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The Ribosome Recycling Step: Consensus or Controversy?

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Abstract

Ribosome recycling, the last step in translation, is now accepted as an essential process for prokaryotes. In 2005, three laboratories showed that ribosome-recycling factor (RRF) and elongation factor G (EF-G) cause dissociation of ribosomes into subunits, solving the long-standing problem of how this essential step of translation occurs. However, there remains ongoing controversy regarding the other actions of RRF and EF-G during ribosome recycling. We propose that the available data are consistent with the notion that RRF and EF-G not only split ribosomes into subunits but also participate directly in the release of deacylated tRNA and mRNA for the next round of translation.

Translation, the last stage of genetic information transfer, is a process carried out by a large macromolecular complex, the ribosome. There are four consecutive steps in the translation: initiation, elongation, termination and recycling (Figure 1a). Each step is controlled and catalyzed by translation factors. During termination, a stop codon in the ribosomal acceptor site (A-site) is recognized by class-1 release factors (RFs) RF1 or RF2 in prokaryotes [1 and 2] and eRF1 in eukaryotes [3]. These factors induce the hydrolysis of peptidyl-tRNA at the peptidyl-tRNA site (P-site) and the nascent polypeptide is released from the tRNA on the ribosome. Class-2 release factor RF3 [4 and 5] possesses GTPase activity and stimulates the release of class-1 RFs from the ribosome [6, 7 and 8], leaving the post-termination complex (Figure 1a). The post-termination complex consists of mRNA with the termination codon at the A-site, tRNAs and the ribosome. The final step of translation – ribosome recycling – is the disassembly of the post-termination ribosomal complex. Historically, the disassembly had been thought to occur spontaneously [9] *in vivo* after the termination step. This idea was consistent with the pioneering work of the Nirenberg group, in which two separate triplets (AUG and UAA) were used to identify the RFs. In their assay, the termination triplet (UAA) was spontaneously released upon action of RF1 and RF3 [5]. In contrast to this concept, it was subsequently shown that the disassembly of the post-termination complex is an active process catalyzed by a novel protein called the ribosome-release factor (acting to release the ribosomes from the mRNA) [10 and 11] (Figure 1b). This factor, later re-named ribosome-recycling factor (RRF) [12], functions with elongation factor G (EF-G) [13] and is essential for the viability of prokaryotes [12].

RRF was discovered [10] by observing the disassembly of polysomes after removal of the nascent peptide group by the antibiotic puromycin [14] (Figure 1b). Each of the ribosomes in the polysome takes the form of the post-termination complex upon treatment with puromycin (model post-termination complex). For the disassembly of this model post-termination complex, EF-G functions with RRF [13].

Controversy over the action of RRF and EF-G exists, and two models have emerged [15, 16, 17, 18 and 19] (Box 1). In model 1 [18], RRF and EF-G not only catalyze the dissociation (splitting) of 70S ribosomes into subunits but they also catalyze the release of mRNA and tRNA. By contrast, in model 2 [15, 16, 17 and 19], RRF and EF-G catalyze only the dissociation of the 70S ribosome into subunits. In this model, initiation factor 3 (IF3) is required for the release of tRNA, and then mRNA is released spontaneously. Here, we offer possible explanations for these differences by focusing on the three activities that are essential for recycling: the release of tRNA and mRNA from the post-termination complex, and the dissociation of the 70S ribosomes into subunits. We show that the different models have emerged from interpretations of the available data, which, in fact, point to one clear-cut conclusion (model 1).

Release of deacylated tRNA for recycling

The exact number of tRNAs on naturally occurring post-termination complexes has not yet been determined. However, two tRNAs are depicted in the post-termination complex of model 1 because the model post-termination complexes are puromycin-treated polysomes containing two tRNAs per ribosome [20] (see Box 1, Figure I). The post-termination complex of model 2 also has two tRNAs [17] based on recent cryo-electron microscopy (cryo-EM) studies by Gao *et al.* [19].

The first step of model 2 is the release of tRNA by RRF, which was proposed by Zavialov *et al.* [17] based on a cryo-EM study [19]. In this study, model post-termination complexes were prepared by incubation of termination complexes with puromycin, and the complexes then incubated with RRF. The authors observed two populations of ribosomes: one with RRF and tRNA, and the other with two tRNAs but no RRF [19]. On this basis, Zavialov *et al.* [17] proposed that one tRNA is released upon binding of RRF to the ribosome. However, it is likely that the two tRNAs per ribosome shown by Gao *et al.* [19] actually represent an ensembled average of tRNAs present in two separate ribosomes, which have tRNAs either in the P/P state (anticodon region of tRNA at the P-site of the 30S subunit and amino-acid-acceptor end at the P-site of the 50S subunit) or in the P/E state [the amino-acid-acceptor end at the exit site (E-site) of the 50S subunit]. Because the densities corresponding to the anticodons of the two tRNAs seem to overlap, tRNAs in these two positions cannot co-exist. In other words, the data presented by Gao *et al.* [19] do not necessarily indicate that the post-termination complex contains two tRNAs (R. Agrawal, personal communications). This means that the initial step in model 2 should be taken as tentative. In fact, we have never observed the tRNA to be released upon the binding of RRF to model post-termination complexes [13 and 21].

Although RRF alone might not release the ribosome-bound tRNA, two groups [13, 17 and 21] have shown that RRF together with EF-G releases the ribosome-bound tRNA. This release occurs in the absence of IF3 (see Box 1, Figure I). Zavialov *et al.* [17] also observed that IF3 does not stimulate the tRNA release induced by RRF and EF-G. This is in contrast to the data they presented earlier (i.e. that IF3, in addition to RRF and EF-G is necessary for tRNA release) [15]. However, it is surprising that the scheme presented by Zavialov *et al.* [17] (model 2) goes against the data in the same paper but, instead, corresponds to the earlier data [15]. This is confusing and should be clarified by the group. The earlier results possibly stemmed from the assay system used: released tRNA was measured by aminoacylation in the same mixture for the disassembly reaction. Because IF3 is known to stabilize the subunits by binding to the 30S subunit, this complicates the interpretation of the results.

Peske *et al.* [16] also showed that 40% of the bound tRNA is released by RRF and EF-G from post-termination complexes without involvement of IF3 [16]. However, in contrast to the lack of IF3 effect shown by Zavialov *et al.* [17], these authors showed that IF3 stimulated the rate of the tRNA release by RRF and EF-G. We cannot reconcile the discrepancy between these two papers. Peske *et al.* [16] propose that IF3 is involved in the manner shown in model 2 because they observed that IF3 stimulated the release of tRNA.

According to Karimi *et al.* [15], the release of deacylated tRNA from 30S ribosome–mRNA complexes requires only IF3. This reaction, however, was not carried out on the intermediate of the disassembly reaction but on the separately made complex of 30S–mRNA–tRNA. Therefore, whether the release of deacylated tRNA by IF3 from the 30S–mRNA complex has anything to do with the recycling step is undetermined. A similar reaction, that is, the release of aminoacyl-tRNA from the 30S–mRNA complex by IF3, was reported prior to these observations [22]. We propose that these reactions by IF3 might serve to prevent accidental initiation of translation from the middle of mRNA [22].

Dissociation of ribosomes into subunits by RRF and EF-G

We suggest that, after the release of tRNA, the complex is ready to be split into subunits because the tRNA probably stabilizes the 70S ribosomes. The stabilization is due to the binding of the tRNA anti-codon region to the 30S–mRNA complex [23] while the CCA end (the amino acid acceptor end) binds to the 50S subunit [24]. Despite the stabilization effect of tRNA, it should be emphasized that the loss of tRNA does not lead to spontaneous splitting of the 70S ribosomes. The splitting of 70S ribosomes has to be catalyzed by RRF and EF-G because even the splitting of vacant 70S ribosomes is dependent on RRF and EF-G [18]. Thus, in agreement with an earlier suggestion by Karimi *et al.* [15], it was shown in 2005 that RRF and EF-G dissociate ribosomes into 30S and 50S subunits [16, 17 and 18]. The dissociation of 70S ribosomes into subunits has been demonstrated by three independent methods: (i) fluorescence resonance energy transfer (FRET) change between fluorescence-labeled subunits [16], (ii) ribosomal subunit exchange between the radioactively labeled ribosomal complex and free subunits [17], and (iii) decrease in the light-scattering of ribosomes [18]. From the initial studies and up until 2004, our laboratory used the sucrose-density-gradient centrifugation (SDGC) technique exclusively to examine the RRF reaction [10, 11, 13, 21 and 25]. We now believe that the SDGC technique [10] permitted the transiently dissociated subunits to re-associate to form 70S ribosomes [17 and 18] (Figure 1b). This re-association of subunits led us to the erroneous conclusion that RRF and EF-G disassemble the post-termination complex into 70S ribosomes, mRNA and tRNA [25]. On this basis, it is understandable why the partial splitting of 70S ribosomes by RRF and EF-G observed by the SDGC technique [15] could not be repeated [17 and 18].

Peske *et al.* [16] observed that RRF together with EF-G induced a FRET change of fluorescence-labeled ribosomes at the rate of $0.3\ \mathrm{s}^{-1}$ – a rate much higher than the tRNA release or mRNA exchange. The FRET change was interpreted to indicate the splitting of the 70S ribosomes. Thus, the subunit-dissociation step is proposed as the first step in model 2 (Box 1). It is noted that the rate of FRET change is ∼60-fold faster than that of the reported physical exchange of ribosomal subunits [17] under similar experimental conditions. It is also much faster than the splitting measured by the decrease in light scattering [18]. Therefore, the FRET change between the subunits might reflect an early event in the splitting or structural (or rotational) changes of ribosomes [19, 26, 27, 28 and 29].

The dissociation of 70S ribosomes into subunits by RRF and EF-G requires GTP hydrolysis; a non-hydrolyzable analog of GTP exhibits no functionality [16, 17 and 18]. This is in contrast to the translocation by EF-G during peptide elongation, which does take place, albeit slowly, with the help of the non-hydrolyzable GTP analog.

In vivo inactivation of a temperature-sensitive RRF at its non-permissive temperature [30] causes the accumulation of 70S ribosomes and the shut-down of bulk protein synthesis [31] except for the translation of leaderless mRNA [32]. The leaderless mRNA is known to be initiated by 70S ribosomes [32 and 33]. These *in vivo* data are consistent with the concept that RRF and EF-G dissociate 70S ribosomes into subunits.

The transiently dissociated subunits are stabilized by IF3, which is known to prevent the association of 30S subunits with 50S subunits [34]. Confirming this, IF3 attaches to the 30S subunit when the 70S ribosome is split by RRF and EF-G [18]. By contrast, an excess of IF3 alone dissociates 70S ribosomes [18 and 35]. A recent report has shown that *Thermus thermophilus* RRF, which does not function in *Escherichia coli*

in vivo [36], can complement the defective *E. coli* RRF if a plasmid expressing IF3 is present [37]. Because *T. thermophilus* RRF, together with *E. coli* EF-G and ribosomes, functions only to release tRNA [38], an excess of IF3 might disassemble the 70S ribosomes left on mRNA without tRNA. These data suggest the possibility that excess IF3 might participate actively in the splitting of 70S ribosomes *in vivo*.

Release of mRNA for recycling

The splitting of ribosomes might occur simultaneously with the release of the ribosome-bound mRNA, which is also destabilized by the loss of tRNA. The bound mRNA must be quickly released from the ribosome during the recycling step with the rate comparable to that of initiation and termination (Figure 1a). In model 2, mRNA is released spontaneously after tRNA is released by IF3 without participation of RRF or EF-G, whereas RRF and EF-G actively release mRNA in model 1 (Box 1).

Important evidence supporting the active release of mRNA by RRF and EF-G (model 1) is the disassembly of model post-termination complexes into monosomes by RRF and EF-G without requiring IF3 [10, 21, 30, 38, 39, 40, 41 and 42] (Figure 1b). The release of mRNA by RRF and EF-G from natural post-termination complexes in the absence of IF3 has also been shown [43]. Furthermore, Zavialov *et al.* [17] recently showed that the ribosome-bound mRNA cleavage by the bacterial toxin RelE disappears upon addition of RRF and EF-G to post-termination complexes. We believe that this is consistent with the notion that mRNA is released by RRF and EF-G.

Peske *et al.* [16] showed that the exchange between the ribosome-bound non-labeled mRNA [without the Shine–Dalgarno sequence (AGGAGGU; the signal for initiation of protein biosynthesis in bacterial mRNA)] with free fluorescence-labeled mRNA (with the Shine–Dalgarno sequence) requires RRF, EF-G and IF3. The authors' interpretation is that the 30S subunit formed by RRF and EF-G remains on the mRNA with tRNA and IF3 releases the bound tRNA from the complex. The ribosome-bound mRNA is then released spontaneously. Thus, the release of mRNA is dependent on IF3 (model 2). However, the data are also compatible with the interpretation that RRF and EF-G release the ribosome-bound mRNA and that the binding of fluorescence-labeled free mRNA to the ribosome is dependent on IF3. Therefore, no 'exchange' occurs without IF3. In fact, Peske *et al.* [16] showed that the binding of the same mRNA to vacant ribosomes is dependent on IF3. A similar interpretation is possible for the earlier data that was thought to support model 2 [15]. Thus, Karimi *et al.* [15] showed that 30S subunits recycle only in the presence of IF3. They interpreted that IF3, but not RRF and EF-G, is responsible for the release of tRNA, and hence mRNA, from the 30S subunits. We suggest the alternative explanation that IF3 is necessary for the initiation of a new round of translation by the 30S subunits released by RRF and EF-G.

The release of mRNA also strictly requires GTP and is inhibited by a non-hydrolyzable GTP analog [13 and 21] in a similar manner to the strict requirement of GTP for splitting 70S ribosomes. These observations suggest that the splitting of 70S ribosomes might occur simultaneously with the release of mRNA.

Translocation during the recycling reaction

Release of mRNA might be related to translocation (i.e. the movement of mRNA and tRNA on the ribosome by EF-G during peptide-chain elongation). However, Peske *et al.* [16] reported that mRNA translocation does not take place upon the addition of RRF and EF-G. By contrast, as mentioned, Zavialov *et al.* [17] suggest that the A-site-situated termination codon might be moved by RRF and EF-G based on the disappearance of the ribosomal A-site-specific cleavage of mRNA by RelE. We believe that these seemingly contradictory conclusions can be explained by the difference in the mRNA used. Peske *et al.* [16] used mRNA with the Shine–Dalgarno sequence 11 nucleotides upstream from the A-site codon, whereas Zavialov *et al.* [17] used mRNA with the Shine–Dalgarno sequence 18 nucleotides upstream from the A-site termination codon. The Shine–Dalgarno sequence nearer the A-site codon might inhibit mRNA movement or release by RRF and EF-G. It is known that the Shine–Dalgarno sequence near the termination codon stabilizes the mRNA on the ribosome [17], and ribosomes are known to be influenced by the sequence surrounding the termination codon [44]. This argument does not go against the finding that the splitting of 70S ribosomes occurs regardless of whether the Shine–Dalgarno sequence is present [16]. It is the mRNA movement that is influenced by the Shine–Dalgarno sequence, not the splitting of ribosomes.

Although we have no specific data on the translocation of mRNA, we have suggested that RRF moves (translocates) [21, 38, 39 and 45] on the ribosome from the A/P-site (RRF covers both the A- and P-sites) [28 and 46] to a second ribosomal site. This is because the deacylated tRNA of the post-termination complex is released by RRF and EF-G [13 and 21], which is analogous with the release of tRNA during the translocation by EF-G in the peptide-elongation step [47]. In support of this notion, we have observed tRNA release induced by RRF and EF-G using a non-hydrolyzable GTP analog [21]. By contrast, Zavialov *et al.* [17] reported that the non-hydrolyzable GTP analog does not function for the release of tRNA [17]. We have no explanation for the difference and it will require future studies to decipher this.

The overall structure of RRF is similar to that of tRNA and consists of two domains, domain I and II [52]. The complex of the 50S subunit with domain I of RRF [48] revealed that the position of RRF is slightly shifted compared with its position on the 70S ribosome [28 and 46]. However, this slight shift cannot account for the large movement of RRF needed to release tRNA. The recent discovery that domain II of RRF on the 50S subunit swings ~60° [19] by no means negates the hypothesis of RRF movement on the 70S ribosome.

In terms of the actual molecular mechanism of the splitting of the 70S ribosomes, the ribosomal RRF-binding site is instructive. Domain I of RRF on the 70S ribosome interacts with helices 69 and 71 of 23S rRNA [19, 28, 46 and 48] (Figure 2a). These helices, together with helix 44 of the 16S rRNA, comprise the inter-subunit bridges B2a and B3, respectively [24]. We propose that the movement (translocation) of RRF by EF-G causes disruption of these inter-subunit bridges and makes it possible for IF3 to bind to other bridge regions such as B2b [49]. The fact that mRNA closely associates with helix 44 of 16S rRNA [50 and 51] (Figure 2b) is consistent with RRF movement (translocation) actively releasing mRNA. We propose, therefore, that the functions of RRF might be caused by the movement of RRF on the ribosome. The structural similarity of RRF to tRNA [52] might be understood because both of these molecules move on the ribosome.

Concluding remarks

In 2005, three laboratories showed that RRF and EF-G split ribosomes into subunits during the recycling of post-termination complexes. The previous observation that RRF and EF-G, in the absence of IF3, release tRNA from the post-termination complex for recycling was confirmed. In terms of the release of mRNA, it was observed back in 1972 that RRF and EF-G cause the release of mRNA from model post-termination complexes in the absence of IF3. This observation is consistent with the recent data suggesting that mRNA is moved by RRF and EF-G without IF3. In addition, the exchange of free fluorescence-labeled mRNA with the ribosome-bound mRNA by RRF, EF-G and IF3 is consistent with the notion that RRF and EF-G release the bound mRNA, and that the binding of free mRNA to the ribosome is dependent on IF3. IF3 helps the ribosome-recycling process mostly by converting transiently dissociated subunits into stable subunits by binding to the 30S subunits. Thus, all the available data support the model that RRF and EF-G actively recycle not only ribosomes but also mRNA and tRNA (see Box 1, Figure Ia). Controversy exists only with the interpretation of 'consensus' data.

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Figure legends
Figure 1. Ribosome recycling.
(a) The recycling step in the bacterial ribosome cycle, exemplified by growing *Escherichia coli*, and four steps of bacterial protein synthesis are shown. In growing *E. coli*, >80% of the total ribosomes are in the form of a polysome (multiple ribosomes on one mRNA), the remaining ～10–20% are present as subunits or 70S particles [53]. The protein synthesis is initiated by the 30S subunit and formylmethionyl (fMet)-tRNA at the initiation codon (AUG) preceded by the Shine–Dalgarno (SD) sequence. To this complex, the 50S subunit joins to form the 70S ribosome, which starts the peptide-elongation process. As the 70S ribosome moves along the reading frame (thick horizontal line) towards the 3′ end of the mRNA, the nascent polypeptide elongates. Elongation is terminated by the release of the synthesized polypeptide when the 70S ribosome reaches the termination codon (UAA). The post-termination complex consisting of mRNA, tRNA and the 70S ribosome is then disassembled by RRF, EF-G and IF3 for recycling. **(b)** Disassembly of the model post-termination complex by RRF and EF-G. The assay used for the discovery of RRF is shown. The polysome from growing *E. coli* is isolated by gel-filtration (i) and the model post-termination complexes are formed by removing the nascent polypeptides with puromycin (Pm) (ii). The model post-termination complex thus made is then incubated with RRF, EF-G and GTP (iii). During incubation, disassembly of the model post-termination complex occurs and the ribosomal subunits are formed transiently. When subjected to sucrose-density-gradient centrifugation (SDGC) containing 8-mM Mg^{2+} and 85-mM NH_4^+ or K^+, the transiently formed subunits separate from RRF and EF-G. This causes association of the subunits to form 70S ribosomes (monosomes) (iv).

Fig. 2 Interaction sites between RRF and the ribosome.

X-ray crystallographic structures of *Thermotoga maritima* RRF [52] and ribosomal subunits [54 and 55] have been fitted into a cryo-EM map of the RRF–ribosome complex [28]. **(a)** RRF directly interacts with helices 69 and 71 (H69 and H71; cyan) of 23S rRNA, which form the inter-subunit bridges B2a and B3 [24] with helix 44 of 16S rRNA, respectively. These bridges might be disrupted by the movement of RRF during disassembly of the post-termination complex. The fitting also shows that helix 80 (H80; cyan) of 23S rRNA is close to the tip of domain I (magenta) of RRF. Domain II of RRF is shown in purple. Certain nucleotides of rRNA that are close to the RRF are depicted as beads. Conserved (red) and semi-conserved (orange) residues of RRF are highlighted. **(b)** The mRNA is closely associated with helix 44 (h44; brown). The position of a segment of mRNA (green) [51] superimposed into the RRF–ribosome structure with helices 43 (H43; cyan) of 23S rRNA, h18 and h44 (brown) of 16S rRNA and ribosomal protein S12 (yellow). Adapted, with permission, from Ref [28]. © (2004) National Academy of Sciences, U.S.A.

Box 1 Actions of RRF and EF-G during ribosome recycling: proposed models
Model 1
The first step of model 1, in which RRF and EF-G release tRNA without IF3, is supported by the investigations of two laboratories [17 and 21] (Figure Ia). In addition,

another laboratory observed that ∼40% of the bound tRNA is released by RRF and EF-G [16]. In the next step, the release of mRNA by RRF and EF-G is supported by the fact that model post-termination complexes are disassembled without IF3 [10, 21, 30, 40, 41 and 42]. The ribosome-dependent cleavage of mRNA by the bacterial toxin RelE is lost upon the addition of RRF and EF-G [17]; we believe that this entails the release of mRNA. The release of bound mRNA and the binding of free mRNA, which has been measured by an exchange reaction, are dependent on RRF, EF-G and IF3 [16]. In our opinion, this is consistent with mRNA release by RRF and EF-G as shown in this proposed model. This is because the data can be interpreted in such a way to indicate that, after the bound mRNA is released by RRF and EF-G, IF3 binds the free mRNA to the ribosome. Regarding the splitting of the 70S ribosome, three laboratories support this reaction [16, 17 and 18]. Evidence for the order of the first two steps comes from the fact that the intermediate complex (mRNA–70S ribosome complex without tRNA) can be isolated in the presence of low concentrations of inhibitor [21] or with the use of *Thermus thermophilus* RRF [38]. However, this does not preclude the possibility that the first two steps might occur simultaneously. In fact, the rates of tRNA release, ribosome splitting and disappearance of the RelE-cleavable ribosome-bound mRNA are similar [17]. For the final step in this model, IF3 has long been known to prevent the association of subunits [34] and IF3 has been detected on the 30S subunit after disassembly [18].

Model 2
The first step of the second model [17] (Figure Ib) – the binding of RRF to cause release of tRNA – is supported by the recent cryo-EM picture [19]. However, the basis of this step is not certain because the cryo-EM picture [19] does not necessarily indicate two tRNAs per ribosome (R. Agrawal, personal communication). The fast rate of the next step measured by FRET change [16] might reflect an early event of splitting or structural (or rotational) changes of ribosomes [19, 26, 27, 28 and 29]. In support of the third step, IF3 stimulated the tRNA release from a model post-termination complex [16], but recent data from another laboratory show no stimulation by IF3 [17]. This step is also supported by an experiment showing the release of tRNA from the 30S subunit–mRNA complex by IF3 [15]. However, this experiment was not done with the isolated intermediate of the disassembly reaction. Therefore, the finding might not be specifically related to disassembly. There is no direct *in vitro* evidence for the diffusion of mRNA in the final step of this model. The intermediates of model 1, but not of model 2, have been isolated.

Fig. I Proposed models for the ribosome-recycling step in prokaryotes.
(**a**) In model 1 [18], tRNAs are released from the post-termination complex by RRF and EF-G (i). Next, mRNA is released and the ribosomes undergo transient dissociation (ii). Steps (i) and (ii) might occur simultaneously. In the next step, IF3 binds to the 30S subunit, preventing the association back to form 70S ribosomes (iii). (**b**) In model 2, there are two starting points. The current scheme of Zavialov et al. [17] starts with a complex with two tRNAs, whereas that of the earlier scheme from the same group [15] and Peske *et al.* [16] starts with a complex with one tRNA. In the first step, the binding of RRF to the post-termination complex releases the E-site-bound tRNA (yellow) (i). This step is depicted in parentheses because it is tentative. The basis for this step is a cryo-EM picture

[19] but it might not represent two tRNAs per post-termination complex (R. Agrawal, personal communication). The next step is the release of the 50S subunit from the post-termination complex by RRF and EF-G, which results in the 30S subunit complexed with mRNA and tRNA (red) (ii). In the following step, IF3 releases tRNA from the 30S subunits (iii). Finally, the bound mRNA diffuses away from the 30S subunit (iv).