Cell, Vol. 107, 115–124, October 5, 2001, Copyright ©2001 by Cell Press

A Posttermination Ribosomal Complex Is the Guanine Nucleotide Exchange Factor for Peptide Release Factor RF3

Andrey V. Zavialov,[1,3] Richard H. Buckingham,[2] and Måns Ehrenberg[1,3]

[1]Department of Cell and Molecular Biology
BMC
Uppsala University
Box 596
S-75124 Uppsala
Sweden

[2]Unité Propre de Recherche 9073 du Centre National de la Recherche Scientifique
Institut de Biologie Physico-Chimique
13 rue Pierre et Marie Curie
Paris 75005
France

Summary

The mechanism by which peptide release factor RF3 recycles RF1 and RF2 has been clarified and incorporated in a complete scheme for translation termination. Free RF3 is in vivo stably bound to GDP, and ribosomes in complex with RF1 or RF2 act as guanine nucleotide exchange factors (GEF). Hydrolysis of peptidyl-tRNA by RF1 or RF2 allows GTP binding to RF3 on the ribosome. This induces an RF3 conformation with high affinity for ribosomes and leads to rapid dissociation of RF1 or RF2. Dissociation of RF3 from the ribosome requires GTP hydrolysis. Our data suggest that RF3 and its eukaryotic counterpart, eRF3, have mechanistic principles in common.

Introduction

The final step in protein synthesis is hydrolysis of the ester bond in peptidyl-tRNA and release of the finished protein. This reaction is induced in prokaryotes by one of the two class I release factors, RF1 or RF2 (Scolnick et al., 1968) and in eukaryotes by the unique class I factor eRF1 (Frolova et al., 1994). Termination of translation is triggered by the class I factors following a translocation step that places a stop codon in the ribosomal A-site and peptidyl-tRNA in the P-site. In prokaryotes, RF1 recognizes the stop codons UAA and UAG, while RF2 recognizes UAA and UGA. In eukaryotes, release factor eRF1 recognizes all three stop codons (reviewed by Kisselev and Buckingham, 2000). It has been suggested that class I release factors are structural mimics of aminoacyl-tRNA (Nakamura et al., 1996), a hypothesis supported by the crystallographic model of human eRF1 (Song et al., 2000). A second class of release factors is found in both prokaryotic (Milman et al., 1969) and eukaryotic organisms (Zhouravleva et al., 1995). These class II factors are G proteins, RF3 in bacteria and eRF3 in higher organisms, which are not required in either case for peptide release (Grentzmann et al., 1994; Mikuni et al., 1994; Zhouravleva et al., 1995; Freistroffer et al., 1997). The role of RF3 is to promote rapid dissociation of RF1 or RF2 from the posttermination complex (Freistroffer et al., 1997), while the function of eRF3 remains undecided (Kisselev and Buckingham, 2000).

Basic features of translation termination in bacteria have remained elusive, since previously obtained experimental results lead to apparently conflicting interpretations. This is in particular true for the role of guanine nucleotides in the cycle of RF3. Early biochemical experiments addressing this question used nucleotide triplets in the ribosomal A- and P-sites and measured fMet release from P-site-bound fMet-tRNA^{fMet}, but release was either enhanced or inhibited by RF3 and GTP depending on triplet concentration and buffer composition (Goldstein and Caskey, 1970). These ambiguous observations resulted in two long-lived and essentially different interpretations of the role of RF3 in translation termination.

According to one hypothesis, RF3 in a GTP-induced conformation forms a complex with RF1 or RF2 off the ribosome and accelerates their association to a stop codon programmed A-site. In this model, RF3•GTP would play a role very similar to EF-Tu•GTP, which binds to aminoacyl-tRNAs and greatly accelerates their binding to ribosomal A-sites programmed with cognate sense codons. This scenario was more recently advocated by Nakamura et al. (1996), supported by the affinity of eukaryotic eRF3 for free eRF1 (Zhouravleva et al., 1995) and a putative structural mimicry between the aminoacyl-tRNA•EF-Tu•GTP complex, elongation factor EF-G (Nissen et al., 1995), and a hypothetical complex between RF3 and RF1 or RF2. Such a scheme would also make room for ribosomal proofreading of RF1 or RF2 with the help of GTP hydrolysis on RF3. This could, in principle, increase the precision by which class I release factors discriminate between nonsense and sense codons, in analogy with how GTP hydrolysis on EF-Tu allows proofreading of aminoacyl-tRNAs (Ruusala et al., 1982b).

According to another hypothesis, RF3 in the presence of GTP induces rapid dissociation of RF1 or RF2 from the ribosome after ester bond cleavage. This view was confirmed by data from an in vitro translation system with purified translation factors and continuous, heteropolymeric mRNAs (Freistroffer et al., 1997). Data obtained from the same system also showed that acceleration of the rate of binding of class I release factors to the ribosome by RF3•GTP is negligible. Furthermore, Pel et al. (1998) found that RF3 in complex with a noncleavable GTP analog bound strongly to the ribosome in the absence of class I release factors, but not in their presence, as required by the mimicry hypothesis (Nakamura et al., 1996). Finally, although the accuracy with which RF1 or RF2 discriminates between sense and nonsense codons is remarkably high, it does not depend on proofreading (Freistroffer et al., 2000; Nakamura et al., 2000). However, these experiments left open the question of how RF3 uses GTP to remove RF1 or RF2 after, but not before, release of polypeptide, and how RF3 can rapidly recycle between ribosomes without

[3]Correspondence: ehrenberg@xray.bmc.uu.se [M.E.]; andrey.zavialov@icm.uu.se [A.V.Z.]

excessive idling GTPase activity. Most G proteins work as molecular switches between an "active" conformation induced by GTP and an "inactive" GDP conformation. The return of monomeric G proteins from the inactive GDP conformation to the active GTP conformation is frequently catalyzed by a guanine exchange factor (or GEF, see Bourne et al., 1991; Sprang and Coleman, 1998). For example, protein elongation factor EF-Tu requires elongation factor EF-Ts for rapid exchange of GDP for GTP. However, no GEF factor has so far been identified for the translation factors IF2, EF-G, RF3, or eRF3. It has been suggested that a domain in eRF3, rather than a separate GEF protein, catalyzes guanine nucleotide exchange during its cycle (Kisselev and Buckingham, 2000).

In this study, we have taken advantage of an in vitro translation system with components of high purity and with fully active ribosomes programmed with heteropolymeric mRNAs (Rodnina and Wintermeyer, 1995; Freistroffer et al., 1997). This has been used to clarify the role of GTP in translation termination and to show that the missing GEF for RF3 is a posttermination ribosomal complex. These findings reconcile many previously incompatible observations. They show that ribosome-dependent GTP hydrolysis on RF3 can be greatly stimulated by RF1 or RF2 and explain how idling GTPase activity of RF3 is avoided in the cell.

Results

Recycling of RF1 or RF2 Requires RF3 and GTP, but Recycling of RF3 Requires GTP Hydrolysis

The development of an in vitro protein synthesizing system with purified components and able to translate short synthetic messengers has allowed the synthesis and purification of release complexes in which the ribosome is paused with a peptidyl-tRNA in the P-site and a stop codon in the A-site. These complexes can be used to study the enzyme kinetics of the termination reaction (Freistroffer et al., 1997; 2000; Pavlov et al., 1997). In the experiments described here, the system has been further improved by the use of 70S ribosomes with near 100% activity (Rodnina and Wintermeyer, 1995). The effects of guanine nucleotides on the recycling times of RF2 were monitored by the rate of tetrapeptide release by RF2 from release complexes with fMet-Phe-Thr-[^{14}C]Ile-tRNA^{Ile} in the P-site and a UAA stop codon in the A-site. The experiments were carried out in the presence or absence of RF3 with release complex in large excess over RF2, such that complete termination required extensive recycling of RF2. The data allowed the recycling time (τ) of RF2 to be calculated in each case (Figure 1).

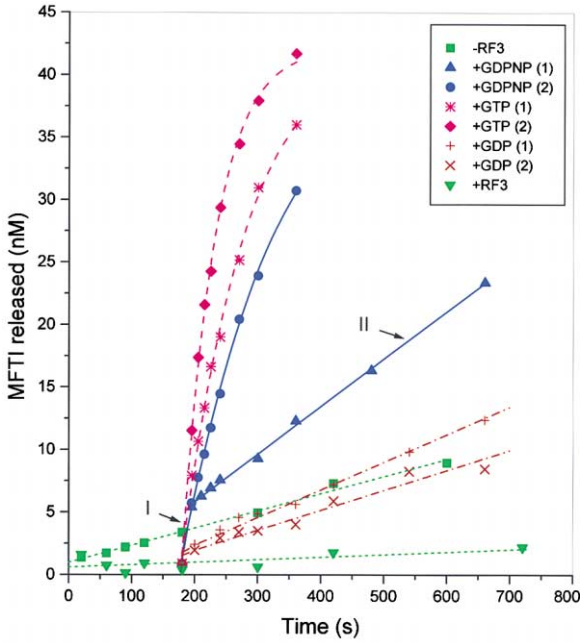
In the absence of RF3, RF2 recycled very slowly (τ = 79 s), reflecting tight binding between RF2 and the ribosome after release of polypeptide. In the presence of RF3, but without guanine nucleotides, recycling of RF2 almost ceased (τ = 920 s), due to formation of a strong complex between RF3 and RF2 on the ribosome (Freistroffer et al., 1997; Pel et al., 1998).

The effects of GTP and its nonhydrolyzable analog GDPNP on the recycling rate of RF2 were studied either with release complex in about 8-fold excess over RF3, where RF3 also had to recycle, or with RF3 in excess over release complex, where only RF2 had to recycle between ribosomes. When RF3 was in excess, GDPNP stimulated recycling of RF2 almost as well as GTP (τ = 3.7 s with GDPNP and τ = 1.4 s with GTP). In contrast, when RF3 was present in a small amount, the cycle time of RF2 was short in the presence of GTP (τ = 2.2 s), but considerably longer (τ = 29 s) in the presence of GDPNP. This suggests, first, that dissociation of RF2 from the ribosome is stimulated by RF3 together with GTP (or GDPNP) and that no GTP hydrolysis is required at this stage. Second, since strong stimulation of RF2 recycling can be carried out by catalytic amounts of RF3 in the presence of GTP but not GDPNP, hydrolysis of GTP must be required for recycling of RF3 itself.

This conclusion is further supported by a more detailed inspection of the curves in Figure 1. When RF2 recycling was monitored in the presence of GDPNP with release complex in excess over RF3, and with RF3 in excess over RF2, there was a large difference between the initial (I) and later (II) parts of the time curve. The extent of RF3-dependent stimulation of release of peptide (part I) corresponds exactly to the added amount of RF3. At longer times (part II), RF2 recycled almost as slowly as in the absence of RF3. This suggests that RF3 in the presence of GDPNP remains ribosome bound for a long time after dissociation of RF2 and, furthermore, that GTP hydrolysis drives RF3 from a conformation with strong binding to the ribosome to one with weak binding. Indeed, in the presence of GDP there was no significant stimulation of RF2 recycling either at high or at low concentration of RF3 (Figure 1), and the inhibition by RF3 of RF2 recycling observed in the absence of guanine nucleotide was abolished. This indicates that the addition of GDP leads to the release of RF3 in a complex with GDP with low affinity for the ribosome. A closely similar set of data to those shown for RF2 in Figure 1 was obtained for RF1 (data not shown).

RF3 Forms a High-Affinity Complex with GDP

Mortensen et al. (1995) estimated the apparent dissociation constants (K_D) for binding of GDP and GTP to RF3 to be 2 and 10 μM, respectively. These numbers suggested that in the cell, RF3 exists in its GTP conformation. However, results from a number of RF2 recycling experiments were difficult to interpret in terms of the K_D values of Mortensen et al. (1995). We therefore remeasured the binding of GDP and GTP to RF3, paying attention to the fact that many G proteins bind GDP with very high affinity and therefore often contain GDP after purification. If this is ignored, experiments designed to measure the binding of GDP or GTP to these proteins may reflect trivial isotope dilution effects, rather than K_D values (Goody et al., 1991). We found that, indeed, active RF3 in our preparations was in 1:1 complex with GDP (Experimental Procedures). RF3 was therefore first equilibrated with [^{3}H]GDP and freed from excess nucleotide by cation-exchange chromatography. After dilution of RF3•[^{3}H]GDP to 1 nM and equilibration with varying amounts of externally added [^{3}H]GDP of the same specific activity, the amount of GDP in complex with RF3 was measured by binding of the protein to nitrocellulose. From a Scatchard plot of the binding equilibrium (Figure 2A), K_D for the binding of GDP to RF3 was estimated to be 5.5 nM. The



Factors added to RF2	Time per cycle s
-RF3	79
+RF3-Nucleotides	920
+RF3+GDPNP(1) Part I	3.7
+RF3+GDPNP(1) Part II	29
+RF3+GDPNP(2)	3.0
+RF3+GTP(1)	2.2
+RF3+GTP(2)	1.4
+RF3+GDP(1)	50
+RF3+GDP(2)	69

Figure 1. The Effects of RF3 and Addition of Various Guanine Nucleotides on the Rate by which RF2 Recycles between Release Complexes (RC), Releasing Peptide

The tetrapeptide (MFTI) released as a function of time from 50 nM RC by 1.1 nM RF2 is shown in the absence of RF3 (■), in the presence of RF3 (6 nM) without guanine nucleotides (▼), in the presence of RF3 (6 nM) with either GTP (*), GDPNP (▲), or GDP (+), and in the presence of RF3 (200 nM) with either GTP (◆), GDPNP (●), or GDP (X). When present, guanine nucleotides were always at 0.2 mM final concentration and were added to RC•RF2•RF3 complexes that had been formed during 180 s.

dissociation rate constant (K_d) of the RF3•GDP complex was estimated by a nucleotide exchange experiment, where the decrease of labeled GDP on RF3 was monitored by nitrocellulose filter binding as a function of time after addition of a large excess of unlabeled GDP. The estimated value of K_d (0.032 s^{-1}), obtained from the slope of the logarithmic plot in the insert of Figure 2A, implies that spontaneous dissociation of GDP takes on average about 30 s. This may be compared to about 90 s for the dissociation of GDP from EF-Tu in the absence of EF-Ts (Ruusala et al., 1982a).

K_D values for binding of GTP and GDPNP to RF3 were estimated from competition experiments, in which the decrease of [^{3}H]GDP•RF3 complex caused by increasing concentrations of unlabeled GTP or GDPNP was monitored by nitrocellulose binding. The data in Figure 2B allowed K_D for GTP and GDPNP to be estimated at 2.5 μM and 8.5 μM, respectively. However, it is possible that the true binding of GTP and GDPNP to RF3 may be even weaker than these estimates, as both the GTP and GDPNP preparations contained a small fraction of GDP (about 0.1%), which could lead to underestimates of the K_D values. This implies that GTP and GDPNP bind at least three orders of magnitude more weakly to RF3 than GDP.

Release Complex Bound to RF1 or RF2 Is the Missing Guanine Nucleotide Exchange Factor (GEF) for RF3

The difference of about a thousand-fold in the affinity of GDP and GTP for free RF3 (Figure 2) means that in vivo RF3 must enter the ribosome in the GDP conformation. To answer the question of how RF3 can recycle so rapidly (Figure 1) in spite of the fact that the spontaneous release of GDP takes as much as 30 s (Figure 2A, insert), more extensive guanine nucleotide exchange experiments were performed. RF3 was first preincubated with [^{3}H]GDP. Unlabeled GDP was then added in large excess in the absence of other factors, in the presence of RF2, in the presence of release complex with UGA (stop codon only for RF2), or in the presence of both this release complex and RF2. The dissociation of labeled GDP from RF3 was monitored by nitrocellulose filter binding. Figure 3A shows that rapid exchange of GDP occurred only when RF3 was presented with its functional target, i.e., a release complex containing RF2 in the A-site. Similar data were obtained when RF1 replaced RF2 in a release complex with UAA (stop codon for RF1 and RF2). It was also demonstrated that RF2 could stimulate exchange of GDP on RF3 when the release complex had UGA but not UAG (stop codon only for RF1). Further, it was shown that RF1 could stimulate exchange of GDP when the release complex had UAG but not UGA (Figure 3B). These results reveal that RF1 and RF2 not only promote the hydrolysis of the ester bond in peptidyl-tRNA but, together with the ribosome, serve as guanine nucleotide exchange factors for RF3 in a codon-specific way.

RF1 and RF2 Stimulate Ribosome-Dependent GTPase Activity of RF3 in a Codon-Dependent Manner

It was previously shown that ribosomes are strictly required for the GTPase activity of RF3, but that addition of RF1 or RF2 does not seem to affect the rate of this reaction (Freistroffer et al., 1997; Grentzmann et al., 1998). These observations suggested a possible fundamental difference between translation termination in prokaryotes and eukaryotes, where the GTPase activity of eRF3 depends on the presence of both eRF1 and ribosomes (Frolova et al., 1996). However, the present finding that rapid GDP exchange on RF3 requires not only release complex but also the presence of RF1 or RF2 (Figure 3) motivated a more detailed analysis of the ribosome-mediated GTPase activity of RF3.

Release complexes with either UGA or UAG in the A-site and tetrapeptidyl-tRNA in the P-site were prepared and incubated with RF3, GTP, and varying con-

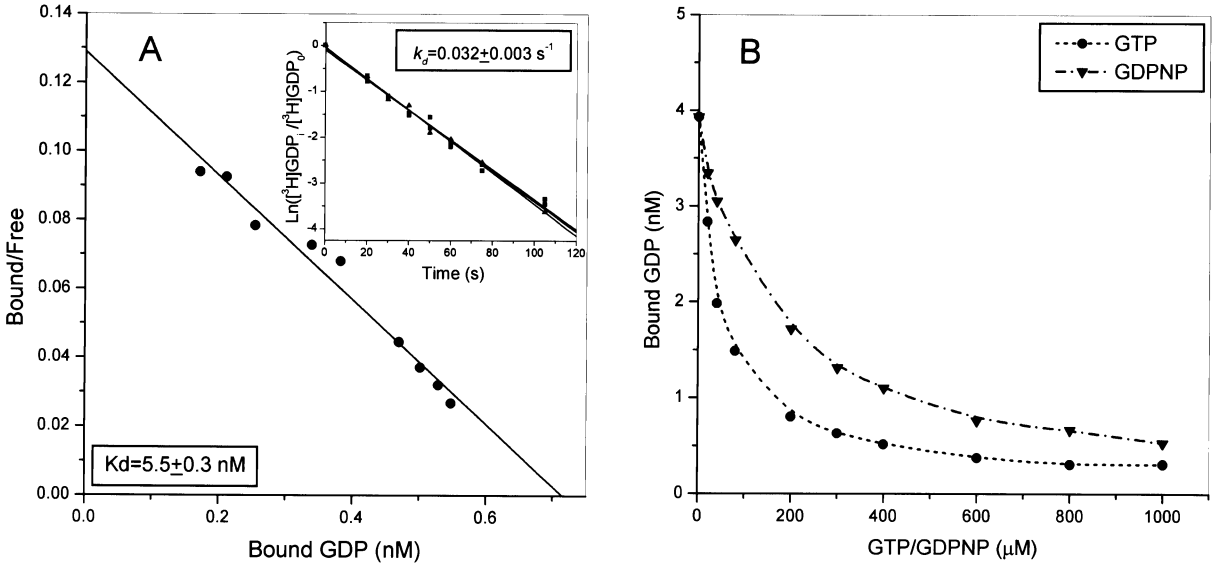


Figure 2. The Binding of GDP, GTP, and GDPNP to RF3

(A) Dissociation constant (K_D) for the RF3•GDP complex. RF3•[^{3}H]GDP (1 nM) was incubated with increasing amounts of added [^{3}H]GDP of the same specific activity as the GDP in the complex. The ratio of the concentrations of factor-bound and -free GDP is plotted as a function of the concentration of factor-bound GDP (Scatchard plot). K_D was estimated from the inverse of the slope of the straight line.

(A, insert) The dissociation rate constant (K_d) for the RF3•GDP complex. To an RF3•[^{3}H]GDP complex (80 nM) was added unlabeled GDP in large excess. The natural logarithm of the ratio of RF3•[^{3}H]GDP$_i$ remaining and RF3•[^{3}H]GDP$_0$ at zero time is shown as a function of time after addition of unlabeled GDP. The K_d value was estimated from the slope of the straight line.

(B) Apparent dissociation constants (K_{DT}) for RF3•GTP and RF3•GDPNP complexes. RF3 (4 nM) was incubated with [^{3}H]GDP (100 nM) at different concentrations of GTP or GDP. The amount of RF3•[^{3}H]GDP bound is shown as a function of the concentration of GTP or GDPNP. Apparent dissociation constants were calculated from $K_{DT} = I_{50}/(1 + [GDP]/K_D)$, where K_D = 5.5 nM, [GDP] = 100 nM and I_{50} is the value of GTP or GDPNP where the amount of RF3•[^{3}H]GDP has dropped to half, i.e., to 2 nM.

centrations of RF1 or RF2. The extent of GTP hydrolysis after the incubation was monitored by direct measurement of the quantities of GTP and GDP separated by anion exchange chromatography. The results (Figure 4) demonstrate that the rate of hydrolysis of GTP catalyzed by RF3 and release complex could be stimulated by an order of magnitude by the presence of RF1 or RF2. The stimulation was codon specific; thus, when the release

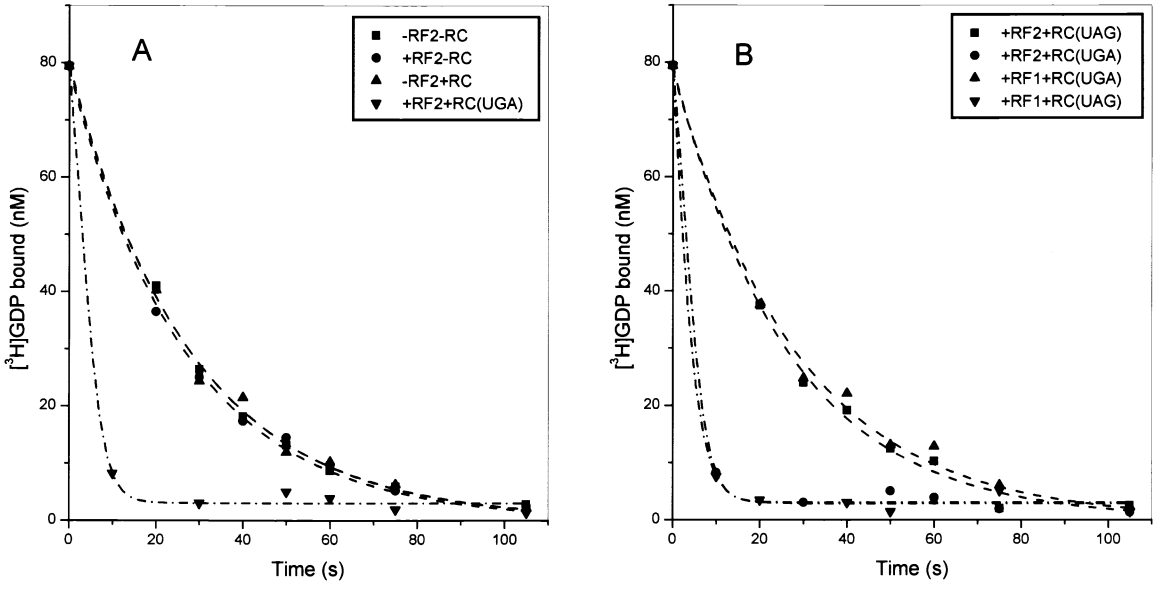


Figure 3. Rapid Exchange of RF3-Bound GDP Requires RC and RF2

(A) To an RF3•[^{3}H]GDP complex (80 nM) was added unlabeled GDP in large excess (0.5 mM) in the absence of other factors (■), in the presence of 15 nM RF2 (●), in the presence of 4 nM RC (stop codon UGA) (▲), and in the presence of RF2 and RC (▼). The concentration of [^{3}H]GDP remaining bound to RF3 is shown as a function of time after the addition of excess unlabeled GDP.

(B) Exchange of [^{3}H]GDP to GDP on RF3 was monitored in the presence of RF2 and RC with UAG (■) or UGA (●) stop codon or in the presence of RF1 and RC with UGA (▲) or UAG (▼) stop codon at the same conditions as in (A).

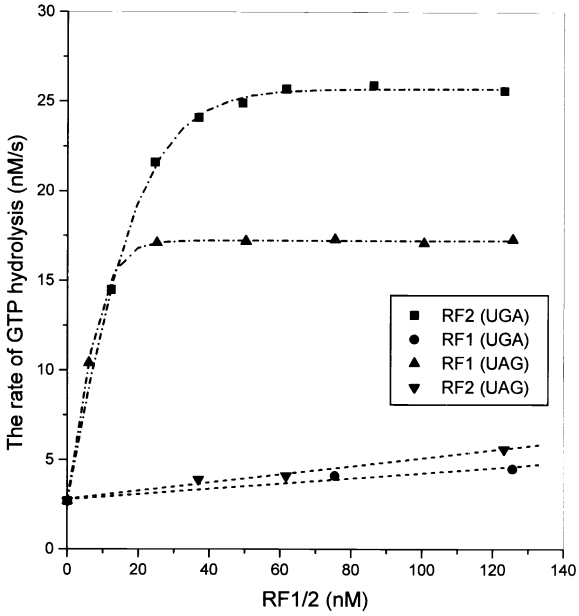


Figure 4. RF1 and RF2 Stimulate Ribosome-Mediated GTP Hydrolysis by RF3

RC (60 nM), containing either UGA or UAG stop codons, was incubated with RF3 (53 nM) and GTP at increasing concentrations of RF1 or RF2. The y axis shows the rate of GTP hydrolysis in the presence of RF2 with stop codon UGA (■) or UAG (▼) and in the presence of RF1 with stop codon UGA (●) or UAG (▲). The x axis shows the concentration of RF1 or RF2.

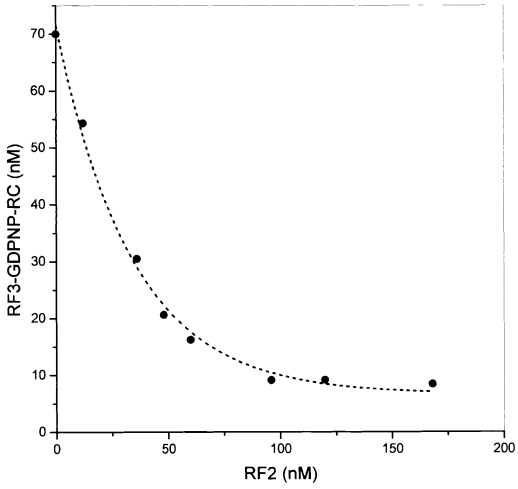


Figure 5. Competition between RF2 and RF3•GDPNP for Binding to RC

RC (80 nM), GDPNP (400 nM), and RF3 (800 nM) were incubated with different concentrations of RF2. The concentration of RF3•GDPNP•RC as obtained from nitrocellulose filter binding is shown as a function of the concentration of RF2.

complex was programmed with UAG in the A-site, RF1, but not RF2 stimulated the GTPase activity of RF3. In contrast, when UGA was present in the A-site, RF2, but not RF1 stimulated the GTPase activity. These findings indicate how the potential problem with idling ribosome-dependent GTPase activity of RF3 is avoided: dissociation of GDP is promoted only when RF3 associates with its natural target, which is a ribosome in posttermination state with a class I release factor in A-site. Therefore, subsequent binding of GTP and GTP hydrolysis become coupled to the removal of RF1 or RF2 and RF3 from the ribosome (Figure 1).

The large stimulatory effect of RF1 or RF2 on the GTPase activity of RF3 is seen (Figure 4) at ratios of RF1 or RF2 to RF3 close to those found in the cell (Adamski et al., 1994; Holst-Hansen et al., 1997). This may explain why stimulation was not observed in experiments where RF3 was present in large excess over RF1 or RF2 and with release complex preparations of much lower activity (Freistroffer et al., 1997) than those used here.

Binding of RF3•GDPNP and RF1 or RF2 to Release Complexes Are Mutually Exclusive

The finding that RF3 and GDPNP can catalyze recycling of RF1 or RF2, provided that RF3 is in excess (Figure 1), suggests that the binding of RF3 in GTP form and RF1 or RF2 to the ribosome are mutually exclusive. This would imply that high concentrations of RF1 or RF2 should inhibit RF3-mediated binding of GDPNP to release complexes. Support for this conclusion has been obtained from experiments in which RF3•GDPNP and RF2 compete for post-release complexes, obtained by the action of puromycin on fMFTI-tRNA•70S release complexes. Post-release complexes were first equilibrated with [^{3}H]GDPNP in the presence of RF3, and then incubated with different concentrations of RF2. The amount of [^{3}H]GDPNP bound to RF3 on the post-release complexes could be detected by nitrocellulose filter binding, since RF3 did not bind [^{3}H]GDPNP in the absence of ribosomes. As seen in Figure 5, RF2 can compete out the RF3•GDPNP complex from the ribosome. This result and the principle of detailed balance imply that the binding of RF3•GDPNP and RF2 to the ribosome are mutually exclusive. Identical results were obtained from competition experiments in which RF1 replaced RF2 (data not shown). This confirms that binding of RF1 or RF2 to the ribosome is destabilized by RF3 in the GTP conformation, and explains how RF3 catalyzes the dissociation of class I release factors from the post-release complex. A remaining question is how negative interference between class I and class II release factors, i.e., that RF3 promotes dissociation of RF1 or RF2 before hydrolysis of the ester bond in peptidyl-tRNA, is avoided.

RF3 in Complex with GDPNP Can Bind to Release Complex after, but Not before, Polypeptide Release

The findings that ribosome binding of RF3•GDPNP and RF1 or RF2 are mutually exclusive and that RF3 enters the ribosome in the GDP conformation raise an important question: when does the "active" GTP conformation appear in the RF3 cycle? To find an answer, we first measured RF3-mediated binding of GDPNP to release complex before and after tetrapeptide release from the ribosome. Release was performed either with puromycin or with catalytic amounts of RF1. RF3 and ribosomes were kept at constant concentrations and [^{3}H]GDPNP was titrated from low to high concentrations. After equilibration of the reactions, the amount of [^{3}H]GDPNP on

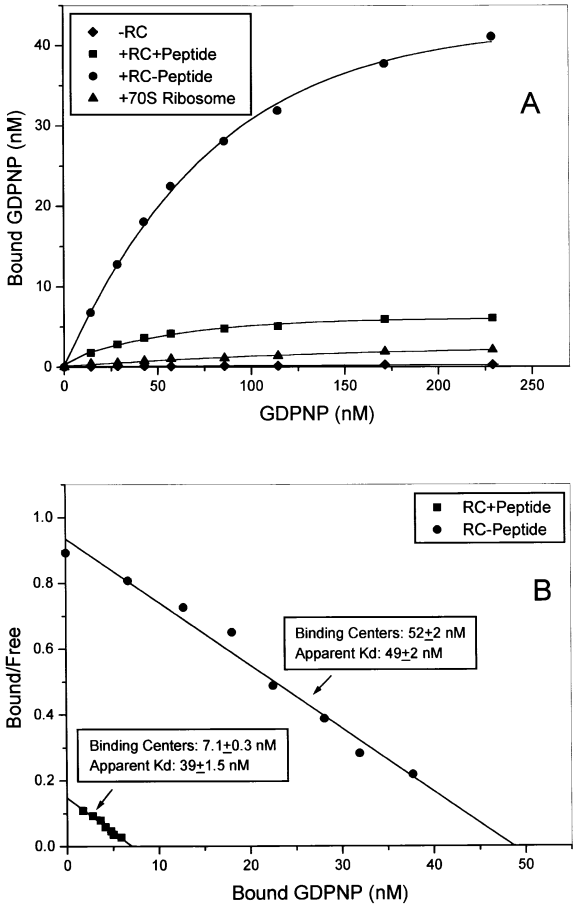


Figure 6. Binding of RF3•GDPNP to Release Complexes and Ribosomes

(A) RF3•GDPNP complex formation on ribosomes or RC (65 nM). RF3 (600 nM) was incubated with increasing concentrations of [^{3}H]GDPNP in the absence of ribosomes (◆), in the presence of naked ribosomes (▲), and in RC with (■) or without (●) tetrapeptide. The concentration of GDPNP bound to RF3 as estimated from nitrocellulose filter binding is shown as a function of the concentration of added GDPNP.

(B) Scatchard plots of GDPNP bound to RF3 on RC with (■) and without (●) peptide. K_D values were estimated from the inverse of the slopes of the straight lines, and the concentration of binding sites for GDPNP on RF3 in complex with ribosomes by their intercepts with the x axis.

RF3 in complex with ribosomes was determined by nitrocellulose binding. The extent of binding of GDPNP in the three cases is shown in Figure 6A, and the corresponding Scatchard plots are displayed in Figure 6B. Extrapolation to the abscissa shows that about 10% of the release complex ribosomes bound RF3•GDPNP at saturating levels of free GDPNP before release of the peptide, and that the number of binding sites increased 7-fold following peptide release. At the same time, the apparent K_D value remained essentially unaffected (39 nM before and 49 nM after removal of the tetrapeptide). We interpret these data to mean that RF3 in the GTP conformation cannot bind to ribosomes with peptidyl-tRNA in the P-site. The residual binding of GDPNP to ribosomes seen before removal of the tetrapeptide may be accounted for by the observation that about 15% of the ribosomes in the release complex preparation did not contain tetrapeptide after the gel filtration step. These results explain how RF3 is prevented from removing RF1 or RF2 before release of peptide from peptidyl-tRNA.

Discussion

In the present study, we have clarified the actual mechanism behind the function of RF3 and we explain how RF3 can remove the class I release factors at the right time point in the termination cycle, with minimal cost from idling GTPase activity and without interfering negatively with the action of RF1 or RF2. Our results have been summarized in a detailed scheme for the whole termination process in bacteria (Figure 7). One difficulty faced by previous models for RF3 action can now be seen to arise from the mistaken assumption that RF3 enters the ribosome in the GTP form (Goldstein and Caskey, 1970; Nakamura et al., 1996; Frolova et al., 1996; Freistroffer et al., 1997; Kisselev and Buckingham, 2000). Another obstacle to progress in the past has been an oversimplified biochemistry with RNA triplets replacing homogeneous, heteropolymeric mRNAs in the release complex. Use of these nonphysiological complexes has led to apparently conflicting observations, e.g., that RF3 in the presence of GTP can both stimulate and inhibit release of peptide (Pel et al., 1998).

Now, we demonstrate that GDP binds three orders of magnitude more strongly to RF3 than GTP, implying that free RF3 must be in the GDP form. Furthermore, we show that rapid exchange of GDP for GTP occurs only when RF3 interacts with a release complex carrying RF1 or RF2, and is sufficient to promote their rapid dissociation. Hydrolysis of the GTP molecule is, on the other hand, necessary for fast dissociation of RF3 itself.

The Mechanism of Translation Termination in Bacteria in Seven Steps

The first step (I) in the scheme of translation termination (Figure 7) is the binding of RF1 or RF2 to a ribosome that carries peptidyl-tRNA in the P-site and is programmed with a stop codon in the A-site. In step II, peptide release occurs, presumably triggered by a direct and stable interaction between RF1 or RF2 and the base triplet in the A-site (Brown and Tate, 1994; Ito et al., 2000). This interaction promotes hydrolysis of the ester bond in peptidyl-tRNA, probably by activation of the peptidyl transferase center in the 50S subunit (reviewed by Kisselev and Buckingham, 2000). Our finding that free RF3 must be in the GDP form (Figure 2) and the outcome of the GDP exchange experiments in Figure 3 show that step III is binding of RF3•GDP to the ribosome and step IV rapid release of GDP. Dissociation of GDP leads to the previously identified stable, guanine nucleotide free complex between RF3 and RF1 or RF2 on the ribosome (Freistroffer et al., 1997; Pel et al., 1998). We can now conclude that this ribosomal state is physiologically relevant, and we suggest that its thermodynamic role is to drive RF3 into a structure that allows rapid dissociation of GDP. Erroneous termination at sense codons by RF1 or RF2 can be significantly stimulated by high concentrations of RF3 (Freistroffer et al., 2000).

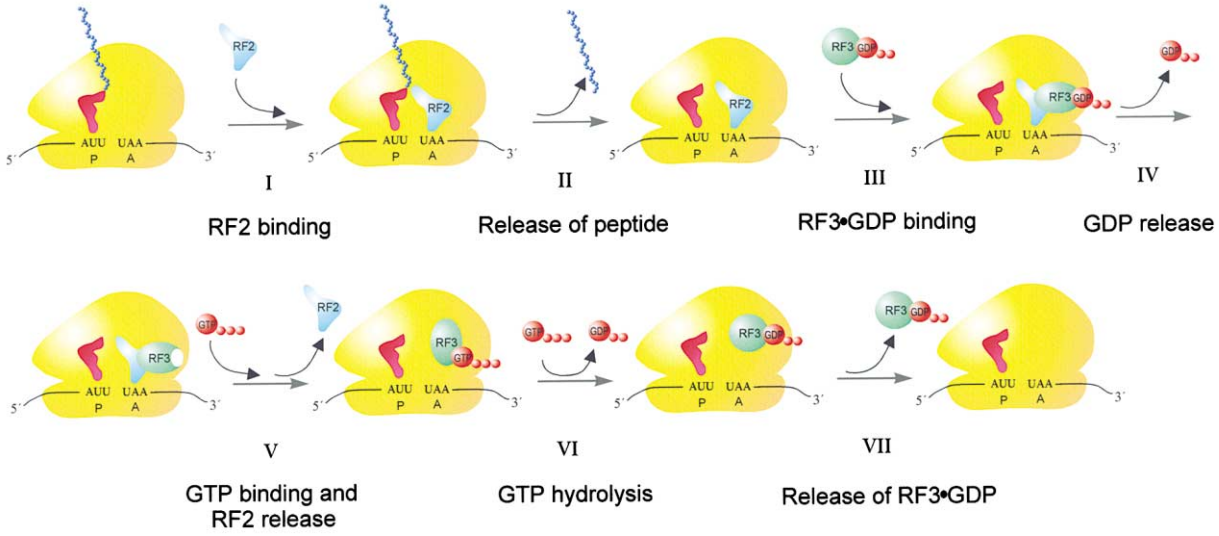


Figure 7. A Scheme in Seven Steps Summarizing the Mechanism of Translation Termination in Prokaryotes (see Discussion for details)

These observations, which do not depend on any stimulatory effect of RF3 on the recycling of RF1 or RF2, suggest that the stable, guanine nucleotide-free complex between RF3 and RF1 or RF2 can be formed on ribosomes containing intact peptidyl-tRNA. Thus, steps III and IV in Figure 7 can be executed before peptide release in step I. This may explain why RF3 enhances the rate of erroneous termination at sense codons, where the binding of RF1 or RF2 to the ribosome is very weak, but does not affect the rate of proper termination at stop codons (Freistroffer et al., 2000), where their binding to the ribosome is sufficiently stable in the absence of RF3. In step V, GTP binds to RF3 and changes its conformation. Since RF3•GDPNP (or RF3•GTP) cannot form a complex with ribosomes containing peptidyl-tRNA in P-site, this step can only be executed after peptide release, which prevents negative interference between the action of RF3 and termination by RF1 or RF2. Step VI is formation of a strong complex between RF3•GTP and the ribosome and rapid dissociation of RF1 or RF2. This follows since RF3•GDPNP binds strongly to the ribosome after peptide release (Figure 6) and binding of RF3•GDPNP and RF1 or RF2 to the ribosome are mutually exclusive (Figure 5). In the final step (VII), GTP is hydrolyzed on RF3, which leads to dissociation of the factor in GDP conformation with low affinity for the ribosome. This ends the termination process and leaves the ribosome in a state from which it can be recycled back to initiation by RRF, EF-G, and IF3 (Karimi et al., 1999).

Resolution of Experimental Paradoxes

The present experiments and those by Freistroffer et al. (1997) were carried out with well-defined release complexes containing a continuous mRNA with a stop codon in the A-site and a peptidyl-tRNA in the P-site. With such intrinsically stable ribosomal complexes, the recycling activity of RF3 can be clearly seen. If, however, separate RNA triplets are used as start and stop codons and termination is defined as deacylation of fMet-tRNA, the release complexes are intrinsically unstable and this reduces the activity of RF1 or RF2. Under these circumstances, the increase in the stability of the release complex by addition of RF3 in the absence, but not in the presence, of guanine nucleotides will stimulate the fMet release activity of RF1 and RF2 (Milman et al., 1969; Goldstein and Caskey, 1970; Pel et al., 1998). This interpretation is supported by the observation that ligation of the initiation and termination triplets, which stabilizes the release complex, leads to RF3-mediated stimulation of termination also in the presence of GTP (Grentzmann et al., 1998). Paradoxically, for release complexes with separate initiation and termination triplets, addition of RF3 in the presence of the nonhydrolyzable analog GDPNP inhibits termination (Pel et al., 1998). The reason, we suggest, is that GDPNP may already bind to RF3 on the less physiological triplet-dependent release complex before termination has taken place. In this way, RF1 or RF2 can be ejected prematurely and translation termination inhibited.

Are the Functions of RF3 and eRF3 Essentially Similar?

Two major functional differences have in the past been identified between RF3 and eRF3. The first is that eRF1 and eRF3 can form a stable complex off the ribosome (Zhouravleva et al., 1995), while no significant affinity between free RF1 or RF2 and RF3 has been demonstrated (Nakamura et al., 1996). However, the importance of the interaction in higher organisms remains unclear (Merkulova et al., 1999), and in fission yeast it appears that C-terminal truncation of eRF1, largely or completely abolishing the interaction, allows cell viability (Ito et al., 1998).

The second difference is that ribosome-dependent GTP hydrolysis on eRF3 requires eRF1 (Frolova et al., 1996), while no stimulation of the ribosome-mediated GTPase activity of RF3 was previously detected upon addition of RF1 or RF2 (Freistroffer et al., 1997; Grentzmann et al., 1998). In this study, we have found that the

GTPase activity of RF3 on ribosomes is, in fact, greatly stimulated by the presence of RF1 or RF2, provided that RF3 is present at sufficiently low concentrations (Figure 4). The reason for the stimulation is that ribosome-bound class I release factors are required for rapid exchange of GDP for GTP on RF3 (Figure 5), followed by their removal from the ribosome (Figure 5), and subsequent GTP hydrolysis that also drives the dissociation of RF3 from the ribosome (Figure 7). This result removes one of the apparent differences between translation termination in pro- and eukaryotes and suggests that the mechanisms may indeed be similar.

RF3 and EF-Tu Have Similar Modes of Action

The best-characterized prokaryotic translation factor has so far been elongation factor EF-Tu (Ruusala et al., 1982a, 1982b; Pape et al., 1998). While EF-Tu brings aminoacyl-tRNA to the ribosome, we now know that RF3 removes RF1 or RF2 from the ribosome (Freistroffer et al., 1997). Therefore, EF-Tu and RF3 carry out quite opposite tasks, but interesting mechanistic parallels can in any case be drawn. First, both factors rapidly leave the ribosome in GDP conformation and, second, have high affinity for GDP and slow spontaneous rate of exchange of GDP for GTP. Third, both use nucleotide exchange factors which seem to function according to similar kinetic principles: elongation factor EF-Ts binds EF-Tu with high affinity in the absence of GDP or GTP and this facilitates rapid exchange of GDP for GTP (Ruusala et al., 1982a). Similarly, a release complex with RF1 or RF2 in the A-site has very high affinity for RF3 in the absence of guanine nucleotides, and this speeds up the rate of exchange of GDP for GTP. Fourth, the GTP conformation is strongly stabilized for both factors by complex formation with another macromolecule. For EF-Tu, the stabilizing partner is aminoacyl-tRNA, and for RF3, it is a ribosome after release of RF1 or RF2. Finally, both EF-Tu and RF3 rapidly hydrolyze GTP when their respective tasks, i.e., delivery of aminoacyl-tRNA to or removal of RF1 or RF2 from the ribosomal A-site, are done. Both EF-Tu and RF3 can now be seen to belong to the subset of small GTPases (Bourne et al., 1991; Sprang and Coleman, 1998) that require guanine nucleotide exchange factors. Experimental observations of the four GTP binding translation factors in bacteria suggest that RF3 and EF-Tu form a natural pair with similar functional properties, clearly distinct from those of EF-G (Pape et al., 1998) and IF2 (Tomsic et al., 2000). The reason for these apparently different strategies among the G proteins in bacterial protein synthesis poses a challenging question for future work.

Experimental Procedures

Chemicals and Buffers

Nucleoside triphosphates were from Pharmacia (Uppsala). Guanilylimidophosphate (GDPNP) and pyruvate kinase (PK) were from Boehringer Mannheim. Phosphoenolpyruvate (PEP), myokinase (MK), putrescine, spermidine, puromycin dihydrochloride, and nonradioactive amino acids were from Sigma. All nucleotides were further purified by ion exchange chromatography on a Mono Q column (Pharmacia) for immediate use or freezing. Radioactive amino acids and nucleotides were from Amersham. All other chemicals were of analytical grade from Merck. All experiments were carried out in polymix buffer, containing at final concentration 5 mM potassium phosphate, 5 mM magnesium acetate, 5 mM ammonium chloride, 95 mM potassium chloride, 0.5 mM calcium chloride, 8 mM putrescine, 1 mM spermidine, and 1 mM dithioerythritol (Jelenc and Kurland, 1979).

Synthetic mRNAs

An MFTI-mRNA used for release complex preparations had the sequence gggaauucgggcccuuguuaacaau*uaaggaggu*auacu AUG UUU ACG AUU (STOP) uugcag(a)$_{21}$. It contained a strong ribosome binding site (italic lower case letters), an open reading frame encoding an MFTI tetrapeptide (capital letters), either one of the stop codons (UAG, UGA, or UAA), and a poly (A) tail used for its purification on a poly(dT) column (Pharmacia). The mRNAs were synthesized by T7 RNA transcription of the corresponding DNA constructs (Pavlov et al., 1997).

Components of the Translation System

Wild-type and fully active ribosomes were prepared from *E. coli* strain MRE600 by sucrose gradient ultracentrifugation according to Rodnina and Wintermeyer (1995), with minor modifications. Initiation factors were purified from overproducing strains according to Soffientini et al. (1994) with minor modifications. Peptide release factors (RF1, RF2, and RF3), Ile-tRNA synthetase, and fMet-tRNA^{Met} were prepared according to Freistroffer et al. (1997). Elongation factors EF-Tu, EF-Ts, EF-G, tRNA-bulk, and Phe-tRNA synthetase were purified according to Ehrenberg et al. (1990). Thr-tRNA synthetase was prepared according to Brunel et al. (1993).

Release Complex Preparation

Ribosomes paused with a stop codon in A-site and a tetrapeptidyl-tRNA in P-site (release complexes, RC) were prepared according to Freistroffer et al. (1997), with modifications. An initiation mix (0.4 ml), containing 6 μM ribosomes, 1.5 μM of IF1, IF2, and IF3, 12 μM MFTI-mRNA, 12 μM fMet-tRNA^{Met}, and an energy regeneration system (50 ng/μl PK, 1 mM GTP, and 6 mM PEP) was incubated at 37°C for 20 min. An elongation mix (0.4 ml), containing 250 μM Phe and Thr, 20 μM [^{14}C]Ile, tRNA bulk (containing 11 μM tRNA^{Ile}), 18 μM EF-Tu, 1.2 μM EF-Ts, 6 μM EF-G, 50 U each of Phe-, Thr- and Ile-tRNA synthetases, as defined in Ehrenberg et al. (1990), and an energy regeneration system (50 ng/μl PK, 1 mM GTP, 10 mM PEP, 7 ng/μl MK, 1 mM ATP) was incubated separately at 37°C for 10 min. Peptide elongation was started by adding together the initiation and elongation mixtures. After 1 min incubation at 37°C, the reaction mixture was cooled on ice and applied to a gel filtration column (Sephacryl S-300 HR, 2 cm^2 × 30 cm, Pharmacia) held at 2°C and connected to an FPLC system (Pharmacia). Pure RC eluted in the first peak and was monitored by OD$_{280}$ and by scintillation counting of [^{14}C]Ile in tetrapeptidyl-tRNA. Fractions of RC were pooled and frozen in liquid nitrogen at concentrations between 0.5 and 1 μM.

Release Complex and Release Factor Activity

The total concentration of ribosomes was obtained from OD$_{260}$ (1 OU corresponds to 23 pmol of ribosomes). The amount of MFTI-tRNA in RC was measured by scintillation counting of [^{14}C]Ile in tetrapeptide after its release from tRNA by RF1 (or RF2). An aliquot of RC was incubated with excess RF1 (or RF2) for 3 min at 37°C in 50 μl. After incubation, 900 μl of ice cold 5% TCA (containing 0.75% casaminoacids) was added. Ribosomes and tRNA were pelleted by centrifugation at 14,000 g for 10 min. The [^{14}C] content in the supernatant from the radioactive peptide was measured by scintillation counting using Aquasafe 300 Plus (Zinser Analytics). The fraction of active RC (about 80%) was measured as the amount of released tetrapeptide normalized to the total amount of ribosome (from OD$_{260}$).

The fraction of active RF1 or RF2 (typically between 30% and 40%) was measured as the amount of tetrapeptide released in the absence of guanine nucleotides with ribosomes and RF3 (to prevent recycling) in excess, normalized to the total concentration of RF1 (or RF2) measured as described by Bradford (1976). Active RF3 was prepared in 1:1 complex with GDP. This was deduced from the observation that the rate of binding of labeled GDP to RF3 was uniform and became equal to the dissociation rate constant for the RF3•GDP complex (Figure 2, insert) when labeled GDP was in

sufficient excess. The active fraction of RF3 (typically 40%) was measured as the amount of [^{3}H]GDP in complex with factor (from nitrocellulose filter binding) with GDP in excess, normalized to the total amount of RF3 measured as described by Bradford (1976). Only active concentrations of RC and release factors are given in the text.

Recycling of Release Factors

Fifty nM RC, 1.1 nM RF2 (or RF1), 6 nM or 200 nM RF3, and 0.2 mM guanine nucleotides (GTP, GDP, and GDPNP) were used for factor recycling experiments. For experiments in the absence of RF3, RC and RF2 (or RF1) were preincubated for 3 min at 37°C in two separate tubes. Peptide hydrolysis was initiated by addition of RF2 (or RF1) to RC. For experiments in the presence of RF3, (RC + RF3) and RF2 were preincubated for 3 min at 37°C in separate tubes. Termination was initiated by addition of RF2 (or RF1) to the RC + RF3 mix. For experiments with addition of guanine nucleotides, (RC + RF3 + RF2) and one of the guanine nucleotides were preincubated for 3 min at 37°C separately. Then the guanine nucleotide was added to the RC + RF3 + RF2 mix. Aliquots (50 μl) were in all cases removed at different time points after the start of the reaction and quenched by addition to ice-cold 5% TCA. The concentration of peptide remaining in the supernatant after centrifugation was determined by scintillation counting.

Dissociation Constant for the RF3•GDP Complex and Inhibition of RF3•GDP Formation by Addition of GDPNP or GTP

Eighty nM RF3 was incubated with 1.5 μM [^{3}H]GDP in 1 ml of buffer A (25 mM Tris·HCl [pH 7.5]) at 37°C for 10 min to allow formation of an RF3•[^{3}H]GDP complex. The mix was cooled on ice and applied to a Mono Q column equilibrated with buffer A. The RF3•[^{3}H]GDP complex was eluted with a 0–0.5 M NaCl gradient in buffer A. To determine the K_D, RF3•[^{3}H]GDP was diluted with polymix buffer to 1 nM and incubated with increasing concentrations of [^{3}H]GDP (1–20 nM). After 10min incubation, the reaction mixture (1 ml) was filtered through nitrocellulose and washed with 1 ml ice-cold polymix buffer. The amount of RF3•[^{3}H]GDP retained on the filter was determined by scintillation counting. The total concentration of [^{3}H]GDP was calculated taking the amount of [^{3}H]GDP contained in the RF3 preparation into account.

To study the inhibition of RF3•GDP complex formation by GDPNP or GTP, 4 nM RF3•[^{3}H]GDP was incubated with 100 nM [^{3}H]GDP in the presence of increasing concentrations of GDPNP or GTP (0.02–1 mM). After incubation, the different mixes (90 μl) were filtered through nitrocellulose and the retained amount of RF3•[^{3}H]GDP was measured by scintillation counting.

GDP Exchange on RF3

RF3 with [^{3}H]GDP (mix A) and unlabeled GDP with different additions (mixes B: ±RF1 (or RF2) ±RC (with UAA, UAG, or UGA stop codons)) were preincubated separately. The exchange reaction was started by adding mix A to mix B and the concentrations of components (when present) in the reaction mix were 80 nM RF3, 4 nM RC, 15 nM RF1 (or RF2), 1μM [^{3}H]GDP, and 0.5 mM GDP (unlabeled). The reaction was stopped by filtering of 45 μl samples through nitrocellulose, and the amount of [^{3}H]GDP retained was determined by scintillation counting.

GTPase Activity of RF3

The GTPase activity of RF3 (53 nM) was studied in the presence of RC (60 nM) containing UGA or UAG stop codons in the A-site with RF1 (or RF2) concentrations in the range 0–130 nM. For each reaction, a factor mix with RF3+RC±RF1 (or RF2) and a GTP mix were preincubated for 3 min at 37°C in two separate tubes. The reaction was started by adding the GTP mix to the factor mix to a final volume of 50 μl. The incubation times (2–10 min) were chosen so that 0.5%–3% of the total GTP was hydrolyzed. The reaction was stopped by addition of 1 ml ice-cold magnesium-free buffer A (25 mM Tris·HCl [pH 7.5]). Samples containing the diluted reaction mixture were kept on ice and subsequently applied to a Mono Q anion exchange column equilibrated with buffer A. Components of the reaction mixture, GTP, and GDP were completely separated by elution with a 4 ml NaCl gradient (0.18–0.32 mM) in buffer A at a flow rate of 1 ml/min. The separation was controlled by an FPLC system (Pharmacia). The rate of GTP hydrolysis was then obtained from the ratio between the GDP peak area and the sum of the GDP and GTP peak areas measured by OD, the total concentration of guanine nucleotides, and the incubation time.

Competition between RF1 and RF3•GDPNP for Binding to Release Complex

A mixture containing 0.8 μM RF3, 80 nM RC, 0.2 mM puromycin, and 0.4 μM [^{3}H]GDPNP was incubated at 37°C for 10 min with different concentrations of RF1 (0–170 nM). The reaction mixtures (45 μl) were then filtered through nitrocellulose. The amount of RF3•GDPNP•RC retained on the filter was determined by scintillation counting of [^{3}H]GDPNP.

Complex Formation between GDPNP and RF3 on Release Complexes and Naked Ribosomes

To determine the apparent dissociation constant and number of binding sites for complex formation between GDPNP and RF3 in RC with intact peptidyl-tRNA or deacylated tRNA in P-site, 600 nM RF3 was incubated for 10 min at 37°C with different concentrations of GDPNP (15–300 nM) in the presence of 65 nM RC. The reaction mixtures (60 μl) were then added to 3 ml ice-cold polymix buffer, filtered through nitrocellulose, and washed with 3 ml buffer. The amount of RF3•GDPNP•RC retained on the filter was determined by scintillation counting of [^{3}H]GDPNP. To remove the tetrapeptide from tRNA, RC was either incubated with 0.2 mM puromycin or with RF1 in catalytic amounts (5 nM). As controls, the binding of GDPNP to RF3 was studied with no additions or in the presence of 65 nM naked ribosomes.

Acknowledgments

We thank Diarmaid Hughes, Tillmann Pape, Harald Putzer, Urmila Rawat, Marina Rodnina, and Miklos de Zamaroczy for helpful suggestions on the manuscript. This work was supported by grants from the Swedish Foundation for Strategic Research, the Swedish Research Council, and the Centre National pour la Recherche Scientifique (UPR9073).

Received July 2, 2001; revised August 27, 2001.

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