Release of Ribosome-bound Ribosome Recycling Factor by Elongation Factor G*

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Elongation factor G (EF-G) and ribosome recycling factor (RRF) disassemble post-termination complexes of ribosome, mRNA, and tRNA. RRF forms stable complexes with 70 S ribosomes and 50 S ribosomal subunits. Here, we show that EF-G releases RRF from 70 S ribosomal and model post-termination complexes but not from 50 S ribosomal subunit complexes. The release of bound RRF by EF-G is stimulated by GTP analogues. The EF-G-dependent release occurs in the presence of fusidic acid and viomycin. However, thiostrepton inhibits the release. RRF was shown to bind to EF-G-ribosome complexes in the presence of GTP with much weaker affinity, suggesting that EF-G may move RRF to this position during the release of RRF. On the other hand, RRF did not bind to EF-G-ribosome complexes with fusidic acid, suggesting that EF-G stabilized by fusidic acid does not represent the natural post-termination complex. In contrast, the complexes of ribosome, EF-G and thiostrepton could bind RRF, although with lower affinity. These results suggest that thiostrepton traps an intermediate complex having RRF on a position that clashes with the P/E site bound tRNA. Mutants of EF-G that are impaired for translocation fail to disassemble post-termination complexes and exhibit lower activity in releasing RRF. We propose that the release of ribosome-bound RRF by EF-G is required for post-termination complex disassembly. Before release from the ribosome, the position of RRF on the ribosome will change from the original A/P site to a new location that clashes with tRNA on the P/E site.

Protein synthesis occurs on ribosomes in three basic steps of initiation (1, 2), elongation (3–5), and termination (6). Both decylated tRNA and mRNA remain bound to the ribosome after the termination step (7–9). In order for the ribosome to enter a new round of translation, it must be “recycled,” a process achieved by the release of both tRNA and mRNA. This “fourth step” of protein synthesis is catalyzed by the concerted action of elongation factor G (EF-G)† and ribosome recycling factor (RRF) (reviewed in Refs. 10 and 11). The simultaneous presence of EF-G and RRF is required (12), and optimal activity occurs when they are present at a 1:1 ratio (13).

RRF is an essential protein for cell growth (14). It is a nearly perfect structural (15) and functional (16) tRNA mimic. However, RRF lies at the ribosomal subunit interface, across the 50 S subunit, in a position that is nearly orthogonal to the A- and P-site bound tRNA (17, 18). These data rule out a straightforward functional mimicry of tRNA by RRF but do not rule out a possible movement of RRF by EF-G (16).

In this paper, we describe how EF-G affects the binding of RRF to ribosomes. RRF readily forms complexes with 70 S ribosomes, 50 S ribosomal subunits, and polysomes (also see Refs. 16 and 17). We demonstrate that, as part of the RRF-dependent post-termination complex disassembly reaction, EF-G releases RRF from ribosomal complexes, and this release activity of EF-G is due to the translocation activity of EF-G.

EXPERIMENTAL PROCEDURES

Buffers—The buffers used were as follows: buffer A, 20 mM Tris-HCl (pH 7.5), 100 mM NH4Cl, 10 mM Mg(OAc)2, 3 mM DTT; buffer B, 20 mM Tris-HCl (pH 7.5), 10 mM Mg(OAc)2, 500 mM NH4Cl, 2 mM DTT; buffer C, 20 mM Tris-HCl (pH 7.5), 50 mM NH4Cl, 10 mM Mg(OAc)2, 2 mM DTT; buffer D, 10 mM Tris-HCl (pH 7.5), 10 mM MgSO4, 50 mM NH4Cl, 0.5 mM DTT; buffer E, 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM DTT; buffer F, 5 mM potassium phosphate buffer (pH 7), 1 mM DTT; buffer G, 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 10 mM Mg(OAc)2, buffer H, 14 mM Tris-HCl (pH 7.4), 12 mM Mg(OAc)2, 66 mM NH4Cl, 0.3 mM DTT; buffer I, 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 7 mM Mg(OAc)2, 8 mM urea; buffer J, 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 7 mM Mg(OAc)2, 300 mM imidazole.

Preparation of 70 S Ribosomes—17 g of Escherichia coli MR600 cells (purchased from the University of Alabama Fermentation Facility) were thawed, suspended in 35 ml of buffer A, and passed once through a French press at ~12,000 p.s.i. The cell suspension was centrifuged twice at 30,000 × g for 15 min, discarding the pellets. The supernatant was layered onto four 15-ml sucrose solutions (20 mM Tris-HCl (pH 7.5), 10 mM Mg(OAc)2, 500 mM NH4Cl, 2 mM DTT, 1.1 mM succrose) and centrifuged at 30,000 rpm in a Ti70 rotor for 22 h. The crude ribosome pellets were resuspended in 45 ml of buffer B (no sucrose), cleared by a 15-min centrifugation step at 30,000 × g, and pelleted at 35,000 rpm in a Ti70 rotor for 2 h. The clearing and pelleting step was repeated once more. The washed ribosome pellet was resuspended in storage buffer (buffer A except 50 mM NH4Cl) and stored at ~80 °C. Analysis of the 70 S ribosomes on 10–30% sucrose gradients (8 mM Mg2+) showed no significant subunit dissociation of the 70 S ribosomes.

Purification of Native RRF—Native RRF was purified from an overproducing strain, DH5α(pRR2) (19), as we have previously described (13). The purified RRF was dialyzed against buffer D and stored at ~80 °C.

Purification of Native EF-G—Native EF-G was purified from an overproducing strain, JMS3(pECG), based on published methods (20, 21). Four grams of cells were lysed in buffer C by passing through a French press at 12,000 p.s.i. The cell suspension was centrifuged once at 30,000 × g for 20 min (pellet discarded) and once at 40,000 rpm in a Ti70 rotor for 2 h. The supernatant was precipitated with 70% saturated (NH4)2SO4 and resuspended in 8 ml of buffer E. The suspension...
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was passed through a Sephadex G100 column equilibrated with buffer E. Fractions containing EF-G were determined by Coomassie-stained SDS gels, pooled, and applied to a 22-ml DEAE-Sephacore column equilibrated in buffer E. EF-G was eluted with a 0–0.6 M KCl gradient in buffer E. Fractions containing EF-G were pooled, dialyzed against buffer F, and applied to a 10-ml hydroxyapatite column equilibrated in buffer F. Purified EF-G was eluted with a 5–500 mM phosphate gradient (pH 7), dialyzed against buffer D, and stored at −80 °C. Purification of Mutant EF-G—BL21(DE3) E. coli cells harboring plasmids expressing His-tagged mutant EF-G, lacking single domains (either domain 1, 4, or 5) or having a single point mutation (H583K) (22–24), were plated on LB medium with the appropriate antibiotic (either 125 μg/ml ampicillin or 30 μg/ml kanamycin). Individual colonies were then grown in LB liquid at 37 °C until an A600 of ~0.6 was reached. Isopropyl-β-D-galactopyranoside was added at a final concentration of 1 mM, and the cells were grown an additional 3.5 h. The cells were harvested and lysed in buffer I. The His-tagged EF-G was bound to Ni2+–nitrilotriacetic acid beads (Qiagen) and washed in buffer I. His-tagged EF-G was refolded by slowly replacing buffer I with buffer J containing 1 M urea. The refolded EF-G was eluted with buffer J, dialyzed against buffer D, and stored at −80 °C.

Preparation of RRF-Ribosome Complexes—70 S ribosomes (0.25–0.5 μM) and RRF (3.75–5 μM) were incubated together for 10 min at room temperature in 40 mM Tris-HCl, 0.6 M KCl, 5 mM MgCl2, and 1 mM GTP at 37 °C. The ribosomes were saturated with RRF and 1:1 complexes were formed. Complexes were separated from free RRF by Microcon-100 (Millipore) ultrafiltration, and isolated complexes were routinely measured for ribosome concentration (1 mM KCl). Freshly made complexes were used right away (within 5 min of preparation).

Preparation of 35S-Labeled tRNA51–tRNA1—tRNA51 was incubated with 150 μCi of [35S]ATP (5000 Ci/mmol) and 20 units of T4 polynucleotide kinase (Invitrogen) in 50 μl of reaction buffer (supplied with the kinase by Invitrogen). The reaction mixture was incubated for 20 min at 37 °C, followed by a heat-inactivating step at 65 °C for 60 min. The mixture was precipitated with 2 volumes of buffer E, −20 °C and pelleted by centrifugation at 15,000 × g for 15 min. The pellet was resuspended with 500 μl of 70% ethanol, air-dried, and resuspended in 200 μl of buffer G.

Preparation of RNA-Ribosome Complexes—70 S ribosomes (0.25 μM) and 35S-labeled tRNA51 (1.25 μM, ~13,500 cpm/pmol) were incubated together for 10 min at room temperature in 40 μl of buffer G with or without one A260 unit of poly(U). During the process of making the complexes of tRNA and ribosomes, no EF-G or RRF were added. Complexes were separated from free tRNA by Microcon-100 ultrafiltration as previously described (17).

Release of RRF–tRNA by EF-G—EF-G was added to 40 μl of freshly prepared RRF-ribosome or tRNA-ribosome complexes in buffer G. Release was allowed to occur for 10 min at 35 °C or room temperature. Released RRF or released tRNA was separated from complexes on Microcon-100 as described for the formation of the complexes (17). The amount of RRF still bound to ribosomes was measured by quantitative Western blot analysis of the complexes using anti-RRF antibody as described (17). The amount of tRNA bound to ribosomes was measured by filtering through nitrocellulose filters and measuring 35S counts on the filter.

Binding of RRF to 70 S Ribosomes in the Presence of EF-G—For studying the effects of the presence of EF-G on the binding of RRF to ribosomes, EF-G (15 or 120 pmol) was mixed with various concentrations of 70 S ribosomes (0.25–0.5 μM) and 1 mM GTP in 40 μl of buffer G. After the reaction, free EF-G and free RRF were removed from bound EF-G and RRF by Nano-Sep 300K ultracentrifugation (Pall Life Sciences). The EF-G-ribosome complexes are not stable enough to withstand high speed centrifugation (about 80% is dissociated (25)), but we found that they are stable enough to withstand isolation by Nano-Sep 300K ultrafiltration (pH 7), dialyzed against buffer H, and stored at −80 °C. The interaction between RRF-G and ribosomes (stabilized with this technique will be published elsewhere). The amounts of RRF and EF-G bound by antibodies were determined by quantitative Western blot using anti-EF-G or anti-RRF antibody.

In experiments where the binding of RRF to preformed complexes of ribosome, EF-G, and thiostrepton were studied, the preformed complexes were prepared by incubating 140 pmol of 70 S ribosomes, 30 pmol of EF-G, 1 mM GTP, and 40 μM thiostrepton in 40 μl of buffer G for 10 min. In the experiments where the binding of RRF to preformed complexes of ribosome, EF-G, and fusidic acid were studied, preformed complexes were prepared by incubating 100 pmol of ribosomes, 250 pmol of EF-G, 1 mM GTP, and 1 mM fusidic acid in 40 μl of buffer G for 10 min. In both cases, free EF-G was removed, bound EF-G was quantified, and the complexes were reacted with RRF as described above.

EF-G Releases Ribosome-bound RRF—Complexes of 70 S ribosomes and RRF were formed by incubating RRF with well washed, vacant ribosomes (17). RRF-ribosome complexes thus formed were isolated, and EF-G and GTP were added at various concentrations. As shown in Fig. 1A, EF-G releases RRF from ribosomes in a dose-dependent manner. Under these conditions, EF-G optimally works at an approximate 1:1 ratio with the complexes. This is consistent with earlier studies that had shown that the optimal rate of ribosome recycling occurs when EF-G and RRF are present at a 1:1 ratio (13), and it demonstrates that EF-G works stoichiometrically with RRF.

One may wonder if EF-G actually releases RRF from the ribosomes or simply affects its rebinding in a rapid equilibrium situation. It should be noted that the Kd value of RRF to the vacant ribosome is 0.2–0.5 μM, and therefore only a fraction (~30%) of the released RRF (~0.2 μM maximum released) should rebind. In this experiment, however, one would expect that no rebinding would take place, because the presence of EF-G would completely remove the bound RRF. Since the Kd value of EF-G to the vacant ribosome is ~0.04 μM, one would expect nearly 100% of ribosomes should have bound EF-G under the experimental conditions. It is possible that very rapid release and binding can take place, but such rapid action cannot be detected with the present technique used. However, in separate experiments with fluorescent RRF and fast kinetic analysis, we did not detect such a rapid action of release and rebinding.

As indicated below, about 20–25% of the ribosomes used in this experiment are inactive with respect to EF-G binding. On the other hand, ~100% of the ribosomes are able to bind RRF (one RRF per ribosome). Hence, about 25% of bound RRF remains on the ribosomes even in the presence of excess EF-G.

In our previous studies, we showed that higher concentrations of NH4Cl inhibit the initial binding of RRF (e.g. 150 mM NH4Cl inhibits ~70% of the initial binding of RRF as compared with 30 mM monovalent ions (17)). It was therefore possible that RRF may be released at physiological monovalent concentrations (150 mM) without EF-G. As shown in Fig. 1B, higher NH4Cl concentrations could not substitute for EF-G activity, indicating that EF-G is required for the release of RRF from ribosomes under physiological monovalent ion conditions.

EF-G Does Not Release tRNA Bound to Nonprogrammed Ribosomes—RRF is a nearly perfect structural mimic of tRNA (15). The effect of RRF on tRNA bound to nonprogrammed ribosomes, poly(U)-programmed ribosomes and polysomes, and vice versa has already been examined (17). We previously proposed that EF-site tRNA would be released by RRF and EF-G (17). Furthermore, we showed that tRNA bound to ribosomes is released by EF-G only if the A-site is occupied (26). Although the way RRF binds to ribosomes is quite different from that of tRNA, it would be of interest to examine whether tRNA is released from ribosomes by EF-G under similar conditions to the release of RRF.

In the experiment described in Fig. 1C, complexes of ribo-
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The release of RRF from ribosomes by EF-G is stimulated by guanine nucleotide, but GTP hydrolysis is not required

| Added          | RRF bound to 70 S ribosomes | RRF bound to 50 S subunits |
|----------------|-----------------------------|----------------------------|
| —             | 100%                        | 100%                       |
| EF-G          | 46 ± 14                     | ND                         |
| EF-G/GTP      | 22 ± 9                      | 106 ± 8                    |
| EF-G/GDP      | 26 ± 0                      | 114 ± 14                   |
| EF-G/GMPPCP   | 10 ± 3                      | 118 ± 4                    |
| —             | 100%                        | 100%                       |

a 100% binding of RRF was defined as the amount of RRF bound in the absence of EF-G (—). b ND, not determined.

somes and tRNA were formed under conditions where tRNA binds to the P- and/or E-sites but not the A-site (26–30). It is clear from Fig. 1C that EF-G alone does not release the deacetylated tRNA from these complexes as it does RRF. It is also noted that the presence or absence of poly(U) did not alter this situation. This is consistent with our earlier observation that the A-site must be occupied for the release of tRNA from the P- and/or E-site of nonprogrammed or homopolymer-programmed ribosomes by EF-G (26), although the situation is different with natural mRNA (16). This experiment was performed to demonstrate that bound tRNA behaves differently from bound RRF upon the addition of EF-G and GTP. The effect of RRF and EF-G together on bound tRNA has already been studied in detail (16).

Release of RRF Is Not Dependent on GTP Hydrolysis—The activity of RRF is routinely checked by a model post-termination complex disassembly assay (31). In this assay, polysomes are treated with puromycin so that each ribosome acts as if it has reached a termination codon. The addition of RRF, EF-G, and GTP then converts the polysomes to monosomes by releasing both tRNA and mRNA. The hydrolysis of GTP is essential for the release of mRNA but not for the release of tRNA from model post-termination complexes (16). We therefore tested whether or not the hydrolysis of GTP by EF-G was also essential for the release of RRF from ribosomes.

As shown in Table I, nonhydrolyzable GTP analogues were effective, indicating that GTP hydrolysis is not required for the release of RRF by EF-G and GTP. As a matter of fact, nonhydrolyzable GTP analogue gave the maximum release of RRF. GMP-PCP freezes EF-G on the ribosomes (32), and this is the most likely reason why it gave the best release. The observation that the release of RRF takes place in the presence of GTP analogue has been confirmed by studies using fluorescent-labeled RRF.

The fact that the release of RRF takes place with nonhydrolyzable GTP analogue is very similar to the findings observed for the RRF-dependent release of tRNA catalyzed by EF-G (16). We previously suggested that the RRF-dependent release of tRNA requires translocation of RRF by EF-G. In a similar fashion, we propose that the release of RRF involves translocation of RRF from its A/P binding site to a new site by EF-G (Fig. 6). Evidence supporting this concept is given below.

EF-G Does Not Release RRF from 50 S Subunits—RRF binds to 50 S subunits with a 10-fold lower affinity than to 70 S ribosomes (16). In contrast to the binding of tRNA to the 30 S subunit (33, 34), the 30 S subunit by itself has practically no

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Fig. 2. Stability of RRF-ribosome complexes and time course of the EF-G-dependent release of RRF. RRF-ribosome complexes (20 pmol, 1:1) were formed and isolated in 40 μl as described under “Experimental Procedures.” The isolated complexes were incubated at ambient temperature for the indicated times in the absence (open circles) or presence of 15 pmol (open squares) or 100 pmol (filled squares) of EF-G and 2.5 mM GTP. The complexes were then reisolated, and the amount of RRF remaining on the ribosomes was measured by quantitative Western blotting.

affinity for RRF (17, 18), although it does play an important role in the binding of RRF to the 70 S ribosome in a similar fashion to the binding of tRNA to the 70 S ribosome (34).

The binding site for EF-G is partially located on the 50 S subunit (35, 36), and EF-G-50 S complexes can be formed (37). Therefore, we tested whether or not EF-G could release RRF from 50 S subunits. Using identical conditions as for the release of RRF from 70 S ribosomes, no release of RRF bound to 50 S subunits could be detected (Table I, second column).

We conclude that the binding sites of EF-G and RRF on the 50 S subunit do not overlap each other. It should be noted that the residual RRF (about 25%) remaining on 70 S ribosomes after treatment with EF-G (Fig. 1A) is not due to the possible binding of RRF to 50 S subunits, because our vacant ribosome preparation does not contain significant amounts of subunits. Furthermore, after the disassembly of post-termination complexes by RRF and EF-G, we do not observe a significant amount of subunit formation (16).

Stability of RRF Complexes and Time Course for Release—

The requirement of EF-G for the release of RRF from ribosomes suggests strongly that RRF-ribosome complexes must be stable. Indeed, as shown in Fig. 2, the amount of RRF bound in the isolated complexes does not significantly change over a 1-h period of incubation, demonstrating that the complexes are stable (open circle).

Based on the apparent \( K_d \) of RRF (0.2–0.5 μM) (17), RRF cannot be constantly released and rebound in the isolated complexes used here, because the affinity of RRF is not high enough otherwise to maintain the 1:1 complexes that we observe. The free RRF concentration will never rise above –0.2 μM, because the concentration of isolated complex is 0.25 μM. Therefore, RRF must be tightly bound to the ribosomes in the isolated complexes in such a way that the \( K_{off} \) rate must be extremely slow and essentially negligible under our conditions and time frame.

In this sense, the binding of RRF would be similar to the binding of tRNA. Complexes of cognate tRNA and programmed ribosomes can be sedimented through sucrose gradient centrifugation without releasing tRNA (38). Thus, tRNA is known to bind in a concentration-dependent manner, yet after codon recognition, correct tRNA binding leads to an essentially irreversible step (\( K_{off} \) rate is less than one-five hundredth of the \( K_{on} \) rate) that is only dependent on the configuration of tRNA on the ribosome (39). Our preliminary fast kinetic analysis of ribosome binding of fluorescent-labeled RRF is consistent with this interpretation.

After the addition of EF-G to the isolated RRF-ribosome complexes, however, RRF is rapidly released (within the first minute of incubation) and then remains released from the ribosome over the 1-h incubation time (Fig. 2, squares). In confirmation of the data presented in Fig. 1A, the amount of RRF released is dependent on the quantity of EF-G. Regardless of the amount of EF-G, RRF remaining on the ribosome is constant for as long as 1 h, again confirming the stability of the ribosome-bound RRF. This observation has also been confirmed by our separate fast kinetic studies with fluorescence-labeled RRF. 2

Effect of Inhibitors—Translocation of tRNA by EF-G can occur without the hydrolysis of GTP, although the rate is slower than with hydrolysis (23). The release of RRF described here is similar to translocation of tRNA in that it occurs with EF-G and can occur without the hydrolysis of GTP (Table I). Therefore, in addition to GMP-PCP, we examined the effects of other EF-G inhibitors, thiostrepton (40, 41), viomycin (23, 42), and fusidic acid (43, 44), as well as the aminoglycoside gentamicin, since aminoglycoside is known to inhibit translocation (42, 45).

None of the antibiotics, in the absence of EF-G, had any significant effect on ribosome-bound RRF (Fig. 3A). As shown in Fig. 3B, most of the EF-G inhibitors did not inhibit the release of RRF by EF-G, although they did inhibit the total disassembly of polyosomes (16). However, thiostrepton did inhibit the release of RRF by EF-G. Approximately 50% inhibition of the release of RRF occurs at a thiostrepton concentration of 16.4 μM (Fig. 3B). This corresponds to the inhibitory concentration of thiopeptin (12.5 μM) effective to stop 50% of the release of mRNA from model post-termination complexes. On the other hand, we showed previously that a higher concentration of thiopeptin (92.3 μM) is required to inhibit 50% of tRNA release (16). Thus, the inhibition of RRF release compares well with the inhibition observed for the complete disassembly of post-termination complexes by EF-G and RRF.

Note, however, that the inhibition by thiopeptin is not due to the inhibition of EF-G binding to ribosomes. As discussed below (see Fig. 4), we have demonstrated that, under our conditions, EF-G binds 70 S ribosomes in the presence of GTP, thiostrepton, viomycin, fusidic acid, or GMP-PCP.

Binding of RRF to 70 S Ribosomes in the Presence of EF-G—

The binding site of RRF covers both the A- and P-sites (referred to in this paper as the RRF A/P binding site) (17, 18). This A/P binding site clearly overlaps with the proposed binding site of post-translocational EF-G (35, 36, 46, 47). It appears then, as observed in Figs. 1–3, that RRF must be moved and/or released from its defined A/P binding site by the action of EF-G on the ribosome.

In the presence of GTP, EF-G binds ribosomes for translocation. In the experiment described in Fig. 1A, we showed that ribosome-bound RRF is released by EF-G in a dose-dependent manner. It appeared that RRF is released by ribosome-bound EF-G in a stoichiometric manner. We then reasoned that concentrations of EF-G that would increase the amount of bound steady state EF-G should reduce the initial binding of RRF.

To test this possibility, in the experiment shown in Fig. 4A, the binding of RRF to ribosomes was studied in the presence and absence of EF-G and GTP. The amount of bound EF-G was also examined. Since the \( K_d \) of EF-G is ~0.04 μM, under the experimental conditions of Fig. 4, one would expect that nearly all of the ribosomes would have bound EF-G. In contrast, however, we found that only 60–70% of ribosomes have bound EF-G. This must be the reason why we do not observe 100%
release of RRF from the isolated RRF-ribosome complexes in Fig. 1A. It is clear from Fig. 4A that the presence of EF-G made it harder for RRF to bind to ribosomes.

These data suggest that the RRF A/P binding sites are occupied by EF-G, and this forces RRF to bind to a weaker binding site different from the original A/P site that overlaps with the post-translocational EF-G binding site. Therefore, even in the presence of EF-G, RRF is able to bind to ribosomes to the same extent, although with weaker affinity. In other words, the second weaker RRF binding site does not overlap with the post-translocational EF-G binding site.

The above interpretation is further supported by the observation that the amount of bound EF-G is not influenced by the amount of bound RRF (Fig. 4A, open squares and triangles). Clearly, RRF can bind to complexes of EF-G and ribosome, although with weaker affinity, because it takes more RRF to bind to this site compared with the A/P binding site of RRF.

A precise calculation of the affinity of RRF to the weaker site follows by further action of EF-G. In other words, the binding reactions contain a mixture of vacant ribosomes (~30%) and ribosomes complexed with EF-G and fusidic acid (~70%). Since RRF binds to vacant ribosomes with a $K_d$ of about 0.2 μM, one can understand the background binding (~25%) even at low concentrations of RRF. It is clear from this figure that the amount of bound EF-G was not influenced by the presence of RRF (Fig. 4B, compare filled circles at zero and high concentrations of EF-G). Although the results were unexpected from the presumable position of fusidic acid/EF-G on the ribosome, they are consistent with the data indicating that the release of RRF by EF-G is not inhibited by fusidic acid (Fig. 3B). These data suggest strongly that EF-G-fusidic acid-ribosome complexes may not be identical to the post-translocation complexes formed with GTP.

Thiostrepton, in contrast to fusidic acid, has been reported to inhibit the binding of EF-G when studied by the high speed centrifugation of ribosomes (25). On the contrary, thiostrepton, even at the high concentration of 100 μM, has also been re-
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Fig. 4. Ribosome-bound EF-G affects the binding of RRF to 70 S ribosomes. A, ribosome-bound EF-G reduces the affinity of ribosomes for RRF. The binding of RRF to 10 pmol of 70 S ribosomes in 40 μl of buffer G was measured in the absence (filled circles) or presence of 15 pmol (filled squares) or 120 pmol (open squares) of EF-G. The presence of EF-G (dotted lines) on the ribosome after the addition of 15 pmol (open circles) or 120 pmol (open triangles) of EF-G was also measured in the presence or absence of RRF. B, binding of RRF to complexes of EF-G, ribosome, and inhibitor. Complexes of ribosomes and EF-G were formed in the presence of 1 mM fusidic acid or 40 μM thiostrepton, and the EF-G-ribosome complexes were isolated as described under "Experimental Procedures." The 40-μl reaction mixture for binding of RRF to the complexes of ribosome and EF-G contained preformed, isolated complexes of 20 pmol of ribosome in buffer G. The mixture was incubated for 10 min at ambient temperature, the ribosome complexes were isolated by Nano-Sep 300K ultrafiltration, and bound EF-G and RRF were measured by specific antibodies. The control reaction mixture (filled circles) did not contain EF-G and showed binding of RRF to vacant ribosomes. Where indicated, 1 mM fusidic acid (×) or 40 μM thiostrepton (+) was added to this mixture, showing that these inhibitors do not influence the binding of RRF to the absence of EF-G. Filled inverted triangles, RRF binding to ribosomes complexed with fusidic acid and EF-G; filled diamonds, RRF binding to ribosomes complexed with EF-G and thiostrepton. The amounts of the ribosome-bound EF-G (dotted lines) in the presence of thiostrepton (open diamonds) or fusidic acid (open inverted triangles) are also indicated. C, Scatchard plots of binding of RRF. The apparent Kd values indicated were determined by nonlinear regression. Circles, RRF binding in the presence of EF-G (Kd ~ 0.2 μM); squares, RRF binding in the presence of 1 mM EF-G and 1 mM GTP (Kd ~ 3.9 μM); triangles, RRF binding in the presence of 3 μM EF-G and 1 mM GTP (Kd ~ 3.0 μM); diamonds, RRF binding to the isolated ribosome complexes of EF-G and thiostrepton (Kd ~ 4.4 μM); asterisks, RRF binding to model post-terminiation complexes (Kd ~ 0.033 μM). Open symbols represent binding that is likely due to ribosomes inactive for EF-G binding.

Despite these controversies, in confirmation of our previous findings, it is clear from Fig. 4B (open circle) that, under our experimental conditions, EF-G can bind ribosomes in the presence of 40 μM thiostrepton. However, ~30% of ribosomes did not bind EF-G. This is not due to the presence of thiostrepton, because a similar percentage of ribosomes did not bind EF-G even in the absence of thiostrepton (Fig. 4A).

The data presented in Fig. 3B (filled circle) indicate that thiostrepton inhibits the release of RRF by EF-G. This suggests that there is still a ribosomal binding site for RRF even in the presence of ribosome-bound EF-G and thiostrepton. In support of this concept, in Fig. 4B (filled diamonds), RRF bound to ribosomes complexed with EF-G in the presence of thiostrepton. However, as shown in Fig. 4C, the affinity of these ribosomal complexes for RRF was clearly reduced. The apparent Kd value as determined by nonlinear regression was ~4.4 μM, compared with 0.2 μM in the absence of EF-G and thiostrepton. The total amount of ribosome-bound RRF in the presence of thiostrepton and EF-G still approaches the maximum level (~1 mol/1 mol of ribosome) observed in the absence of EF-G. It is noted in Fig. 4B again that the EF-G bound to ribosomes remains constant (open diamonds) regardless of the presence of bound RRF. Clearly, RRF and EF-G can co-exist on the ribosome in the presence of thiostrepton.

It should be pointed out that, as shown in Fig. 4B, neither 1 mM fusidic acid (×) nor 40 μM thiostrepton (+) significantly affected the binding of RRF in the absence of EF-G, indicating that these effects are not direct effects on the RRF binding sites of the ribosome.

Release of RRF from Model Post-termination Complexes—The physiological substrate of RRF for the disassembly reaction is a ribosome complexed with mRNA (with a stop codon at the A-site) and decayed tRNA(s) bound at the P/E sites (18, 50). To establish that the release of RRF from vacant ribosomes by EF-G detailed above is indeed representative of part of the natural reaction catalyzed by RRF, the possible release of RRF from model post-termination complexes (7, 31) was examined.

In the experiment described in Table II, complexes of RRF and model post-termination ribosomes were prepared. When EF-G and GTP were added to these complexes, complete dis-
**Table II**

| Added         | RRF bound to ribosomes | Disassembly |
|---------------|------------------------|-------------|
| —             | 100                    | 0           |
| EF-G/GTP      | 32 ± 1                 | 98          |
| EF-G/GDP      | 37 ± 1                 | 0           |
| EF-G/GMPPCP   | 30 ± 2                 | 0           |

*Binding of RRF was defined in relation to the amount of RRF bound in the absence of EF-G (*—*): ~8 pmol of RRF per 0.6 A260 units of polysome (corresponding to ~13.8 pmol of ribosomes), which was set as 100%.

*Polysome disassembly reactions as described in Ref. 16.

assembly of the model post-termination complexes took place as expected from the routine assay conditions (12). In this process, about ~70% but not 100% of the bound RRF was released, as shown in Table II. Since the concentration of RRF in this experiment (0.18 μM) is close to the Kd value of RRF to 70 S ribosomes, this is understandable. Since the amount of bound RRF is much higher before the disassembly reaction, the data suggest that RRF has a higher affinity to post-termination complexes than to washed, vacant ribosomes. This was confirmed, as shown in Fig. 4C (compare the asterisks with circles). The Kd value for RRF and polysomes was measured as 0.033 μM as compared with 0.2 μM for RRF and vacant 70 S ribosomes.

These considerations suggest that during the active disassembly of model post-termination complexes, most if not all of the ribosome-bound RRF is released. In a similar manner to the assembly of model post-termination complexes, mutants of EF-G that have impaired release RRF, due possibly to configurational changes of the EF-G is used to actually move RRF on the ribosome or simply suggested that RRF may be moved on the ribosome prior to its release (11, 16). However, whether the translocation activity of EF-G is used to actually move RRF on the ribosome or simply release RRF, due possibly to configurational changes of the ribosome induced by EF-G (51), has not been established. To demonstrate that the translocation activity of EF-G is somehow related to the release of RRF and the disassembly of post-termination complexes, mutants of EF-G that have impaired translocation activity were used in the experiments described in Table III. The EF-G mutants used here lack domain 1 (EF-Gα1), domain 4 (EF-Gα4), or domain 5 (EF-Gα5) or have a single point mutation in domain 4 (H583K) (22–24).

**EF-G Mutants Lacking Various Domains Are Unable to Disassemble Post-termination Complexes: Relationship to the Release of RRF**—Clearly, RRF is released from ribosomes during the disassembly of model post-termination complexes, and this release is dependent on the action of EF-G. We have previously suggested that RRF may be moved on the ribosome prior to its release (11, 16). However, whether the translocation activity of EF-G is used to actually move RRF on the ribosome or simply release RRF, due possibly to configurational changes of the ribosome induced by EF-G (51), has not been established. To demonstrate that the translocation activity of EF-G is somehow related to the release of RRF and the disassembly of post-termination complexes, mutants of EF-G that have impaired translocation activity were used in the experiments described in Table III. The EF-G mutants used here lack domain 1 (EF-Gα1), domain 4 (EF-Gα4), or domain 5 (EF-Gα5) or have a single point mutation in domain 4 (H583K) (22–24).

EF-Gα4 and EF-Gα5 reduce translocation of tRNA by 1000-fold as compared with wild-type EF-G (24). Consistent with this finding, we observed, as shown in Table III, impairment of the release of RRF (10 and 14% activity for domain 4 and 5 mutations, respectively) as well as the release of tRNA from post-termination complexes (27 and 41% activity for domain 4 and 5 mutations, respectively). The release of RRF was impaired more than that of tRNA. These mutations are also believed to inhibit the release of EF-G from ribosomes after GTP hydrolysis, because turnover of GTPase is very low (24). These mutant EF-Gs completely fail to disassemble the post-
termination complexes. This indicates that the partial reactions of disassembly, release of tRNA and RRF from ribosomes, are much less sensitive to the mutations of EF-G than the total disassembly reaction measured by the release of mRNA from ribosomes.

EF-GΔ1 allows for “partial translocation” of tRNA on the 50 S subunit but may prevent the proper positioning of domain 4 for translocation on the 30 S subunit, thus losing all activity required for the release of tRNA (22). Indeed, this is the only mutant that gave a more severe effect upon tRNA release (90% loss) than upon RRF release (80% loss) (Table III).

The H583K mutation, located at the tip of domain 4, also reduces translocation at least 100-fold in comparison with wild-type EF-G (24). We see a significant impairment of RRF (92% loss) and tRNA (70% loss) release from post-termination complexes with this mutant.

Because all of these mutations simply slow the rate of tRNA translocation, they are expected to translocate 100% of complexes if given sufficient time. Therefore, the data presented in Table III represents a time point in which the mutants show lower activity in releasing RRF and tRNA. Since our assay method cannot measure the real time kinetics, the effects of these mutations are much more moderate on our partial reactions than the values reported from fast kinetic measurements of translocation, except for the mRNA release, which showed almost complete inhibition due to the mutations.

These data are consistent with the notion that the release of RRF by EF-G is dependent on the translocation activity of EF-G. All mutations impaired the overall disassembly more severely than any of the partial reactions. Further analysis of the data is presented in the discussion below.

**DISCUSSION**

After RRF and EF-G disassemble a post-termination complex, they must leave the ribosome. The EF-G-dependent release of RRF described in this paper represents a partial but essential step of the disassembly reaction. In support of this concept, inhibition of RRF release by thiostrepton (an EF-G inhibitor) (Figs. 3B and 5) or mutation of EF-G (which impairs EF-G activity), results in failure of the disassembly (16) (Table III).

We propose that, for release of ribosome-bound RRF, RRF is moved from its initial high affinity A/P site to a site that has much lower affinity for RRF. During this motion, RRF may move like a piston, parallel to the interface of ribosomal subunits at the cavity surrounded by helix 69 and 71 of 23 S rRNA (18). This motion would presumably lead to the release of tRNA at the P/E site and prepare RRF to be released from the

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**Fig. 6. Model of the disassembly of post-termination complexes by RRF and EF-G: Effect of inhibitors.** The post-termination complex (PTC) is composed of ribosome (marked with the three tRNA binding sites A, P, and E), mRNA (black), and tRNA (gold) at the hybrid P/E site. In step A, RRF (red) binds to its high affinity A/P site (17, 18), forming “complex a.” In step B, EF-G (multicolored) binds to the ribosome, forming “complex b.” The exact order of binding is unknown, but RRF is shown to bind first, because the presence of EF-G interferes with the tight binding of RRF (Fig. 4A). In step C, EF-G moves RRF, causing the release of tRNA and forming “complex c.” The position of RRF after this step is purely speculative. In the following step D, EF-G, mRNA, and RRF are released, but the order of release of each component is not known. Three inhibitory pathways (designated as I) are shown. Thiostrepton, as shown in I₁, prevents the release of RRF and mRNA but not tRNA, forming “complex i₁.” EF-G is shown on the ribosome as lightened color to indicate that it still binds (Fig. 4B), but the affinity of EF-G for ribosomes is reduced in the presence of thiostrepton (25). As shown in I₂, fusidic acid and GMP-PCP prevent the release of EF-G and mRNA but allow for the release of RRF by EF-G (Fig. 3A). EF-G is shown more horizontally in “complex i₂” to show some overlap with the weaker binding site of RRF (after moving from the high affinity A/P site), because RRF can no longer bind to ribosomes in the presence of fusidic acid and EF-G (Fig. 4B). Viomycin (I₃) prevents the release of mRNA but not the release of RRF (Fig. 3A). EF-G is shown to be on the ribosome of “complex i₃,” because our data using 70 S ribosomes suggest that EF-G remains on the ribosome even in the presence of viomycin (Fig. 4B). In the complexes b, c, and i₁, both RRF and EF-G are present on the ribosome as shown in Fig. 4, A and B.
EF-G Releases RRF

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Table III

EF-G mutants that are translocation-impaired have reduced activity in releasing RRF and tRNA and fail to disassemble post-termination complexes

| EF-G added | mRFP (polysome conversion) | tRNA release | RRF release |
|------------|-----------------------------|--------------|-------------|
| wtEF-G     |                            |              |             |
| EF-GΔ1     | 18                         | 11           | 22          |
| EF-GΔ4     | 14                         | 27           | 10          |
| EF-GΔ5     | 2.6                        | 41           | 14          |
| EF-GH583K  | -1.8                       | 32           | 8           |

* Polysome disassembly is measured by the conversion of polysome to monosomes catalyzed by RRF and EF-G (16); wild-type EF-G (wtEF-G) is considered to have 100% activity. The puromycin-treated polysomes used for the disassembly reaction assay contained 66% polysome, 34% monosome. After the disassembly reaction, it contained 6% polysome, 94% monosome, indicating 91% conversion of model post-termination complexes.

b tRNA release is expressed as the percentage of tRNA released from polysomes as compared with the amount released by wild-type EF-G (wtEF-G), defined as having 100% activity. wtEF-G released tRNA that could be charged with an average of 1873 dpm of 14C amino acids. For RRF release, the substrate complex had ~0.69 pmol of RRF per pmol of ribosome in the form of post-termination complex. Wild-type EF-G released an average of 61% of this bound RRF. This activity was regarded as 100%, and the mutant EF-G activities are expressed as percentages of this activity.

c wtEF-G, wild-type EF-G; all other EF-Gs are either lacking (Δ) the domain indicated or have the point mutation indicated.

Ribosome. We summarize below the evidence supporting this proposal.

First, the release of RRF is dependent on EF-G, an enzyme dedicated to translocate (move) tRNA and mRNA on the ribosome (Fig. 1A, Tables I and II). Second, GTP hydrolysis is not required for the EF-G-dependent release of RRF (Tables I and II). Thus, GMP-PCP, which fixes EF-G on the ribosome (32), allows the release of RRF. This is reminiscent of the observation that one round of slow translocation of tRNA is not dependent on GTP hydrolysis. Related to this observation is that another inhibitor of EF-G, fusidic acid, which allows for a single round of tRNA translocation (52), does not inhibit the release of RRF (Fig. 3B).

Third, mutants of EF-G that are translocation-impaired are also impaired for both the release of RRF and the complete disassembly of post-termination complexes. Likewise, these mutants are also partially deficient for the RRF-dependent release of tRNA from post-termination complexes (regarded as an indication of translocation (26)) (Table III). In fact, in all cases, impairment of the release of RRF leads to failure of disassembly.

Fourth, viomycin, a translocation inhibitor, fails to inhibit the release of RRF. This observation may give the false impression that it contradicts our hypothesis that translocation activity is required for the release of RRF because viomycin is a known inhibitor of EF-G (23, 52). However, Cabanas and Modolell (53) observed that viomycin does not inhibit translocation of noncognate tRNA. Structurally, RRF is similar to tRNA except that it does not have an anti-codon region (15), and ribosomal binding of RRF is not influenced by mRNA (17, 18). Therefore, RRF movement on the ribosome may represent a similar situation to the movement of noncognate tRNA on the ribosome.

Fifth, the affinity of RRF for complexes of EF-G and ribosome in the presence of GTP was at least 20-fold less than the affinity for vacant ribosomes (Fig. 4, A and C). Direct hydroxyl radical probing studies have shown that domain I of RRF overlaps with the A-site and the position of post-translocation EF-G (18, 35, 46, 47). Recent cryoelectron microscopy studies of ribosome-bound RRF obtained in collaboration with the Frank laboratory agree with this notion. Thus, RRF must be moved from its initial, high affinity binding site (A/P site) to a weaker binding site that does not overlap with the binding site of post-translocational EF-G.

It has been widely assumed that EF-G stabilized on the ribosome through fusidic acid is at a post-translocation position (35, 36, 43, 47). We therefore expected that complexes of EF-G, fusidic acid, and ribosome would behave similarly to complexes of EF-G and the ribosome formed with GTP. Unexpectedly, no RRF was bound to the fusidic acid complex. The amount of ribosome-bound EF-G was not influenced by fusidic acid or RRF. This suggests the possibility that the fusidic acid complex may not be identical to the natural post-translocation complex. In contrast, the thiostrepton-EF-G complexes still allowed for the binding of RRF, although with very weak affinity (Fig. 4, B and C). However, thiostrepton still inhibited the release of ribosome-bound RRF (Figs. 3B and 5). Therefore, moving RRF to a weaker binding site is not sufficient to release it from the ribosome. EF-G must act to release RRF after RRF changes the binding site to a second site with weaker affinity.

On the basis of the findings so far available, we propose the following scheme for the action of RRF and the site of action for various inhibitors that interfere with post-termination complex disassembly. First, RRF binds to a post-termination complex at the high affinity A/P site (Fig. 6, step A). Then EF-G binds to this complex (Fig. 6, step B). The reason why RRF has to bind first is that the binding of EF-G prevents the initial binding of RRF to the higher affinity A/P site (Fig. 4A). We postulate that EF-G binding will move RRF from its initial position as shown in this scheme, because the reported pretranslocation position of EF-G (46, 54) seems to clash with the initial binding position of RRF. This movement of RRF caused by the binding of EF-G alone probably does not release tRNA. In support of this notion, EF-G mutants, although they bind ribosomes, do not efficiently release RRF or tRNA (Table III).

The next step (step C) is the release of P/E site bound tRNA. We postulate that this step involves movement of RRF to a lower affinity site (Fig. 4A) by the translocation activity of EF-G catalyzed by GTP hydrolysis (16). Thiostrepton appears to keep RRF at this position (step 1; Figs. 3, 4B, and 5). Fusidic acid, viomycin, or GMP-PCP allows this step, although some of them may slow down the rate of the movement. The last step (step D) involves the release of RRF, EF-G, and mRNA. Viomycin (I1), fusidic acid, or GMP-PCP allows this step, although some of them may slow down the rate of the movement. The exact timing of the release of RRF relative to the timing of the release of EF-G and mRNA as shown in step D remains obscure until careful fast kinetic studies with fluorescent labeling is conducted on this step. Such studies are currently in progress.

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