Release factor RF3 in *E.coli* accelerates the dissociation of release factors RF1 and RF2 from the ribosome in a GTP-dependent manner

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Ribosomes complexed with synthetic mRNA and peptidyl-tRNA, ready for peptide release, were purified by gel filtration and used to study the function of release factor RF3 and guanine nucleotides in the termination of protein synthesis. The peptide-releasing activity of RF1 and RF2 in limiting concentrations was stimulated by the addition of RF3 and GTP, stimulated, though to a lesser extent, by RF3 and a non-hydrolysable GTP analogue, and inhibited by RF3 and GDP or RF3 without guanine nucleotide. With short incubation times allowing only a single catalytic cycle of RF1 or RF2, peptide release activity was independent of RF3 and guanine nucleotide. RF3 hydrolysis of GTP to GDP + P_i was dependent only on ribosomes and not on RF1 or RF2. RF3 affected neither the rate of association of RF1 and RF2 with the ribosome nor the catalytic rate of peptide release. A model is proposed which explains how RF3 recycles RF1 and RF2 by displacing the factors from the ribosome after the release of peptide.

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Introduction

The termination of protein synthesis occurs when there is a peptidyl-tRNA in the P-site and a stop (nonsense) codon in the A-site of the ribosome. In prokaryotes, hydrolysis of the ester bond between peptide and tRNA is triggered by release factors RF1 (reading UAG, UAA in the A-site) and RF2 (reading UGA, UAA in the A-site) with help from the G-protein RF3 (Ganoza, 1966; Capecchi, 1967; Scolnick *et al.*, 1968). In eukaryotes, release factor eRF1 reads all three stop codons and is aided by the G-protein eRF3 (Zhouravleva *et al.*, 1995).

The role of RF3 in termination has remained elusive in spite of considerable efforts to uncover its secrets. One problem has been to clarify the role of guanine nucleotides in the termination process. It has been known for a long time that they affect the release of polypeptide (Milman *et al.*, 1969). At the same time, the classical *in vitro* assay for termination (Caskey *et al.*, 1968) indicates that addition of GTP inhibits, rather than stimulates, the catalytic effect

of RF3 on peptide release from the ribosome (Capecchi and Klein, 1969; Milman *et al.*, 1969; Grentzmann *et al.*, 1995). The expected stimulatory effect of GTP on polypeptide release in the presence of RF3 has only been observed under particular conditions with respect to tri-nucleotide concentrations and cations (Goldstein and Caskey, 1970).

The first attempt to explain the role of RF3 in translation termination proposed a dual function, first to stimulate the binding of RF1 and RF2 (RF1/2) to the ribosome, and secondly to catalyse the dissociation of the factors from the ribosome after hydrolysis of peptidyl-tRNA in a reaction dependent on guanine nucleotide, thereby stimulating the recycling of RF1/2 between ribosomes (Goldstein and Caskey, 1970).

Recently, an extended version of the first action of RF3 proposed by Goldstein and Caskey (1970) has been strongly propagated (Ito *et al.*, 1996; Nakamura *et al.*, 1996): the 'ternary complex model'. This proposal was inspired by the recent discovery of structural similarity between EF-G on one hand and the ternary complex between EF-Tu-GTP and aminoacyl-tRNA on the other (Ævarsson *et al.*, 1994; Czworkowski *et al.*, 1994; Nissen *et al.*, 1995). Domain 4 in EF-G structurally mimics the anticodon stem in tRNA, and domains I (the G-domain) and II in EF-G have sequence similarity with EF-Tu. The model was supported further by sequence similarities between RF3 on one hand and EF-Tu and EF-G on the other, as well as by sequence similarities between the part of EF-G that mimics the tRNA anticodon stem, and RF1/2. From these data, it was suggested that RF3, GTP and RF1 or RF2 may form a ternary complex like that between EF-Tu, GTP and aminoacyl-tRNA, either off or on the ribosome (Ito *et al.*, 1996; Nakamura *et al.*, 1996). It was proposed further that RF3 helps RF1 or RF2 to promote hydrolysis of peptidyl-tRNA by enhancing the rate of association of the latter two factors with the ribosome, in analogy with how EF-Tu accelerates the binding of aminoacyl-tRNA to the A-site (Kaziro, 1978).

It was shown recently in eukaryotes that eRF3 and eRF1 can form a complex off the ribosome (Zhouravleva *et al.*, 1995). Further, it was found that eRF3 stimulates the action of eRF1 in a GTP-dependent manner and that eRF3 is a GTPase, hydrolysing GTP to GDP and P_i, dependent on eRF1 and the ribosome (Zhouravleva *et al.*, 1995; Frolova *et al.*, 1996). Insofar as the action of eRF3 is similar to that of RF3 in prokaryotes, these findings indirectly support the model (Ito *et al.*, 1996; Nakamura *et al.*, 1996) that RF3 forms a ternary complex with RF1/2 and GTP.

It has been found *in vivo* that the level of RF3 in the cell affects the readthrough of stop codons by a suppressor tRNA ternary complex (Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994). The interpretation of these and similar *in vivo*

experiments (Grentzmann *et al.*, 1995) depends crucially on a correct understanding of RF3 action. If the factor acts after hydrolysis of peptidyl-tRNA, as in the recycling model (Goldstein and Caskey, 1970), differences in readthrough due to changes in the RF3 pool must be caused by variations in the RF1/2 pools. If, in contrast, RF3 can actively enhance the rate of association of RF1/2 with the ribosome (Ito *et al.*, 1996; Nakamura *et al.*, 1996), then differences in readthrough may instead be interpreted as different kinetic efficiencies of RF1/2 due to variations in the extent of their interaction with RF3.

In the present work, we have clarified biochemical aspects of the role of RF3 in termination of translation. Our results were obtained with the help of a new *in vitro* termination assay based on an optimized translation system (Pavlov and Ehrenberg, 1996). Termination complexes, containing tetrapeptidyl-tRNA in the ribosomal P-site and a stop codon in the A-site, were prepared by the translation of a small heteropolymeric mRNA in the absence of release factors. Ribosomes pausing at a stop codon were purified from all translation components and used subsequently in the termination experiments.

Our results show that RF3 and GTP have no influence on the action of RF1/2 before hydrolysis of peptidyl-tRNA, and demonstrate that RF3 and GTP catalyse the removal of RF1/2 from the ribosome after termination. Our experiments are not compatible with the 'ternary complex model' (Ito *et al.*, 1996; Nakamura *et al.*, 1996) but favour instead the recycling scheme by Goldstein and Caskey (1970). We present a new model for the action of RF3 that explains all our experimental data and suggests that RF3 may have some functional properties in common with myosin (Block, 1996; Holmes, 1996).

Results

Isolation of stable ribosomal complexes stalled at a stop codon

Stable ribosomal complexes containing [³H]fMet-Phe-Thr-[¹⁴C]Ile-tRNA^{Ile} bound at the P-site and stalled with a stop codon in the A-site (release complexes) were separated by gel filtration from unbound tRNA, nucleotide, amino acids and other factors as judged from optical density and from scintillation counting of ³H and ¹⁴C (Materials and methods). These complexes were produced on a large scale and saved in aliquots at -80°C for termination experiments.

Hydrolysis of peptidyl-tRNA by RF1 or RF2 in titration and recycling experiments

Purified ribosome termination complexes containing fMet-Phe-Thr-Ile-tRNA^{Ile} bound at the P-site were used as substrate for RF1. Figure 1 shows the amount of tetrapeptidyl-tRNA that remains after 2 min incubation in titrations with RF1 with or without GTP in the absence or presence of RF3. When both GTP and RF3 are present, the rate of hydrolysis of peptidyl-tRNA is very fast, even at low concentrations of RF1. In the absence of RF3, the hydrolysis rate is slower and does not depend on GTP. In the absence of GTP, RF3 slightly inhibits the rate of termination.

The recycling of RF1 was studied under conditions where ribosomes (2.5 pmol active) are in large excess

Fig. 1. Hydrolysis of peptidyl-tRNA in the P-site by RF1 with a UAG codon in the A-site. RF1 was titrated in the absence of RF3 plus 0.2 mM GTP (◇) or minus GTP (△) and in the presence of 90 pmol of RF3 plus 0.2 mM GTP (□) or minus GTP (○). The incubation time for the reaction was 2 min.

Fig. 2. Hydrolysis of peptidyl-tRNA, as in Figure 1, measured as a function of time with the amount of RF1 fixed at 0.25 pmol. The experiments were carried out in the absence of RF3 plus 0.2 mM GTP (◇) or minus GTP (△) and in the presence of 90 pmol of RF3 plus 0.2 mM GTP (□) or minus GTP (○).

over RF1 (0.25 pmol), so that RF1 has to cycle many times to release peptide from all ribosomes. The amount of tetrapeptidyl-tRNA remaining after different incubation times was measured in the presence or absence of RF3 and GTP. Figure 2 shows that the rate of hydrolysis of peptidyl-tRNA is very fast in the presence of both RF3 and GTP. In the absence of RF3, the rate is intermediate and does not depend on GTP. In the absence of GTP, RF3 strongly inhibits the recycling rate of RF1. A similar inhibition by RF3 is also found in the presence of GDP at concentrations up to 2 mM (see below).

The results in both Figures 1 and 2 apparently contradict earlier findings using the classical trinucleotide assay for termination (Milman *et al.*, 1969; Goldstein and Caskey, 1970; Mikuni *et al.*, 1994; Grentzmann *et al.*, 1995),

which show that RF3 in the presence of GTP inhibits, rather than stimulates, RF1-dependent termination and that RF3 in the absence of GTP stimulates, rather than inhibits, termination.

Experiments similar to those in Figures 1 and 2 were performed for RF1 and RF2 with UAA and for RF2 with UGA as stop codons, with very similar results (not shown). It may be noted that under the conditions of these experiments, RF3 stimulates the recycling rates of RF1 and RF2 by the same factor. This is in contrast to an earlier conclusion by Grentzmann *et al.* (1995), who suggested that RF3 may preferentially stimulate the action of RF2.

Hydrolysis of peptidyl-tRNA by RF1 and RF2 in single cycle experiments

RF3 might stimulate the recycling rates of RF1 and RF2 (see Figure 2) by several distinct mechanisms: by enhancing the rates of association of the factors with the ribosome, by increasing the catalytic rate (k_{cat}) of hydrolysis of peptidyl-tRNA or by catalysing the release of RF1 and RF2 from the ribosome after hydrolysis of peptidyl-tRNA. To discriminate between the latter two alternatives, experiments were designed to study the rate of hydrolysis of peptidyl-tRNA where the action of RF1 and RF2 is restricted to a single cycle of hydrolysis. Single cycle operation of RF1 was achieved in titration experiments similar to those in Figure 1, except that the extent of peptidyl-tRNA hydrolysis was measured after incubation for 4 s instead of 2 min. Figure 3A shows that under these conditions the RF1-catalysed rate of hydrolysis of peptidyl-tRNA at the UAG stop codon is the same in the presence and absence of both RF3 and GTP. In all four cases displayed in Figure 3A, the k_{cat} value for hydrolysis of peptidyl-tRNA is $\sim 0.25 \text{ s}^{-1}$ (see Materials and methods).

Similar experiments employing RF2 and a UGA stop codon are presented in Figure 3B. Three of the curves are identical (–RF3 + GTP, –RF3 –GTP and +RF3 –GTP), but the extent of hydrolysis of peptidyl-tRNA after 4 s is somewhat larger for low concentrations of RF2 when both RF3 and GTP are present. However, the effect of adding RF3 and GTP is much smaller after 4 s of incubation than after 2 min (not shown). The catalytic rate (k_{cat}) of hydrolysis of peptidyl-tRNA induced by RF2 is faster than 0.5 s^{-1} (see Materials and methods). It is therefore possible that RF2, in contrast to RF1 ($k_{\text{cat}} = 0.25 \text{ s}^{-1}$), may recycle to some extent even during a 4 s incubation, which may explain why the rate of hydrolysis of peptidyl-tRNA is somewhat faster in the presence of RF3 and GTP.

Hydrolysis of peptidyl-tRNA by RF1 and RF2 in association rate-controlled experiments

The experiments described so far show that RF3 does not influence single cycle rates for RF1 or probably for RF2 either in the presence or absence of GTP. Therefore, the main action of RF3 cannot be to enhance the k_{cat} value for hydrolysis of peptidyl-tRNA. RF3 must, instead, facilitate dissociation of RF1/2 from the ribosome after hydrolysis of peptidyl-tRNA or enhance the effective association rate constant ($k_{\text{cat}}/K_{\text{m}}$) for the interaction between the ribosome and RF1/2. To discriminate between these two alternatives,

Fig. 3. Hydrolysis of peptidyl-tRNA, as in Figure 1, for RF1 (A) and for RF2 (B) titrations at short incubation time (4 s). For RF2 in (B) the stop codon is UAA. The experiments were carried out in the absence of RF3 plus 0.2 mM GTP ($\blacklozenge$) or minus GTP ($\triangle$) and in the presence of RF3 plus 0.2 mM GTP ($\blacksquare$) or minus GTP ($\circ$).

experiments were designed in which the influence of RF3 on the $k_{\text{cat}}/K_{\text{m}}$ of RF1/2 could be studied.

The rate of peptide release was therefore studied in experiments where termination complex and RF1 or RF2 were diluted to decreasing concentrations, while the concentration of RF3 was kept constant. Figure 4A shows how the rate of peptide release depends on the concentration of RF1 with and without RF3 using termination complex with a UAG stop codon. It may be seen that the presence of RF3 and GTP does not affect the $k_{\text{cat}}/K_{\text{m}}$ for RF1. The $k_{\text{cat}}/K_{\text{m}}$ for RF1 was estimated to be $\sim 3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (from a Lineweaver–Burke type plot, not shown) which is the same as the $k_{\text{cat}}/K_{\text{m}}$ for the cognate ternary complex on the ribosome (Bilgin and Ehrenberg, 1994; Pavlov and Ehrenberg, 1996). Using this value for $k_{\text{cat}}/K_{\text{m}}$ and the k_{cat} value (0.25 s^{-1}) obtained from the experiment in Figure 3A, the K_{m} value is calculated to be $8 \times 10^{-9} \text{ M}$.

Figure 4B shows a similar experiment performed with RF2 acting at the UAA stop codon. The experiment

Fig. 4. Hydrolysis rate of peptidyl-tRNA, as in Figure 3, as a function of RF1 (**A**) and RF2 (**B**) concentration at a fixed incubation time (10 s) and with 0.2 mM GTP. Experiments were done in the presence ($\square$) and in the absence ($\diamond$) of RF3.

demonstrates that RF3 does not affect the effective rate constant (k_{cat}/K_m) for the binding of RF2 to the termination complex, since the rates of hydrolysis of peptidyl-tRNA in the absence and presence of RF3 converge to the same value at high dilutions, where the reactions are controlled by k_{cat}/K_m . At higher concentrations of RF2 and ribosomes, in contrast, the rate of peptide release is higher in the presence than in the absence of RF3, and this, we suggest, is caused by residual RF3-catalysed recycling of RF2.

RF3 inhibits the recycling of RF1 in the presence of GDP and stimulates RF1 recycling in the presence of a non-cleavable GTP analogue

Figure 5 shows how the extent of hydrolysis of peptidyl-tRNA, in an RF1 recycling experiment as in Figure 2, depends on RF3 in the presence of GDP or the GTP analogue guanylyl-imidodiphosphate (GMP-PNP). For comparison, RF1 recycling experiments with GTP and with or without RF3 are included in Figure 5. The slowest

Fig. 5. Hydrolysis rate of peptidyl-tRNA in the presence of 0.25 pmol of RF1 as in Figure 1 as time curves. Experiments were done in the presence of 90 pmol of RF3 plus 0.2 mM GTP ($\circ$), plus 0.2 mM GMP-PNP ($\square$), plus 0.2 mM GDP ($\diamond$) and in the absence of RF3 but with 0.2 mM GTP ($\triangle$).

recycling of RF1 occurs in the presence of RF3 and GDP. This means that RF3 inhibits dissociation of RF1 from the ribosome, not only in the absence of guanine nucleotide (Figure 2), but also in the presence of 2 mM GDP, as seen by comparing the two upper curves in Figure 5. When RF3 is present together with GMP-PNP, the recycling of RF1 is faster (the third curve from the top in Figure 5) than in the absence of RF3, but not as fast as when RF3 acts together with GTP (the lower curve in Figure 5).

The dependence of the rate of hydrolysis of peptidyl-tRNA on GMP-PNP, GDP and GTP was also studied in single cycle experiments, where either RF1 or RF2 were titrated with 4 s incubation times as in Figure 3 (not shown). In this short time range, the peptidyl-tRNA hydrolysis rates were the same in all cases. Hydrolysis of GTP on RF3 is therefore not necessary for hydrolysis of peptidyl-tRNA to occur. The result also implies that although RF3 with GDP almost stops the recycling of RF1 and RF2, there is no inhibition of single cycle action of RF1 by RF3 and GDP.

RF3 hydrolysis of GTP requires ribosomes but not RF1/2

The inhibition of RF1/2 recycling by RF3 and GDP was surprising, and led us to characterize the GTPase activity of RF3 in more detail. By analogy with the role of hydrolysis of GTP by the translation factor EF-Tu (Kaziro, 1978), the GDP form of RF3 would be expected to have low affinity for the ribosome, whereas the inhibition of RF1/2 recycling observed (Figure 4) suggests a high affinity. The GTPase activity of RF3 was studied by TLC analysis of incubations containing [^3H]GTP and other components (Figure 6). The presence of ribosomes was an absolute requirement for hydrolysis. However, the rate of GDP production was similar whether the incubations contained naked ribosomes, termination complexes or termination complexes with RF1. These results show a difference between prokaryote RF3 and eukaryote eRF3

Fig. 6. Time curves illustrating GTP hydrolysis by RF3. Experiments were carried out with RF3, release complexes and RF1 (□), with naked ribosomes, RF3 and RF1 (◇), with naked ribosomes and RF3 without RF1 (△) and with release complexes only (○). RF3 or ribosomes alone exhibited no GTPase activity (not shown).

action, since GTP hydrolysis by eRF3 requires both ribosomes and eRF1 (Frolova *et al.*, 1996).

Discussion

Progress in understanding the detailed mechanism of translation termination has been hampered by the lack of an *in vitro* system sufficiently similar to the *in vivo* process to allow physiologically significant conclusions to be drawn and yet capable of being constructed from highly purified components. The isolation of ribosomal complexes paused at a stop codon and their systematic use in studies of termination, as described here, represents a significant advance, and has allowed the major function of release factor RF3 to be defined. In contradiction to recent models (Ito *et al.*, 1996; Nakamura *et al.*, 1996), the role of RF3 does not appear to be one of transporting RF1 or RF2 to the ribosome in a fashion analogous to that of EF-Tu in the ternary complex aminoacyl-tRNA·EF-Tu·GTP. Thus, RF3·GTP affects neither the maximal rate of hydrolysis (k_{cat}) of peptidyl-tRNA by RF1 or RF2 nor the Michaelis-Menten parameter k_{cat}/K_m for RF1- or RF2-promoted peptide release. If a complex between RF3 and RF1/2 is formed off the ribosome, or if RF3 binds before peptide release, then one must assume that the presence of RF3 is virtually neutral with respect to the kinetics of RF1 or RF2 action, a possibility that appears highly unlikely.

Instead, we show that RF3 in the presence of GTP strongly stimulates the rate of recycling of both RF1 and RF2 between ribosomal complexes awaiting peptidyl-tRNA hydrolysis. The results suggest that the only function of prokaryotic RF3 is to accelerate the dissociation of RF1 and RF2 from ribosomes after hydrolysis of peptidyl-tRNA, which is one of the roles for RF3 initially suggested by Goldstein and Caskey (1970). The recycling of RF1 and RF2 is also stimulated by RF3 in the presence of the non-hydrolysable GTP analogue GMP-PNP, though to a lesser extent.

The inhibitory effect on RF1/2 recycling by RF3 without guanine nucleotide or with GDP was the most surprising aspect of our observations, and may be the key to an understanding of the role GTP hydrolysis in the RF3 cycle. A convenient starting point to describe our model for the action of RF3 is a state where there is free RF3·GTP and a ribosome containing deacylated tRNA in its P-site and RF1/2 in its A-site (see Figure 7). Subsequently RF3·GTP binds to the ribosome in an 'encounter complex', where hydrolysis of GTP induces a structural change in RF3 from its GTP to its GDP configuration. Since the ribosome-induced GTP hydrolysis on RF3 does not depend on the presence of RF1/2 (see Figure 7), we suggest that there is no contact between RF1/2 and RF3 in the 'encounter complex'. The change in RF3 configuration then leads to a 'high affinity complex' between RF3·GDP, RF1/2 and the ribosome, from which GDP dissociates rapidly (see Figure 7). We will assume that RF3 and RF1/2 are in direct contact in the 'high affinity complex'. RF3 is then forced into its GTP configuration by the binding of a new GTP molecule from the solvent, and an unstable 'dissociation complex' is formed, consisting of RF3·GTP, RF1/2 and ribosome. This complex has the same molecular composition as the 'encounter complex' described above, but has a different conformation, which favours dissociation of RF3 and RF1/2 rather than hydrolysis of GTP on RF3.

In this model, the release of RF1/2 from the ribosome is driven by two GTP-dependent steps. In the first, GTP hydrolysis in the 'encounter complex' leads to the rapid formation of the 'high affinity complex' by a conformational change in RF3. In the second, the exchange of GDP for GTP rapidly forces the system into the 'dissociation complex' in a way similar to how the association of ATP with myosin leads to dissociation of the myosin-actin complex in muscle movement (Block, 1996; Holmes, 1996). Since our model postulates that GTP hydrolysis precedes the action of RF3, it has some similarities also with a recent model concerning the action of EF-G (Rodnina *et al.*, 1997).

Our model explains why RF3 inhibits the recycling of RF1/2 in the presence of GDP by the postulated 'high affinity complex' (Figure 7). RF3 inhibits recycling of RF1/2 equally in the absence of guanine nucleotide and in the presence of GDP. From this, we predict that the RF3·GDP structure will turn out to be similar to the RF3 structure in the absence of guanine nucleotide, and these two configurations will be different from the RF3·GTP structure.

The model can also explain why RF3 stimulates the recycling of RF1/2 in the presence of a non-cleavable GTP analogue but to a lesser extent than in the presence of GTP. The reason is, we suggest, that the 'dissociation complex' (Figure 7) can also be reached directly from the 'encounter complex'. This pathway is the only one available for non-cleavable GTP analogues and is, by hypothesis, considerably less efficient than the normal route via the 'high affinity complex', and this explains the reduced efficiency of RF3 action in the presence of GMP-PNP (Figure 5).

The observation of GTP hydrolysis by RF3 and ribosomes in the absence of RF1/2 is in clear contrast to the eukaryotic system, which requires eRF1 as well as eRF3

Fig. 7. Model explaining how RF3 may accelerate the dissociation of RF1/2 from the ribosome after the hydrolysis of peptidyl-tRNA (see Discussion).

and ribosomes (Frolova *et al.*, 1996). A further difference lies in the mutual affinity between eRF1 and eRF3, which readily form a complex in the absence of ribosomes (Zhouravleva *et al.*, 1995). Attempts to demonstrate a similar complex between the prokaryotic factors were unsuccessful (Ito *et al.*, 1996). However, even in the eukaryotic case, it is not clear that a complex between eRF1 and eRF3 is necessary or indeed important for eRF1 function. When present in sufficient amounts, eRF1 alone is sufficient for Met release from ribosomes (Zhouravleva *et al.*, 1995), and shows anti-suppressor activity both *in vivo* (Le Goff *et al.*, 1997) and in a cell-free protein synthesis system *in vitro* (G.Drugeon, X.Le Goff, L.Frolova, O.Jean-Jean, M.Philippe, L.Kisselev and A.-L.Haenni, personal communication). Furthermore, the concentration of ribosome-bound eRF3 in yeast is independent of the eRF1 concentration, pointing to the possibility of independent binding of eRF1 and eRF3 to ribosomes (Stansfield *et al.*, 1996).

The mechanism of action of RF3 proposed here is relevant to observations *in vivo* concerning translation termination in *prfC* mutant strains, from which RF3 is absent. Increased readthrough or suppression of UGA stop codons (Grentzmann *et al.*, 1994, 1995; Mikuni *et al.*, 1994) or UAG and UAA stop codons (Mikuni *et al.*, 1994) has been reported and indeed served for the identification of the *prfC* gene. The relevant kinetic parameters being compared in these assays are k_{cat}/K_m for RF1/2 multiplied by its concentration and k_{cat}/K_m for a competing ternary complex (suppressor) multiplied by its concentration. We have shown that RF3 does not affect the Michaelis-Menten parameter k_{cat}/K_m for catalysis of peptide release by RF1/2, but only the rate of dissociation of the factors from terminated ribosomes. With our data in mind, the simplest explanation for the observed increase in nonsense codon readthrough or suppression is that *prfC* inactivation leads to a reduction in the pools of free RF1/2, due to sequestration of the factors on terminated ribosomes. More complex explanations cannot, however, be excluded.

Our results also suggest that the observed suppression of defects in temperature-sensitive RF1 and RF2 by overexpression of RF3 (Matsumura *et al.*, 1996) may be caused by changes in the pools of free RF1 and RF2. The thermosensitive mutations affecting RF1/2 may, at elevated temperatures, decrease the overall amounts of the factors in the cell, or decrease the pools of free factors due to an

altered factor-ribosome interaction. An increase in the cytoplasmic concentration of RF3 may improve recycling of the altered RF1/2 sufficiently to partially revert thermosensitivity.

Materials and methods

Chemicals and buffers

ATP, UTP, CTP, GTP and GDP were from Pharmacia. GMP-PNP was from Boehringer Mannheim. Phosphoenolpyruvate (PEP), putrescine, spermidine and non-radioactive phenylalanine, methionine, isoleucine and threonine were from Sigma. [8-³H]GTP, L-[methyl-³H]methionine and L-[U-¹⁴C]isoleucine were from Amersham. All other chemicals were of analytical grade from Merck. Polymix buffer was made according to Jelenc and Kurland (1979).

Release factor purifications

All protein purification procedures were carried out at 4°C. SDS-PAGE was run according to Laemmli (1970) with 10% acrylamide. Purified RF1 and RF3 were quantified according to Bradford (1976) using bovine serum albumin (BSA) as a standard. The effective concentration of RF1 used in experiments was determined by stoichiometric titrations (as in Figures 1 and 3A).

RF1 was purified from the overproducing strain pFJU335 (Jørgensen *et al.*, 1993). Six g of induced cells were suspended in 20 ml of buffer A [Na-MES 30 mM pH 6.0, EDTA 1 mM, phenylmethylsulfonyl fluoride (PMSF) 100 µM, dithioerythritol (DTE) 2 mM] and 10 µg of DNase I (Sigma). The cells were lysed in a French press and the debris was removed by centrifugation in a Sorvall SS-34 rotor at 17 000 r.p.m. The supernatant was removed, diluted to 150 ml with buffer A and applied to an S-Sepharose Fast Flow column (5 cm² × 40 cm, Pharmacia) equilibrated with buffer A. The column was washed with 100 ml of buffer A and a gradient was applied 600 + 600 ml from 0 to 400 mM NaCl in buffer A. RF1 protein was followed by SDS-PAGE (10%). Cell lysate from the induced cells was used as a marker for RF1. Samples containing RF1 were pooled and precipitated in 55% saturated ammonium sulfate, centrifuged in a SS-34 rotor at 17 000 r.p.m. for 30 min, dissolved in a minimal volume of buffer B (50 mM potassium phosphate, PMSF 100 µM, DTE 2 mM, pH 7.5) and applied to an Aca44 column (Sepharose, 5 cm² × 150 cm) equilibrated with buffer B. Protein was eluted at 25 ml/h. Samples containing RF1 (SDS-PAGE, 10%) were pooled and applied directly to DEAE-cellulose, DE-52 (2 cm² × 12 cm, Whatman) equilibrated with buffer B. The RF1 was eluted with a gradient 300 + 300 ml buffer B to 250 mM potassium phosphate, PMSF 100 µM, DTE 2 mM, pH 6.6. RF1 was once more identified with SDS-PAGE (10%), pooled and precipitated in 55% saturated ammonium sulfate, centrifuged in a SS-34 rotor at 17 000 r.p.m. for 30 min, dissolved in a minimal volume of polymix buffer, dialysed and stored at -80°C.

A crude preparation of RF2 was made in the following way: 10 g of *Escherichia coli* MRE600 paste from cells grown in TY2 media supplemented with 0.4% glucose (Holme *et al.*, 1970) and harvested in late log phase was suspended in 20 ml of buffer C (Tris-HCl 30 mM, PMSF 100 µM, DTE 2 mM, pH 8.0) with 10 µg of DNase I. The cell suspension was run through a French press and the debris was removed

by centrifugation in a Sorvall SS-34 rotor at 17 000 r.p.m. for 30 min. The supernatant was diluted to 150 ml with buffer C and applied to a DEAE CL6B column (5 cm² × 30 cm, Pharmacia) equilibrated with buffer C. The column was rinsed with one column volume of buffer C, and a gradient, 500 + 500 ml, 0–600 mM KCl in buffer C was applied. RF2, eluting late in the gradient, was detected by an activity assay (see Release assays below) and by SDS–PAGE. Active fractions were pooled, precipitated in 60% saturated ammonium sulfate and centrifuged in a SS-34 rotor at 17 000 r.p.m. for 30 min. The pellet was dissolved and dialysed against 2× polymix buffer. Glycerol was added up to 50% and the protein solution was stored at –20°C. The final concentration was ~2 pmol/μl as measured by a stoichiometric titration (see Figure 3B).

RF3 was prepared from an osmo-expression vector (Mortensen *et al.*, 1995) with minor modifications, and a gel filtration step was added at the end of the procedure in the following way. RF3-containing fractions from S-Sepharose chromatography were precipitated in 80% saturated ammonium sulfate, centrifuged in an SS-34 rotor at 17 000 r.p.m. for 30 min, dissolved in 50 mM Na-MES pH 6.8, 10 mM MgCl₂, 100 mM KCl, 2 mM DTE, and applied to the gel filtration column AcA44 (Sepracor, 5 cm² × 150 cm) in the same buffer. Protein was eluted at 25 ml/h. Electrophoretically pure samples were pooled, precipitated in 80% saturated ammonium sulfate and centrifuged in a SS-34 rotor at 17 000 r.p.m. for 30 min. The pellet was dissolved and dialysed against 2× polymix buffer. Glycerol was added up to 50% and the purified protein was stored at –20°C.

Other protein factors

RNA guard (porcine) was from Pharmacia. Pyruvate kinase (PK) was from Boehringer. Myokinase (MK) was from Sigma. Wild-type *E. coli* 70S ribosomes 017, were prepared according to Jelenc (1980) and stored at –80°C in polymix buffer. Initiation factors were prepared according to Soffientini *et al.* (1994) with minor modifications. Elongation factors Tu, Ts, G, tRNA bulk and phenylalanyl-tRNA synthetase were purified according to Ehrenberg *et al.* (1990).

Isoleucyl-tRNA synthetase was purified as part of a preparation of EF-Tu. After the preliminary fractionation of proteins on DEAE CL6B, the fractions containing isoleucyl-tRNA synthetase activity were pooled and then precipitated in 80% saturated ammonium sulfate and the precipitate was collected and dialysed against buffer D (30 mM Tris–HCl, 10 mM MgCl₂, PMSF 100 μM, DTE 2 mM, 0.02% Na₂S₂O₅, pH 7.5). Next, the enzyme fraction was applied to a Q-Sepharose Fast Flow column (5 cm² × 30 cm, Pharmacia) and eluted with a gradient of 0–300 mM NaCl in buffer D. The synthetase was detected by tRNA isoleucine charging activity and pooled accordingly. The protein was precipitated in 80% saturated ammonium sulfate, collected by centrifugation and applied, in a minimum volume of buffer B, to AcA44 gel filtration (see above). Enzyme was pooled according to tRNA isoleucine charging activity, applied to DEAE–cellulose and run in the same way as RF1 above. Again, the isoleucyl-tRNA synthetase was pooled by activity, precipitated in 80% saturated ammonium sulfate, collected by centrifugation, dissolved and dialysed against 2× polymix buffer. Glycerol was added up to 50% and the protein solution was stored at –20°C. Threonyl-tRNA synthetase was prepared from MRE600 according to Brunel *et al.* (1993) with minor modifications. tRNA^{Met} was purified according to Seno *et al.* (1968). The charging and formylation reactions were carried out as in Dubnoff and Maitra (1971). The fMet-tRNA^{Met}–Met-tRNA^{Met} mixture was then applied to a BD-cellulose column in 10 mM sodium acetate, pH 5.1 and eluted with a 0–1.5 M NaCl gradient in the same buffer. The purity of the amino acid obtained by deacylating the tRNA preparation was >90% fMet, as analysed by HPLC on a C18 column (Merck) eluted with 10% methanol in 0.1% trifluoroacetic acid.

DNA constructs

A plasmid pUC18 with the XR7-UAG sequence was constructed as a base for the XR7 DNA constructs, containing the promoter region for T7 RNA polymerase and the ribosomal binding site of the 002 mRNA (Calogero *et al.*, 1988), in the following way: two oligonucleotides: 5'-ctctctGGTACCGAAATTAATACGACTCACTATAGGGAATTCGGG-CCCTGTGTTAAACAATTAAGGAGG3' and 5'-ttttttttttttttttttCTGCAGCTAAATCGTAAACATAGTATACCTCCTTAATTGTTAAACAAGGGC-CCG3' were synthesized in an Applied Biosystems 392 DNA/RNA synthesizer. They were annealed (underlined sequence), and PCR (Mullis *et al.*, 1986) was performed to fill in the remainder of the sequence. The DNA was then cloned into pUC18 using the *Kpn*I and *Mlu*I restriction sites, and the plasmid gene sequence was confirmed by acrylamide/urea gel sequencing. DNA to be used in the T7 transcription reaction was made from *Bst*II-linearized plasmid by PCR using the oligonucleotide

5'-GGTACCGAAATTAATACGACTCACTATAGG3' and one of the following, depending on the sequence desired: 5'-ttttttttttttttttttCTGCAG3' for XR7-UAG; 5'-ttttttttttttttttttCTGCAGTCAAATCGTAAACATAGT-ATACCTCC3' for XR7-UGA; and 5'-ttttttttttttttttttCTGCAGTTAAATCGTAAACATAGTATACCTCC3' for XR7-UAA. The resulting DNA fragment was used in the T7 transcription reaction.

mRNA XR7

The mRNA used throughout, called XR7, was transcribed from PCR-generated DNA by the T7 RNA polymerase reaction and purified by the poly(A) tail (Pavlov and Ehrenberg, 1996). The sequence of the mRNA was: CGGGCCCCUUGUUAACAAUUAAGGAGGUUAUCUAUGUUU-UACGAUU(STOP)CUGCAG(A)₂₁, where the AUG start codon is underlined and the stop codon is in bold.

Release complexes

The substrate for the *in vitro* release reaction is factor-free ribosomes with mRNA bound (the A-site codon is a stop codon) and fMet-Phe-Thr-Ile-tRNA^{Ile} bound to the P-site. This substrate is made by initiating and elongating the peptide using a fully purified system of components and then purifying the ribosome fraction away from proteins, RNA, nucleotides, etc., on a gel filtration column. The signal measured in our assays is peptide remaining on peptidyl-tRNA after exposure to release factors by cold trichloroacetic acid (TCA) precipitation. Peptide is detected by a ¹⁴C label on the final amino acid in the peptide: isoleucine.

The ribosomes stalled on the stop codon, together with bound [¹⁴C]peptidyl-tRNA and mRNA, here called release complexes, were produced by combining a ribosome mix consisting of 5 nmol of 70S ribosomal subunits, 2 nmol of initiation factor 1 (IF1), 250 pmol of IF2 and 1.5 nmol of IF3 with an initiation mix containing 1 mM GTP, 7500 pmol of mRNA XR7 with the specified stop codon, and 10 nmol of fMet-tRNA^{Met} in 400 μl total, and incubating for 30 min at 37°C. This reaction mixture was then added to an elongation mix, containing 10 mM PEP, 1 mM ATP, 1 mM GTP, 150 μM phenylalanine, 150 μM threonine, 50 μl (8 nmol) of high specific activity [¹⁴C]isoleucine, 300 nmol of tBULK, 2 nmol of EF-G, 2 nmol of EF-Ts, 6 nmol of EF-Tu, 6 μg of MK, 100 μg of pyruvate kinase and 500 U each of phenylalanyl-, threonyl- and isoleucyl-tRNA synthetases, as defined in Ehrenberg *et al.* (1990), which had been pre-incubated at 37°C for 5 min. Peptide elongation was allowed to take place for 30 s. The release complexes were then cooled on ice and purified on a gel filtration column (Sephacryl S-300HR, 5 cm² × 45 cm, Pharmacia), and eluted in polymix at 4°C. The first peak (ribosomes), detected by OD₂₈₀ and by scintillation counting, was pooled and frozen in aliquots at –80°C for later use. The peptide bound in the P-site of the ribosome has the sequence fMet[³H]Phe-Thr-Ile[¹⁴C].

GTP hydrolysis

GTP hydrolysis experiments and calculations were carried out using TLC to separate GDP and GTP (Ehrenberg *et al.*, 1990). Fifty pmol of [³H]GTP, 20 pmol of total ribosomes or release complexes, 0.25 pmol of RF1 and 25 pmol of RF3 were used for each 100 μl point.

Release assays

Release complexes (20–50 μl) were added to release factors (as described in the figure legends), pre-incubated with GTP, GDP or GMP-PNP, PEP 10 mM, 0.3 μg of MK and 5 μg of PK in polymix, and incubation was carried out at 37°C in a total of 100 μl for each time or titration point. In the reactions with GDP, the energy regeneration system (PK, MK, PEP) was omitted. The reaction was quenched with ice-cold 5% TCA containing 0.75% casamino acids and filtered onto glass fibre GF/C filters (Whatman). The filters were dried and scintillation counted using Quickszint 701 (Zinser Analytic).

k_{cat}/K_m experiments

These experiments were carried out as dilution experiments. For experiments containing RF1, 5 pmol of active RF1 was diluted in various volumes (3–10 ml) of factor mix containing 10 mM PEP, 0.2 mM GTP, 50 μg of PK, 3 μg of MK and 1000 pmol of RF3 per ml in polymix and pre-incubated at 37°C for 5 min. Next, 4 pmol of release complex was added to start the reaction. The incubation time was 10 s. For experiments containing RF2, 1.2 μl of crude RF2 was used for each experimental point, and the dilution volumes used were from 100 μl to 3 ml.

Calculation of k_{cat} for single turnover assays

The amount of peptidyl-tRNA decays exponentially, $C_t = C_0 \cdot e^{-kt}$, and at saturating RF1 concentrations $k = k_{cat}$. The background in the

experiment in Figure 3A is 0.44 pmol. Subtracting this from all points gives $C_0 = 2.3$ pmol and $C_1 = 0.89$ pmol, which gives $k_{\text{cat}} = 0.25 \text{ s}^{-1}$ since $t = 4 \text{ s}$.

The k_{cat} for RF2 was estimated to be $>0.5 \text{ s}^{-1}$. This is because the peptide was at least 90% released, down to the background, in Figure 3B. When $C_1/C_0 = 0.1$ and $t = 4 \text{ s}$, k_{cat} is $\sim 0.5 \text{ s}^{-1}$.

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