provided the basis for measurement of EF-Tu-mediated hydrolysis of labeled GTP virtually independently of the EF-G-catalyzed hydrolysis of exogenous unlabeled GTP by addition to the system of the preformed ternary complex instead of the separate components (provided the incubation period is shorter than 1 min).

To determine the stoichiometry of the EF-Tu-dependent GTP hydrolysis during elongation, the amount of [γ-^{32}P]GTP hydrolysed during 1 min of elongation and the rates of poly(Phe) or poly(Leu) synthesis were measured in parallel, in the same incubation mixture. The background of [γ-^{32}P]GTP hydrolysis in the mixture without ribosomes was also measured. The results are summarized in table 1. It was found that only one GTP molecule per amino acid residue was expended in the EF-Tu-promoted binding of Phe-tRNA during codon-specific elongation. At the same time, the expen-

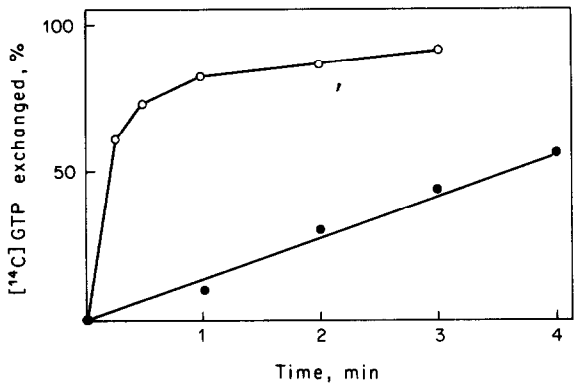

Fig.2. The rate of exchange of labeled GTP in the binary complex EF-Tu·[^{14}C]GTP (—○—) and in the ternary complex Phe-tRNA·EF-Tu·[^{14}C]GTP (—●—) with exogenous unlabeled GTP at 37°C. Experimental conditions are described in section 2.

Table 1

Stoichiometry of the hydrolysis of GTP of the preformed aminoacyl-tRNA·EF-Tu·[γ-^{32}P]GTP complex during elongation in the Sepharose-bound poly(U) translation system (6 mM $MgCl_2$, 37°C)

Expt no.	^{14}C-labeled amino acid (Aa)	Special conditions	Aa polymerized (pmol/min)	[γ-^{32}P]GTP hydrolyzed (pmol/min)[a]	GTP cleaved per peptide bond
1	Phe	–	29.91	35.14	1.2
		–	27.75	23.84	0.9
2	Leu	–	0.54	11.70	21.7
		+ [γ-^{32}P]GTP[b]	0.51	10.75	21.1
3	Leu	–	0.48	10.61	22.1
		+ [γ-^{32}P]GTP[b]	0.44	9.25	21.0
4	Leu	–	0.64	9.82	15.3
		+ SM[c]	1.60	2.59	1.6
5	Leu	–	0.32	5.58	17.4
		+ SM[c]	1.29	1.51	1.2

[a] Background of [γ-^{32}P]GTP cleavage (in the mixture without ribosomes) was usually not higher than 0.5 pmol GTP/min; values in this column are given without its subtraction

[b] To eliminate the contribution from EF-G-catalyzed cleavage of free [γ-^{32}P]GTP present in the incubation mixture, excess unlabeled GTP (400 μM) was added to all incubation assays, except this special case, where 50 μM [γ-^{32}P]GTP was added instead of the unlabeled GTP (see text)

[c] Streptomycin (SM) at 5×10^{-6} M was present

diture of GTP in the EF-Tu-promoted binding of Leu-tRNA, i.e. during misreading, is about 20-times higher.

Two kinds of controls are also included in table 1. First, in some parallel experiments the excess of unlabeled GTP in the incubation mixture was replaced by labeled GTP, in order to estimate the possible additional contribution of EF-G to the GTP cleavage during misreading. It can be seen that the additional GTP cleavage by the EF-G-promoted translocation is within a ± 10% error of the EF-Tu-dependent GTP cleavage measurements in the misreading system, i.e. it seems to correspond to about one GTP molecule per amino acid residue. Second, streptomycin, which is known to inhibit the proof-reading step in the EF-Tu-promoted aminoacyl-tRNA binding [4,10–12], was added to some assays. As expected, the GTP expenditure was reduced by streptomycin to almost one molecule per amino acid residue in the EF-Tu-promoted binding of the non-cognate aminoacyl-tRNA.

4. DISCUSSION

Hopfield [13] was the first to propose that the error correction during elongation can proceed at the stage of EF-Tu-promoted binding of aminoacyl-tRNA and is coupled with additional expenditure of GTP [13]. Using systems of poly(U) translation with different sets of elongation factors, such as a complete one, including both EF-Tu and EF-G, the EF-Tu-promoted one without EF-G, and the EF-G-promoted one without EF-Tu, as well as the factor-free translation system, the role of EF-Tu in reducing the error level during elongation has been demonstrated experimentally [5]. Direct measurements of the GTP stoichiometry in the poly(U)-dependent translation showed that misreading consumes an order of magnitude more GTP than the codon-specific elongation [2–4]. The latter results were consistent with the idea of an error-correcting function of EF-Tu and EF-Tu-mediated GTP hydrolysis during elongation. Strictly speaking, however, a possible correcting

role of the EF-G-dependent GTP hydrolysis and coupled translocation [14–16] could not be discounted in these experiments. Our results demonstrate that all GTP excessively hydrolyzed in the course of mistranslation (at least, under the given conditions) is expended at the stage of EF-Tu-dependent binding of non-cognate aminoacyl-tRNA.

From the present results it can be deduced that under our experimental conditions only one out of 20 Leu-tRNA molecules interacting with the translating ribosomes is finally found to be accepted by the ribosome and thus to become incorporated into a peptide. The other Leu-tRNA molecules are withdrawn from the ribosome after EF-Tu-mediated GTP hydrolysis. Such a mechanism is called proof-reading. Streptomycin has been shown to inhibit this mechanism, so that almost every one of the Leu-tRNAs interacting with the ribosome remains in the poly(U)-translating ribosome after GTP hydrolysis and is incorporated into the peptide; as a result, the rate of miselongation is stimulated [4].

ACKNOWLEDGEMENTS

We are very grateful to Professor A.S. Spirin for attention, help and discussions, and to V.P. Burmistrova for excellent technical assistance.

REFERENCES

[1] Kakhniashvili, D.G., Smailov, S.K., Gogia, I.N. and Gavrilova, L.P. (1983) Biokhimiya 48, 959–969.
[2] Smailov, S.K., Kakhniashvili, D.G. and Gavrilova, L.P. (1984) Biokhimiya 49, 1868–1873.
[3] Gavrilova, L.P., Kakhniashvili, D.G. and Smailov, S.K. (1984) FEBS Lett. 178, 283–287.
[4] Smailov, S.K. and Gavrilova, L.P. (1985) FEBS Lett. 192, 165–169.
[5] Gavrilova, L.P., Perminova, I.N. and Spirin, A.S. (1981) J. Mol. Biol. 149, 69–78.
[6] Gavrilova, L.P. and Rutkevitch, N.M. (1980) FEBS Lett. 120, 135–140.
[7] Miller, D.L. and Weissbach, H. (1974) Methods Enzymol. 30, 219–231.
[8] Nishizuka, Y. and Lipmann, F. (1966) Arch. Biochem. Biophys. 116, 344–351.
[9] Kaziro, Y., Inoue-Yokosawa, N. and Kawakita, M. (1972) J. Biochem. (Tokyo) 72, 853–863.
[10] Yates, J.L. (1979) J. Biol. Chem. 254, 11550–11554.
[11] Thompson, R.C., Dix, D.B., Gerson, R.B. and Karim, A.M. (1981) J. Biol. Chem. 256, 6676–6681.
[12] Ruusala, T. and Kurland, C.G. (1984) Mol. Gen. Genet. 198, 100–104.
[13] Hopfield, J.J. (1974) Proc. Natl. Acad. Sci. USA 71, 4135–4139.
[14] Menninger, J.R. (1976) J. Biol. Chem. 251, 3392–3398.
[15] Menninger, J.R. (1978) J. Biol. Chem. 253, 6808–6813.
[16] Caplan, A.B. and Menninger, J.R. (1984) Mol. Gen. Genet. 194, 534–538.