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Sequence of Steps in Ribosome Recycling as Defined by Kinetic Analysis

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Summary

After termination of protein synthesis in bacteria, ribosomes are recycled from posttermination complexes by the combined action of elongation factor G (EF-G), ribosome recycling factor (RRF), and initiation factor 3 (IF3). The functions of the factors and the sequence in which ribosomal subunits, tRNA, and mRNA are released from posttermination complexes are unclear and, in part, controversial. Here, we study the reaction by rapid kinetics monitoring fluorescence. We show that RRF and EF-G with GTP, but not with GDPNP, promote the dissociation of 50S subunits from the posttermination complex without involving translocation or a translocation-like event. IF3 does not affect subunit dissociation but prevents reassociation, thereby masking the dissociating effect of EF-G-RRF under certain experimental conditions. IF3 is required for the subsequent ejection of tRNA and mRNA from the small subunit. The latter step is slower than subunit dissociation and constitutes the rate-limiting step of ribosome recycling.

Introduction

Protein synthesis in bacteria takes place in four phases: initiation, elongation, termination, and recycling. During initiation, the first aminoacyl-tRNA (aa-tRNA), fMet-$tRNA^{fMet}$ in bacteria, binds to an initiation codon in the ribosomal P site aided by three initiation factors: IF1, IF2, and IF3. In the elongation step, aa-tRNAs bind to the ribosome with the help of elongation factor Tu (EF-Tu), and the peptide bond is formed yielding deacylated tRNA in the P site and peptidyl-tRNA in the A site. Subsequently, EF-G promotes the translocation of peptidyl-tRNA together with the mRNA from the A site to the P site, and deacylated tRNA moves out of the P site and dissociates from the ribosome. When a termination codon appears in the decoding site, the release of the nascent polypeptide is carried out by release factor RF1 (or RF2), resulting in a posttermination ribosome complex that has deacylated tRNA bound to the mRNA in the P site and an empty A site. The turnover of RF1 and RF2 is promoted by RF3. Ribosomes, tRNA, and mRNA are released from the posttermination complex by the concerted action of EF-G, RRF, and IF3.

The mechanism of ribosome recycling is not clear. Based on biochemical and genetic evidence, two conflicting models were proposed that differed in important features. According to the model proposed by Kaji and colleagues (Janosi et al., 1996; Kaji et al., 2001; Selmer et al., 1999), EF-G promotes a translocation-like rearrangement of the posttermination complex with RRF bound to it, during which RRF moves out of its initial binding site toward the P site, and the tRNA moves out of the P site and dissociates from the ribosome; subsequently, the mRNA dissociates spontaneously due to the loss of stabilizing interactions with tRNA. According to that model, the ribosome is not dissociated into subunits during the first phase promoted by EF-G and RRF, and subunit dissociation requires the action of IF3. In stark contrast to that model, the models put forward by Ehrenberg et al. (Karimi et al., 1999) and Nakamura et al. (Fujiwara et al., 2001; Fujiwara et al., 2004) present another order of events and different functions for the factors. First, EF-G and RRF promote the dissociation of the posttermination complex into ribosomal subunits, whereas mRNA and tRNA remain bound to the 30S subunit. Subsequently, deacylated tRNA is removed from the 30S subunit by the action of IF3. Mechanism and timing of the release of the mRNA was not directly examined and inferred only indirectly.

Crystal and solution structures of RRF have been solved from five different organisms (Agrawal et al., 2004; Kim et al., 2000; Nakano et al., 2002; Nakano et al., 2003; Selmer et al., 1999; Toyoda et al., 2000; Yoshida et al., 2001). The factor comprises two domains connected by a flexible hinge; mutational analysis of the hinge region suggested that the flexibility of relative arrangement of RRF domains is important for RRF function in vivo (Toyoda et al., 2000). The binding site and orientation of RRF on the ribosome was identified by chemical footprinting on ribosomal RNA (Lancaster et al., 2002), cryo-electron microscopy (cryo-EM) (Agrawal et al., 2004), and crystallography (Wilson et al., 2005). RRF interacts mainly with elements of the 50S ribosomal subunit, in particular with two intersubunit bridges, B2a (helix 69 of 23S rRNA) and B3 (helix 71), nucleotides 2253–2255 in the P loop, A2602, and ribosomal proteins L16 and L27 at the peptidyltransferase center. The structural data are compatible with either functional model and do not clarify the sequence of events during ribosome recycling.

Several key questions remain unanswered: Is there translocation or a translocation-like event during disassembly of the posttermination complex? Which factors are required for subunit dissociation, two (RRF and EF-G) or three (RRF, EF-G, and IF3)? When is the tRNA released from the ribosome, prior to or after 50S dissociation? And finally, when is the mRNA released?

To answer these questions for the *Escherichia coli* system, we have applied fluorescence and fluorescence resonance energy transfer (FRET) assays that allowed us to monitor partial reactions of ribosome recycling directly and to resolve the time course of reactions by rapid kinetic measurements using the stopped-flow technique. Translocation, i.e., movement of the tRNA-mRNA complex within the ribosome by one

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mRNA codon, and mRNA exchange on the ribosome were monitored by a fluorescence change of a reporter group at the 3′ end of the mRNA. Subunit dissociation was measured by changes in FRET efficiency between dyes attached to the 30S and 50S subunits. Finally, tRNA release was measured by nitrocellulose filtration. Our results show that EF-G does not promote mRNA-tRNA-RRF translocation. Furthermore, our results suggest a sequence of recycling events that is inconsistent with the model proposed by Kaji and colleagues (Janosi et al., 1996; Kaji et al., 2001; Selmer et al., 1999) and in agreement with the models put forward by Ehrenberg, Nakamura, and colleagues (Fujiwara et al., 2001; Fujiwara et al., 2004; Karimi et al., 1999; Nakamura and Ito, 2003). The seemingly profound discrepancies between the conflicting models are explained by limitations of the experimental approaches used by the different groups.

Results

EF-G Does Not Promote mRNA-tRNA Translocation on the Ribosome

One key question in ribosome recycling is whether EF-G-promoted mRNA-tRNA translocation constitutes part of the mechanism of posttermination complex disassembly, in particular for the release of deacylated tRNA from the P site. To address this question, we used a fluorescence-based mRNA-translocation assay (Peske et al., 2004; Savelsbergh et al., 2003). To monitor the movement of mRNA, we used an mRNA fragment that was labeled with fluorescein at the 3′ end (nucleotide +14 after the A(+1)UG codon) and reported the movement of the dye toward the ribosome by an increase in fluorescence (Figure 1A). This was verified by performing a control experiment in which EF-G·GTP was added to pretranslocation complexes with peptidyl-tRNA in the A site and deacylated tRNA in the P site (Figure 1B); translocation of peptidyl-tRNA was rapid and took place on 85% of the ribosomes, as monitored by reaction with puromycin (Peske et al., 2004). When Phe-tRNA^{Phe} was bound to the A site of the posttermination complex (by adding the ternary complex Phe-tRNA^{Phe}·EF-Tu·GTP), the same, albeit slower, fluorescence increase was observed upon addition of EF-G·GTP, again indicating translocation at the lower rate known for Phe-tRNA^{Phe} (Semenkov et al., 2000). In contrast, absolutely no fluorescence change was observed with the posttermination complex in the presence of RRF and EF-G, indicating no movement of the mRNA through the ribosome. Radioactively labeled tRNA remained bound to the ribosome, as verified by nitrocellulose filtration (see below, Figure 4). Thus, EF-G·GTP in the presence of RRF does not promote mRNA-tRNA translocation nor release of tRNA.

RRF and EF-G Promote Dissociation of Ribosomes into Subunits

Another important question in ribosome recycling is whether RRF and EF-G are sufficient for subunit dissociation or whether IF3 is required as well. So far, subunit dissociation was studied by sucrose-gradient centrifugation (Hirokawa et al., 2002b; Karimi et al., 1999; Kiel et al., 2003), a method that is inherently slow and does not allow studying the kinetics of the reaction or the identification of transient intermediates. To overcome this limitation, we developed a stopped-flow FRET assay with 50S subunits labeled with fluorescein as a donor [50S(Flu)] and 30S subunits labeled with a fluorescence quencher, QSY9, as an acceptor [30S(QSY)]. In 70S ribosomes, the donor and the acceptor are close to each other, and the fluorescence of fluorescein is quenched. Upon subunit dissociation, the distance between fluorophore and quencher is increased, resulting in higher fluorescence intensity (Figure 2A). The R_0 value for the donor-acceptor couple fluorescein and QSY9, i.e., the distance at which the FRET efficiency is 50%, is 64 Å, well within the useful range for the present purpose.

The FRET assay was validated in a subunit association experiment (Figure S1 available in the Supplemental Data with this article online). When 50S(Flu) subunits were mixed with 30S(QSY) subunits, a fluorescence decrease was observed, whereas no effect was found with unlabeled 30S or upon mixing with buffer. Furthermore, no fluorescence change was observed when the factors EF-G, RRF, and IF3 were added to 50S(Flu) in complex with unlabeled 30S subunits at conditions of

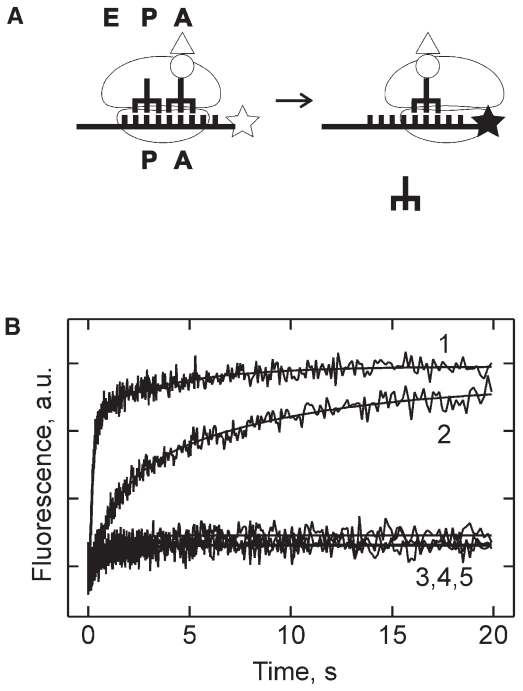

Figure 1. mRNA Translocation

(A) Experimental approach. The movement of mRNA during translocation is monitored by the fluorescence of fluorescein attached to the 3′ end of an mRNA fragment; the fluorescence is low prior to translocation (open star) and increases upon translocation (filled star).

(B) Time courses. Initiation complex with fMet-tRNA^{fMet} in the P site (1) or posttermination complex with deacylated tRNA^{fMet} in the P site (2, 3, 4, and 5) was mixed with ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} and EF-G·GTP (1 and 2), RRF and EF-G·GTP (3), EF-G·GTP (4), or buffer (5). For experimental details, see the Experimental Procedures.

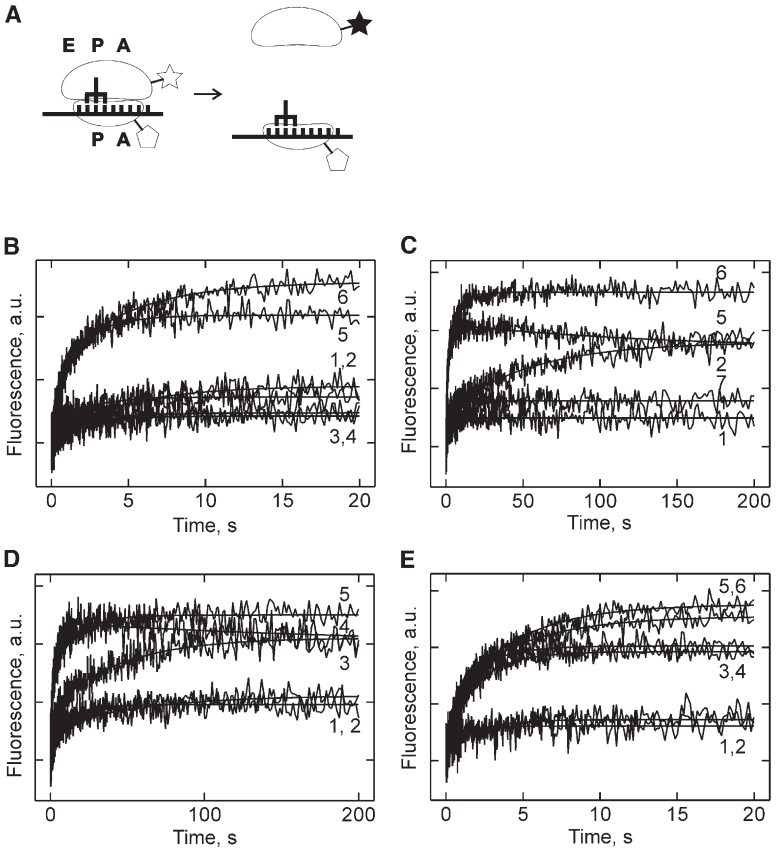

Figure 2. Subunit Dissociation of Posttermination Complexes

(A) Experimental approach. Subunit dissociation is monitored by FRET between fluorescein (donor) attached to the 50S subunit, 50S(Flu) (star), and the quencher QSY9 (acceptor) attached to the 30S subunit 30S(QSY) (pentagon). In the posttermination complex, the fluorescence of the donor is quenched (open star). Upon subunit dissociation, the donor fluorescence increases (closed star) due to separation of donor and acceptor and release of FRET. For the validation of the assay, see Figure S1.

(B) Time course of subunit dissociation. Posttermination complex was mixed with buffer (1); IF3 (2); RRF (3); EF-G·GTP (4); RRF and EF-G·GTP (5); or RRF, EF-G·GTP, and IF3 (6).

(C) Long-time window of experiments 1, 2, 5, and 6 shown in (B). Additional experiment: RRF, EF-G·GDPNP, and IF3 (7).

(D) Subunit dissociation at different factor concentrations. Posttermination complexes containing 30S(QSY) and 50S(Flu) (0.05 μM final concentration) were mixed with unlabeled 30S subunits (0.25 μM) (1); RRF (0.2 μM) and EF-G·GTP (0.5 μM) (2); RRF (0.2 μM), EF-G·GTP (0.5 μM), and unlabeled 30S subunits (3); RRF (5 μM) and EF-G·GTP (2 μM) (4); or RRF (5 μM), EF-G·GTP (2 μM), and unlabeled 30S subunits (0.25 μM) (5).

(E) Influence of the SD sequence on subunit dissociation. Subunit dissociation was measured with posttermination complexes containing 30S(QSY) and 50S(Flu) programmed with mRNA with (1, 3, and 5) or without (2, 4, and 6) an SD sequence. Posttermination complexes were mixed with buffer (1 and 2); RRF and EF-G·GTP (3 and 4); or RRF, EF-G·GTP, and IF3 (5 and 6).

subunit dissociation (see below), suggesting that the fluorescence of 50S(Flu) was not directly affected by factor binding and function (Figure S1). Thus, the only reaction monitored by FRET between 50S(Flu) and 30S(QSY) was subunit association and dissociation.

Next, the effect of factors on subunit dissociation was studied by FRET. RRF and EF-G·GTP were sufficient to dissociate posttermination complexes (Figure 2B). The intensity increase due to subunit dissociation followed single-exponential kinetics with a rate of 0.3 ± 0.1 s^{-1}. At constant RRF concentration (5 μM), the rate of subunit dissociation increased with the concentration of EF-G and reached saturation at 0.7 ± 0.2 s^{-1} (data not shown). Upon prolonged incubation, the fluorescence effect was reversed (Figure 2C), suggesting subunit reassociation, presumably due to the depletion of GTP caused by GTP hydrolysis by EF-G (see below). The addition of IF3 in the presence of RRF and EF-G had no effect on the rate of subunit dissociation but rather prevented subunit reassociation (Figures 2B and 2C), as indicated by the larger amplitude of the fluorescence change and the lack of the fluorescence decrease at longer times.

Addition of thiostrepton, an antibiotic that inhibits EF-G function in translocation (Rodnina et al., 1999) and RRF and tRNA release from the ribosome (Hirokawa et al., 2002b; Kiel et al., 2003), blocked subunit dissociation (data not shown). Furthermore, no subunit dissociation was observed when a nonhydrolyzable GTP analog, GDPNP, was used instead of GTP (Figure 2C), indicating that GTP hydrolysis was required for the function of EF-G in subunit dissociation. IF3 alone was also able to promote the dissociation of the posttermination complex but at a very low rate, 0.02 ± 0.01 s^{-1} (Figure 2C). Neither EF-G·GTP nor RRF alone were able to induce subunit dissociation. Thus, the presence of RRF and EF-G·GTP together is necessary and sufficient to induce rapid dissociation of the posttermination complex into subunits. At this step of recycling, IF3 acts as an antiassociation factor and prevents the reassociation of the separated subunits.

These results are at variance with previous reports that were based on sucrose gradient analyses and suggested that all three factors were necessary for subunit dissociation (Hirokawa et al., 2002b). Given that subunit dissociation appears to be reversible in the absence of IF3, subunits may be expected to reassociate during the long incubation times required for sucrose gradient centrifugation. To test this possibility, we measured the dissociation of subunits by FRET under the conditions used by Kiel et al. (2003) (Figure 2D). Indeed, at these (lower) factor concentrations, practically no separated

subunits were observed in the presence of RRF and EF-G alone, suggesting that either subunit dissociation was not induced by RRF and EF-G, or dissociation was transient at these experimental conditions and followed by rapid reassociation. To distinguish between the two possibilities, we carried out the experiment in the presence of an excess of unlabeled 30S subunits. If rapid dissociation took place, the unlabeled 30S subunits were expected to compete with 30S(QSY) for binding to the 50S(Flu); in this case, an increase of fluorescence was expected, because reassociation of 50S(Flu) with unlabeled 30S subunits did not quench fluorescence. If, on the other hand, there was no subunit dissociation, the presence of the unlabeled subunits should have no effect on the reaction. As shown in Figure 2D, the addition of unlabeled 30S subunits at low concentrations of RRF and EF-G resulted in an increase of 50S(Flu) fluorescence to the level observed with high factor concentrations. Thus, subunit dissociation is induced also at low concentrations of RRF and EF-G, although under these conditions, the extent of dissociation is small and—in the absence of IF3—reassociation predominates. This masks the transient dissociation of the subunits, unless IF3 keeps the subunits apart, giving rise to the incorrect conclusion that all three factors were necessary for subunit dissociation.

It has been argued that the discrepancies in the factor requirement for subunits dissociation may be attributed to the use of model mRNAs that contained a Shine-Dalgarno (SD) sequence shortly upstream of the termination codon (Hirokawa et al., 2002b). In the polysome-breakdown assay used by Hirokawa et al., the posttermination ribosomes were not bound to an SD sequence, whereas mRNAs containing an SD sequence were used in the present experiments or in previous work (Karimi et al., 1999). To test whether the presence of an SD sequence affects the factor requirement for subunit dissociation, we compared the reactions on posttermination complexes containing mRNA with or without an SD sequence (Figure 2E). Identical results were obtained with the two mRNAs: (i) RRF and EF-G·GTP were sufficient to induce rapid subunit dissociation, and (ii) IF3 was not required for subunit dissociation but prevented reassociation. These results show that the SD sequence has no influence on the mechanism of ribosome recycling.

Specificity of RRF for Ribosome Complexes

In the posttermination complex, which is the natural substrate for RRF and EF-G, deacylated tRNA is bound to the P site, whereas the A site is empty. In order to test whether RRF and EF-G generally act on ribosomes with an empty A site, we examined vacant ribosomes and posttranslocation complexes, i.e. 70S ribosomes with peptidyl-tRNA in the P site and an empty A site, again by using the FRET assay. RRF and EF-G·GTP induced efficient dissociation of vacant ribosomes (Figure 3); IF3 was not required for dissociation but inhibited the reassociation of subunits. In contrast, ribosome complexes with peptidyl-tRNA in the P site did not dissociate even in the presence of all three factors: RRF, EF-G, and IF3 (Figure 3). Thus, the dissociating activity of EF-G-RRF appears to be restricted to posttermination (or vacant) ribosomes.

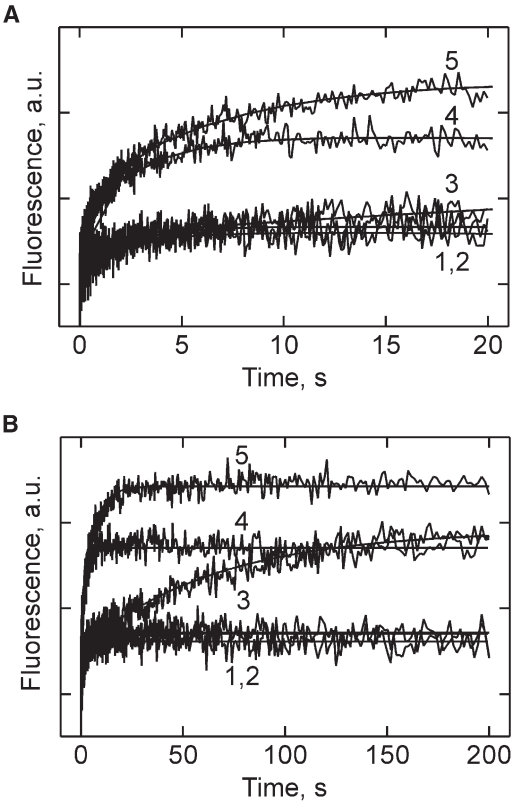

Figure 3. Substrate Specificity of Recycling

(A) Vacant ribosomes (1, 3, 4, and 5) or posttranslocation complexes (2) containing 30S(QSY) and 50S(Flu) subunits were mixed with buffer (1); RRF, EF-G·GTP, and IF3 (2 and 5); IF3 (3); or RRF and EF-G (4).

(B) Long-time window of the experiments in (A).

tRNA Dissociation Requires IF3

To determine the timing and factor requirement for tRNA release from the posttermination complex, we monitored the release of radioactively labeled tRNA by nitrocellulose filtration. None of the factors alone induced tRNA dissociation from the posttermination complex within the time of the experiment (Figure 4). In

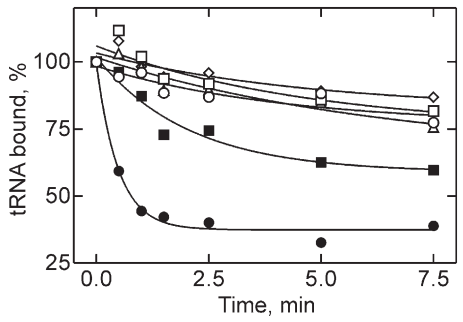

Figure 4. tRNA Dissociation from the Posttermination Complex

Ribosome bound [5′-^{32}P]tRNA^{Phe} was measured by nitrocellulose filtration. Posttermination complexes were incubated with buffer (open circle); RRF (open square); EF-G·GTP (open triangle); IF3 (open diamonds); RRF and EF-G·GTP (filled squares); or RRF, EF-G·GTP, and IF3 (filled circles). The addition of RRF and IF3 or of EF-G·GTP and IF3 had no effect (data not shown).

the presence of only RRF and EF-G·GTP, tRNA dissociation was slow. Rapid tRNA dissociation was observed only when all three factors, RRF, EF-G·GTP, and IF3, were present. The same release kinetics were obtained for $tRNA^{Phe}$ (Figure 4) and $tRNA^{fMet}$ (data not shown). It is important to note that upon prolonged incubation, the amount of tRNA released in the presence of only RRF and EF-G·GTP may become as large as that ejected by RRF, EF-G·GTP, and IF3 together. This explains why in the assays of Hirokawa et al. (2002b), IF3 did not appear to be necessary for tRNA release. However, in the time window relevant for ribosome recycling in vivo, the reaction is strongly dependent on IF3. Thus, IF3 has two functions during ribosome recycling, i.e., preventing the subunits from reassociation and promoting tRNA ejection from the 30S subunit after disassembly of the posttermination complex.

The rate of tRNA dissociation was 0.04 ± 0.01 s^{-1} in the presence of the three factors, about 8-fold slower than subunit dissociation under the same conditions (0.3 s^{-1}), implying that the tRNA was released from the 30S subunit rather than from the 70S ribosome complex. Dissociation of tRNA from the 30S subunit was shown to require IF3 only (Karimi et al., 1999). This suggests that ribosome recycling proceeds in two steps, the dissociation of subunits induced by RRF and EF-G·GTP followed by the release of tRNA from the 30S subunit catalyzed by IF3.

mRNA Exchange in the Posttermination Complexes

Ribosome recycling is followed by initiation complex formation on a new mRNA in the next translation cycle. In order to monitor the transition of ribosomes from the posttermination complex into the initiation complex, we developed a reprogramming assay that entailed the exchange of unlabeled mRNA with fluorescein-labeled mRNA and of deacylated $tRNA^{Phe}$ with radioactively labeled f[^{3}H]Met-$tRNA^{fMet}$ (Figure 5A). Upon formation of the 70S initiation complex, the fluorescence of the labeled mRNA increased concomitantly with the binding of f[^{3}H]Met-$tRNA^{fMet}$ to the ribosome as monitored by nitrocellulose filtration (data not shown). Early events of initiation, such as factor-independent mRNA binding to the ribosome or the 30S subunit, or conformational adjustments during initiation (La Teana et al., 1995) were not reported by the fluorescence label.

First, the assay was validated by studying initiation complex formation with vacant 70S ribosomes (Figure 5B). Initiation complex formation was dependent on the presence of fMet-$tRNA^{fMet}$ and all three initiation factors, as no fluorescence increase was found in the presence of mRNA alone (trace 1) or of fMet-$tRNA^{fMet}$, IF1, IF2 only (no IF3) (trace 2). The latter result reflects the dual role of IF3 in translation, acting not only to remove tRNA from the P site during recycling but also to facilitate initiation by vacant 70S ribosomes. The rate of initiation complex formation was 0.01 ± 0.002 s^{-1} in the presence of mRNA, fMet-$tRNA^{fMet}$, and the three initiation factors (trace 3). Interestingly, the presence of RRF and EF-G increased the rate about 4-fold, to 0.04 ± 0.01 s^{-1} (trace 4). This suggests that the rate of initiation without RRF and EF-G to some extent was limited by subunit dissociation, which was rather slow with IF3 alone (0.02 s^{-1}; Figure 3B). RRF and EF-G·GTP accelerated the dissociation of vacant 70S ribosomes (0.3 s^{-1}; Figure 3A), thereby indirectly increasing the rate of initiation.

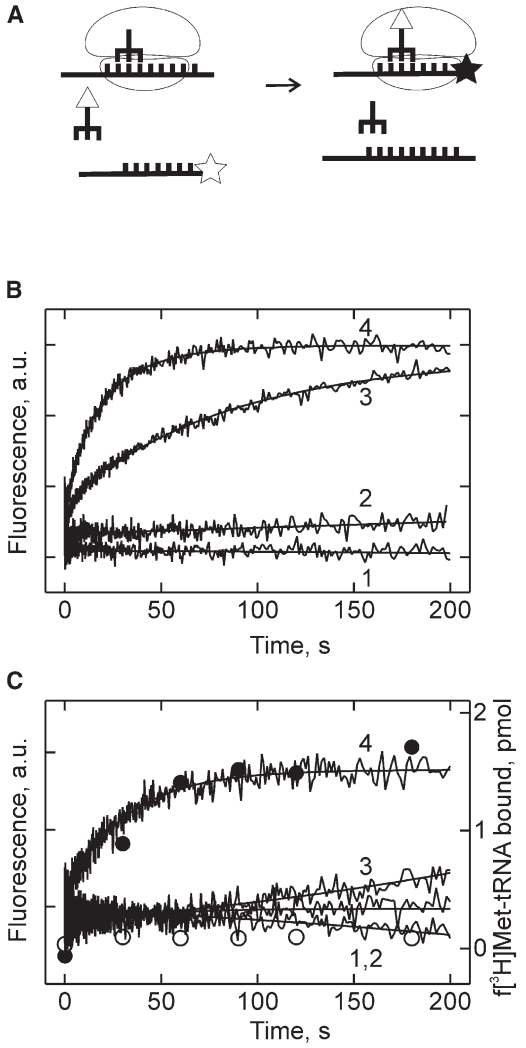

Figure 5. mRNA Exchange in the Posttermination Complex

(A) Experimental approach. Posttermination complexes carrying nonlabeled mRNA without an SD sequence and $tRNA^{Phe}$ in the P site were rapidly mixed with RRF, EF-G, IF1, IF2, IF3, fMet-$tRNA^{fMet}$ (tRNA with triangle representing fMet), GTP, and fluorescein-labeled mRNA containing an SD sequence (open star indicates low fluorescence). Exchange of unlabeled mRNA for fluorescein-labeled mRNA and initiation were monitored by an increase of fluorescence (closed star).

(B) Initiation complex formation with vacant ribosomes. Vacant 70S ribosomes were mixed with fluorescein-labeled mRNA alone (1) or additionally with IF1, IF2, and fMet-$tRNA^{fMet}$ (2); IF1, IF2, IF3, and fMet-$tRNA^{fMet}$ (3); or IF1, IF2, IF3, fMet-$tRNA^{fMet}$, RRF, and EF-G (4).

(C) Reprogramming of posttermination ribosomes. Experiment as in (B) but with posttermination complex mixed with fluorescein-labeled mRNA alone (1); or additionally with IF1, IF2, IF3, and fMet-$tRNA^{fMet}$ (2); IF1, IF2, fMet-$tRNA^{fMet}$, RRF, and EF-G (3); or IF1, IF2, IF3, fMet-$tRNA^{fMet}$, RRF, and EF-G (4). f[^{3}H]Met-$tRNA^{fMet}$ binding to the ribosome was measured with IF1, IF2, f[^{3}H]Met-$tRNA^{fMet}$, RRF, and EF-G (open circles) and the same plus IF3 (closed circles); full conversion of posttermination to initiation complexes corresponds to 2 pmol f[^{3}H]Met-$tRNA^{fMet}$ bound.

Figure 6. Model of Ribosome Recycling

In the first step, RRF and EF-G·GTP bind to the posttermination complex and, after GTP hydrolysis, promote the dissociation of the 50S subunit from the 30S-tRNA-mRNA complex. In the second step, IF3 promotes the dissociation of deacylated tRNA, which is followed by spontaneous mRNA release, rendering the 30S subunit free for initiation on a new mRNA.

To study reprogramming, posttermination complexes programmed with unlabeled mRNA were mixed with a 2-fold excess of fluorescein-labeled mRNA, IF1, and IF2 in the presence of various combinations of RRF, EF-G, and IF3. Reprogramming of posttermination complexes was observed only when all initiation factors, RRF, and EF-G were present (Figure 5C). The rate of reprogramming, 0.03 s^{-1}, was identical to the rate of tRNA release from the posttermination complex (cf. Figure 4). The binding of f[^{3}H]Met-tRNA to the ribosome, as monitored by nitrocellulose filtration, took place at the same rate. These results demonstrate that the initiation complex was formed on a new mRNA and indicate that after subunit dissociation by RRF and EF-G and tRNA ejection from the 30S subunit by IF3, the dissociation of the mRNA takes place rapidly, thus allowing rapid reprogramming and initiation complex formation.

Discussion

Sequence of Steps in Ribosome Recycling

The present data together with previous biochemical and structural data suggest the following mechanism of the ribosome recycling (Figure 6). RRF and EF-G·GTP bind to the posttermination complex and—after GTP hydrolysis—promote the dissociation of the 50S subunit from the 30S-tRNA-mRNA complex. tRNA-mRNA translocation does not take place, and deacylated tRNA as well as mRNA remain bound to the 30S subunit in a rather stable fashion. In the second step, IF3 stimulates tRNA dissociation from the 30S subunit, followed by the release of mRNA, which renders the ribosome free for the next round of translation.

These results are in agreement with the models proposed by Ehrenberg and Nakamura and coauthors (Fujiwara et al., 2001; Fujiwara et al., 2004; Karimi et al., 1999) but are inconsistent with the model proposed by Kaji et al. (2001). However, apparent discrepancies are explained by our results, as described in the following text.

(1) It has been reported that upon posttermination complex disassembly by EF-G and RRF, 70S ribosomes, not subunits, were formed (Hirashima and Kaji, 1972; Kaji et al., 2001). In that work, the disassembly of posttermination complexes was studied by the breakdown of polysomes in the presence of EF-G and RRF, and the appearance of monosomes in sucrose gradients was taken as to indicate the release of 70S ribosomes from posttermination complexes (Hirashima and Kaji, 1972). In contrast, our results show that RRF and EF-G·GTP do promote rapid subunit dissociation (Figure 2B), in agreement with the results of Karimi et al. (1999). The time courses of disassembly clearly show that the formation of 70S ribosomes in this assay is due to the reassociation of subunits formed by the action of EF-G·GTP and RRF, presumably because the dissociation activity of EF-G decreases due to GTP depletion during longer incubation times. Additionally, during sucrose gradient centrifugation, the factors, which are weakly bound to the ribosomal subunits, will probably be separated from the ribosomes, leaving the subunits free for reassociation. Thus, the appearance of 70S monosomes, rather than subunits, in the polysome-breakdown assay as analyzed on sucrose gradients is explained as a secondary effect caused by long incubation times under conditions favoring subunit association.

(2) According to our results, IF3 is required for tRNA ejection from 30S subunits, in contrast to a model in which IF3 affects the dissociation of vacant ribosomes into subunits after the release of tRNA and mRNA (Kaji et al., 2001; Kiel et al., 2003). Although slow tRNA dissociation takes place during prolonged incubations in the absence of IF3 (Figure 4), consistent with the results of Hirokawa et al. (2002b), IF3 strongly accelerates tRNA release (Figure 4; [Karimi et al., 1999]). Furthermore, in the presence of RRF, EF-G·GTP, and IF3, subunit dissociation is more than ten times faster than tRNA ejection, demonstrating that subunit dissociation takes place before tRNA release from the ribosome.

IF3 has several functions during translational initiation (Gualerzi et al., 2001). Binding of IF3 to the 30S subunit may change the orientation of the head relative to the shoulder of the subunit, thereby affecting the structure of the P site-decoding center, decrease the affinity of tRNA for the P site, and increase its dissociation rate constant (Gualerzi et al., 2001). Thus, IF3 has a dual role in promoting tRNA dissociation and inhibiting reassembly of the subunits into 70S ribosomes. In ribosome recycling, the effect of IF3 as an inhibitor of subunit association explains why IF3, in addition to RRF and EF-G, seemed to be necessary for subunit dissociation upon prolonged incubation times (Hirashima and Kaji, 1972; Kaji et al., 2001). In agreement with the data of Kiel et al. (2003), we did not observe dissociated subunits in our time-resolved FRET assay when working at the factor concentrations used in their experiments. However, subunit competition experiments (Figure 2D) showed that also under those conditions, the posttermination complexes did dissociate into subunits, albeit transiently, apparently because the conditions favored the reassembly of 30S and 50S subunits to 70S ribosomes. The strong dependence of subunit reassociation on factor concentrations and incubation

times may explain the different results obtained by different groups using the sucrose gradient technique (Hirokawa et al., 2002b; Karimi et al., 1999).

Our model predicts that tRNA release should be more efficient with GTP, compared with a nonhydrolyzable GTP analog, because GTP hydrolysis is required for subunit dissociation that precedes tRNA release. In fact, very little tRNA release from the ribosome was observed when GTP was replaced with GDPNP (Karimi et al., 1999). In contrast, it has been reported that the same amount of tRNA was released from polysomes with GTP or GDPCP (Kiel et al., 2003). It is possible that, even though EF-G requires GTP hydrolysis for efficient subunit dissociation, the binding of EF-G·GDPCP to the ribosome was sufficient to affect tRNA release within the incubation time of 15 min (Kiel et al., 2003). Thus, the seemingly contradictory tRNA release results obtained by Karimi et al. (1999) and Kiel et al. (2003) may be explained by the long incubation times used in the latter work.

(3) The shape of RRF resembles that of tRNA, prompting the proposal that RRF binds to the vacant A site of the posttermination complex and, during EF-G-dependent translocation, is moved to the P site, resulting in the ejection of tRNA and, subsequently, mRNA (Selmer et al., 1999). The idea of RRF binding to the A site in a tRNA-like fashion was refuted by footprinting and cryo-EM studies (Agrawal et al., 2004; Lancaster et al., 2002), but a number of biochemical findings seemed to be consistent with the notion of RRF translocation by EF-G. Several antibiotics known to be translocation inhibitors affected ribosome recycling as well (Hirokawa et al., 2002a; Hirokawa et al., 2002b; Kiel et al., 2003). EF-G mutants that were defective in their ability to catalyze translocation were reported to be impaired in tRNA and RRF release and monosome formation from polysomes, suggesting a translocation-like event during posttermination complex disassembly (Kiel et al., 2003). However, for a number of translocation-deficient EF-G mutants, the effect on translocation did not correlate with recycling deficiency (Fujiwara et al., 2004; Ito et al., 2002). The present experiments directly show that the position of the mRNA relative to the ribosome does not change during posttermination complex disassembly catalyzed by EF-GTP and RRF, excluding the possibility that the reaction involves translocation or a translocation-like event.

(4) It has been argued (Hirokawa et al., 2002b) that the apparent differences in the order of recycling events may be attributed to the presence of an SD sequence shortly upstream of the termination codon, precluding tRNA-mRNA release in the experiments of Pavlov et al. (1997). However, we have observed the same order of events, i.e., subunit dissociation preceding tRNA and mRNA release, by using short mRNAs with or without an SD sequence. Furthermore, the rate of subunit dissociation was the same on posttermination complexes containing these different mRNAs and on vacant ribosomes bearing no mRNA at all (Figures 2B, E and 3). Thus, the first step of ribosome recycling, subunit dissociation, does not depend on the nature of mRNA. Reprogramming of ribosomes by a new mRNA takes place rapidly after tRNA ejection from the 30S subunit (Figure 5) and appears to be rapid and spontaneous.

Cryo-EM and crystal structures suggest that the binding site of RRF on the 50S subunit includes both A and P sites (Agrawal et al., 2004; Wilson et al., 2005). According to footprinting data, the 3′ end of deacylated tRNA in the posttermination complex is likely to reach into the E site on the 50S subunit (Lancaster et al., 2002), i.e., to assume the P/E hybrid state (Moazed and Noller, 1989), whereas peptidyl-tRNA is bound in the P/P state only. Thus, unlike deacylated tRNA, P site peptidyl-tRNA is expected to exclude the binding of RRF to the ribosome, explaining the observed specificity of RRF and EF-G function toward ribosomes in the posttermination state.

Role of EF-G in tRNA Translocation and RRF-Dependent Recycling

Translocation entails the coordinated movement of tRNA and mRNA within the ribosome. Kinetic analyses indicated that EF-G, after GTP hydrolysis, induces a conformational rearrangement of the ribosome ("unlocking") that is required for tRNA-mRNA translocation and is driven by GTP hydrolysis (Katunin et al., 2002; Rodnina et al., 1997; Savelsbergh et al., 2003). The reactions of EF-G on the ribosome, including EF-G·GTP binding, GTP hydrolysis, Pi release, and dissociation of EF-G·GDP, are not influenced by the presence or absence of tRNA in either the P or A site (Savelsbergh et al., 2003), indicating that there is no direct interaction between the A site tRNA and EF-G and that tRNA movement is catalyzed by EF-G indirectly, by either acting on movable parts of the ribosome or trapping an open conformation of the ribosome that allows the tRNAs to move by diffusion (Savelsbergh et al., 2003).

The parallel effects on translocation and recycling observed for some antibiotics and some mutations in EF-G indicate that EF-G functions similarly in subunit dissociation and translocation. For example, the antibiotic thiostrepton, which binds to the L11 region of the 50S subunit, effectively blocks both translocation and ribosome recycling (Hirokawa et al., 2002b; Kiel et al., 2003; Rodnina et al., 1999; Seo et al., 2004), most likely because of the inhibition of an EF-G-induced conformational change of the ribosome that is essential for both reactions (Kiel et al., 2003; Rodnina et al., 1999; Seo et al., 2004). This suggests that, in the presence of RRF, EF-G induces a conformational change of the ribosome that resembles the unlocking rearrangement preceding translocation (Savelsbergh et al., 2003), and by cooperation between EF-G and RRF, leads to subunit dissociation rather than to tRNA-mRNA movement. RRF interacts with elements of the 50S subunit that are involved in the formation of two central intersubunit bridges, B2a (helix 69 of 23S rRNA) and B3 (helix 71) (Agrawal et al., 2004; Lancaster et al., 2002; Wilson et al., 2005). The position of helix 69 in the complex is shifted toward helix 44 of the 30S subunit (Wilson et al., 2005), suggesting that EF-G and RRF may induce subunit dissociation by altering the position of the subunit bridges, notably of B2a (helix 69), and their contacts with the 30S subunit and the P-site tRNA.

RRF and EF-G interact with each other, as indicated

by genetic complementation experiments (Ito et al., 2002; Rao and Varshney, 2001). Gain-of-function mutations were identified in domain IV of EF-G and in domain II of RRF, indicating residues that participate in the interaction (Ito et al., 2002). Structural models (Wilson et al., 2005) suggest that moderate adjustments of domain II suffice to accommodate RRF on the ribosome in the presence of EF-G in its posttranslocation state. The flexibility of domain II of RRF, which is bound to the 50S subunit through interactions with domain I, may allow for the interaction with domain IV of EF-G also in the pretranslocation state, possibly even through the same set of contacts. Upon establishing the interactions with RRF, EF-G is likely to shift RRF toward the 30S subunit (Wilson et al., 2005), thereby weakening the association between subunits by the interactions of RRF with the bridges connecting the subunits (Agrawal et al., 2004; Wilson et al., 2005). Alternatively, binding of domain I of RRF to the 50S subunit and its effect on the subunit bridges may increase the frequency of spontaneous subunit dissociation; in such a case, EF-G and domain II of RRF may prevent rebinding of subunits by rapidly occupying the locations necessary for reassociation. It remains to be elucidated how the difference in the function of EF-G and the outcome of the reaction, i.e., subunit dissociation rather than translocation, are related to specific interactions of RRF with EF-G and the ribosome.

Experimental Procedures

Materials

Experiments were carried out in buffer A (50 mM Tris-HCl [pH 7.5], 70 mM NH_4Cl, 30 mM KCl, and 7 mM $MgCl_2$) at 37°C. Ribosomes from *E. coli* MRE600, initiation factors, EF-Tu, EF-G, f[^{3}H]Met-tRNA^{fMet}, and [^{14}C]Phe-tRNA^{Phe} were prepared as described (Rodnina et al., 1999; Rodnina et al., 1994; Rodnina and Wintermeyer, 1995). [^{14}C]tRNA^{fMet} was prepared by using [^{14}C]ATP and polynucleotidyltransferase (Silberklang et al., 1977). [5′-^{32}P]tRNA^{Phe} was prepared by dephosphorylation of tRNA with alkaline phosphatase and subsequent labeling using [γ-^{32}P]ATP and T4 polynucleotide kinase. mRNAs and fluorescein-labeled mRNAs were prepared as described (Peske et al., 2004; Savelsbergh et al., 2003). The mRNA used for all experiments (except the mRNA exchange experiment) had a length of 54 nucleotides (14 coding nucleotides including AUG), an SD sequence (5′-AAGGAGGU-3′) followed by a spacer of five nucleotides, and a coding sequence starting with fMetPhe. For the mRNA exchange experiment, the fluorescence-labeled mRNA had a length of 52 nucleotides (12 coding nucleotides including AUG). mRNA without an SD had the same coding sequence but no SD (m003-mRNA [Calogero et al., 1988]); the plasmid construct was kindly provided by C.O. Gualerzi (Camerino, Italy).

RRF Preparation

The plasmid coding for wild-type RRF, pET-[ATG] ecoRRF No-tag was kindly provided by K. Ito and Y. Nakamura, University of Tokyo, Japan. The protein was expressed in *E. coli* BL21(DE3)pLysS cells and purified in the following way. 5 g of cell pellet were lysed by sonification in 15 ml buffer A with 100 μM PMSF; after centrifugation, the supernatant was diluted to 50 ml with low-salt buffer (20 mM Tris-HCl [pH 7.5], 20 mM KCl) and applied to a Q-Sepharose column, and RRF was collected in the flowthrough. The flowthrough was diluted with 800 ml of 50 mM MOPS (pH 6.4) and applied to an SP-Sepharose column; the flowthrough contained purified RRF (>95% purity). The protein was concentrated by ultrafiltration using 10 kDa Vivaspin concentrators (Sartorius) and stored in buffer A containing 50% glycerol.

Fluorescence Labeling of Ribosomal Subunits

70S ribosomes (4.5 μM) were labeled at surface lysine residues by reacting with either fluorescein succinimidyl ester (0.1 mM) or QSY9 succinimidyl ester (0.1 mM) in 50 mM HEPES (pH 7.5), 100 mM KCl, and 15 mM $MgCl_2$ for 30 min at 37°C. To remove unreacted dye, ribosomes were centrifuged through 400 μl of 1.1 M sucrose in buffer A with 20 mM Mg^{2+} for 2 hr at 259,000 × g in a Sorvall M120GX centrifuge. To dissociate the ribosomes into subunits, the pellets were resuspended in dissociation buffer (20 mM Tris-HCl [pH 7.6], 60 mM NH_4Cl, 1.5 mM magnesium acetate, and 3 mM 2-mercaptoethanol) and dialyzed against the same buffer for 4 hr at 4°C. Subunits were separated by centrifugation through a 10%–38% sucrose gradient in dialysis buffer in a Beckman SW28 rotor at 19,000 rpm at 4°C for 19 hr. Gradients were fractionated, and subunits were collected according to the absorbance at 260 nm and pelleted in a Beckman TI 50.1 rotor at 50,000 rpm for 19 hr at 4°C. Pellets were resuspended in buffer A. Subunits (260 nm), fluorescein (495 nm), and QSY 9 (562 nm) were quantitated photometrically. Labeled 30S and 50S subunits both contained three to five dye molecules per subunit. For the subunit-dissociation assay, fluorescein-labeled 50S and QSY 9-labeled 30S subunits were used. Prior to use, subunits were activated by incubation with 20 mM $MgCl_2$ in buffer A for 1 hr at 37°C.

Preparation of Ribosome Complexes

Initiation complexes were prepared by incubating 70S ribosomes (or an equimolar mixture of the 30S and 50S subunits) (1 μM) with a 2-fold excess of mRNA; 1.5 μM initiation factors IF1, IF2, and IF3; 1.5 μM f[^{3}H]Met-tRNA^{fMet}; and 1 mM GTP in buffer A for 1 hr at 37°C. Ternary complexes, EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe}, were prepared by incubating 10 μM EF-Tu with 1 mM GTP, 3 mM phosphoenol pyruvate, and 0.1 mg/l pyruvate kinase for 15 min at 37°C, followed by the addition of 5 μM [^{14}C]Phe-tRNA^{Phe}. Pretranslocation complex carrying f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} in the A site was prepared by adding ternary complex to the initiation complex and incubating for 30 s at 20°C. Subsequently, the Mg^{2+} concentration was adjusted to 20 mM to stabilize fMetPhe-tRNA^{Phe} in the A site. To form posttranslocation complexes, translocation was induced by incubating the pretranslocation complexes with EF-G (0.2 μM) for 10 min at 37°C. Posttranslocation complexes were purified by ultracentrifugation through 400 μl of a 1.1 M sucrose cushion in buffer A with 20 mM Mg^{2+} for 2 hr at 259,000 × g in a Sorvall M120GX centrifuge. [^{14}C]Phe and [^{3}H]Met bound to ribosomes were determined by nitrocellulose filtration by applying aliquots of the reaction mixture to the filters (0.45 μm, Sartorius) and subsequent washing with 5 ml of buffer A. Filters were dissolved, and radioactivity was measured in QS361 scintillation cocktail (Zinsser Analytic). >95% of the ribosomes carried tRNAs as indicated. The extent of translocation (>85%) was determined by reaction with puromycin (1 mM puromycin, 10 s, 37°C). The reaction with puromycin was quenched with 500 μl 1.5 M sodium acetate saturated with $MgSO_4$. After addition of 750 μl ethyl acetate, extraction for 5 min at room temperature, and phase separation by centrifugation, 500 μl of the organic phase was taken for counting in Luma Safe Plus (LumacLSC).

Posttermination complex with tRNA^{Phe} in the P site was prepared by releasing the dipeptide fMetPhe from posttranslocation complexes (3–5 μM) with puromycin (0.1 mM, 10 min, 37°C). Alternatively, posttermination complexes were prepared by directly incubating ribosomes (1 μM) with mRNA (2 μM) and [^{32}P]tRNA^{Phe} or [^{14}C]tRNA^{fMet} (1.5 μM) in buffer A for 1 hr at 37°C; tRNA occupancy was controlled by nitrocellulose filtration. In the mRNA-reprogramming assay, the posttermination complex was prepared with mRNA lacking an SD sequence and mixed with initiation factors, EF-G, RRF, fMet-tRNA^{fMet}, and SD-containing mRNA with fluorescein attached to position +12 including the AUG codon.

Rapid Kinetics

Fluorescence stopped-flow measurements were performed, and the data were evaluated as described previously (Rodnina et al., 1997; Savelsbergh et al., 2003). Fluorescein fluorescence was excited at 470 nm and measured after passing a KV500 cut-off filter (Schott). Experiments were performed in buffer A with 1 mM GTP at

37°C by rapidly mixing equal volumes (55 μl) each of the ribosome complex and factors, mRNA, and fMet-tRNA^{fMet} as indicated. The following concentrations of factors were used: RRF (5 μM), EF-G (2 μM), and IF3 (2 μM), if not stated otherwise. In translocation experiments (Figure 1), ribosome complexes (0.1 μM) and ternary complexes (0.25 μM) were used. In subunit dissociation experiments (Figures 2 and 3), ribosome complexes were 0.05 μM. In the mRNA reprogramming experiments, ribosome complexes (0.05 μM), IF1, IF2, and IF3 (0.1 μM each), mRNA (0.1 μM), and f[^{3}H]Met-tRNA^{fMet} (0.2 μM) were used; f[^{3}H]Met-tRNA^{fMet} binding to the ribosome was measured by nitrocellulose filtration. Stopped-flow traces were evaluated by one- or two-exponential fitting by using TableCurve software (Jandel Scientific).

tRNA Release

Release of [^{14}C]tRNA^{fMet} or [^{32}P]tRNA^{Phe} from the ribosome was monitored by nitrocellulose filtration. Posttermination complexes (0.1 μM) were incubated with RRF (5 μM), EF-G (2 μM), and IF3 (2 μM) as indicated with 1 mM GTP in buffer A at 37°C with or without an excess of unlabeled competing tRNA.

Supplemental Data

Supplemental Data include one figure and are available with this article online at http://www.molecule.org/cgi/content/full/18/4/403/DC1/.

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References

Agrawal, R.K., Sharma, M.R., Kiel, M.C., Hirokawa, G., Booth, T.M., Spahn, C.M., Grassucci, R.A., Kaji, A., and Frank, J. (2004). Visualization of ribosome-recycling factor on the Escherichia coli 70S ribosome: functional implications. Proc. Natl. Acad. Sci. USA *101*, 8900–8905.

Calogero, R.A., Pon, C.L., Canonaco, M.A., and Gualerzi, C.O. (1988). Selection of the mRNA translation initiation region by *Escherichia coli* ribosomes. Proc. Natl. Acad. Sci. USA *85*, 6427–6431.

Fujiwara, T., Ito, K., and Nakamura, Y. (2001). Functional mapping of ribosome-contact sites in the ribosome recycling factor: a structural view from a tRNA mimic. RNA *7*, 64–70.

Fujiwara, T., Ito, K., Yamami, T., and Nakamura, Y. (2004). Ribosome recycling factor disassembles the post-termination ribosomal complex independent of the ribosomal translocase activity of elongation factor G. Mol. Microbiol. *53*, 517–528.

Gualerzi, C.O., Brandi, L., Caserta, E., Garofalo, C., Lammi, M., La Teana, A., Petrelli, D., Spurio, R., Tomšic, J., and Pon, C.L. (2001). Initiation factors in the early events of mRNA translation in bacteria. In The Ribosome (Cold Spring Harbor Symposia Quantitative Biology, Volume 66), B. Stillman, ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 363–376.

Hirashima, A., and Kaji, A. (1972). Factor-dependent release of ribosomes from messenger RNA. Requirement for two heat-stable factors. J. Mol. Biol. *65*, 43–58.

Hirokawa, G., Kiel, M.C., Muto, A., Kawai, G., Igarashi, K., Kaji, H., and Kaji, A. (2002a). Binding of ribosome recycling factor to ribosomes, comparison with tRNA. J. Biol. Chem. *277*, 35847–35852.

Hirokawa, G., Kiel, M.C., Muto, A., Selmer, M., Raj, V.S., Liljas, A., Igarashi, K., Kaji, H., and Kaji, A. (2002b). Post-termination complex disassembly by ribosome recycling factor, a functional tRNA mimic. EMBO J. *21*, 2272–2281.

Ito, K., Fujiwara, T., Toyoda, T., and Nakamura, Y. (2002). Elongation factor G participates in ribosome disassembly by interacting with ribosome recycling factor at their tRNA-mimicry domains. Mol. Cell *9*, 1263–1272.

Janosi, L., Hara, H., Zhang, S., and Kaji, A. (1996). Ribosome recycling by ribosome recycling factor (RRF)—an important but overlooked step of protein biosynthesis. Adv. Biophys. *32*, 121–201.

Kaji, A., Kiel, M., Hirokawa, G., Muto, A., Inokuchi, H., and Kaji, H. (2001). The forth step of protein synthesis: disassembly of post-termination complex is catalyzed by elongation factor G and ribosome recycling factor, RRF, a near-perfect mimic of tRNA. In The Ribosome (Cold Spring Harbor Symposia Quantitative Biology, Volume 66), B. Stillman, ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 515–530.

Karimi, R., Pavlov, M.Y., Buckingham, R.H., and Ehrenberg, M. (1999). Novel roles for classical factors at the interface between translation termination and initiation. Mol. Cell *3*, 601–609.

Katunin, V.I., Savelsbergh, A., Rodnina, M.V., and Wintermeyer, W. (2002). Coupling of GTP hydrolysis by elongation factor G to translocation and factor recycling on the ribosome. Biochemistry *41*, 12806–12812.

Kiel, M.C., Raj, V.S., Kaji, H., and Kaji, A. (2003). Release of ribosome-bound ribosome recycling factor by elongation factor G. J. Biol. Chem. *278*, 48041–48050.

Kim, K.K., Min, K., and Suh, S.W. (2000). Crystal structure of the ribosome recycling factor from *Escherichia coli*. EMBO J. *19*, 2362–2370.

La Teana, A., Gualerzi, C.O., and Brimacombe, R. (1995). From stand-by to decoding site. Adjustment of the mRNA on the 30S ribosomal subunit under the influence of the initiation factors. RNA *1*, 772–782.

Lancaster, L., Kiel, M.C., Kaji, A., and Noller, H.F. (2002). Orientation of ribosome recycling factor in the ribosome from directed hydroxyl radical probing. Cell *111*, 129–140.

Moazed, D., and Noller, H.F. (1989). Intermediate states in the movement of transfer RNA in the ribosome. Nature *342*, 142–148.

Nakamura, Y., and Ito, K. (2003). Making sense of mimic in translation termination. Trends Biochem. Sci. *28*, 99–105.

Nakano, H., Uchiyama, S., Yoshida, T., Ohkubo, T., Kato, H., Yamagata, Y., and Kobayashi, Y. (2002). Crystallization and preliminary X-ray crystallographic studies of a mutant of ribosome recycling factor from Escherichia coli, Arg132Gly. Acta Crystallogr. D Biol. Crystallogr. *58*, 124–126.

Nakano, H., Yoshida, T., Uchiyama, S., Kawachi, M., Matsuo, H., Kato, T., Ohshima, A., Yamaichi, Y., Honda, T., Kato, H., et al. (2003). Structure and binding mode of a ribosome recycling factor (RRF) from mesophilic bacterium. J. Biol. Chem. *278*, 3427–3436.

Pavlov, M.Y., Freistroffer, D.V., MacDougall, J., Buckingham, R.H., and Ehrenberg, M. (1997). Fast recycling of Escherichia coli ribosomes requires both ribosome recycling factor (RRF) and release factor RF3. EMBO J. *16*, 4134–4141.

Peske, F., Savelsbergh, A., Katunin, V.I., Rodnina, M.V., and Wintermeyer, W. (2004). Conformational changes of the small ribosomal subunit during elongation factor G-dependent tRNA-mRNA translocation. J. Mol. Biol. *343*, 1183–1194.

Rao, A.R., and Varshney, U. (2001). Specific interaction between the ribosome recycling factor and the elongation factor G from *Mycobacterium tuberculosis* mediates peptidyl-tRNA release and ribosome recycling in *Escherichia coli*. EMBO J. *20*, 2977–2986.

Rodnina, M.V., and Wintermeyer, W. (1995). GTP consumption of elongation factor Tu during translation of heteropolymeric mRNAs. Proc. Natl. Acad. Sci. USA *92*, 1945–1949.

Rodnina, M.V., Semenkov, Y.P., and Wintermeyer, W. (1994). Purification of fMet-tRNA(fMet) by fast protein liquid chromatography. Anal. Biochem. *219*, 380–381.

Rodnina, M.V., Savelsbergh, A., Katunin, V.I., and Wintermeyer, W. (1997). Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome. Nature *385*, 37–41.

Rodnina, M.V., Savelsbergh, A., Matassova, N.B., Katunin, V.I., Semenkov, Y.P., and Wintermeyer, W. (1999). Thiostrepton inhibits turnover but not GTP hydrolysis by elongation factor G on the ribosome. Proc. Natl. Acad. Sci. USA *96*, 9586–9590.

Savelsbergh, A., Katunin, V.I., Mohr, D., Peske, F., Rodnina, M.V., and Wintermeyer, W. (2003). An elongation factor G-induced ribosome rearrangement precedes tRNA-mRNA translocation. Mol. Cell *11*, 1517–1523.

Selmer, M., Al-Karadaghi, S., Hirokawa, G., Kaji, A., and Liljas, A. (1999). Crystal structure of *Thermotoga maritima* ribosome recycling factor: a tRNA mimic. Science *286*, 2349–2352.

Semenkov, Y.P., Rodnina, M.V., and Wintermeyer, W. (2000). Energetic contribution of tRNA hybrid state formation to translocation catalysis on the ribosome. Nat. Struct. Biol. *7*, 1027–1031.

Seo, H.S., Kiel, M., Pan, D., Raj, V.S., Kaji, A., and Cooperman, B.S. (2004). Kinetics and thermodynamics of RRF, EF-G, and thiostrepton interaction on the *Escherichia coli* ribosome. Biochemistry *43*, 12728–12740.

Silberklang, M., Gillum, A.M., and RajBhandary, U.L. (1977). The use of nuclease P1 in sequence analysis of end group labeled RNA. Nucl. Acids Res. *4*, 4091–4108.

Toyoda, T., Tin, O.F., Ito, K., Fujiwara, T., Kumasaka, T., Yamamoto, M., Garber, M.B., and Nakamura, Y. (2000). Crystal structure combined with genetic analysis of the *Thermus thermophilus* ribosome recycling factor shows that a flexible hinge may act as a functional switch. RNA *6*, 1432–1444.

Wilson, D.N., Schluenzen, F., Harms, J.M., Yoshida, T., Ohkubo, T., Albrecht, R., Buerger, J., Kobayashi, Y., and Fucini, P. (2005). X-ray crystallography study on ribosome recycling: the mechanism of binding and action of RRF on the 50S ribosomal subunit. EMBO J. *24*, 251–260.

Yoshida, T., Uchiyama, S., Nakano, H., Kashimori, H., Kijima, H., Ohshima, T., Saihara, Y., Ishino, T., Shimahara, H., Yokose, K., et al. (2001). Solution structure of the ribosome recycling factor from *Aquifex aeolicus*. Biochemistry *40*, 2387–2396.