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GTP consumption of elongation factor Tu during translation of heteropolymeric mRNAs

(ribosomes/frameshift/protein synthesis/translocation/tRNA)

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ABSTRACT **The stoichiometry of elongation factor Tu (EF-Tu) and GTP in the complex with aminoacyl-tRNA and the consumption of GTP during peptide bond formation on the ribosome were studied in the *Escherichia coli* system. The ribosomes were programmed either with two different heteropolymeric mRNAs coding for Met-Phe-Thr-Ile... (mMFTI) or Met-Phe-Phe-Gly... (mMFFG) or with poly(U). The composition of the complex of EF-Tu, GTP, and Phe-tRNA^{Phe} was studied by gel chromatography. With equimolar amounts of factor and Phe-tRNA^{Phe}, a pentameric complex, (EF-Tu·GTP)$_2$·Phe-tRNA^{Phe}, was observed, whereas the classical ternary complex, EF-Tu·GTP·Phe-tRNA^{Phe}, was found only when Phe-tRNA^{Phe} was in excess. Upon binding of the purified pentameric complex to ribosomes carrying fMet-tRNA^{fMet} in the peptidyl site and exposing a Phe codon in the aminoacyl site, only one out of two GTPs of the pentameric complex was hydrolyzed per Phe-tRNA bound and peptide bond formed, regardless of the mRNA used. In the presence of EF-G, the stoichiometry of one GTP hydrolyzed per peptide bond formed was found on mMFTI when one or two elongation cycles were completed. In contrast, on mMFFG, which contains two contiguous Phe codons, UUU-UUC, two GTP molecules of the pentameric complex were hydrolyzed per Phe incorporated into dipeptide, whereas the incorporation of the second Phe to form tripeptide consumed only one GTP. Thus, generally one GTP is hydrolyzed by EF-Tu per aminoacyl-tRNA bound and peptide bond formed, and more than one GTP is hydrolyzed only when a particular mRNA sequence, such as a homopolymeric stretch, is translated. The role of the additional GTP hydrolysis is not known; it may be related to frameshifting of peptidyl-tRNA during translocation.**

In the classical model, elongation factor Tu (EF-Tu) completes one GTP hydrolysis cycle for every elongation cycle of the ribosome (1, 2). Early studies performed in partial translation systems lacking EF-G suggested that the number of GTP molecules hydrolyzed on EF-Tu per peptide bond was close to one (1, 3, 4). Also in complete translation systems, one GTP was reported to be hydrolyzed by EF-Tu per cognate peptide bond formation (5, 6). In contrast, Ehrenberg *et al.* (7, 8) and Bilgin *et al.* (9) developed a burst technique to separate the GTP hydrolysis by EF-Tu and by EF-G and showed that two molecules of GTP were hydrolyzed by EF-Tu per incorporated phenylalanine on poly(U)-programmed ribosomes. Recently, the hydrolysis of two nucleoside triphosphates per incorporated phenylalanine was also found by Weijland and Parmeggiani (10), who worked with mutant EF-Tu (D138N) that used XTP rather than GTP. This is another approach to avoid the problem inherent to complete protein synthesis systems—i.e., the GTPase activity of EF-G, which is strongly stimulated by ribosomes and not necessarily coupled to peptide bond formation and translocation. On the other hand, the mutation which changes the nucleotide specificity may change other properties of the factor as well.

The molecular basis for the observed 2:1 stoichiometry is found in the composition of the EF-Tu·GTP complex with aminoacyl-tRNA that was reported to be (EF-Tu·GTP)$_2$·Phe-tRNA ("pentameric complex") (7, 11). However, the issue is controversial, since the pentameric complex was not observed by others, albeit under different conditions (12).

As an alternative approach to selectively measure the GTP consumption by EF-Tu, we have prepared the complex of EF-Tu, GTP, and aminoacyl-tRNA under conditions where the pentameric complex is formed and have purified the complex by gel filtration. In this way the problem of the GTPase of EF-G is avoided, since radioactively labeled GTP is present only in the stable EF-Tu complex. Equally important for obtaining unambiguous stoichiometry data is the fact that the ribosomes we have used were fully active in all partial reactions of initiation and elongation and that ribosomal complexes were prepared by initiation on heteropolymeric mRNAs.

MATERIALS AND METHODS

Buffers and Reagents. Buffer A was 50 mM Tris·HCl, pH 7.5/70 mM NH_4Cl/30 mM KCl/7 mM $MgCl_2$/1 mM dithioerythritol/0.5 mM EDTA. Buffer B was 25 mM Tris·HCl, pH 7.5/90 mM NH_4Cl/30 mM KCl/8 mM $MgCl_2$/3 mM ATP/1 mM dithioerythritol/0.5 mM EDTA. Buffer C was 10 mM Tris·HCl, pH 7.5/60 mM NH_4Cl/5 mM magnesium acetate/0.25 mM EDTA/3 mM 2-mercaptoethanol. The experiments were performed at 20°C, if not stated otherwise.

Biochemicals were from Boehringer Mannheim. ^{3}H-labeled amino acids, [^{3}H]GTP, and [γ-^{32}P]GTP were from Amersham, and ^{14}C-labeled amino acids were from NYCOM (Prague, Czech Republic).

tRNAs, Ribosomes, and Factors. Ac[^{3}H]Phe-tRNA^{Phe} (2330 dpm/pmol, 1750 pmol/A_{260} unit) and [^{14}C]Phe-tRNA^{Phe} (790 dpm/pmol, 1500 pmol/A_{260} unit) were prepared from *Escherichia coli* tRNA^{Phe} (Subriden RNA) and purified as described (13). tRNA^{fMet} was a gift from V. Makhno (St. Petersburg Nuclear Physics Institute, Russia). f[^{3}H]Met-tRNA^{fMet} (1830 dpm/pmol, 1760 pmol/A_{260} unit) was prepared as described (14). tRNA^{Thr} was partially purified from *E. coli* tRNA by FPLC on a phenyl-Superose (Pharmacia) column with a gradient of 1.7 to 1 M $(NH_4)_2SO_4$ in 50 mM potassium phosphate/pH 7.0). For aminoacylation of tRNA^{Thr}, an S-200 extract (200,000 × *g*) purified on Whatman DE-52 was used. Charging of tRNA^{Thr} was 30%, and specific activity was 984 dpm of [^{14}C]Thr per pmol or 1120 dpm of [^{3}H]Thr per pmol. Pure EF-Tu·Thr-tRNA^{Thr} complex was obtained by gel filtration (see below).

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Abbreviations: EF, elongation factor; IF, initiation factor; A site, aminoacyl site.

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Tight-coupled 70S ribosomes were prepared as follows. Frozen *E. coli* MRE600 cells (50 g, wet weight) were opened by alumina grinding in 100 ml of 20 mM Tris·HCl, pH 7.5/100 mM NH_4Cl/10 mM magnesium acetate/0.5 mM EDTA/3 mM 2-mercaptoethanol containing DNase I (RNase-free; Boehringer Mannheim,) at 3 μg/ml. All operations were at 4°C. The S-30 fraction was layered in portions of 16 ml on 9 ml of 1.1 M sucrose in 20 mM Tris·HCl, pH 7.5/0.5 M NH_4Cl/10 mM magnesium acetate/0.5 mM EDTA/3 mM 2-mercaptoethanol. After centrifugation for 16 hr at 33,000 rpm in a Beckman Ti 50.2 rotor, the ribosomes were washed by dissolving the pellets in 200 ml of the same buffer, incubating for 2 hr, and sedimenting portions of 20 ml through 1.5 ml of 1.1 M sucrose in the same buffer (Ti 50.2, 6 hr, 45,000 rpm). The washing step was repeated twice, and the final centrifugation was made in a Beckman SW-28 rotor for 20 hr at 28,000 rpm. Pellets were resuspended in buffer C containing 5% sucrose. The 70S tight couples were isolated by zonal centrifugation in a Beckman Ti 15 rotor (17 hr, 28,000 rpm) on a convex exponential gradient from 10% to 37% sucrose (1.4 liters) in buffer C. The 70S peak was collected, and the ribosomes were pelleted (Ti 50.2, 20 hr, 45,000 rpm), resuspended in buffer A, frozen in small portions in liquid nitrogen, and stored at −80°C. Ribosome concentrations were determined from absorption measurements on the basis of 23 pmol/A_{260} unit. The activities of the ribosomes in all partial reactions of initiation and elongation were ≥90% (see *Results*).

EF-Tu was prepared from *E. coli* K-12 as described (15) with modifications (13). The activity of EF-Tu was >95% in binding [^{3}H]GDP as well as Phe-tRNA^{Phe}, as judged from the absence of any unbound factor in analytical Superdex 75 (Pharmacia) chromatography or on nondenaturing gel electrophoresis in the presence of excess Phe-tRNA^{Phe}. EF-G was prepared and assayed as described (15).

The initiation factors (IF1, -2, and -3) from *E. coli*, a gift from C. Gualerzi (University of Camerino, Italy) were homogeneous according to SDS/PAGE.

Nucleotide-Free EF-Tu. EF-Tu containing 26 μM GDP [determined by nucleotide exchange (15)] was incubated with 1.5 μCi (55.5 kBq) of [^{3}H]GDP for 15 min at 37°C to allow the equilibration of EF-Tu-bound GDP with ^{3}H-labeled GDP. Then GDP was converted to GTP by adding 1 mM ATP, 3 mM phospho*enol*pyruvate, and pyruvate kinase (0.1 μg/ml) and incubating for 15 min at 37°C. To dissociate GTP from EF-Tu, 7.5 mM EDTA was added, and the incubation was continued for 30 min. The nucleotide was separated from EF-Tu by gel chromatography on a NAP10 column (Pharmacia) in buffer A without magnesium. Individual fractions (0.4 ml) were monitored for tryptophan fluorescence, and [^{3}H]GTP was measured in each fraction. The main fraction of EF-Tu contained <1% GTP relative to protein and was used for further experiments.

mRNAs. Two different mRNAs, mMFTI and mMFFG, each about 120 nucleotides long with Shine–Dalgarno sequence (ribosome binding site) and coding sequence AUGUUUACGAUU... (Met-Phe-Thr-Ile...) or AUGUUUUUCGGC... (Met-Phe-Phe-Gly...), respectively, were used. The gene coding for mMFTI was inserted into the polylinker region of plasmid pTZ18R via *Eco*RI and *Hin*dIII sites as described (16); the plasmid (pXR022) was provided by C. Gualerzi. The plasmid bearing the sequence for mMFFG was prepared from pXR001 (16) by replacing the sequence between the *Acc* I and *Pst* I sites of the polylinker with the respective DNA fragment. The plasmids were linearized with *Hin*dIII and the respective mRNA was obtained by T7 RNA polymerase run-off transcription (16). The transcripts were purified by FPLC on Mono Q (Pharmacia).

Preparation of (EF-Tu·GTP)$_2$·Phe-tRNA^{Phe} Complex. EF-Tu (7 nmol) was preincubated for 10 min at 37°C with 0.2 mM [γ-^{32}P]GTP (400 dpm/pmol) in 0.08 ml of buffer A. tRNA^{Phe} (3 nmol) was charged in 0.15 ml of buffer B containing 0.1 mM L-[^{14}C]phenylalanine (800 dpm/pmol) and 0.5 unit of purified phenylalanyl-tRNA synthetase. After 30 min at 37°C, the mixture was added to EF-Tu·[γ-^{32}P]GTP and the incubation was continued for 5 min. The complex was purified by FPLC on Superdex 75 (Pharmacia; two HR 10/30 columns in tandem) in buffer A at 20°C. Fractions of 0.4 ml were collected, and 10-μl aliquots were taken for counting of radioactivity to determine the elution position of the complex. The complex was completely separated from unbound Phe-tRNA^{Phe}, EF-Tu, tRNA^{Phe}, and [γ-^{32}P]GTP. The final concentration of the complex (1.0–1.5 μM) was calculated on the basis of the radioactivity of [^{14}C]Phe-tRNA^{Phe}. For the nonlabeled complexes, the concentration was determined by measuring tryptophan fluorescence calibrated with a solution of complex at known concentration.

The complex (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Thr-tRNA^{Thr} was prepared analogously, except that [^{14}C]Thr-tRNA^{Thr} was used at a lower concentration; the final concentration of the complex after purification was 0.3–0.5 μM. Analytical experiments were performed in the same way, except that the amounts of EF-Tu and Phe-tRNA were smaller, as indicated.

Initiation Complex Formation and Elongation Assays. To prepare the initiation complex, 10 pmol of 70S ribosomes was incubated with 15 pmol each of IF1, IF2, IF3, and f[^{3}H]Met-tRNA^{fMet}, 30–50 pmol of mRNA, and 1 mM GTP in 20 μl of buffer A for 30 min at 20°C. When the formation of the dipeptide fMet-Phe was to be measured, the preformed initiation complex was mixed with purified (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Phe-tRNA^{Phe} complex as indicated and incubated for 10 sec. This incubation time was fully sufficient for the completion of aminoacyl (A)-site binding and peptide bond formation. For the synthesis of the tripeptide fMet-Phe-Phe, 10 pmol of EF-G was added to the initiation complex prior to the addition of the EF-Tu complex. To get the tripeptide fMet-Phe-Thr, the complexes EF-Tu·GTP·Phe-tRNA^{Phe} and EF-Tu·GTP·Thr-tRNA^{Thr} were added together with EF-G.

For peptide analysis, the reaction was stopped in aliquots by the addition of 1 M KOH, and the aliquots were incubated for 30 min at 37°C and neutralized with acetic acid. The peptides fMet-Phe, fMet-Phe-Thr, fMet-Phe-Phe, and AcPhe-Phe were separated by HPLC on RP8 (Merck) with an acetonitrile gradient in 0.1% trifluoroacetic acid. ^{3}H and ^{14}C radioactivity was measured in a Packard 2500 scintillation counter using a double label program. To measure the amount of f[^{3}H]Met-, or [^{14}C]aminoacyl-, or peptidyl-tRNA bound to the ribosome, the samples were rapidly filtrated through nitrocellulose filters (Sartorius) without prior dilution, and the filters were washed extensively with 30 ml of buffer A. Before scintillation counting, the filters were dissolved in QS361 (Zinsser, Frankfurt).

To measure [γ-^{32}P]GTP hydrolysis, the reaction was stopped in parallel aliquots by adding an equal volume of 1 M $HClO_4$/3 mM potassium phosphate; $^{32}P_i$ was extracted (17) and ^{32}P radioactivity was measured by scintillation counting.

RESULTS

Stoichiometry of EF-Tu·GTP Binding to Aminoacyl-tRNA. Complexes were formed at fixed concentrations of EF-Tu and [γ-^{32}P]GTP and various concentrations of [^{14}C]Phe-tRNA^{Phe} and studied. In the presence of EF-Tu in 2-fold excess the stoichiometry of EF-Tu·[γ-^{32}P]GTP to [^{14}C]Phe-tRNA^{Phe} was 2:1 (Fig. 1*A*). This indicates the presence of the pentameric complex (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Phe-tRNA^{Phe}, since any unbound EF-Tu·[γ-^{32}P]GTP would be separated from the complex (Fig. 1*D*). Also at a 1:1 ratio of EF-Tu to aminoacyl-tRNA in the incubation mixture, a pentameric complex was formed (Fig. 1*B*), and only at a 2.5-fold excess of [^{14}C]Phe-tRNA^{Phe} was formation of the ternary complex EF-Tu·[γ-^{32}P]GTP·[^{14}C]Phe-tRNA^{Phe} observed (Fig. 1*C*). These data clearly show that either pentameric or ternary complexes can be formed, depending on the ratio of EF-Tu to aminoacyl-tRNA in the incubation mixture.

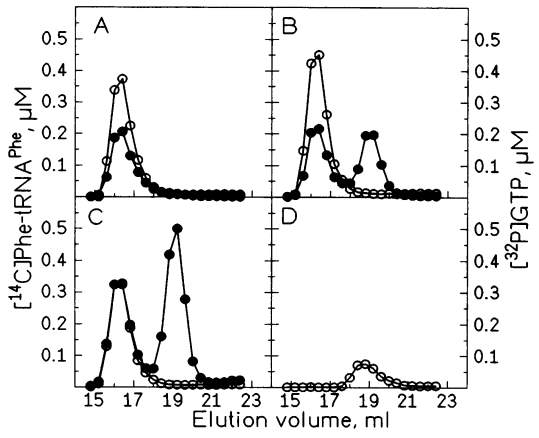

FIG. 1. Composition of the EF-Tu·GTP complex with Phe-tRNA^{Phe} as determined by gel filtration on Superdex 75. Nucleotide-free EF-Tu (5 μM) was incubated with [γ-^{32}P]GTP (50 μM) in buffer A for 20 min at 20°C. Then the complex was incubated for 5 min at 20°C with [^{14}C]Phe-tRNA^{Phe} at 2.5 μM (EF-Tu/tRNA ratio of 1:0.5) (*A*), 5 μM (1:1) (*B*), or 12.5 μM (1:2.5) (*C*) or in the absence of aminoacyl-tRNA (*D*). ●, ^{14}C; ○, ^{32}P.

It is apparent from Fig. 1 that Superdex 75 chromatography allows the isolation of complexes of defined composition which are sufficiently stable to be used to measure the stoichiometry of GTP hydrolysis by EF-Tu on the ribosome. The exchange of labeled GTP from the complex was found to be slower than 10^{-3} sec^{-1} under the conditions used for the following experiments (data not shown).

One GTP Is Hydrolyzed upon A-Site Binding of Pentameric Complex. To determine how many molecules of GTP were hydrolyzed for one aminoacyl-tRNA bound to the A site, we used the purified pentameric complex (EF-Tu·GTP)$_2$·Phe-tRNA^{Phe} (Fig. 2*A*). Ribosomes were programmed with the heteropolymeric mRNAs mMFTI (Fig. 2*B*) or mMFFG (Fig. 2*C*) in the presence of IFs and f[^{3}H]Met-tRNA^{fMet}. The incubation time was kept at 10 sec to minimize the contribution of GTP exchange from the complex and of the intrinsic GTPase of EF-Tu; blank values of GTP hydrolyzed in the absence of ribosomes (2–6% of added EF-Tu complex) were subtracted. In the absence of either initiation factors or mRNA, essentially no tRNA binding was found (<0.1 pmol per pmol of ribosomes). It is apparent from Fig. 2 *B* and *C* that the ribosomes used were fully active with respect to binding of f[^{3}H]Met-tRNA^{fMet}, binding of aminoacyl-tRNA from the EF-Tu complex, and peptide bond formation, as monitored by nitrocellulose filtration and analysis of fMet-Phe dipeptides. Clearly, the reaction resulted in the hydrolysis of one GTP molecule (out of two available in the pentameric complex), on both mMFTI and MFFG mRNAs. Subsequent translocation induced by the addition of EF-G did not change the GTP stoichiometry (Fig. 2*B Inset*).

The titration of a fixed amount of the EF-Tu complex with various amounts of the ribosome complex also revealed that the EF-Tu complex was fully active, in that all aminoacyl-tRNA present in the complex was bound to the A site and yielded dipeptide and that the stoichiometry of GTP hydrolysis relative to aminoacyl-tRNA binding or peptide formation did not exceed 1:1 (data not shown).

Since the results obtained with the heteropolymeric mRNAs were in obvious discrepancy with the results of both Ehrenberg *et al.* (7–9) and Weijland and Parmeggiani (10), who worked with poly(U)-programmed ribosomes, we also performed binding experiments with poly(U). For experiments with the full initiation system, we took advantage of the possibility that an initiation complex can be formed on poly(U) using AcPhe-tRNA^{Phe} and initiation factors (18). The ribosomal complexes

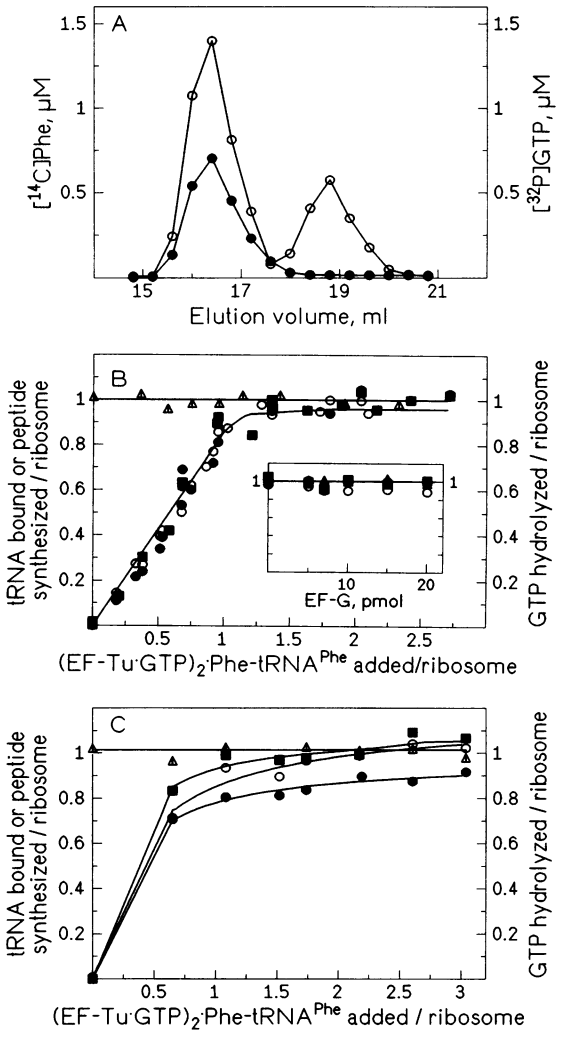

FIG. 2. Stoichiometry of GTP hydrolysis upon A-site binding of pentameric complex. (*A*) Isolation of the (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Phe-tRNA^{Phe} complex by FPLC on Superdex 75. ●, ^{14}C; ○, ^{32}P. (*B*) A-site binding on mMFTI mRNA. Ribosomes were programmed with mRNA in the presence of IFs and f[^{3}H]Met-tRNA^{fMet}. The final incubation mixtures (60 μl) contained 10 pmol of initiation complex and various amounts of (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Phe-tRNA^{Phe} complex. After 10 sec at 20°C, the incubation mixtures were filtrated through nitrocellulose filters or the reaction was stopped by addition of $HClO_4$ or KOH. (*Inset*) Effect of EF-G on the stoichiometry of GTP hydrolysis. The incubation mixtures (80 μl, buffer A) contained 10 pmol of initiation complex programmed with mMFTI, 20 pmol of (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Phe-tRNA^{Phe}, and various amounts of EF-G. (*C*) A-site binding on mMFFG mRNA. The experiments were performed as described under *B*. The amounts of [γ-^{32}P]GTP hydrolyzed (■), f[^{3}H]Met-[^{14}C]Phe formed (●), and of f[^{3}H]Met-tRNA^{fMet} (△) or [^{14}C]Phe in f[^{3}H]Met-[^{14}C]Phe-tRNA^{Phe} (○) bound per ribosome were determined as described in *Materials and Methods*.

were formed with poly(U) instead of heteropolymeric mRNA, and the incubation mixture either contained the initiation system (Fig. 3*A*), or was prepared without IFs and GTP (Fig. 3*B*). In the latter case, Ac[^{3}H]Phe-tRNA^{Phe} was preincubated with ribosomes and poly(U) at 15 mM $MgCl_2$, and subsequently the Mg^{2+} concentration was adjusted to 7 mM immediately before the addition of the EF-Tu complex. Only one molecule of GTP was hydrolyzed per Phe-tRNA^{Phe} bound or dipeptide formed, independent of the presence or absence of external GTP or the components of the initiation system (Fig. 3).

Tripeptide Formation Does Not Increase GTP Consumption per Peptide Bond. The experiments on A-site binding and

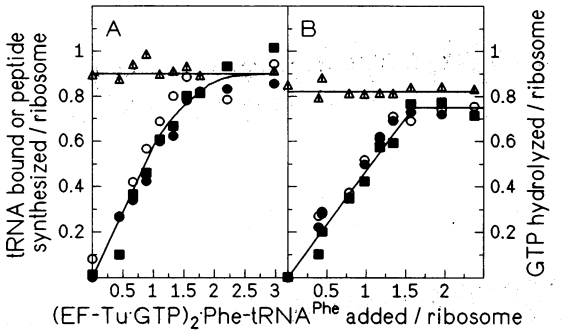

FIG. 3. Stoichiometry of GTP hydrolysis upon A-site binding of pentameric complex on poly(U)-programmed ribosomes with (*A*) or without (*B*) IFs. Ribosomes were programmed with poly(U) in the presence of IFs, GTP and Ac[^{3}H]Phe-tRNA^{Phe} in buffer A (*A*), or ribosomes were preincubated with poly(U) and Ac[^{3}H]Phe-tRNA^{Phe} at 15 mM $MgCl_2$ for 15 min at 20°C, and then the incubation mixture was diluted to 7 mM $MgCl_2$ with buffer A without $MgCl_2$ (*B*). In both experiments, the final mixtures (60 μl) contained 10 pmol of ribosome complex and various amounts of purified (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Phe-tRNA^{Phe}. Incubation was for 10 sec at 20°C. [γ-^{32}P]GTP hydrolysis (■), and Ac[^{3}H]Phe-[^{14}C]Phe (●), Ac[^{3}H]Phe-tRNA^{Phe} (△), and [^{14}C]Phe in peptidyl-tRNA (○) bound per ribosome were measured.

translocation clearly showed the consumption of only one GTP (of two available in the pentameric complex) per dipeptide formed. To verify whether this was also the case for one more round of elongation, we determined the GTP consumption during the synthesis of the tripeptide fMet-Phe-Thr on mMFTI mRNA. First, the complexes (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Phe-tRNA^{Phe} and (EF-Tu·GTP)$_2$·[^{3}H]Thr-tRNA^{Thr} were prepared, and the hydrolysis of GTP from the first EF-Tu complex was studied upon addition of increasing amounts of the second. For these experiments, nonlabeled fMet-tRNA^{fMet} was used to form the initiation complex. No additional GTP hydrolysis in (EF-Tu·[γ-^{32}P]GTP)$_2$·Phe-tRNA^{Phe} was observed upon binding of [^{3}H]Thr-tRNA^{Thr} and formation of the tripeptide fMet-[^{14}C]Phe-[^{3}H]Thr (Fig. 4*A*).

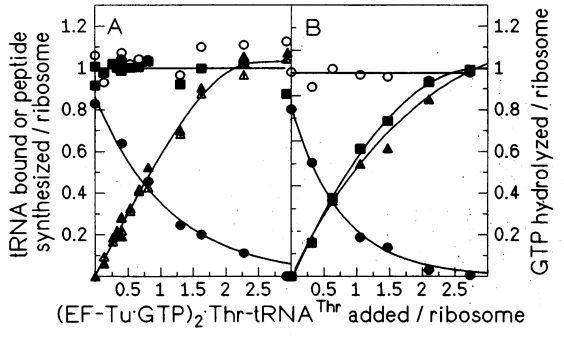

FIG. 4. Stoichiometry of GTP hydrolysis during synthesis of the tripeptide fMet-Phe-Thr. (*A*) The incubation mixture (125 μl) contained 10 pmol of initiation complex programmed with mMFTI in the presence of nonlabeled fMet-tRNA^{fMet}, 10 pmol of EF-G, 22.6 pmol of (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Phe-tRNA^{Phe}, and various amounts of (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{3}H]Thr-tRNA^{Thr}. The reaction was initiated by the addition of the premixed EF-Tu complexes to the mixture of ribosomes and EF-G. Incubation was for 10 sec at 20°C. [γ-^{32}P]GTP hydrolysis (■) and fMet[^{14}C]-Phe (●), fMet[^{14}C]Phe-[^{3}H]Thr (▲), and [^{14}C]Phe (○) or [^{3}H]Thr (△) in peptidyl-tRNA bound per ribosome were measured. (*B*) The incubation mixture (125 μl) contained 10 pmol of initiation complex programmed with mMFTI and f[^{3}H]Met-tRNA^{fMet}, 10 pmol of EF-G, 23.5 pmol of (EF-Tu·GTP)$_2$·Phe-tRNA^{Phe}, and various amounts of (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Thr-tRNA^{Thr}. The reaction was initiated by the addition of the two EF-Tu complexes. Incubation was for 10 sec at 20°C. [γ-^{32}P]GTP hydrolysis (■) and f[^{3}H]Met-Phe (●), f[^{3}H]Met-Phe-[^{14}C]Thr (▲), and f[^{3}H]Met-tRNA^{fMet} (○) bound per ribosome were measured.

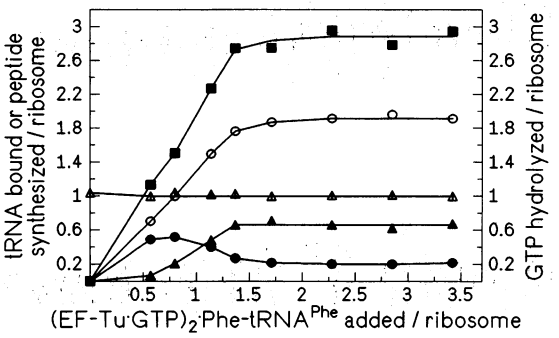

FIG. 5. Stoichiometry of GTP hydrolysis during synthesis of the dipeptide fMet-Phe and the tripeptide fMet-Phe-Phe in the presence of EF-G. The reaction mixture (60 μl) contained 10 pmol of initiation complex programmed with mMFFG and f[^{3}H]Met-tRNA^{fMet}, 10 pmol of EF-G, and various amounts of (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Phe-tRNA^{Phe} complex. Incubation was for 10 sec at 20°C. [γ-^{32}P]GTP hydrolysis (■) at f[^{3}H]Met-[^{14}C]Phe (●), f[^{3}H]Met-[^{14}C]Phe-[^{14}C]Phe (▲), f[^{3}H]Met-tRNA^{fMet} (△), and [^{14}C]Phe in peptidyl-tRNA (○) bound per ribosome were measured.

In further experiments, we studied the hydrolysis of [γ-^{32}P]GTP upon binding of (EF-Tu·[γ-^{32}P]GTP)$_2$·[^{14}C]Thr-tRNA^{Thr} to ribosomes programmed with mMPTI and carrying f[^{3}H]Met-Phe-tRNA^{Phe} in the peptidyl (P) site from a previous round of A-site binding, peptide bond formation, and translocation (Fig. 4*B*). Again, one GTP was hydrolyzed for every molecule of Thr-tRNA^{Thr} bound or fMet-Phe-Thr formed. Thus, neither translocation nor a second round of elongation changed the 1:1 stoichiometry of GTP hydrolysis on EF-Tu.

Two GTPs per Peptide Bond Are Hydrolyzed When a Stretch of Uridines Is Translated. The consumption of GTP was further tested in the translation of mMFFG, which carries two contiguous Phe codons, UUU-UUC, following the initiation codon. As shown above (Fig. 2*C*), without EF-G one molecule of Phe-tRNA^{Phe} was bound to the ribosome, the dipeptide fMet-Phe was formed, and one molecule of GTP was hydrolyzed. In the presence of EF-G, the hydrolysis of [^{32}P]GTP on EF-Tu exceeded the 1:1 stoichiometry during synthesis of the dipeptide (Fig. 5). A 2:1 stoichiometry was clearly seen at the beginning of the titration when ribosomes were in excess over EF-Tu complex and mostly the dipeptide fMet-Phe was formed, whereas only a small amount of the tripeptide fMet-Phe-Phe was formed. Hence, on mMFFG the addition of EF-G—i.e., the translocation of the dipeptidyl-tRNA—introduced the hydrolysis of the second GTP from the pentameric complex.

After translocation, the formation of tripeptide by incorporation of the second phenylalanine gave rise to the hydrolysis of only one GTP, following from the finding that at saturation 2.9 mol of GTP were hydrolyzed per 1.9 mol of phenylalanine found in ribosome-bound di- and tripeptidyl-tRNA; i.e., on the average the ratio was 1.5 (Fig. 5). Compared with the filtration data, on HPLC somewhat less [^{14}C]Phe was found in dipeptide plus tripeptide, probably due to an incomplete recovery of the tripeptide fMet-Phe-Phe. Thus, the determination of the GTP stoichiometry for tripeptide formation in the experiment of Fig. 5 is based on the data from the binding assay.

DISCUSSION

Both Pentameric and Ternary EF-Tu·Aminoacyl-tRNA Complexes Can Be Formed, Depending on the Conditions. The finding of a 2:1 complex of EF-Tu·GTP and Phe-tRNA (7), which challenged the classic, seemingly well-established concept of the ternary complex (19), was not generally accepted. According to our present results, obtained by gel filtration, the pentameric complex, (EF-Tu·GTP)$_2$·Phe-tRNA^{Phe}, at least at

20°C, was formed predominantly at ratios of factor to tRNA down to 1:1. The ternary complex, EF-Tu·GTP·Phe-tRNA^{Phe}, was observed only when Phe-tRNA^{Phe} was in two-fold excess over EF-Tu.

Although the ternary complex is functional in ribosome binding of Phe-tRNA^{Phe} followed by peptide bond formation (data not shown), EF-Tu titrations of Phe-tRNA^{Phe} binding to the ribosome are significantly sigmoidal (7), also under our conditions (data not shown). These results and the results of the titrations on the gel filtration column suggest that the pentameric complex is formed by binding of EF-Tu·GTP to the preexisting ternary complex and that it is functional on the ribosome. This is also suggested by the observation that, under certain conditions, both GTP molecules of the complex are hydrolyzed on the ribosome.

Binding of the Pentameric Complex to the A Site Is Accompanied by the Hydrolysis of One GTP. Although it is the pentameric complex, (EF-Tu·GTP)$_2$·Phe-tRNA^{Phe}, that is binding to the ribosome, in no case have we observed the hydrolysis of more than one GTP per molecule of Phe-tRNA bound to the A site and forming a dipeptide. Regardless of the mRNA used—mMFTI, mMFFG, or poly(U)—A-site binding and peptide bond formation was accompanied by the hydrolysis of strictly one GTP. This result is at variance with the results of Bilgin *et al.* (9) and of Weijland and Parmeggiani (10). At present, there is no entirely consistent explanation for the discrepancy. Since the ribosome system we are using is fully active in all partial reactions, we are confident that the 1:1 stoichiometry we observe is the correct one.

The Presence of EF-G Does Not Generally Increase GTP Hydrolysis on EF-Tu, Except on a Stretch of Uridines. The completion of the elongation cycle by EF-G-dependent translocation does not increase the GTP consumption by EF-Tu, as long as heteropolymeric mRNA sequences are translated. We conclude that generally only one out of the two GTPs in the pentameric complex is hydrolyzed during one full elongation cycle. However, both GTPs of the pentameric complex are hydrolyzed upon incorporation of the first Phe when adjacent Phe codons, UUU-UUC, are translated in the presence of EF-G. This result is in keeping with the 2:1 stoichiometry obtained when poly(U) is translated (7, 10). It is likely that the hydrolysis of the additional GTP is due to the translation of the stretch of uridines.

The present results show that (*i*) the hydrolysis of two molecules of GTP per amino acid incorporated is the exception rather than the rule and (*ii*) the hydrolysis, when it occurs on a homopolymeric stretch of the mRNA, requires the action of EF-G; i.e., it is related to the translocation that follows A-site binding and peptide bond formation. Thus, the hydrolysis of the second GTP is not likely to be involved in the mechanism of A-site binding or in controlling the accuracy of aminoacyl-tRNA selection, as proposed previously (20).

Potential Role of EF-Tu in Controlling the Reading Frame. The molecular mechanism that controls the reading frame during translation is not known (21). There is evidence suggesting that EF-Tu has a role in frameshift suppression (22) and, furthermore, that mutations in both EF-Tu genes of *E. coli, tufA* and *tufB*, result in enhanced translational frameshifts, indicating that a cooperation of two EF-Tu molecules may be involved in reading-frame control (23). A consistent—though at present speculative—explanation of our results would be that mRNA sequences prone to +1 frameshifts favor aberrant translocation and that the second EF-Tu·GTP restores the correct frame in an as yet unknown way.

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