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Accelerated Publications

Stoichiometry for the Elongation Factor Tu•Aminoacyl-tRNA Complex Switches with Temperature†

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ABSTRACT: In bacterial protein synthesis binding of an aminoacyl-transferRNA (aa-tRNA) to the ribosomal acceptor site (A-site) is catalyzed by elongation factor Tu (EF-Tu). Two guanosine triphosphates (GTPs) are hydrolyzed on EF-Tu for every bound aa-tRNA. This was rationalized by the notion of an extended ternary complex, consisting of two EF-Tu•GTPs bound to a single aa-tRNA. In this work, we combine fast kinetics with RNase A protection experiments to measure the stoichiometry between EF-Tu•GTP and aa-tRNA at 37 °C, where the binding is weak. We find a 2:1 stoichiometry between EF-Tu•GTP and aa-tRNA at 37 °C, but at 0 °C, under otherwise similar conditions, the stoichiometry of the complex is close to 1:1. These results suggest alternative pathways for aa-tRNA binding to ribosomes, since two GTPs are hydrolyzed per peptide bond at both temperatures. At 37 °C, aa-tRNA enters the A-site in a pentameric complex with two EF-Tu's on which two GTPs are hydrolyzed in synchrony, when cognate codon−anticodon contact is established. This pentameric model also explains how two GTPs can be hydrolyzed on EF-Tu, without rejection of 50% of the cognate aa-tRNAs in proofreading. At 0 °C, in contrast, two ordinary ternary complexes may form a pentameric complex on, rather than off, the ribosome. When the two EF-Tu bound GTPs are hydrolyzed, one aa-tRNA enters the A-site, and the other dissociates to the free state.

According to text books, hydrolysis of two molecules of GTP is required for each amino acid incorporated during the elongation cycle of protein synthesis. One of the GTPs is bound to elongation factor Tu (EF-Tu) in a ternary complex with aminoacyl-tRNA which enters the ribosomal A- (acceptor) site. When the anticodon of aminoacyl-tRNA matches the mRNA codon, GTP is hydrolyzed on EF-Tu and peptide bond formation occurs after EF-Tu•GDP has left the ribosome. The second GTP hydrolysis is associated with elongation factor G (EF-G), which catalyzes translocation of peptidyl-tRNA from A- to P- (peptidyl) site after peptidyl transfer and hydrolyzes GTP during this process (Kaziro, 1978).

Two novel experimental findings have lead to a revison of this commonly accepted view: first, it was shown that there are two GTPs hydrolyzed on EF-Tu for each peptide bond (Ehrenberg et al., 1990). Second, it was demonstrated that two complexes of EF-Tu•GTP can bind to one molecule of aminoacyl-tRNA and form a pentameric complex (Ehrenberg et al., 1990; Tapio et al., 1990). With support from these observations it was suggested that aminoacyl-tRNA enters the ribosome as a pentameric complex together with two EF-Tu's and two GTPs. Subsequently, when aminoacyl-tRNA is delivered to the ribosome, two GTPs are hydrolyzed on two EF-Tu molecules (Ehrenberg et al., 1990).

The new pentameric model for aminoacyl-tRNA binding to the ribosome, followed by hydrolysis of two GTPs on

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EF-Tu, did not receive immediate acceptance. The proposed mechanism was at variance with some of the earlier measurements with partial translation systems, showing that one GTP is hydrolyzed on EF-Tu per peptide bond (Gordon, 1967; Miller & Weissbach, 1977; Thompson et al., 1977). In addition, 1:1 stoichiometry for the complex between EF-Tu•GTP and aminoacyl-tRNA was subsequently reported, apparently refuting the notion of a pentameric complex (Bensch et al., 1991). However, alongside these contradictory reports, supporting evidence for a 2:1 stoichiometry of GTP hydrolysis to peptide bond formation has also been given (Chinali & Parmeggiani, 1980; Thompson et al., 1981; Eccleston et al., 1985; Hornig et al., 1987). In addition, indirect support for higher order structures between EF-Tu molecules and aminoacyl-tRNA comes from *in vivo* observations of synergistic interactions between EF-Tu's from mutated genes (Vijgenboom et al., 1985; Vijgenboom & Bosch, 1989; Anborgh et al., 1991).

A more recent report from Weijland and Parmeggiani (1993), based on experiments with an altered EF-Tu, supported our original findings (Ehrenberg et al., 1990) that two GTPs are hydrolyzed on EF-Tu per peptide bond. By substituting Asn138 in EF-Tu with Asp, which changes the substrate specificity of EF-Tu from GTP to xantosine triphosphate (XTP), these authors demonstrated that two molecules of XTP are hydrolyzed on EF-Tu for each amino acid incorporated into poly(Phe). They suggested that the second EF-Tu may be used in a (proofreading) mechanism, where "two GTPase reactions taking place sequentially would allow the ribosome to build up a higher level of accuracy". They also proposed that a pentameric complex may form on rather than off the ribosome (Weijland & Parmeggiani, 1993).

To understand why two EF-Tu bound GTPs are required to bring one aminoacyl-tRNA to the A-site, confirmation or rejection of the hypothesis of an extended ternary complex is essential.

In the present work we give new experimental evidence that at growth-optimal physiological conditions (37 °C) two EF-Tu•GTP molecules bind one aminoacyl-tRNA. We also show that at low temperature (0 °C) the complex between EF-Tu•GTP and aminoacyl-tRNA has near 1:1 stoichiometry.

EXPERIMENTAL PROCEDURES

Purification of EF-Tu. EF-Tu was isolated from frozen *Escherichia coli* MRE 600 cells, harvested during logarithmic growth according to Ehrenberg et al. (1989). The isolation procedure includes one anion exchange chromatography (DEAE-Sepharose CL6B, Whatman) and two gel filtration chromatographies on ACA-44 (IBF). EF-Tu was homogeneous as judged by SDS−PAGE. The factor was extensively dialyzed against polymix buffer (Jelenc & Kurland, 1979) containing 10 μM GDP [Polymix buffer: 5 mM magnesium acetate, 0.5 mM calcium chloride, 95 mM potassium chloride, 5 mM ammonium chloride, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate (pH 7.3) and 1 mM 1,4-dithioerythritol] and stored at −80 °C.

Purification of tRNA^{Phe}. tRNA^{Phe} was purified from *E. coli* MRE 600 cells. tRNA^{bulk} was prepared by phenol extraction, NaCl extraction, and DEAE-cellulose chromatography (DE-52, Whatman). tRNA^{Phe} was purified from tRNA^{bulk} using Benzoylated-DEAE cellulose (Boehringer) according to Gillam et al. (1967) and by Sepharose 4B (Pharmacia) chromatography according to Holmes et al. (1975). tRNA^{Phe} was extensively dialyzed against polymix buffer and stored at −80 °C. tRNA^{Phe} purified this way can be aminoacylated to about 1100 pmol of Phe-tRNA^{Phe}/OD$_{260}$ units.

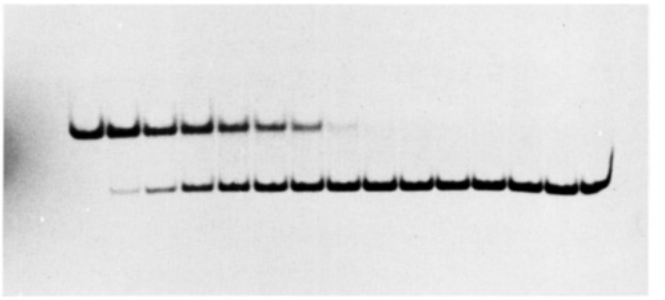

FIGURE 1: Formation of EF-Tu•GTP•aminoacyl-tRNA complex analyzed by an autoradiogram following nondenaturing polyacrylamide gel electrophoresis. In the presence of 200 pmol of ^{35}S-labeled EF-Tu, Phe-tRNA^{Phe} was varied (from left to right: 0, 20, 40, 60, 80, 90, 100, 120, 140, 160, 200, 250, 300, 400, and 600 pmol, respectively) under the conditions described in Experimental Procedures.

Other Enzymes. Phe-tRNA synthetase (PRS) was purified from *E. coli* MRE 600 cells according to Ehrenberg et al. (1989). RNase A (EC 3.1.27.5, type X-A) and myokinase (EC. 2.7.4.3) were purchased from Sigma. Pyruvate kinase (EC 2.7.1.40) was purchased from Boehringer.

Determination of EF-Tu Concentration. Four methods were used to determine EF-Tu concentration: Protein content of the EF-Tu solution was determined colorimetrically according to Bradford (1976) as well as by amino acid analysis of the EF-Tu hydrolyzate. Determination of EF-Tu concentration by [^{3}H]GDP binding to EF-Tu at varying EF-Tu concentrations (nitrocellulose filter assay) and by conversion of [^{3}H]GDP on EF-Tu to [^{3}H]GTP as a function of time (nucleotide exchange assay) have been described in detail by Ehrenberg et al. (1989). All four methods were in good agreement. This means that all EF-Tu, as measured from protein content, is capable of binding GDP and GTP. Nondenaturing gels have showed that all EF-Tu is fully active also in aminoacyl-tRNA binding (see Figure 1).

Nondenaturing Polyacrylamide Gel Electrophoresis (PAGE). EF-Tu•GTP•aminoacyl-tRNA complex was formed in polymix buffer (80 μL) containing 0.1 μmol of ATP, 1 μmol of PEP, 0.1 μmol of GTP, 30 nmol of [^{14}C]- or [^{3}H]Phe, 5 μg of pyruvate kinase, 0.3 μg of myokinase, 3 units of PRS (one unit aminoacylates one pmol of tRNA per second) and EF-Tu (as indicated). tRNA^{Phe} was added at varying concentrations (as indicated). Complex was formed during 15 min at 37 °C, during which time essentially all GDP (more than 99.99%) on EF-Tu is replaced by GTP. This is achieved by the energy regenerating system converting free GDP to GTP. tRNA^{Phe} is fully charged to Phe-tRNA^{Phe} in a short fraction of this time. After complex formation the samples were cooled and kept on ice. The extent of aminoacylation was determined by precipitating 50 μL of the samples in ice-cold 10% TCA and then filtering through glass-fiber filters (GF/C, Whatman). The filters were washed twice with 5 mL of 5% ice-cold TCA and then with 5 mL of ice-cold isopropanol, dried for 15 min at 105 °C, and counted in 5 mL of a toluene based scintillation coctail containing 10% tissue solubilizer (TS-1, Zinsser). For PAGE, 20 μL of the

samples was mixed with 2 μL of 50% glycerol (containing bromphenol blue) and applied to 5% gels. PAGE was for 2 h at 2 °C with a circulating electrophoresis buffer (50 mM Tris-HCl, pH 6.5, 10 mM magnesium acetate, 65 mM ammonium acetate, 1 mM Na-EDTA, 1 mM DTE, and 10 μM GTP).

RNase A Protection Experiments. Protection of aminoacyl-tRNA by EF-Tu from RNase A digestion was as described by Tanada et al. (1981). All reactions were in polymix buffer and contained (per 100 μL) 0.1 μmol of ATP, 1 μmol of PEP, 0.1 μmol of GTP, 30 nmol of [^{14}C]- or [^{3}H]-Phe, 5 μg of pyruvate kinase, 0.3 μg of myokinase, and 10 units of PRS. EF-Tu and tRNA^{Phe} were added as indicated in the figure legends. Complex formation was for 15 min at 37 °C. That all EF-Tu enters the ternary complex was verified by nondenaturing gel runs (above). Samples were either directly treated with RNase A or precooled to reaction temperatures if indicated. RNase A was in polymix buffer. The amount of RNase A used and reaction times are indicated in figure legends. The reactions were stopped by cold TCA precipitation and processed as described above to measure the extent of aminoacylation. Control experiments performed in the absence of EF-Tu showed that, under the conditions of our experiments, the amount of noncleaved aminoacyl-tRNA is negligible in relation to the amount protected by EF-Tu.

RESULTS

To assess the stoichiometry of the complex between EF-Tu•GTP and aminoacyl-tRNA, conditions that are relevant for living bacteria are of particular interest. This motivates stoichiometry determinations at 37 °C, where the ternary complex is relatively unstable, so that column and gel techniques are difficult to apply. We have chosen an RNase A protection assay (Tanada et al., 1981) in combination with quench-flow techniques to obtain high temperature data on the stoichiometry of the ternary complex.

For reliable stoichiometric titrations, precise measurements of the EF-Tu concentration (Experimental Procedures) as well as of the fraction of EF-Tu that is active in aminoacyl-tRNA binding are crucial. To determine the fraction of active EF-Tu, we developed nondenaturing gel methods as well as a Superdex 75 (Pharmacia) chromatography technique to separate ternary complex from free EF-Tu and aminoacyl-tRNA. Both techniques showed EF-Tu to be fully active in aminoacyl-tRNA binding (see Figure 1 for the gel result).

With these accurately determined concentrations of active EF-Tu, classical RNase protection experiments (Tanada et al., 1981) were first performed at 0 °C. When aminoacyl-tRNA was kept at a constant, high concentration and EF-Tu was varied, the amount of aminoacyl-tRNA protected from RNase digestion increased linearly with the amount of added EF-Tu•GTP until full protection was achieved at excess EF-Tu. From the slope of the straight line in the beginning of this titration, a 1:1 stoichiometry between EF-Tu and aminoacyl-tRNA was calculated (Figure 2).

RNAse A protection experiments were also performed at 37 °C, where a stoichiometry of two GTPs hydrolyzed on EF-Tu per formed peptide bond was originally reported (Ehrenberg et al., 1990; Tapio et al., 1990). Here, due to the rapid dissociation rate (0.2 s^{-1}) of aminoacyl-tRNA from

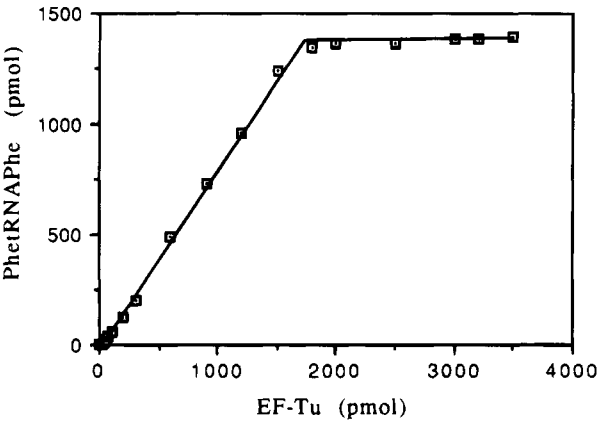

FIGURE 2: RNase A protection experiment at 0 °C at varying EF-Tu concentrations. Complex formation was as described in Experimental Procedures, and a sample contained, in 90 μL, 1390 pmol of Phe-tRNA^{Phe}. EF-Tu was varied from 0 to 3500 pmol. After 15 min at 37 °C, samples were placed on ice for 5 min to cool, and then 20 μg of RNase A was added in 10 μL of polymix buffer and the reaction proceeded for 15 s on ice. The extent of aminoacylation was determined as described in Experimental Procedures.

EF-Tu•GTP, the amount of aminoacyl-tRNA protected from RNase A decreases rapidly with time (Figure 3). Therefore, full time curves must be recorded for each concentration of EF-Tu, so that the amount of protected aminoacyl-tRNA can be determined by extrapolation to zero time from log plots. By this method, a stoichiometry of about two EF-Tu's to one aminoacyl-tRNA was measured at 37 °C (Figure 3).

Such extrapolations to zero time are ambiguous if the RNase A activity decreases with incubation time, due to increasing competition with free EF-Tu•GTP. In a control experiment the amount of protected aminoacyl-tRNA, estimated from the intercept at the *y*-axis, did not vary when the concentration of RNase A increased (Figure 4). This shows that, under our conditions, the RNase A concentration is sufficiently high to make negligible the probability that a free aminoacyl-tRNA rebinds an EF-Tu rather than becomes digested by RNase A.

The rapid dissociation of the complex at 37 °C makes long extrapolations back to zero time necessary, and this tends to reduce the precision of the estimated intercepts. Therefore, a rapid mixing quench-flow apparatus was used to follow the amount of protected aminoacyl-tRNA also at short incubation times. The same reaction mixes, with aminoacyl-tRNA in excess over EF-Tu, were used in two parallel experiments: one performed at 37 °C and the other at 0 °C. For the same amount of EF-Tu, about 2-fold more aminoacyl-tRNA was protected at 0 °C than at 37 °C. The calculated EF-Tu to aminoacyl-tRNA stoichiometries were 1.9 for 37 °C and 1.2 at 0 °C (Figure 5). Another control experiment demonstrated that over a broad range the high stoichiometry at 37 °C is independent of the concentrations of aminoacyl-tRNA and EF-Tu (data not shown). This shows that the results faithfully reflect the true stoichiometry of the complex and not weak binding between EF-Tu•GTP and aminoacyl-tRNA. That our measurements were performed under stoichiometric conditions is also indicated by previous estimates of the dissociation constant between aminoacyl-tRNA and EF-Tu•GTP (Tapio et al., 1990). This is about 10^{-7} M, which is far lower then the concentrations

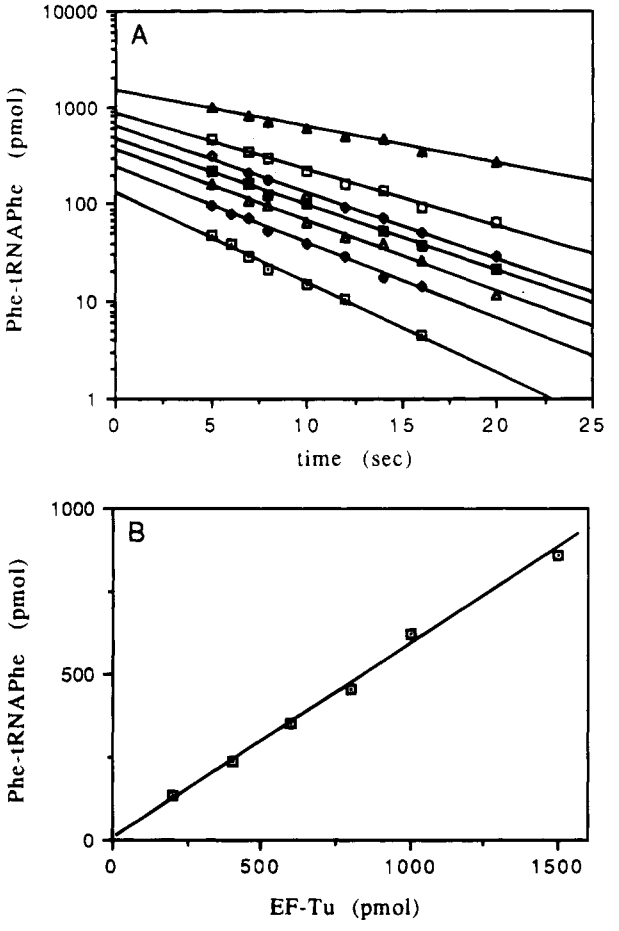

FIGURE 3: RNase A protection at 37 °C. (A). The amounts of Phe-tRNA^{Phe} protected after different times following the addition of RNase A are plotted for different EF-Tu concentrations: 200 (⊡), 400 (♦), 600 (△), 800 (■), 1000 (◇), 1500 (□), and 4000 (▲) pmol. The experimental conditions are as described in Experimental Procedures with 1500 pmol of Phe-tRNA^{Phe} present in the reaction mix. RNase A kept at 37 °C was added directly after ternary complex formation. (B). The *y*-intercepts from panel A are plotted against the corresponding EF-Tu concentrations. The slope of the straight line is used for stoichiometry determination.

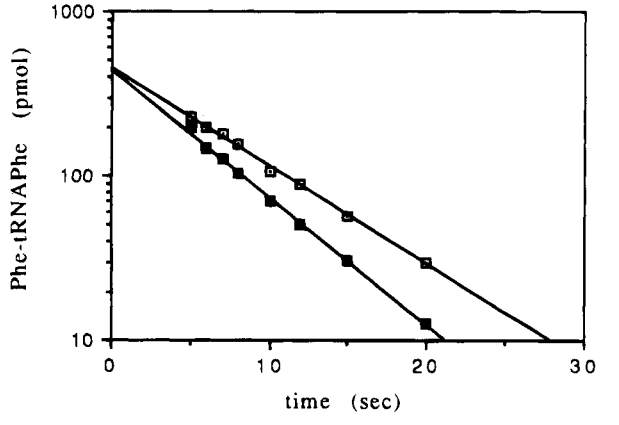

FIGURE 4: RNase A protection at 37 °C at different RNase A concentrations. 1500 pmol of Phe-tRNA^{Phe} and 800 pmol of EF-Tu were present (in 90 μL) under the reaction conditions described in Experimental Procedures. After 15 min at 37 °C, 10 μg (open symbols) or 50 μg (closed symbols) of RNase A was added in 10 μL, and the reaction was stopped at different times. The extent of aminoacylation in the samples was determined as described in Experimental Procedures.

of aminoacyl-tRNA (about 10^{-5} M) used in the present assays.

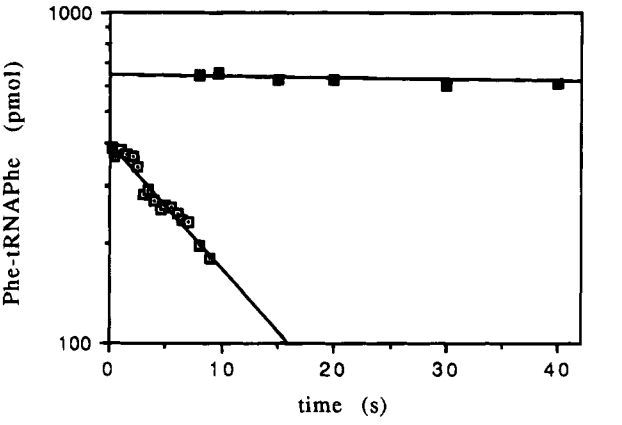

FIGURE 5: RNase A protection at 0 and 37 °C at short times. Ternary complexes were prepared as described in Experimental Procedures containing, per 100 μL, 800 pmol of EF-Tu and 1000 pmol of Phe-tRNA^{Phe}. After complex formation for 15 min at 37 °C, the samples were added to one of the reservoir syringes of the quench flow apparatus (Hi-Tech, Salisbury). The other reservoir syringe contained, per 100 μL, 50 μg of RNase A in polymix containing 1 mM ATP and 1 mM GTP. After rapidly mixing, the samples were quenched by 750 μL of 20% TCA after different incubation times at 37 °C (open symbols). Then, the reservoir syringes were cooled for the 0 °C reactions (closed symbols). The quenched samples were processed as described in Experimental Procedures to determine the extent of aminoacylation. Recovery of the quenched material is 96% as calculated from Phe-tRNA^{Phe} recovered in the absence of RNase A. This corresponds to 768 pmol of EF-Tu for each reaction. From the intercepts at the *y*-axis it is calculated that 640 pmol of Phe-tRNA^{Phe} at 0 °C, and 402 pmol of Phe-tRNA^{Phe} at 37 °C, is initially in complex with EF-Tu, corresponding to, respectively, 1.2 and 1.9 EF-Tu in complex with one Phe-tRNA^{Phe}.

DISCUSSION

We have used a combination of fast kinetics (quench-flow) and classical RNase A protection experiments to demonstrate that there is a pentameric complex at 37 °C, consisting of two molecules of EF-Tu•GTP and one aminoacyl-tRNA. These results confirm previous observations, obtained with other techniques (Ehrenberg et al., 1990; Tapio et al., 1990). With the same methods we have also established that at 0 °C the stoichiometry between EF-Tu•GTP and aminoacyl-tRNA is close to one to one, in accordance with findings by Bensch et al. (1991). This means that there is a change in the quaternary organization of the complex between EF-Tu•GTP and aminoacyl-tRNA, from a ternary complex at 0 °C to an extended ternary complex at 37 °C.

The present results strongly support the original suggestion (Ehrenberg et al., 1990) that at 37 °C aminoacyl-tRNA normally enters the ribosomal A-site in complex with two EF-Tu•GTPs. This would, in fact, explain in a natural way how two GTPs are hydrolyzed per peptide bond in EF-Tu function, at least at 37 °C (Ehrenberg et al., 1990; Bilgin et al., 1992). It would also explain how two XTPs are hydrolyzed per peptide bond in an altered EF-Tu, specifically binding XTP rather than GTP (Weijland & Parmeggiani, 1993).

Accordingly, the presence of a pentameric complex at 37 °C argues against the hypothesis that the reason two GTPs are hydrolyzed per peptide bond is because a cognate aminoacyl-tRNA has only a 50% probability of reaching peptidyl transfer due to agressive proofreading (Ehrenberg et al., 1990, 1994).

The notion of the extended ternary complex also rationalizes the finding that at 37 °C the two GTPs on EF-Tu seem to be hydrolyzed in synchrony, with a single rate constant of 100 s^{-1}, when a complex of EF-Tu•GTP and aminoacyl-tRNA interacts with the A-site (Bilgin et al., 1992). It is likely that sequential binding of two ordinary ternary complexes, where one aminoacyl-tRNA is discarded by proofreading or for other reasons, would lead to sequential, rather than synchronous, GTP-hydrolysis reactions.

The switch in stoichiometry of the ternary complex, from 1:1 at low to 2:1 at high temperature, raises the question of what the GTP to peptide bond ratio is at those low temperatures where the complex between EF-Tu and aminoacyl-tRNA has the "classical" 1:1 configuration. The answer is that also at 0 °C there are two GTPs hydrolyzed per peptide bond (Dinçbaş et al., 1994; Weijland et al., 1994). This implies that two EF-Tu•GTP cycles are used to bring one aminoacyl-tRNA to the A-site irrespective of the stoichiometry of the free ternary complex. We suggest, therefore, that an extended ternary complex is formed *on* the ribosome at low but *off* the ribosome at high temperature. This would mean that aminoacyl-tRNA enters the ribosome along different routes, depending on temperature.

With respect to the situation *in vivo*, laboratory strains of *E. coli* have their maximum growth rate at 37 °C, suggesting that the extended ternary complex is highly relevant for translation in living bacteria.

The present work does not explain *why* two molecules of EF-Tu are used for the apparently simple task of bringing one aminoacyl-tRNA to the A-site. It is easy to understand why one GTP is involved in the proofreading of tRNAs (Hopfield, 1974; Ninio, 1975). Its hydrolysis provides the thermodynamic driving force that prevents substrates from entering at the proofreading discard steps (Kurland, 1978; Ehrenberg & Blomberg, 1980; Blomberg et al., 1980). However, for the observed accuracy enhancement by proofreading (Ruusala et al., 1984; Bilgin et al., 1992; 1994), the driving force provided by one GTP is more than enough (cf. Blomberg et al., 1980).

The extension of ternary complexes by an extra EF-Tu increases the protein burden of growing bacteria. We propose that this additional cost is traded for a significant increase in their fitness and suggest that the extended ternary complex is used to increase the speed of protein elongation on ribosomes.

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