Two GTPs are consumed on EF-Tu per peptide bond in poly(Phe) synthesis, in spite of switching stoichiometry of the EF-Tu·aminoacyl-tRNA complex with temperature

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Abstract Recent observations indicate that the stoichiometry for the complex between EF-Tu · GTP and aminoacyl-tRNA (aa-tRNA) changes with temperature. At 37°C two EF-Tu·GTPs bind one aa-tRNA in an extended ternary complex, but at 0°C the complex has 1:1 stoichiometry. However, the present experiments show that there are two GTPs hydrolyzed on EF-Tu per peptide bond in poly(Phe) synthesis at 37°C as well as at 0°C. This indicates two different pathways for the enzymatic binding of aa-tRNA to the A-site on the ribosome.

Key words: GTP hydrolysis; Translation; Poly(Phe) synthesis; Elongation factor Tu; Ternary complex

1. Introduction

Recently, two novel features of *E. coli* translation were identified [1]: hydrolysis of two GTPs on EF-Tu was observed for every peptide bond in poly(Phe)-synthesis; in addition, two EF-Tu·GTPs bound to a single aminoacyl (aa)-tRNA [1,2]. These in vitro experiments were performed under conditions where the ribosome behaves as if in vivo with respect to rate and accuracy [3–5]. From these observations a revision of the classical description of prokaryotic translation was suggested: aminoacyl-tRNA enters the A-site in an extended ternary complex together with two EF-Tu's and two GTPs. When a cognate codon–anticodon contact is established, both GTPs in the complex are hydrolyzed and subsequently two EF-Tu·GDPs leave the ribosome. Quench-flow experiments indicate that the two GTPs are hydrolyzed simultaneously [5]. The classical view of *E. coli* translation is, in contrast, that aa-tRNA forms a 1:1 complex with EF-Tu·GTP and that one GTP is dissipated per peptide bond in EF-Tu-function, as reviewed by Kaziro [6].

The conclusion that two GTPs are hydrolyzed per peptide bond was also supported by experiments with an altered EF-Tu, which uses XTP (xanthosine triphosphate) instead of GTP as the energy source in translation [7,8]. According to these observations two XTPs are hydrolyzed per peptide bond in poly(U) translation. At the same time, hydrolysis of only one GTP on EF-G was observed per translocated peptidyl-tRNA [7], as previously shown by Richter and Kurland [9].

The 2:1 stoichiometry between EF-Tu and aa-tRNA, observed at 37°C, has been questioned [7,8,11,12]. However, this controversy about the extended ternary complex has recently been resolved [10,13]: RNAse A protection assays in combination with the quench-flow technique show 1:1 stoichiometry at 0°C and 2:1 at 37°C, which demonstrates that the stoichiometry of the ternary complex changes with temperature.

In the present work we investigated if the stoichiometry of the ternary complex determines the GTP consumption per peptide bond. If this were the case, one would expect hydrolysis of one GTP per peptide bond at low temperatures and of two GTPs at high temperatures. We show this not to be the case. We find that there are always two GTPs used in EF-Tu function per elongation cycle, irrespective of the stoichiometry of the ternary complex. Some consequences of this finding are discussed.

2. Materials and methods

2.1. Chemicals

Poly(U), ATP, GTP and GDP were purchased from Pharmacia, Sweden. Putrescine, spermidine, phosphoenolpyruvate (PEP), myokinase (MK), pyruvate kinase (PK) were from Sigma. [^{3}H]Phenylalanine, [^{14}C]phenylalanine and [^{3}H]guanosine-5′-diphosphate were obtained from Amersham (Buckinghamshire).

2.2. Buffers

Polymix buffer [2] contained 5 mM magnesium acetate, 5 mM ammonium chloride, 95 mM potassium chloride, 0.5 mM calcium chloride, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phoshate, 1 mM dithioerythritol.

2.3. Purifications and preparations

Elongation factors EF-Tu and EF-G, tRNA^{Phe} and Phe-tRNA synthetase (PheS) were purified from *E. coli* MRE-600 cells as reviewed by Ehrenberg et al. [1]. *E. coli* 017 cells were used to prepare ribosomes according to Jelenc [14]. NAc-Phe-tRNA^{Phe} was prepared as described by Wagner et al. [3]. All enzymes, ribosomes and tRNAs were dialyzed against and stored in polymix buffer.

2.4. Assay for the number of GTPs hydrolyzed per peptide bond (f_n)

The assay described by Ehrenberg et al. [1] was used to measure GTP hydrolysis on EF-Tu during elongation. Factor mix contained (in 40 μl balanced polymix buffer) ATP (0.1 μmol), PEP (1 μmol), PK (5 μg), MK (0.3 μg), [^{14}C]Phe (30 nmol, 7 cpm/pmol), PheS (100 units), tRNA^{Phe} (500 pmol), EF-G (100 pmol). The EF-Tu mix was prepared as [^{3}H]GDP (90 pmol) and EF-Tu (400 pmol or 250 pmol for 37°C or 0°C, respectively) in 10 μl balanced polymix. Two EF-Tu mixes were prepared in parallel, one with an additional amount of unlabeled GDP (1578 pmol or 635 pmol for 37°C or 0°C, respectively). The ribosome mix contained (in 50 μl balanced polymix) 70 S ribosomes (50 pmol,

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Abbreviations: EF-Tu, elongation factor Tu; EF-G, elongation factor G; EF-Ts, elongation factor Ts; aa-tRNA, aminoacyl transfer RNA; Nac-Phe-tRNA^{Phe}, *N*-acetyl-phenyl-tRNA^{Phe}; TCA, trichloroacetic acid; RNase A, ribonuclease A.

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corresponding to 10 pmol active), [^{3}H]NAc-Phe-tRNA^{Phe} (60 pmol), poly(U) (20 μg), GTP (0.1 μmol). The factor mix and the EF-Tu mixes were incubated separately at 37°C for 10 min in order to aminoacylate tRNA^{Phe} in the factor mix as well as to equilibrate labeled and unlabeled GDP on EF-Tu. The factor mix was then added to the EF-Tu mixes and incubated for 10 min at 37°C to form the ternary complex. The ribosome mix was also incubated for 10 min at 37°C to initiate the ribosomes. For the reaction at 0°C, ribosome and factor mixes were subsequently cooled to 0°C for 5 min. Samples from the factor mixes were withdrawn into cold TCA (5%) to measure charging levels and into cold HCOOH (20%) to measure the initial ratio of [^{3}H]GDP to [^{3}H]GTP. The reaction was started by adding equal volumes of the two mixes. At different times, samples were taken into 5% TCA to measure poly(Phe) synthesis and into 20% HCOOH for GTP hydrolysis measurements. Following TCA precipitation, the samples were boiled at 95°C to hydrolyze aa-tRNA and peptidyl-tRNA, filtered through glass fiber filters (GF/C, Whatman), dried and the radioactivity counted in toluene containing 0.5% PPO (Fluka A.G.), 0.0125% bisMSB (Beckman), and 10% Biolute-S (Zinsser Analytic GmbH), to measure the extent of poly(Phe) synthesis.

The samples taken into HCOOH were centrifuged at 15,000 rpm for 10 min to precipitate the proteins. 10 μl samples were withdrawn from the supernatants and applied to PEI plates (Schleicher and Schull). 1 μl of unlabeled GTP and GDP (10 mM each) was applied at every position to identify the spots under $UV_{254\ nm}$ light after chromatography in 0.5 M KH_2PO_4 buffer (pH 3.5). The spots were cut out and counted in toluene containing 0.5% PPO and 0.0125% bisMSB.

3. Results

The number of GTPs hydrolyzed on EF-Tu per peptide bond in poly(U) translation was measured at 37, 20 and 0°C. Our technique to separate the action of EF-Tu from that of EF-G [1,13] takes advantage of the slow exchange rate of GTP in the ternary complex: ^{3}H-labeled GTP is used for EF-Tu and a high concentration of unlabeled GTP is used to drive the cycle of EF-G. The experiments are performed in the absence of EF-Ts and the amount of GTP hydrolyzed is obtained by isotope dilution [1,13] (see Fig. 1b).

At 37°C there is 191 pmol of poly(Phe) synthesized during the burst phase of the experiment (intercept at y-axis in Fig. 1a). At the same time 416 pmol of GTP is hydrolyzed on EF-Tu (Fig. 1b, calculation explained in figure legend). From this it follows that there are 2.2 GTPs hydrolyzed per peptide bond (f_c = 416/191 = 2.2). After the burst phase, poly(Phe) synthesis continues at a slow rate C (C = 2.554 s^{-1} from the slope in Fig. 1a). C is determined by the amount of active EF-Tu (416 pmol), the rate of exchange of GDP from EF-Tu in the absence of EF-Ts (k_d), and by the number of EF-Tu cycles per peptide bond (f_c). According to Fig. 1b, $k_d = 1.27 \times 10^{-2}$ s^{-1}. From this f_c is 2.1 (f_c = EF-Tu × k_d/C = 416 × 0.0127/2.554 = 2.1). This means that the number of GTPs consumed per peptide bond is the same during the two phases of the experiment, in accordance with previous results [1].

At 0°C, 131 pmol of poly(Phe) is made during the burst phase (Fig. 2a) and there is a concomitant consumption of 248 pmol of GTP (Fig. 2b). Here f_c is 1.9, which is similar to the burst stoichiometry at 37°C (Fig. 1). A direct calculation of f_c from the slow phase, with C determined from the slope of the upper straight line in Fig. 2a, gives a lower f_c value. However, at low temperatures, the uncatalyzed, direct binding of aa-tRNA to the ribosome cannot be neglected (lower curve in Fig. 2a; the non-linearity of the curve at short times is caused by poor precipitability of short poly(Phe)-chains). When this background synthesis of poly(Phe) has been subtracted from the

Table 1
Number of GTPs hydrolyzed per peptide bond (f_c) at two different temperatures

Temperature (°C)	f_c (slow phase)	f_c (burst phase)
37	2.2	2.1
0	1.9	2.2

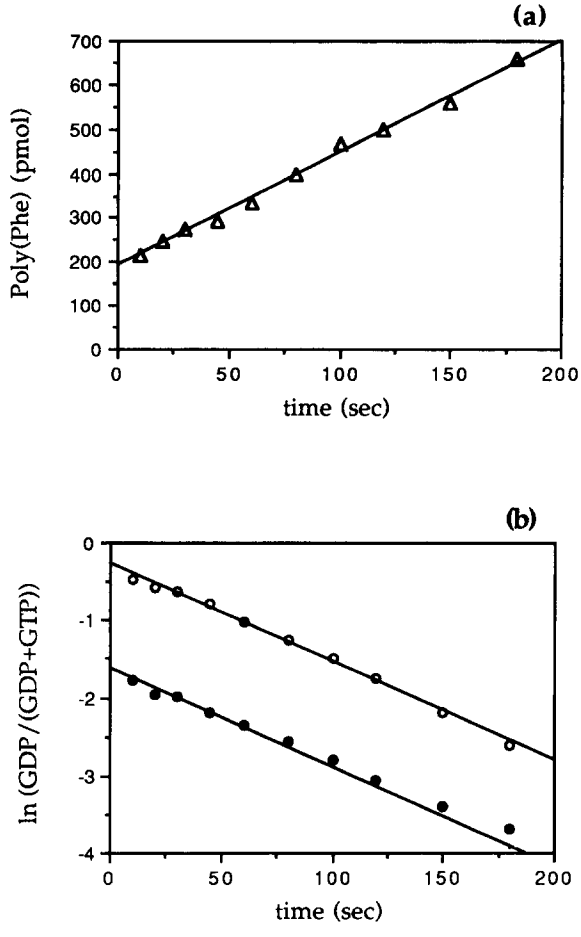

Fig. 1. (a) Poly(Phe) synthesis at 37°C. The intercept at the y-axis gives 191 pmol poly(Phe) made during the burst phase. The slope C of the curve is 2.554 pmol·s^{-1}. (b) Natural logarithm of the ratio (r) between [^{3}H]GDP and ([^{3}H]GDP + [^{3}H]GTP) at different reaction times without ($r_1(t)$; ○) and with ($r_2(t)$; ●) addition of 1578 pmol unlabeled GDP to the factor mix. The extra addition is made as GDP, and during preincubation all guanine nucleotide in the factor mix is turned into GTP by the energy pump (see section 2). GTP_0 and (GTP_0 + 1578), respectively, are the total amounts of guanine nucleotide without or with the extra addition in the factor mix. When translation starts, all [^{3}H]GTP on active EF-Tu is rapidly turned into [^{3}H]GDP, while free [^{3}H]GTP remains as GTP. With increasing incubation time, the [^{3}H]GDP on EF-Tu dissociates and is subsequently converted to [^{3}H]GTP by the energy pump. From the y-axis intercepts $\ln r_1(0) = -0.27$ and $\ln r_2(0) = -1.63$, we calculate that $r_1(0) = 0.762$ and that $r_2(0) = 0.196$. When x is the total amount of GTP on EF-Tu that is hydrolyzed to GDP during the burst phase of the incubation, then $x = r_1(0) \times GTP_0$ and $x = r_2(0) \times (GTP_0 + 1578)$, since this amount is the same for the two curves. From these expressions we get $GTP_0 = 1578/(r_1(0)/r_2(0)-1) = 545$ pmol and $x = 0.762 \times 545 = 416$ pmol. The slope of the line (dissociation rate k_d of GDP from EF-Tu in the absence of EF-Ts) is 1.27×10^{-2} s^{-1}. f_c = 416/191 = 2.18 (burst phase). f_c = $416 \times (1.27 \times 10^{-2}/2.554) = 2.07$ (slow phase).

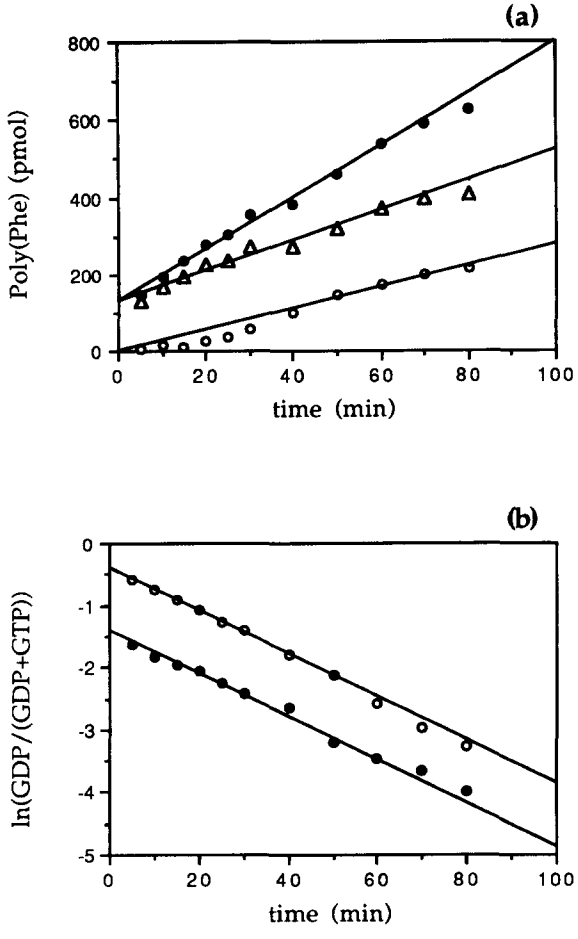

Fig. 2. (a) Poly(Phe) synthesis at 0°C. The intercept at the y-axis gives 131 pmol poly(Phe) synthesis during the burst phase. The upper curve (●) shows elongation in presence of EF-Tu. The lower curve (○) shows poly(Phe) synthesis in the absence of EF-Tu. Subtraction of this background from the upper curve gives the middle line (△), with slope $C = 3.89$ pmol·min^{-1}. (b) Natural logarithm of the ratio (r) between [^{3}H]GDP and ([^{3}H]GDP + [^{3}H]GDP) at different reaction times without (○) and with (●) addition of 635 pmol unlabeled GDP. The upper curve intercepts the y-axis at $\ln r_1(0) = -0.4$ ($r_1(0) = 0.67$) and the lower at $\ln r_2(0) = -1.4$ ($r_2(0) = 0.246$). The total amount of guanosine nucleotide in the factor mix is found to be 370 pmol, and the amount of GTP on EF-Tu that is hydrolyzed to GDP in the burst is 248 pmol. The calculations were as described in Fig. 1b. The slope of the line (k_d) is 3.46×10^{-2} min^{-1}. $f_c = 248/131 = 1.89$ (burst phase). $f_c = 248 \times (3.46 \times 10^{-2}/3.89) = 2.2$ (slow phase).

upper curve, the f_c value calculated from the corrected middle curve is 2.2 (Fig. 2a): at 0°C two GTPs are also consumed per peptide bond in the burst as well as in the slow phase of the experiment. The results of the experiments (Figs. 1 and 2) are summarized in Table 1.

Experiments at an intermediate temperature (20°C) give a similiar burst stoichiometry. In one experiment, 487 pmol GTP is hydrolyzed on EF-Tu leading to synthesis of 230 pmol poly(Phe) (not shown). Accordingly, f_c is 2.11 which is close to the results at 0 and 37°C (Table 1).

4. Discussion

Figs. 1 and 2 appear almost identical. The number of GTPs hydrolyzed per peptide bond during the burst phase is very close to two, both at 0 and 37°C. During the slow phase, when there is only a small amount of ternary complex with most of the EF-Tu trapped as EF-Tu·GDP, there are also two GTPs consumed per peptide bond at both temperatures. One difference is the time scale, which is about one hundred times longer at 0 than at 37°C (Figs. 1 and 2 and Table 1).

More important is that the similarity between the two figures is deceptive: there is a fundamental, mechanistic difference between the two cases. This is implied by the recent observation [10] that the stoichiometry of the ternary complex switches with temperature under conditions identical to those for the burst phase in Figs. 1 and 2. At 0°C one EF-Tu·GTP binds one Phe-tRNA^{Phe}, but at 37°C two EF-Tu·GTPs form an extended ternary complex with aa-tRNA [1,10,13].

For the higher temperature it is therefore natural to assume that aa-tRNA enters the A-site with two molecules of EF-Tu as well as of GTP [1]. However, this scheme appears less likely at the lower temperature.

Since all EF-Tu binds aa-tRNA [10] and since aa-tRNA is present in good excess (see section 2), there is almost no free EF-Tu·GTP in the experiments illustrated in Figs. 1 and 2. This strongly suggests the following scenario for what happens at 0°C: first, a ternary complex with 1:1 stoichiometry between EF-Tu and aa-tRNA arrives at the A-site. It waits for another ternary complex to come, and when this happens the first one 'borrows' an EF-Tu·GTP from the second. Subsequently, an extended ternary complex is formed at the A-site, which catalyzes the entry of aa-tRNA to the ribosome at the expense of two GTPs. We suggest, in other words, that the extended ternary complex is formed *on* the ribosome at low temperatures but *off* the ribosome at high temperature. This hypothesis is now being tested in our laboratory.

One intriguing question is how the two EF-Tu's interact with one aa-tRNA, either on or off the ribosome. One option is that the aa-tRNA is 'sandwiched' between the two elongation factors. Another, that one EF-Tu binds the aa-tRNA as well as the second EF-Tu. Structural data on the topographical location of aa-tRNA in the extended complex are now required to settle this question.

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