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Peptidyl-tRNA Regulates the GTPase Activity of Translation Factors

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Summary

Rapid protein synthesis in bacteria requires the G proteins IF2, EF-Tu, EF-G, and RF3. These factors catalyze all major steps of mRNA translation in a GTP-dependent manner. Here, it is shown how the position of peptidyl-tRNA in the ribosome and presence of its peptide control the binding and GTPase activity of these translation factors. The results explain how idling GTPase activity and negative interference between different translation factors are avoided and suggest that hybrid sites for tRNA on the ribosome play essential roles in translocation of tRNAs, recycling of class 1 release factors by RF3, and recycling of ribosomes back to a new round of initiation. We also propose a model for translocation of tRNAs in two separate steps, which clarifies the roles of EF-G·GTP and GTP hydrolysis in this process.

Introduction

There is a G protein involved in each of the four major steps in bacterial protein synthesis. These GTPases belong to a large class of G proteins that switch between active GTP and inactive GDP forms and participate in diverse cellular processes (Bourne et al., 1991). In eubacteria, the four GTPases are initiation factor IF2, elongation factor EF-Tu, elongation factor EF-G, and peptide release factor RF3. Their GTPs are hydrolyzed when the factors contact the ribosomal GTPase center (see Ramakrishnan, 2002). IF2·GTP strengthens the binding of fMet-tRNA^{fMet} to the 30S subunit (Laalami et al., 1991) and association of the two ribosomal subunits (A. Antoun, submitted). EF-Tu·GTP forms a "ternary complex" with aminoacyl-tRNA, brings it rapidly to the A site of the ribosome and subsequently dissociates from the ribosome in the GDP form. The GTPase activity of EF-Tu is also used to enhance the accuracy of codon recognition by proofreading (Thompson and Stone, 1977; Rusala et al., 1982). Translocation of peptidyl-tRNA from the A to the P site and deacylated tRNA from the P to the E site is catalyzed by EF-G. According to the "classical model," EF-G in the GTP form drives the translocation movement and then GTP hydrolysis allows for rapid dissociation of EF-G (Inoue-Yokosawa et al., 1974). Recently, it was suggested that GTP hydrolysis precedes translocation (Rodnina et al., 1997). In termination of protein synthesis, a class 1 release factor (RF1 or RF2) induces release of the peptide from the P-site bound tRNA and is then recycled by RF3 in a GTP-dependent manner (Zavialov et al., 2001, 2002). Finally, recycling of ribosomes from their posttermination state to a new round of initiation is catalyzed by EF-G and ribosome-recycling factor RRF (Karimi et al., 1999; Hirokawa et al., 2002).

All four GTPases in bacterial protein synthesis share essentially the same binding pocket on the ribosome (Cameron et al., 2002). One would therefore expect the existence of a control system that makes the binding and action of each G protein happen at the correct moment in protein synthesis and that minimizes idling GTPase activities.

In line with this anticipation, it was recently shown that the peptide on the P-site bound tRNA regulates the GTPase activity of peptide release factor RF3 (Zavialov et al., 2002). A class 1 release factor in the ribosomal A site is the guanine nucleotide exchange factor (GEF) for RF3 as it enters the ribosome in the GDP form. When there is a peptidyl-tRNA in the P site, the GDP to GDP, but not the GDP to GTP, exchange is allowed. Release of the peptide leads to fast GDP to GTP exchange on RF3 and rapid removal of the class 1 release factor from the ribosome. It was postulated that peptide release allows for a structural change of the ribosome from a state with low to one with high affinity for RF3·GTP (Zavialov et al., 2002).

In this work, we show how the activities of the four G proteins in bacterial protein synthesis depend on the positioning of tRNAs on the ribosome and whether these tRNAs carry a peptide.

Our results suggest interpretations of several major steps in bacterial protein synthesis that place hybrid sites for tRNAs in the foreground, as anticipated by Bretscher (1968). The action of RF3 and the actions of EF-G in both translocation and recycling of ribosomes are all controlled by the peptide on the P-site tRNA; its removal allows for a change in the relative position of the ribosomal subunits that we propose is essential for these three major functions. From our data we propose, in particular, that translocation of tRNAs takes place in two distinct steps; the first is driven by EF-G in the GTP form and the second by EF-G in the GDP form. Cryo-EM observations reinforce our interpretations and reveal how the same relative motion of the ribosomal subunits can play essential roles in termination, translocation, and ribosomal recycling (Valle et al., 2003 [this issue of *Cell*]).

Results

Ribosome-Dependent GTPase Activities of IF2, EF-G, and RF3 Are Controlled by the Peptide on the tRNA in the P Site

To study how the peptide on the tRNA in the ribosomal P site affects the GTPase activities of initiation factor IF2, elongation factor EF-G, and peptide release factor

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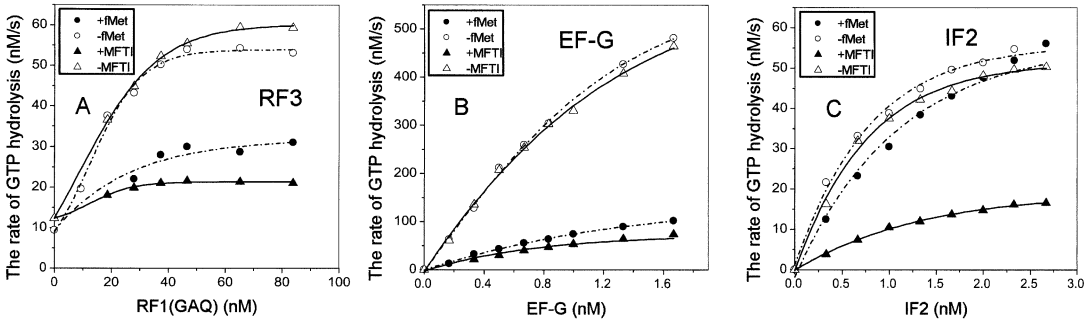

Figure 1. Ribosome Dependent GTPase Activity of Translation Factors Is Regulated by the Absence or Presence of a Tetrapeptide (MFTI) or fMet on the P-Site Bound tRNA

(A) The rate of GTP hydrolysis by RF3 on RC (IC) stimulated by RF2 (GAQ) in the presence or absence of fMet or MFTI.
(B–C) The rate of GTP hydrolysis by EF-G and IF2 on RC (IC) with and without MFTI or fMet on the A-site tRNA.
In (A), ribosomes (65 nM with fMet and 68 nM with MFTI) were incubated 4 min with RF3 (66 nM) at increasing concentrations of RF2 (GAQ). In (B) and (C), ribosomes (46 nM with fMet and 54 nM with MFTI) were incubated for 4 and 10 min with increasing concentrations of EF-G or IF2, respectively. In all experiments, the GTP concentration was 0.4 mM and peptide removal was accomplished by addition of 20 μM puromycin.

RF3, highly active ribosome complexes were made from *E. coli* components of high purity (Zavialov et al., 2001). Three types of complexes were separated from all free components by gel filtration. The initiation complexes (ICs) contained fMet-tRNA^{fMet} in the P site and an mRNA encoding either fMet-Phe-Thr-Ile-Stop (IC1) or fMet-Stop (IC2) (Figure 7A). The release complex (RC) contained fMet-Phe-Thr-Ile-tRNA^{Ile} in the P site and a stop codon in the A site (Figure 7G; Zavialov et al., 2001). The stop codon was always UAA, the peptide was radiolabeled for detection purposes and the extent of GTP hydrolysis was monitored by OD after separation of GTP from GDP with anion exchange chromatography (Experimental Procedures). When required, the peptide on the P-site tRNA was removed by addition of the aminoacyl-tRNA analog antibiotic puromycin (pur). This treatment resulted in "ribosomal-recycling complexes" (RRC) that can be recycled by RRF and EF-G in the presence of GTP (Karimi et al., 1999).

We first studied stimulation of the ribosome dependent GTPase activity of RF3 by the mutant class 1 release factor RF1 (GAQ). This mutant has a strong affinity to ribosomes with a peptidyl-tRNA in P site and the stop codon UAA or UAG in A site, but promotes peptide release very poorly. Therefore, its stimulatory effects can be studied both in the absence and presence of peptide (Zavialov et al., 2002). Little stimulation by RF1 (GAQ) was obtained for RC with intact peptide, as previously observed (Zavialov et al., 2002), and a similar result was now obtained with the ribosomal complex IC2 with fMet-tRNA^{fMet} in the P site. Removal of the tetrapeptide or fMet by pur from RC or IC2, respectively, significantly enhanced the RF1(GAQ)-dependent GTPase activity of RF3 (Figure 1A).

Then, the ribosome dependent GTPase activity of EF-G was studied with IC1 and RC complexes. The rate of GTP hydrolysis on EF-G was strongly stimulated by removal of the tetrapeptide from RC, and similarly by removal of fMet from IC1 (Figure 1B). The fact that removal of the peptide from the tRNA in the ribosomal P site affected the RF1 dependent GTP hydrolysis on RF3 and the GTP hydrolysis on EF-G in a similar fashion, suggests that the GTPase activities of these two translation factors are regulated by the same mechanism. Furthermore, GTP hydrolysis on both translation factors was inhibited to an equal extent by a tetrapeptide or fMet on the tRNA in the P site. This suggests that the conformation of the ribosome with P-site bound peptidyl-tRNA, but not the peptide itself, affects the GTPase activity of these two factors and that removal of the peptide activates GTP hydrolysis via a conformational change of the ribosome.

We also found that removal of the tetrapeptide from the P-site tRNA strongly stimulated the GTPase activity of IF2, but no such stimulation was found by removal of fMet from the IC complex. These data suggest the same control system for the GTPase activities of RF3 and EF-G, but a different control mechanism for IF2.

To further clarify these issues, we went on to study how the binding of IF2, EF-G, and RF3 in their GTP forms was affected by removal of the tetrapeptide or fMet from P-site bound tRNAs.

Binding of IF2, EF-G, and RF3 in the GTP Form to the Ribosome Is Prohibited by a Peptidyl-tRNA in the P Site

RF3, EF-G, or IF2 was incubated with an IC or RC complex or with an RRC complex, formed by removal with pur of the fMet from the IC or the tetrapeptide from the RC, in the presence of varying concentrations of the nonhydrolyzable GTP analog GDPNP. The amount of ribosome bound factor was in each case quantified by the extent of radiolabeled GDPNP retained on nitrocellulose after filtering (Figure 2; Experimental Procedures).

Neither RF3 (Figure 2A) nor EF-G (Figure 2B) in the GTP form bound to ribosomes with tetrapeptidyl-tRNA or fMet-tRNA in the P site. However, removal of fMet or tetrapeptide led to high affinity complexes for EF-G·GDPNP (Figure 2B) and RF3·GDPNP (Figure 2A). The present data show that the binding of EF-G in its GTP form to the ribosome depends on removal of the peptide from the P-site tRNA, similar to the binding of RF3, and that also fMet-tRNA in the ribosomal P site prevents the binding of both factors in the GTP conformation to

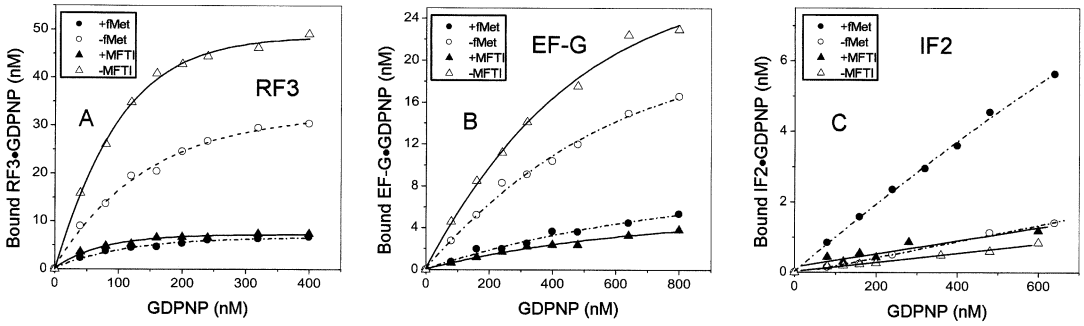

Figure 2. The P-Site tRNA Controls the Binding of Translation Factors in the GTP Conformation to the Ribosome

(A–C) Binding of RF3·GDPNP, EF-G·GDPNP, and IF2·GDPNP to RC or IC with peptidyl-tRNA or deacylated tRNA in the P site. The ribosomes (69 nM with fMet and 79 nM with MFTI) were incubated with 400 nM RF3, 2 μM EF-G, or 2 μM IF2 in the presence of increasing concentrations of [^{3}H]GDPNP with or without puromycin (100 μM).

the ribosome. These results support the inference in the previous section, that the peptide on the P-site tRNA does not affect the actions of RF3 and EF-G directly, but by a conformational change of the ribosome. Concerning IF2, our experiments show that the GTP form of this factor can bind to ribosomes neither with a tetrapeptidyl-tRNA nor a deacylated tRNA in the P site (Figure 2C). It was only when the P site was occupied with fMet-tRNA^{fMet} that significant binding of IF2·GDPNP to the ribosome could be observed (Figure 2C). This result follows naturally if it is assumed that IF2 directly and specifically interacts with fMet in the initiation complex as proposed by Wu and RajBhandary (1997).

EF-G·GDP and Fusidic Acid in Complex with Ribosomes Carrying Peptidyl-tRNA or Deacylated tRNA in the P Site

The antibiotic fusidic acid (fus) can block EF-G in complex with GDP on naked ribosomes (Agrawal et al., 2000; Stark et al., 2000), and it has been proposed that fus freezes EF-G in its GTP form even after hydrolysis of GTP, thereby preventing the factor from leaving the ribosome after translocation (Burns et al., 1974). Here, we studied the effects of the drug on the binding of EF-G·GDP to functional ribosomal complexes containing tetrapeptidyl-tRNA (RC), fMet-tRNA (IC), or deacylated tRNA (RRC) in the P site.

In the presence of GDP and fus, EF-G formed a high-affinity complex both with the RC (peptidyl-tRNA in the P site) and its corresponding RRC (deacylated tRNA in the P site) complex (Figure 3A). Therefore, EF-G·GDP·fus must have a different structure on the ribosome than EF-G·GDPNP, since the former could bind to the RC but not the latter (Figure 2B). EF-G·GDP could also bind to the RC and the RRC without fus, but with much lower affinity than in the presence of fus (Figure 3A).

The binding of EF-G to ribosomal complexes was also studied in the presence of fus at varying concentrations of GTP, rather than GDP. It is known that fus does not inhibit GTP hydrolysis on EF-G (Stark et al., 2000), but much less binding of EF-G·GDP to the RC was observed in the presence of GTP (Figure 3B), than in the presence of GDP (Figure 3A). Removal of the peptide with pur strongly stimulated the binding of EF-G·GDP to the RRC complex in the presence of fus (Figure 3B), to the same level as obtained in the GDP-titration with fus in Figure 3A. In GTP titrations, a similar result was obtained for the binding of EF-G·GDP and fus to an IC with fMet-tRNA or deacylated initiator tRNA in the P site (Figure 3B). The presence of fMet was prohibitive for EF-G·GDP binding in this case, and removal of fMet stimulated the factor binding considerably, albeit to a lower saturation level than observed by removal of the tetrapeptide in the RC (Figure 3B). The difference between the results obtained in the GTP (Figure 3B) and GDP (Figure 3A) titrations can be explained by postulating that the kinetic path to a stable EF-G·GDP·fus·ribosome complex is via an EF-G·GTP·ribosome complex in the former, but not in the latter case. Since EF-G·GTP cannot interact with ribosomes carrying fMet-tRNA or peptidyl-tRNA in the P site (Figures 1B and 2B), the formation of a stable EF-G·GDP·fus·ribosome complex was blocked in the

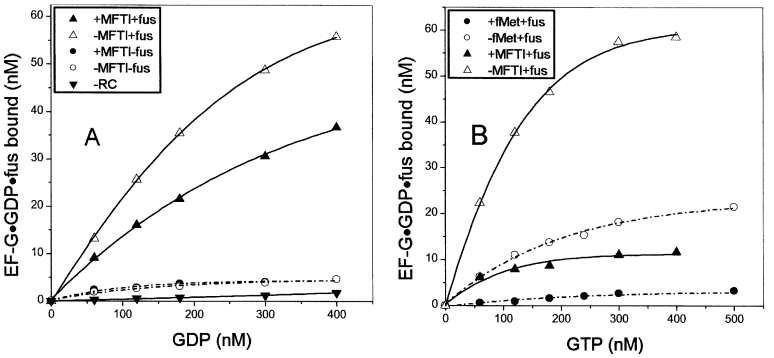

Figure 3. Binding of EF-G·GDP to the Ribosome in the Presence of Fusidic Acid (fus)

(A) Binding of EF-G and GDP to RC Before and After Release of Peptide from the P-site tRNA with and without fus. Release complex (108 nM) was incubated with 2 μM EF-G in the presence of increasing concentrations of [^{3}H]GDP with or without addition of 100 μM puromycin and 0.3 mM fus.

(B) Binding of EF-G to RC or IC with peptidyl-tRNA or deacylated tRNA in the P site in the presence of GTP and fus. 88 nM RC or 77 nM IC were incubated together with 1 μM EF-G, 0.2 mM fus, and different concentrations of [^{3}H]GTP in the presence or absence of puromycin (0.1 mM).

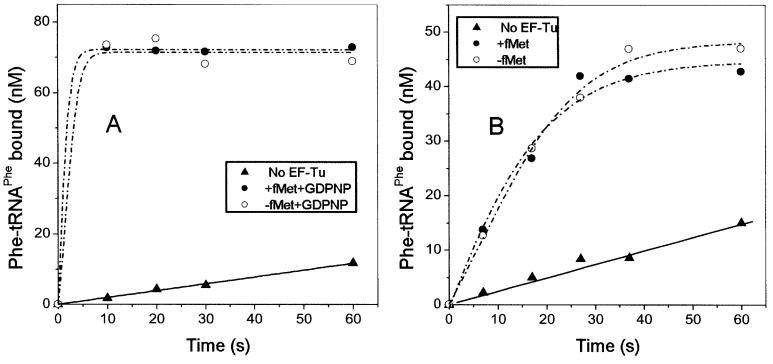

Figure 4. Binding of EF-Tu·GTP·aa-tRNA to the Ribosome Does Not Depend on the Presence or Absence of a Peptide on the P-Site tRNA

(A) Time dependent binding of EF-Tu·GDPNP·[^{14}C]Phe-tRNA to the ribosomes with and without fMet on the P-site tRNA. Samples of the reaction mixture containing 97 nM IC, 1 μM EF-Tu, 200 nM EF-Ts, 0.5 mM GDPNP with and without 100 μM puromycin were withdrawn at different times and the amount of [^{14}C]Phe-tRNA in the A site was measured using nitrocellulose filtration.

(B) Binding of [^{14}C] Phe-tRNA to the ribosome with tRNA^{fMet} or fMet-tRNA^{fMet} in the P site in the presence of a catalytic amount of EF-Tu. At different time points, aliquots of the reaction mixture containing 97 nM IC, 20 nM EF-Tu, 20 nM EF-Ts, 1 mM GTP with and without 100 μM puromycin were withdrawn and filtered throw nitrocellulose to determine the [^{14}C]Phe-tRNA^{Phe} occupancy in the A site.

GTP-titrations (Figure 3B). However, when the peptide or fMet has been removed, this allows for initial binding of EF-G·GTP (Figures 1B and 2B), which by GTP hydrolysis leads to the EF-G·GDP·fus·ribosome complex (Figure 3B). When, in contrast, free EF-G carries GDP it can directly form strong, fus-dependent complexes with ribosomes carrying peptidyl-tRNA as well as deacylated tRNA in the P site (Figure 3A).

EF-Tu·GTP·aa-tRNA Binds to Ribosomes with Either Peptidyl-tRNA or Deacylated tRNA in the P Site

In the next series of experiments we studied the rate of binding of Phe-tRNA^{Phe} catalyzed by a small amount of EF-Tu in recycling mode with EF-Ts, to ribosomes with either fMet-tRNA^{fMet} or deacylated tRNA^{fMet} in the P site and also observed the extent of binding of EF-Tu·GDPNP·Phe-tRNA^{Phe} to the same ribosomal complexes. A-site bound Phe-tRNA^{Phe} was in both cases monitored by nitrocellulose filter binding and fMet was removed from fMet-tRNA^{fMet} in the IC by pur, which cannot attack A-site bound Phe-tRNA^{Phe} (Experimental Procedures). The binding of EF-Tu·GDPNP·Phe-tRNA^{Phe} to the two complexes was the same (Figure 4A), as was the rate of Phe-tRNA^{Phe} binding catalyzed by recycling EF-Tu (Figure 4B). Binding of Phe-tRNA^{Phe} to ribosomes with either fMet-tRNA^{fMet} or tRNA^{fMet} in P site aided by EF-Tu in recycling mode resulted in ribosomal complexes with tRNA^{fMet} in the P site and either fMet-Phe-tRNA^{Phe} or Phe-tRNA^{Phe}, respectively, in the A site. The rate of Phe-tRNA^{Phe} binding to the A site was similar in both cases, showing that EF-Tu·GTP·Phe-tRNA^{Phe} binding to the ribosome is insensitive to the presence of peptide on the P-site bound tRNA.

Translocation with EF-G in the Presence of GTP, GDPNP, or GDP

Our studies on EF-G binding to ribosomes with an empty A site and either deacylated tRNA or peptidyl-tRNA in the P site (Figures 1B and 2B) were complemented with direct experiments on EF-G dependent translocation. For this, elongation complexes (ECs) containing [^{3}H]fMet-[^{14}C]Ile-tRNA^{Ile} in the A site and tRNA^{fMet} in the P site were assembled and purified from other components by gel filtration (Experimental Procedures). Using Ile-tRNA^{Ile} rather than Phe-tRNA^{Phe} as a receptor in the peptidyl transfer reaction resulted in a more stably bound peptidyl-tRNA in the A site, which was technically advantageous. Translocation of fMet-Ile-tRNA^{Ile} in these complexes was carried out by EF-G in the presence of GTP, GDP, or GDPNP (Figure 5A). The reaction was monitored by addition of either pur, which is an mRNA independent acceptor in the peptidyl transfer reaction also when EF-G is present on the ribosome (Katunin et al., 2002), or by addition of release factor RF2, which can hydrolyze the ester bond in peptidyl-tRNA when a stop codon has been moved into the A site and EF-G has dissociated from the ribosome (Experimental Procedures).

Addition of EF-G to the EC in the presence of GTP resulted in very rapid removal of the dipeptide with both pur and RF2 (Figure 5A), suggesting very fast translocation and dissociation of EF-G from the ribosome. When GTP was replaced by GDP, removal of the dipeptide by either pur or RF2 was very slow, suggesting very slow translocation in this case. In the presence of EF-G and GDPNP, removal of the peptide with pur was much faster than with RF2, but significantly slower than in the GTP case. The same type of experiments, where the fraction of dipeptidyl-tRNA remaining in ribosomal complex was monitored by nitro cellulose filtration, gave very similar results (Figure 5B). There was no drop-off of peptidyl-tRNA during the incubation time (see GDP case) and the translocation efficiency was close to 100% in our assay system.

One interpretation of the results in Figures 5A and 5B is that EF-G·GDPNP rapidly drove the ribosome into an intermediate translocation state, which had intermediate puromycin reactivity but was unable to accommodate RF2. Subsequent dissociation of EF-G·GDPNP, possibly accelerated by the presence of RF2, eventually allowed for exposure of the stop codon in the A site and termination by RF2. To study this further, we also incubated ECs in the presence of EF-G·GDPNP but without pur and RF2. Then, either pur (Figure 5C) or RF2 (Figure 5D) was added at different time points and the release of peptide monitored as a function of time as before. Now, peptide release was biphasic; first there was rapid release with both pur and RF2 (as with GTP in Figure 5A) and then slow release (as with GDPNP) as in Figure 5A. The amplitude of the fast peptide release reaction increased with the time during which the ECs

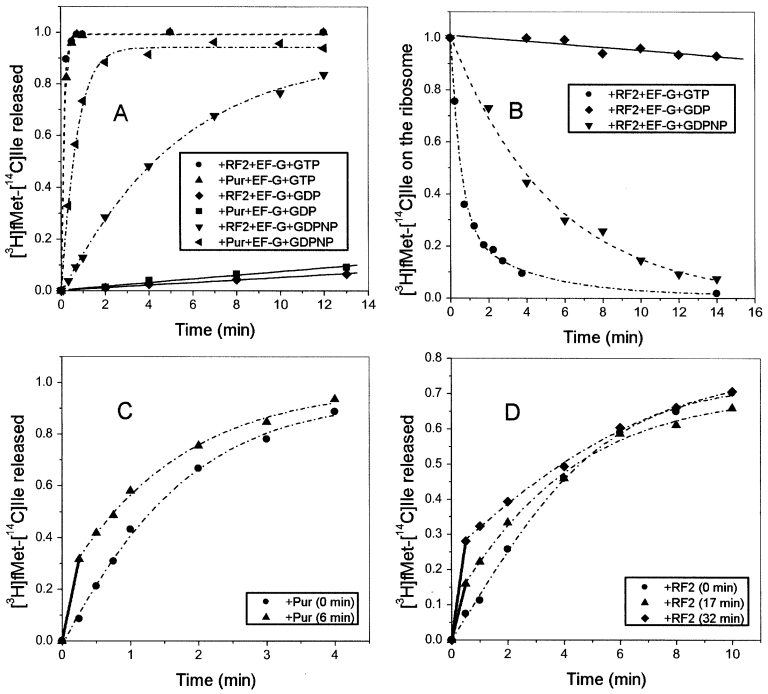

Figure 5. Translocation of A-Site Bound Peptidyl-tRNA

(A) Release of peptide from 50 nm EC by 0.4 mM puromycin or 1 μM RF2 in the presence of 1.4 μM EF-G and 0.3 mM GTP, GDP, or GDPNP and elongation complexes with of [^{3}H]fMet-[^{14}C]Phe-tRNA^{Phe} in the A site.

(B) The amount of [^{3}H]fMet-[^{14}C]Phe-tRNA^{Phe} remaining on the ribosome, under conditions as in (A).

(C–D) Release of fMet-Phe by puromycin (C) or RF2 (D) from elongation complexes preincubated with EF-G·GDPNP for different times.

were incubated with EF-G·GDPNP. These results suggest that EF-G·GDPNP was slowly dissociating from a high-affinity complex with the ribosome, in which fMet-Ile-tRNA^{Ile} had moved to an intermediate translocation state. This was characterized by relatively low puromycin reactivity (Figures 5A and 5C) and very slow termination by RF2 (Figures 5A and 5D), suggesting that the stop codon had not moved into the A site.

Discussion

The two large subunits (50S and 30S) of the bacterial ribosome are connected via a network of inter-subunit RNA-RNA and RNA-protein interactions (Cate et al., 1999; Gabashvili et al., 2000). In the working 70S ribosome, peptidyl-tRNA constitutes an additional contact between the subunits by connecting the mRNA on the 30S subunit with the peptidyl-transferase center on the 50S subunit. Previous cryo-EM studies of EF-G bound to ribosomes in the pre- and posttranslocation states showed that the translocation step is associated with a large conformational change of the ribosome (Agrawal et al., 2000; Stark et al., 2000; Frank and Agrawal, 2000). The present work describes how the presence or absence of a peptide on the P-site bound tRNA regulates the action of three out of four translation factors: EF-G, RF3, and IF2, while the action of the fourth factor, EF-Tu, was insensitive to the peptide. Binding of tRNAs to the partial E site in the 50S subunit requires a free CCA end (Lill et al., 1986). Peptide control of the GTPase activities of translation factors could therefore be associated with a structural rearrangement of the ribosome that is allowed if a P/P-site bound tRNA can be moved to P/E state and forbidden if it cannot. This inference is directly confirmed by cryo-EM data (Valle et al., 2003 [this issue of *Cell*]) in an important special case, directly relevant to ribosomal recycling by RRF and EF-G. The data show that a deacylated tRNA, originally in P/P state of a posttermination ribosomal complex, was moved into P/E state by the action of EF-G·GDPNP and how this was achieved by a relative movement of the ribosomal subunits (Figure 6). Other cryo-EM studies (B.P. Klaholz, A.V.Z., A.G. Myasnikov, M.E., and M. van Heel, unpublished data; U. Rawat, A.V.Z., M.E., J. Frank, unpublished data), suggests that also RF3·GDPNP can drive a P/P bound-deacylated tRNA in the same type of posttermination ribosomal complex into the P/E site.

Our data suggest that peptide controlled hybrid site formation is essential for the action of EF-G in transloca-

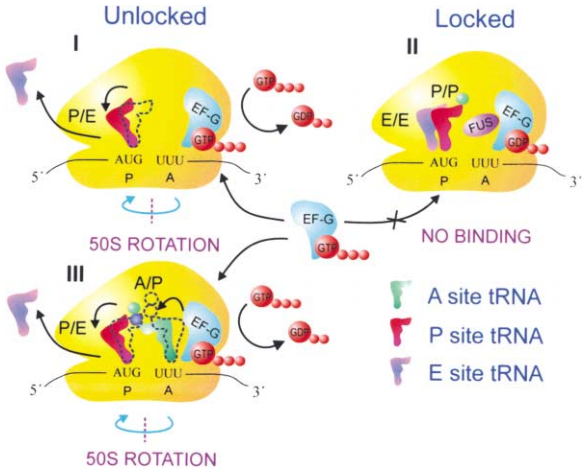

Figure 6. Binding of EF-G to Different Ribosomal States

Formation of a tRNA hybrid state is required to activate the GTPase activity EF-G. The dashed contour shows position of the tRNAs before EF-G binding to the ribosome.

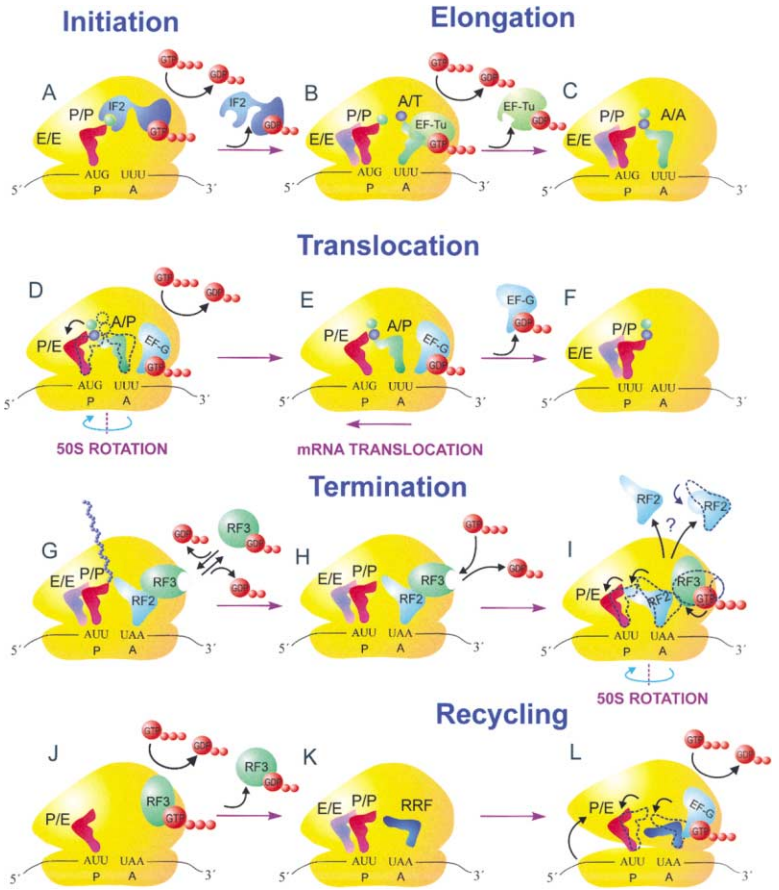

Figure 7. A Model Explaining Regulation of the Activities of the G-Proteins in Bacterial Protein Synthesis

tion of tRNA and in recycling of ribosomes back to initiation after termination as well as for the action of RF3 in recycling of class 1 release factors. The GTPase activity of IF2, in contrast, appears to be controlled by a different mechanism.

We observed how ribosomes with an empty A site and a tetrapeptidyl-tRNA or an fMet-tRNA^{fMet} in the P site promoted GTP hydrolysis on both RF3 and EF-G very poorly, while GTP hydrolysis on IF2 was slow in the former but not in the latter case (Figure 1). Furthermore, that these ribosomal complexes were unable to bind RF3 or EF-G in the presence of GDPNP and that only the fMet-tRNA^{fMet} containing complex had affinity to IF2 and GDPNP (Figure 2). Removal of the tetrapeptide or fMet with puromycin stimulated GTP hydrolysis (Figure 1) and led to stoichiometric ribosomal binding of both EF-G and RF3 in complex with GDPNP (Figure 2). Removal of fMet from the initiator tRNA did not change the high rate of GTP hydrolysis on IF2 (Figure 1C), but abolished the binding of IF2 to the ribosome in the presence of GDPNP (Figure 2C). Removal of the tetrapeptide stimulated GTP hydrolysis on IF2 (Figure 1C), but not its affinity to the ribosome.

Initiation of Protein Synthesis and Enzymatic Binding of Aminoacyl-tRNA

In the ribosomal initiation complex, a single, formylated amino acid is attached to the initiator tRNA^{fMet} (Figure 7A). The only G protein that continuously hydrolyzes GTP in the ribosomal initiation complex is initiation factor IF2 itself (Figures 1C). When, in contrast, there is a tetrapeptidyl-tRNA in the ribosomal P site, binding of IF2 in the GTP form (Figure 2C) and GTP hydrolysis (Figure 1C) are abolished. These data show how the bacterial ribosome has evolved to avoid crosstalk between RF3 and EF-G on one hand and IF2 on the other. The former two are inactive on initiation complexes and the latter is inactive on ribosome complexes with peptidyl-tRNA in the P site. Since IF2 forms a stable complex with GDPNP on initiation complexes (Figure 2C), GTP hydrolysis and release of IF2·GDP are required for the binding of EF-Tu·GTP·aa-tRNA to the ribosomal A site and formation of the first peptide bond in protein synthesis. At the same time, EF-Tu·GTP dependent binding of aa-tRNA to the ribosome (Figure 7B) does not depend on whether there is a peptidyl-tRNA or a deacylated tRNA in the ribosomal P site (Figures 4A and 4B). The binding step is, in other words, only controlled by the identity of the codon in the A site. Since the binding of IF2 to the ribosome requires the presence of fMet-tRNA^{fMet} in the P site, the stimulation of the GTPase activity of IF2 by posttermination ribosomes is insignificant (Figure 1A) and, hence, IF2 cannot interfere with EF-G and RRF during ribosomal recycling (Karimi et al., 1999) back to initiation.

Translocation of tRNAs by the Action of Elongation Factor EF-G

According to the classical model for translocation (Inoue-Yokosawa et al., 1974), which does not take the E

site into account, EF-G in the GTP form drives the peptidyl-tRNA from the A site to the P site and concomitantly releases the deacylated tRNA originally bound in the P site from the ribosome. Following GTP hydrolysis, EF-G rapidly dissociates from the ribosome. If this were true, ribosomes in the posttranslocation state would be expected to stimulate rapid binding of EF-G·GTP and release of EF-G·GDP in an idling GTPase activity. Since the classical model was formulated, the view of translocation has been revised in two important ways.

Firstly, Moazed and Noller (1989) suggested from footprinting experiments that transfer of a nascent peptide chain from peptidyl-tRNA in the P site to aminoacyl-tRNA in the A site immediately brings the ribosome into a ribosomal state with the peptidyl-tRNA in a hybrid A/P site and with the deacylated tRNA in a hybrid P/E site (reviewed in Noller et al., 2002). This would mean that the first half of the translocation cycle is completed already by peptidyl transfer and that the role of EF-G is merely to bring tRNAs in hybrid sites to the posttranslocation state with peptidyl-tRNA in P site and deacylated tRNA in E site. Secondly, Rodnina et al. (1997) discovered that GTP hydrolysis on EF-G after binding of the factor to a pretranslocation ribosome is much faster than translocation, as monitored by the change in fluorescence from a label in the peptidyl-tRNA. This would mean that EF-G promotes translocation after GTP hydrolysis and thereby acts like a motor protein (Rodnina et al., 1997) rather than a small G protein (Bourne et al., 1991). Assuming that the EF-G dependent part of the translocation cycle starts with the ribosome in hybrid site conformation (Moazed and Noller; 1989), Rodnina et al. (1997) used their data to propose an alternative model for translocation.

However, another result from cryo-EM microscopy indicates that also the translocation model by Rodnina et al. (1997) must be revised. Valle et al. (2003 [this issue of *Cell*]) found peptidyl-tRNA in the A/A site after peptidyl transfer, suggesting that under physiological conditions EF-G moves peptidyl-tRNA all the way from A/A to P/P state, as in the classical model, rather than only halfway from A/P to P/P state. This proposition is in line with data from Borowski et al. (1996), showing that the ribosome requires EF-G to become puromycin reactive after peptidyl-transfer but before mRNA translocation, and gets further support from observations by Wower et al. (2000), demonstrating that the CCA end of the P-site tRNA does not move spontaneously into the E site after peptidyl-transfer.

In our study of EF-G dependent translocation in the presence of GTP, GDPNP, or GDP (Figures 5A–5D), we started from well-defined pretranslocation complexes (Experimental Procedures) with peptidyl-tRNA in A/A site and deacylated tRNA in P/P site, prepared exactly as the ones analyzed by Valle et al. (2003 [this issue of *Cell*]). Translocation was monitored either by peptidyl transfer to puromycin, which requires that peptidyl-tRNA is in the P site of the large subunit, or by termination with RF2. The latter reaction requires, in addition, that the stop codon has been moved into the A site of the small ribosomal subunit.

We found very fast reactions with puromycin and RF2 when translocation was performed with GTP, but in contrast to the results by Rodnina et al. (1997), the translocation rate in the presence of GDP was insignificant (Figures 5A and 5B). In the presence of GDPNP, the puromycin reaction was slower than in the GTP case, but much faster than the termination reaction with RF2. This result suggests that EF-G in the presence of GDPNP drives the ribosome into an intermediate state, which is puromycin but not RF2 reactive. The intermediate rate of peptidyl transfer to puromycin could correspond to a peptidyl-tRNA in the A/P site, and the much slower termination reaction could correspond to subsequent movement of the ribosome into its posttranslocation state. This idea was tested in experiments where translocation was first performed with EF-G and GDPNP and then puromycin or RF2 was added. In this case, both peptidyl transfer to puromycin and termination by RF2 displayed biphasic kinetics (Figures 5C and 5D); there was a rapid phase, similar to the one obtained with GTP (Figure 5A), and a slow phase, as when puromycin or RF2 was present from the beginning of the translocation reaction (Figure 5A). This experiment suggests, firstly, that translocation can go to completion also in the presence of a noncleavable analog of GTP, albeit much more slowly than in the presence of GTP. Secondly, the fact that the amplitude for rapid termination with RF2 increased with translocation time (Figure 5D) suggests that EF-G·GDPNP slowly dissociated from the ribosome, thereby allowing for docking of RF2 and subsequent cleavage of the ester bond in peptidyl-tRNA.

From this, we suggest that EF-G·GDPNP has high affinity to a translocation intermediate, with hybrid sites for the ribosome bound tRNAs. As long as EF-G·GDPNP is present, transition to the posttranslocation state is not allowed. It can be reached if GDPNP dissociates, and could then be catalyzed by guanine nucleotide free EF-G. The posttranslocation state can also be reached in the absence of EF-G, in which case it would be spontaneous. When the ribosome has reached posttranslocation state, EF-G in the GTP form can no longer bind (Figure 2B) or hydrolyze GTP (Figure 1B).

The results in Figures 1, 2, and 5 and in the accompanying paper by Valle et al., 2003 [this issue of *Cell*] suggest the following, "semiclassical" model for translocation in eubacteria (Figures 7D–7F).

In the first step, EF-G in the GTP form brings the peptidyl-tRNA in the A/A site and the deacylated tRNA in the P/P site (pretranslocation state) to their hybrid A/P and P/E sites, respectively (translocation-intermediate) (Figure 7D). This movement is strongly favored by the high affinity of EF-G in the GTP form to the intermediate translocation state (Figure 5C and 5D). Since EF-G in the GTP form has low affinity to the posttranslocation ribosome (Figures 2B, 5C, and 5D), the last translocation step requires GTP hydrolysis. It is likely that EF-G in the GDP form stabilizes the transition state for the second translocation step, since EF-G·GDP and fusidic acid, but not EF-G in the GTP form or EF-G in the GDP form, can form a stable complex with ribosomes in their posttranslocation state (Figure 3). This second step is, we suggest, also facilitated by a high affinity of peptidyl-tRNA to the posttranslocation state of the ribosome (Lill et al., 1986).

The α-ϵ model for protein elongation (reviewed by Marquez et al., 2002) postulates a structural "conveyor" element on the ribosome that shuttles two tRNAs from their A/A and P/P sites (pretranslocation) to their P/P and E/E sites, respectively (posttranslocation). The model

explains in terms of this ribosomal shuttle why occupation of the A site by aminoacyl-tRNA or, subsequently, peptidyl-tRNA is prohibitive for E-site binding. It also excludes hybrid site formation for tRNAs during the elongation cycle. The findings that there are three tRNAs on the ribosome after peptidyl-transfer (Valle et al., 2003 [this issue of *Cell*]) and that translocation is likely to occur via an intermediate state with hybrid sites for tRNAs (Figure 5; Valle et al., 2003 [this issue of *Cell*]) do not support the α-ϵ model.

Recycling of Class 1 Release Factors by RF3

Release of peptide from a peptidyl-tRNA in the P/P site is induced by one of the class 1 release factors RF1 or RF2 (Rawat et al., 2003; Klaholz et al., 2003; Figure 7G). Then, the class 1 release factor is removed from the ribosome (Figures 7G–7J) with the help of a class 2 release factor, the G protein RF3 (Kisselev et al., 2003). Free RF3 in the GDP form enters the ribosome, and the GDP on RF3 rapidly dissociates when the posttermination ribosomal complex contains a class 1 release factor (Zavialov et al., 2001). This leads to a stable complex between the ribosome, the class 1 release factor and a guanine nucleotide-free form of RF3 (Zavialov et al., 2001). As long as the peptide remains on the tRNA in the P/P site, the complex can only be resolved by the binding of another GDP molecule, in which case RF3 in the GDP form can dissociate (Figure 7G). However, when the peptide has been cleaved off the peptidyl-tRNA, GTP will rapidly bind to RF3 (Figure 7H), which leads to swift dissociation of the class 1 release factor followed by hydrolysis of GTP and dissociation of RF3 in the GDP form from the ribosome (Zavialov et al., 2002, Figures 7I and 7J).

We now propose that when the peptide is gone from tRNA in the P/P site, RF3 in the GTP form drives the ribosome into the same conformation as EF-G·GTP (Figure 7I). In this ribosomal state with strong binding to RF3·GTP, RF2 or RF1 loses their affinity to the 50S subunit and rapidly dissociates from the ribosome (Rawat et al., 2003). After release of RF3·GDP the ribosome might spontaneously flip back to its original posttermination conformation (Figure 7H), to which RF2 or RF1 rebinds again (Zavialov et al., 2002), or be directly recycled by EF-G and RRF (Figures 7K and 7L).

Recycling of Ribosomes by EF-G and RRF

After the termination of protein synthesis, ribosomes are recycled back to a new round of initiation by the joint action of RRF and EF-G (Hirokawa et al., 2002; Karimi et al., 1999), but the exact mechanism for this event remains obscure. Ribosomes with a deacylated tRNA in the P site are targets for recycling (Figure 7K), and we now know that they have strong affinity for EF-G in the GTP form (Figure 2B). We also know that EF-G in the GTP form drives the ribosome into a state with hybrid site for the deacylated tRNA, originally in P site, by a ratchet-like rotation of the ribosome structure (Valle et al., 2003 [this issue of *Cell*]). Formation of this structure is, we suggest, the starting point for the dissociation of the ribosomal subunits that eventually will allow mRNA and tRNA to leave the small subunit (Karimi et al., 1999). New structural information about the binding of RRF to the ribosome reveals that the recycling factor binds very differently to a peptidyl-tRNA in the A site in the interface between the subunits (Lancaster et al., 2002). It is therefore not likely that the recycling step depends on the translocation of deacylated tRNA from the P to the E site and RRF from the A to the P site (Hirokawa et al., 2002). Instead, we suggest that ribosome splitting occurs rapidly when both EF-G in the GTP form and RRF are present together on the ribosome with a deacylated tRNA in the P/E site (Figure 7L; Karimi et al., 1999).

Conclusions

The combination of biochemical data of this work and cryo-EM reconstructions by Valle et al. (2003 [this issue of *Cell*]) supports a different view of the role of hybrid sites for tRNAs on the ribosome than previously suggested (Noller et al., 2002). Removal of the peptide from the peptidyl-tRNA in the P site of the ribosome by peptidyl-transfer or ester bond hydrolysis allows for its subsequent "unlocking" and formation of a changed structure with hybrid tRNA sites. This conformational change depends on the action of EF-G or RF3 in the GTP form and plays a fundamental role in translocation (EF-G), recycling of class 1 release factors (RF3), and dissociation of ribosomal subunits after termination (EF-G and RRF). The binding of IF2 in the GTP form to the ribosome is also regulated by the peptide on the tRNA in P site, but through direct interactions rather than by unlocking of the ribosome and hybrid site formation.

Experimental Procedures

Components of the In Vitro System for *E. coli* Protein Synthesis

Ribosomes of high activity; a polymix buffer; initiation factors IF1, IF2, IF3; elongation factors EF-Tu, EF-Ts, EF-G, tRNA bulk, PheRS, Thr-RS, IleRS, $tRNA^{Phe}$, $tRNA^{Ile}$, fMet-$tRNA^{fMet}$, RF1, and RF3 were prepared as previously described (Zavialov et al. 2001 and references therein). The RF1 (GAQ) mutant was prepared according to Mora et al. (2003). The *MFTI*-mRNA used for release complex (RC) preparations had the sequence gggcccuuguuaacaauuaaggaggu**au** acu AUG UUU ACG AUU **UAA** uugcag$(a)_{21}$. It contained a strong ribosome binding site (underlined lower case letters), an open reading frame encoding an MFTI tetrapeptide (capital letters), a stop codon **UAA** and a poly (A) tail for purification on a poly (dT) column (Amersham Biosciences). The mRNAs were synthesized by T7 RNA polymerase transcription of the corresponding DNA constructs (Pavlov et al., 1997). *fMet*-, *MF*- and *MI*-mRNAs had an open reading frame encoding fMet (AUG stop), MF (AUG UUU stop), and MI (AUG AUU stop), respectively.

Ribosomal Complexes and Translation Factor Activities

Release complexes with a stop codon in the A site and a tetrapeptidyl-tRNA in the P site were prepared according to Zavialov et al. (2001). Initiation complexes (ICs) were obtained similarly, but in the absence of translation factors. The fraction of active RC or IC ($\sim$80%) was measured as the amount of MFT[^{14}C]I tetrapeptide or [^{3}H]fMet incorporated into the ribosomes and normalized to the total amount of ribosomes (measured from OD_{260}) as described (Zavialov et al., 2001). Alternatively, increase in the rate of EF-G dependent GTP hydrolysis after removal of peptide from RC or IC with RFs or puromycin (Figure 2B) was used to estimate ribosomal complexes activity when MFTI or fMet were not labeled. The fraction of active RF3 and RF1 (GAQ) was determined as described in Zavialov et al. (2002). For EF-Tu, EF-G, and IF2 a total protein concentration (Bredford method) was used.

GTPase Activity of RF3

The GTPase activity of RF3 was studied by, first, preincubating RF3 and RC or IC (*fMet*-mRNA) with or without puromycin (mix A1 and A2, respectively) and RF1 (GAQ) with GTP (mix B) separately during 4 min at 37°C. The reaction was initiated by adding mix B to either one of the A mixes. The resulting reaction mix contained 66 nM RF3, 54 nM RC (46 nM IC), 17 μM puromycin (when present), 0.4 mM GTP, and 0–84 nM RF1 (GAQ). The reaction was stopped after 5 min by addition of 1 ml ice-cold magnesium-free buffer A (25 mM Tris-HCl, [pH 7.5]) to the reaction mixture (60 μl). Components of the reaction mixture, GTP and GDP were separated on a Mono Q column (Amersham Biosciences) by elution with 0.18–0.32 mM NaCl gradient in buffer A. The rate of GTP hydrolysis was obtained from the ratio between the GDP peak area and the sum of the GDP and GTP peak areas measured by OD (254 nm), the total concentration of guanine nucleotides and the incubation time (Zavialov et al. 2001, 2002).

GTPase Activities of EF-G and IF2

The GTPase activity of EF-G (IF2) was studied by, first, preincubating EF-G (IF2) and RC or IC (*MFTI*-mRNA) with or without puromycin (mix A1 and A2, respectively) and GTP (mix B) separately during 4 min at 37°C. The reaction was initiated by adding mix B to either one of the A mixes. The resulting reaction mix (60 μl) contained 0–1.67 μM EF-G (0–3.33 μM IF2), 43 nM RC (37 nM IC), 20 μM puromycin (when present), and 0.4 mM GTP. The reaction was stopped after 5 min (10 min for IF2) by addition of 1 ml ice-cold magnesium-free buffer and the rate of GTP hydrolysis was obtained as described for RF3.

Complex Formation Between GDPNP and Translation Factors on Release and Initiation Complexes

To form a complex between GDPNP and translation factors RF3, EF-G, or IF2 on RC or IC (*MF*-mRNA) with intact peptidyl-tRNA or deacylated tRNA in P site, a translation factor was incubated for 10 min at 37°C with RC (IC) and different concentrations of GDPNP in the presence or absence of puromycin. The mixtures contained 0–400 nM GDPNP (RF3 binding) or 0–800 nM (EF-G and IF2 binding); 0.57 μM RF3 (RC and IC binding), 2 μM IF2 (RC and IC binding), 2 μM EF-G (IC binding), and 1 μM EF-G (RC binding); 55 nM IC, 55 nM RC (RF3 binding), and 63 nM RC (EF-G and IF-2 binding); and 0.1 mM puromycin (if present). After the incubation 45 μl of the reaction mixtures was filtered through nitrocellulose and washed with 1 ml of ice-cold polymix buffer. The amount of factor·[^{3}H]GDPNP·RC(IC) complex was determined by scintillation counting of [^{3}H]GDPNP retained on the filter.

Formation of EF-G·GDP·fus·RC Complex in the Presence of GDP

To form a complex between EF-G, GDP, fusidic acid (fus) and a RC 2 μM EF-G, 80 nM RC, 0.3 mM fus, and 60–400 nM GDP were incubated for 11 min at 37°C with or without addition of 0.2 mM puromycin (all concentrations apply to the final mix). After the incubation 45 μl of the reaction mixture was filtered through nitrocellulose (NC) and washed with 1 ml of ice-cold polymix buffer. The amount of EF-G·GDP bound to RC was determined by scintillation counting of [^{3}H]GDP retained on the filter.

Formation of EF-G·GDP·fus·RC (IC) Complex in the Presence of GTP

To form a complex between EF-G, GDP, fus, and a RC or IC, 1 μM EF-G, 88 nM RC, or 77 nM IC (*MF*-mRNA), 0.2 mM fus, 60–500 nM GTP, 2 mM phosphoenolpyruvate (PEP), 10 μg/ml pyruvate kinase (PK) were incubated for 10 min at 37°C with or without addition of 0.1 mM puromycin (all concentrations apply to the final mix). After the incubation 45 μl of the reaction mixture was filtered through nitrocellulose and washed with 1 ml of ice-cold polymix buffer. The amount of EF-G·GDP bound to RC (IC) was determined by scintillation counting of [^{3}H]GDP retained on the filter.

Phe-tRNA^{Phe} Binding to the A Site

To study the binding of Phe-tRNA^{Phe} to the A site two mixes containing [^{14}C] Phe-tRNA (mix A) and IC (MF mRNA) with factors (mix B1–B4) were preincubated separately for 10 min at 37°C. Then mix A was added into mix B and 45 μl samples were taken and filtered through the NC filters at different times. The filters were washed with 1 ml of polymix buffer and the amount of [^{14}C] Phe-tRNA incorporated into the ribosome was determined from the filter counting. Mix B1 contained IC; mix B2: IC, EF-Tu, EF-Ts, GTP, PEP, PK, and puromycin; mix B3: IC, EF-Tu, EF-Ts, GTP, PEP, PK and puromycin (if present); mix B3: IC, EF-Tu, EF-Ts, GDPNP, and puromycin (if present). The concentration of components after A and B mixing was: 97 nM IC, 1 μM EF-Tu, 200 nM EF-Ts, 10 μg/ml PK, 2 mM PEP, 1 mM GTP, 0.5 mM GDPNP, and 100 μM puromycin. In the experiment with EF-Tu recycling, the concentrations of EF-Tu and EF-Ts were 20 nM.

Translocation of fMet-Ile-tRNA From the A Site to the P Site

To make elongation complexes (EC), initiation and elongation mixtures were prepared. The initiation mixture (500 μl) contained 4.3 μM ribosomes; 3 μM of each of the initiation factors IF1, IF2, and IF3; 12 μM *MI*-mRNA; 8.3 μM [^{3}H]fMet-tRNA^{fMet}; 1 mM GTP, 2 mM PEP and 20 μg/ml pyruvate kinase. The elongation mixture (500 μl) contained 17.5 μM [^{14}C] Ile, 11 μM tRNA^{Ile}, 150 U Ile-tRNA^{Ile} synthetase, 11.5 μM EF-Tu, 2.4 μM EF-Ts, 1 mM GTP, 2 mM PEP, 20 μg/ml pyruvate kinase, 1 mM ATP, 8 μg/ml myokinase, and 0.5 mU pyrophosphatase. The initiation and elongation mixtures were preincubated separately at 37°C for 40 and 10 min, respectively. Then they were added together (1:1 volumes), mixed and immediately applied to a Sephacryl S-300 HR column (1.6 × 30 cm) kept at 2°C. The fractions containing the elongation complex (EC), free from ternary complexes and nucleotides, were pooled and stored on ice.

To monitor translocation of fMet-Ile-tRNA^{Ile} (Figures 5A and 5B) a mixture A, containing EF-G, RF2, or puromycin and one of the nucleotides (GTP, GDP, or GDPNP), was preincubated for 3 min at 37°C. Then a mixture B, containing EC, was added and 45 μl aliquots were withdrawn and added to 800 μl 20% formic acid or filtered through NC filters and washed with 1 ml of polymix buffer. The amount of [^{3}H]fMet-[^{14}C]Ile released from the ribosome was determined by scintillation counting of the supernatant after centrifugation (formic acid precipitation). The amount of peptide remaining on the ribosome was determined by nitrocellulose filter counting. The concentration of components (when present) in the final mixture was 50 nM EC, 1.4 μM EF-G, 1 μM RF2, 0.4 mM puromycin, and 0.3 mM GTP, GDP, or GDPNP.

The experiments in Figures 5C and 5D were done in the same way, except that RF2 was added to the final mixture 0, 17, and 32 min and puromycin 0 and 6 min after incubation start.

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