Stoichiometry of GTP hydrolysis in a poly(U)-dependent cell-free translation system

Determination of GTP/peptide bond ratios during codon-specific elongation and misreading

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The stoichiometry of GTP hydrolysis during peptide elongation in the processes of codon-specific translation and misreading of polyuridylic acid was determined in a cell-free system in which all ribosomes were active in peptide synthesis. Ribosomes carrying oligophenylalanine presynthesized on poly(U) covalently bound to Sepharose were used. In the codon-specific translation of poly(Phe) on poly(U)-Sepharose at optimal Mg^{2+} concentration (6 mM $MgCl_2$), the ratio of GTP cleaved to Phe polymerized was found to be about 2 ($\pm$ 0.1). Under the same conditions but during misreading (elongation of polyleucine on poly(U)-Sepharose) the GTP/Leu ratio increased 10 times (from 16 to 25 in different experiments).

GTP hydrolysis *Translation* *Cell-free translation system* *Misreading* *Matrix-bound poly(U)* *Functionally active ribosome*

1. INTRODUCTION

For a long time it was practically impossible to measure the GTP expense of peptide synthesis proceeding on ribosomes, as ribosome preparations usually consist of no more than 10–50% active ribosomes. The other 50–90% of ribosomes in ribosome preparations are incapable of synthesizing peptide in vitro, but can carry out uncoupled factor-dependent GTP hydrolysis. As yet there are no means of measuring correctly the factor-dependent GTP hydrolysis which is uncoupled with peptide synthesis and takes place on inactive ribosomes.

Here, the GTP expense during peptide synthesis was determined under conditions in which all the ribosomes were active and engaged in peptide synthesis. Active ribosomes were separated from inactive ones using poly(U) covalently bound to Sepharose [1–3]. Peptide synthesis proceeded on the ribosomes carrying oligophenylalanine presynthesized on poly(U)-Sepharose [4]. The GTP expense was measured during peptide elongation both in the process of codon-specific translation (polyphenylalanine synthesis on poly(U)-Sepharose) and during misreading (poly(U)-directed polyleucine synthesis) [5]. It was found that the ratio of hydrolyzed GTP to polymerized Phe was about 2 for the codon-specific translation of poly(U)-Sepharose at 6 mM $MgCl_2$ which is an optimum for the system. Under the same conditions the GTP/Leu ratio was about 20 during elongation of polyleucine on poly(U)-Sepharose.

2. MATERIALS AND METHODS

Ribosomes were isolated from *Escherichia coli* MRE-600 and purified from GTPase activity by 4 washings with 1 M NH_4Cl [6,7]. The ribosome preparations hydrolyzed not more than 2–7 pmol GTP/pmol ribosomes during 1 h at 37°C. EF-Tu and EF-G were obtained as in [8]. EF-Ts was

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prepared as in [9]. EF-T was formed by mixing equimolar parts of EF-Tu and EF-Ts. 1 pmol EF-T hydrolyzed 0.5–1.0 pmol GTP, and 1 pmol EF-G hydrolyzed 0.7 pmol GTP during 1 h at 37°C. *E. coli* tRNA (Serva) was acylated with [^{14}C]phenylalanine (spec. act. 496 Ci/mol) or [^{14}C]leucine (spec. act. 330 Ci/mol, Amersham). After cetavlon precipitation [10] the aminoacyl-tRNA preparations were purified by phenol deproteinization [11] to eliminate low GTPase activity. The ^{14}C-labelled aminoacyl-tRNA preparations hydrolyzed not more than 3–15 pmol GTP/100 μg tRNA during 1 h at 37°C. Poly(U) with $s_{20,w}$ = 3.4–6.0 S was used which corresponds to 200–500 residues of uridylic acid [12]. The poly(U) was oxidized with periodate and covalently bound to Sepharose 2B or 6B hydrazide [3]. Poly(U)-Sepharose was free from GTPase activity.

Elongation of poly(Phe) or poly(Leu) on poly(U)-Sepharose was done as follows. At the first step oligo(Phe) on poly(U)-Sepharose was presynthesized [4]. Then elongation of the oligo(Phe) with phenylalanine or leucine proceeded under conditions in which all the components except the complex [^{14}C]oligo(Phe)–tRNA·ribosome·poly(U)-Sepharose were in excess [4]. Each 0.1 or 0.2 ml aliquot contained 0.5–2.0 A_{260} units of the oligo(Phe)–tRNA·ribosome·poly(U)-Sepharose complex, 3 or 10 μg EF-T, 3–6 μg EF-G, 0.05 mM [γ-^{32}P]GTP (170–250 Ci/mol, Amersham), 200–400 μg total *E. coli* tRNA acylated with [^{14}C]phenylalanine or [^{14}C]leucine, 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 3–10 mM $MgCl_2$. Incubation was done at 37°C. Elongation of the oligo(Phe) presynthesized was determined by measuring the increase of either [^{14}C]Phe or [^{14}C]Leu label in a hot 5% trichloroacetic acid precipitate [4,8]. [γ-^{32}P]GTP hydrolysis to GDP and P_i was measured as in [13,14].

3. RESULTS

To determine the stoichiometry of GTP hydrolysis during peptide elongation on ribosomes in codon-specific translation or misreading of polyuridylic acid, the following were measured in each experiment: (i) kinetics of poly(U)-directed polyphenylalanine or polyleucine elongation, respectively; (ii) kinetics of GTP hydrolysis which accompanied the peptide elongation, and (iii) kinetics of background GTP hydrolysis in the mixture where all components except ribosome (i.e., of EF-T, EF-G, poly(U)-Sepharose, total tRNA acylated with [^{14}C]leucine or [^{14}C]phenylalanine, pyruvate kinase and phospho*enol*pyruvate) were present.

Fig.1 shows typical experimental kinetic curves at 37°C and 6 mM $MgCl_2$ (Mg^{2+} optimum for polyphenylalanine elongation on poly(U)-Sepharose). In all cases elongation started from oligophenylalanine presynthesized in the complex oligo(Phe)–tRNA·ribosome·poly(U)-Sepharose. The rates of either polyphenylalanine or oligophenylalanine-initiated polyleucine elongation and GTP hydrolysis were calculated from the linear parts of the kinetic curves.

The results are summarized in table 1. It is seen that the GTP/Phe ratio was 1.9–2.1 for polyphenylalanine elongation on poly(U)-Sepharose at the Mg^{2+} optimum of the system (6 mM). Under the same conditions, but during misreading of poly(U) (elongation of polyleucine on poly(U)-Sepharose), the GTP/Leu ratio was 16–25 in different experiments.

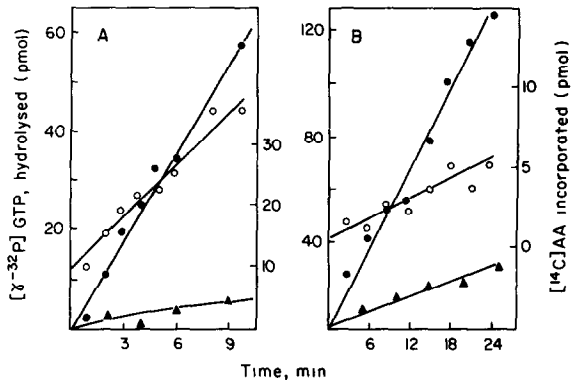

Fig.1. Typical kinetics of peptide elongation and GTP hydrolysis used for calculation of stoichiometry of GTP expense per peptide bond in the cell-free system with poly(U)-Sepharose at 6 mM $MgCl_2$, 37°C. Peptide elongation (○—○); GTP hydrolysis during peptide synthesis (●—●); GTP hydrolysis with all components of the system except ribosomes (▲—▲). (A) Polyphenylalanine, (B) polyleucine.

Table 1

Stoichiometry of GTP hydrolysis during peptide elongation in the ribosome·poly(U)-Sepharose system at 6 mM $MgCl_2$, 37°C

^{14}C-labelled amino acid (AA)	AA, polymerized (pmol/min)	[γ-^{32}P]GTP hydrolysed (pmol/min)		Total GTP cleaved per peptide bond	GTP cleaved minus background per peptide bond
		During peptide elongation	Background of the system without ribosomes		
[^{14}C]Phe	2.58	5.97	0.78	2.31	2.01
	2.80	5.81	0.59	2.09	1.88
	3.48	8.15	0.87	2.34	2.09
	2.28	6.51	2.20	2.86	1.89
[^{14}C]Leu	0.31	6.70	1.41	21.61	17.06
	0.30	6.25	1.13	20.83	17.06
	0.25	7.26	0.37	29.04	25.56
	0.28	5.44	1.04	19.43	17.71

4. DISCUSSION

The stoichiometry of GTP hydrolysis during peptide synthesis on ribosomes has been studied by 3 research groups [13,15,16]. However, in the traditional cell-free system it is very difficult to measure the stoichiometric ratio correctly because of a significant contribution of uncoupled GTP hydrolysis performed by a fraction of ribosomes not engaged in peptide synthesis.

The point is that the elongation factors EF-Tu and/or EF-G are capable of interacting with ribosomes which are not engaged in peptide synthesis. It is known that an uncoupled EF-Tu-dependent GTP hydrolysis can appear in the mixture EF-Tu + ribosomes + poly(U) + tRNA [11,17–19]. An uncoupled EF-G-dependent GTP hydrolysis also results from the interaction of EF-G and GTP with free ribosomes [13,16].

During elongation an uncoupled factor-dependent GTP hydrolysis occurs on free inactive ribosomes not engaged in the peptide synthesis. In a control mixture where there is no peptide synthesis, uncoupled factor-dependent GTP hydrolysis can proceed both on inactive and on potentially active, but free ribosomes. Therefore, the GTP expense in the control mixture is very often higher than that in the experimental one [4,16]. This is why it is hardly possible to measure correctly the contribution of the uncoupled GTP hydrolysis in the traditional cell-free translation systems.

Here stoichiometry of GTP hydrolysis during peptide synthesis was determined in the system where all ribosomes were active and engaged in the complex oligo(Phe)–tRNA·ribosome·poly(U)-Sepharose. The GTP expense was measured during peptide elongation. The experiments were carried out at 6 mM $MgCl_2$, which is the Mg^{2+} optimum for elongation of the peptide corresponding to template specificity (elongation of polyphenylalanine on poly(U)-Sepharose). The GTP/Phe ratio was found to be 1.9–2.1. If only one GTP molecule is assumed to be hydrolyzed at each translocation step then only one GTP molecule is expended for every Phe-tRNA binding. This means that practically every Phe-tRNA bound to oligo(Phe)–tRNA·ribosome·poly(U)-Sepharose as a Phe-tRNA·EF-Tu·GTP complex does not dissociate from the ribosome after GTP hydrolysis and almost inevitably forms a peptide bond with the presynthesized oligophenylalanine.

The stoichiometry of GTP hydrolysis during misreading of poly(U) was also determined. The model system of polyleucine synthesis on poly(U) as a template was used [5]. At 6 mM $MgCl_2$ the

GTP/Leu ratio measured was significantly higher than that of GTP/Phe and was 16–25 in different experiments. It is shown, however, that during misreading of poly(U), the GTP/Leu ratio changes with change in Mg^{2+} concentration: it increases with decreasing Mg^{2+} concentration in the experimental mixture, and vice versa [20].

In [21] it was assumed that the GTP expense during misreading of the RNA template should be higher than that at codon-specific elongation. Some indirect data supporting this idea were obtained in [22,23]. Earlier results [24,25] showed that the GTP excess could be spent during binding of a non-cognate aminoacyl-tRNA to the peptidyl-tRNA·ribosome·template complex. It is likely that the EF-Tu participates in this excess GTP cleavage and thus contributes to the correction of aminoacyl-tRNA binding [26]. If all of the GTP excess is spent only for the correction of the binding stage the results obtained here suggest that during poly(U) misreading under the present conditions only one of the 20 Leu-tRNAs bound can form a peptide bond; the other 19 Leu-tRNAs after binding to the ribosome (in the form of the Leu-tRNA·EF-Tu·GTP complex) should leave it just after GTP hydrolysis.

In accordance with the accepted point of view, GTP hydrolysis during ribosomal protein synthesis is coupled both with EF-Tu and with EF-G functioning. Therefore, it cannot be excluded that the excess GTP cleavage observed during misreading of poly(U) is spent not only at the binding step of the elongation cycle to which EF-Tu contributed but also at the EF-G-promoted translocation step. This could not be determined in our experiments. Nevertheless, it is not unlikely that a second correction may be realized at the translocation step of the elongation cycle, with participation of EF-G. At this step EF-G with GTP could promote dissociation of non-cognate tRNA-carrying peptidyl-tRNA from the ribosome. At present there is some evidence in favour of the appearance of abortive peptidyl-tRNAs in the process of protein synthesis [27–29].

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