

# The role of GTP in transient splitting of 70S ribosomes by RRF (ribosome recycling factor) and EF-G (elongation factor G)

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## ABSTRACT

**Ribosome recycling factor (RRF), elongation factor G (EF-G) and GTP split 70S ribosomes into subunits. Here, we demonstrated that the splitting was transient and the exhaustion of GTP resulted in re-association of the split subunits into 70S ribosomes unless IF3 (initiation factor 3) was present. However, the splitting was observed with sucrose density gradient centrifugation (SDGC) without IF3 if RRF, EF-G and GTP were present in the SDGC buffer. The splitting of 70S ribosomes causes the decrease of light scattering by ribosomes. Kinetic constants obtained from the light scattering studies are sufficient to account for the splitting of 70S ribosomes by RRF and EF-G/GTP during the lag phase for activation of ribosomes for the log phase. As the amount of 70S ribosomes increased, more RRF, EF-G and GTP were necessary to split 70S ribosomes. In the presence of a physiological amount of polyamines, GTP and factors, even 0.6  $\mu$ M 70S ribosomes (12 times higher than the 70S ribosomes for routine assay) were split. Spermidine (2 mM) completely inhibited anti-association activity of IF3, and the RRF/EF-G/GTP-dependent splitting of 70S ribosomes.**

## INTRODUCTION

Translation (protein synthesis) from aminoacyl tRNA consists of four consecutive steps: initiation, elongation, termination and recycling of the machinery of the protein synthesis for the next round of translation. Each step is controlled and catalyzed by translation factors (1). During the termination step, the stop codon in the ribosomal acceptor site (A-site) is recognized by class-1 release factors (RFs); RF1 or RF2 in prokaryotes (2,3) and eRF1 in

eukaryotes (4). These factors bind to the A-site of the ribosome in response to the termination codon and induce the ribosome to hydrolyze peptidyl-tRNA at the peptidyl-tRNA site (P-site) and then the nascent polypeptide is released from the tRNA on the ribosome. Class-2 release factors RF3 (5,6) and eRF3 (7) possess GTPase activity and stimulate the release of class-1 RFs from the ribosome (8,9), leaving the post-termination complex. The post-termination complex consists of three components, mRNA with the termination codon at the A-site, tRNA at the P/E-site (10–12) and the 70S ribosome. The next step is the disassembly of the post-termination ribosomal complex. This step in prokaryotes is an active process catalyzed by a protein called the ribosome-recycling factor (RRF) together with elongation factor G (EF-G) and GTP (13,14).

The disassembly step includes one of the crucial steps of protein synthesis, the splitting of 70S ribosomes into subunits (15). Although there are some exceptions (16–19), the splitting of ribosomes is well accepted as an essential necessary step before the canonical translation initiation process (20). In bacteria, the splitting of the vacant 70S ribosome has been thought to be catalyzed by initiation factor 3 (IF3) (21) and initiation factor 1 (IF1) (22) without involving RRF and EF-G/GTP for a long time. This concept is originated because IF3 alone splits 70S ribosomes to some extent and IF1 assists this process (23). However, the splitting of vacant 70S ribosomes by IF3 and IF1 is slower (24) and of lesser extent (23) than that by RRF and EF-G. Furthermore, splitting of vacant ribosomes by IF3 alone requires a concentration of IF3 much higher than that *in vivo* (23). The fact that RRF is involved in splitting of post-termination complex was first suggested by an indirect method of measuring peptide synthesis in a limited amount of subunits (25). Only recently, three different laboratories showed RRF-dependent splitting of 70S ribosomes with various direct methods (26).

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Briefly, Hirokawa *et al.* showed that RRF and EF-G/GTP split vacant 70S ribosomes using the decrease of ribosomal light scattering upon dissociation into subunits. In addition, they showed splitting of post-termination complex directly in sucrose density gradient centrifugation (SDGC) in the presence of IF3. On the other hand, Zavialov *et al.* (27) presented SDGC evidence that the exchange of the 30S subunits of the post-termination complexes with free 30S subunits were dependent on RRF, EF-G and GTP. Furthermore, Peske *et al.* (28) used fluorescence resonance energy transfer (FRET) of fluorescence-labeled subunits to show the splitting of 70S ribosomes by RRF and EF-G/GTP. The FRET method, however, may not be suitable for studying the actual physical splitting of subunits [for more discussion of this matter, see (26)]. IF3 prevents reassociation of the split subunits. Recent evidence suggests that IF3 may participate more directly in the disassembly by RRF and EF-G/GTP (29,30). This view was however recently refuted (24).

The splitting of 70S ribosomes is not limited to the recycling process. When cells are in the stationary phase, or shift-down conditions, the protein synthesis is lowered or stopped and most of the ribosomes take the form of 100S (31) or 70S ribosomes. In this paper, we concentrate mostly on the splitting of the vacant 70S ribosomes.

Despite the aforementioned evidence for the involvement of the translocation factor in the splitting of ribosomes, Umekage and Ueda (32) recently suggested that RRF and EF-G/GTP may not be involved in the splitting of 70S ribosomes *in vivo*. Their evidence was based on the finding that 0.6–1.2  $\mu\text{M}$  70S ribosomes [closer to the natural concentration (20  $\mu\text{M}$ ) than the conventional ribosome concentration (0.05  $\mu\text{M}$ ) used in our *in vitro* system] could not be split by RRF and EF-G/GTP in their buffer containing 2 mM spermidine. They showed that 70S ribosomes in lower concentrations were split into subunits by RRF and EF-G/GTP in the conventional buffer without spermidine. Although there has been no systematic studies of the effect of polyamines on the ribosome recycling, the polymix buffer (33), containing 1 mM spermidine and 8 mM putrescine (Ptc) could still support the RRF- and EF-G/GTP-dependent splitting of 70S ribosomes (23).

We showed, in this paper, the critical role of GTP for keeping the ribosomal subunits separate and the exhaustion of GTP converted subunits back to 70S ribosomes when IF3 was not present. As soon as IF3 was added, the consumption of GTP energy was not necessary for keeping the subunits separate. The increased 70S ribosome concentration resulted in less subunit dissociation. However, this effect was overcome by increasing GTP and RRF/EF-G concentrations. The *in vitro* velocity of the splitting of the vacant ribosomes was sufficient to account for the conversion of 70S ribosomes into subunits during the lag phase. Furthermore, we showed that 0.6  $\mu\text{M}$  ribosome could be split by physiological concentrations of EF-G (34,35), RRF (36) and GTP (51) in the presence of polyamines present *in vivo* (37,38), supporting the notion that RRF/EF-G-dependent splitting takes place *in vivo*.

## MATERIALS AND METHODS

### Buffers

Buffer R: 10 mM Tris-HCl, pH 7.4, 8.2 mM MgSO<sub>4</sub>, 84 mM NH<sub>4</sub>Cl, 0.2 mM DTT (dithiothreitol). Buffer P: 10 mM Tris-HCl, pH 7.6, 8.2 mM MgSO<sub>4</sub>, 80 mM NH<sub>4</sub>Cl, 0.3 mM spermidine (Spd), 15 mM Ptc, 1 mM DTT. Umekage buffer (32): 5 mM K-phosphate, pH 7.6, 6 mM Mg(OAc)<sub>2</sub>, 150 mM  $\kappa$ -glutamate, 30 mM K(OAc)<sub>2</sub>, 2 mM Spd, 1 mM DTT. Low magnesium buffer: 10 mM Tris-HCl, pH 7.6, 1 mM Mg(OAc)<sub>2</sub>, 50 mM NH<sub>4</sub>Cl, 1 mM DTT. Association buffer: 10 mM Tris-HCl, pH 7.6, 6 mM Mg(OAc)<sub>2</sub>, 49.5 mM NH<sub>4</sub>Cl, 1 mM DTT. SDGC-Umekage buffer: 20 mM HEPES-KOH, pH 7.6, 6 mM Mg(OAc)<sub>2</sub>, 30 mM NH<sub>4</sub>Cl, 7 mM  $\beta$ -mercaptoethanol and 2 mM Spd. Buffer U: 10 mM Tris-HCl, pH 7.4, 5 mM MgSO<sub>4</sub>, 84 mM NH<sub>4</sub>Cl, 0.2 mM DTT.

### Ribosomes and factors

From *Escherichia coli* MRE600 (purchased from University of Alabama Fermentation Facility, Birmingham, AL, USA) 70S ribosomes were prepared as described (39). The prepared sample was named as '70S ribosome preparation'. As shown in Figure 2B, the 70S ribosome preparation contained some subunits (about 25% of total ribosome) detectable by SDGC. RRF and EF-G were purified as described (14,39) from *E. coli* DH5 $\alpha$  harboring plasmid pRR2 (40) and *E. coli* JM83 with plasmid pCEG (41) (kindly supplied by Dr P. March), respectively. His-IF3 was purified from *E. coli* XL1-blue, having plasmid expressing His-IF3 (42) (kindly supplied by Dr T. Ueda) according to the method described for His-EF-G (43). His-EF-G was prepared from strain XL-1/pQE70 (*fusA*) (43). Native IF3 was a kind gift of Dr Claudio Gualerzi.

### Ribosome dissociation assay by sedimentation through sucrose gradient

In Figures 1, 2 and 4B, the 70S ribosome preparation (14.7 pmol) was incubated in 275  $\mu\text{l}$  with factors as specified in the figures at 30°C in buffer R. The volumes of the reaction mixture for Figure 4C–F were as follows: Figure 4C, 147  $\mu\text{l}$ ; Figure 4D, 73  $\mu\text{l}$ ; Figure 4E, 29  $\mu\text{l}$ ; and Figure 4F, 14.5  $\mu\text{l}$ . These reaction mixtures were placed on sucrose gradients (15–30% in buffer R) and were centrifuged in Beckman SW50.1 rotor at 40 000 r.p.m. for 2.5 h at 4°C and monitored at 254 nm with ISCO UA-6 spectrophotometer. Under our experimental conditions, it was the concentration of the ribosomes and factors that determined how much complex of ribosome/factors was made. Therefore, the volume of the reaction mixture did not matter.

In Figure 3A, the reaction mixture without IF3 was centrifuged as in Figure 1, except that fractions were collected from the bottom of the tube and the OD<sub>260</sub> was measured on each fraction. In Figure 3B, sucrose gradient was in buffer R containing RRF (1  $\mu\text{M}$ ), EF-G (1  $\mu\text{M}$ ) and GTP (0.36 mM). The reaction mixture without IF3 was placed on this gradient and subjected to ultracentrifugation as in Figure 3A.

For Figure 5, the 70S ribosome preparation (40 pmol) was incubated with factors in 40  $\mu$ l of buffer R. A portion of the reaction mixture (8  $\mu$ l) was subjected to SDGC in buffer R. For Figure 7A right three profiles, the 70S ribosome preparation (24 pmol) was incubated in 40  $\mu$ l of the Umekage buffer and was analyzed with SDGC containing the SDGC-Umekage buffer. For Figure 7A left three profiles, the splitting reaction was performed in buffer P and the mixture was sedimented through SDGC in buffer P. In Figure 7B and C, the reaction was carried out in buffer P and 8  $\mu$ l of the reaction mixture was subjected to SDGC in buffer R. An additional  $MgSO_4$  equivalent to 1.5 times concentration of GTP was added to compensate for the  $Mg^{2+}$  chelating effect of GTP when the GTP concentration was 0.7 mM or higher. For Figure 8, the buffer conditions for SDGC were the same as those ionic conditions of the reaction mixtures except for the omissions of polyamines and IF3.

#### **Inhibitory effect of 2 mM Spd on the anti-association activity of IF3**

The 70S ribosomes preparation (7.5 pmol) was incubated in 110.6  $\mu$ l of the low magnesium buffer at 30°C for 5 min (profile 1 of Figure 8). Subunits thus formed were further incubated in 137.4  $\mu$ l of the association buffer for 10 min at 30°C (profile 2). For profile 3, the subunits were incubated with 2 mM Spd at 30°C for 5 min followed by incubation in the association buffer. For profile 4, the subunits were incubated as for profile 3 without Spd but with 4.5  $\mu$ M IF3, and then incubated in the association buffer. For profile 5, the subunits were incubated with 4.5  $\mu$ M IF3 followed by incubation with 2 mM Spd, and then incubated in the association buffer. The reaction conditions for profiles 6 and 7 were as described in the legend to Figure 8.

#### **Dissociation of ribosomes measured with the ribosomal light scattering decrease**

The ribosomal light scattering was measured at room temperature with a spectrofluorometer (Photon Technology International, incoming slits: 1 mm  $\times$  0.1 mm, outgoing slits: 0.55 mm  $\times$  1 mm, wave-length: 436 nm, angle: 90°). Mixture A (180  $\mu$ l) containing factors in buffer U as specified in the figure legend was mixed manually with mixture B (20  $\mu$ l) containing 1.5  $\mu$ M of the 70S ribosome preparation in buffer U. The mixture (200  $\mu$ l) was placed in a cuvette quickly and the intensity of the scattering light (436 nm) was continuously recorded beginning at 20 s after the mixing. The change of the amount of light scattered by ribosomes was measured. The values were converted to the remaining 70S ribosome concentrations using the change of the ribosomal light scattering as 100%, upon exposing the ribosomes from 8 mM to 1 mM  $Mg^{2+}$ , which dissociates the 70S ribosomes completely. During the recording of scattered light, the reaction mixture was not stirred. The apparent rate constant ( $k_{app}$ ) of ribosomal splitting was obtained using the IGOR Pro (version 6.03) software (OR, USA) by fitting data to the single exponential equation. The remaining 70S ribosomes ( $\mu$ M) at time  $t = Y = A1\exp(-A2 * (t)) + A3$ . In this

equation,  $t$  represents time in seconds plus 20 s after the mixing of A and B.

## **RESULTS**

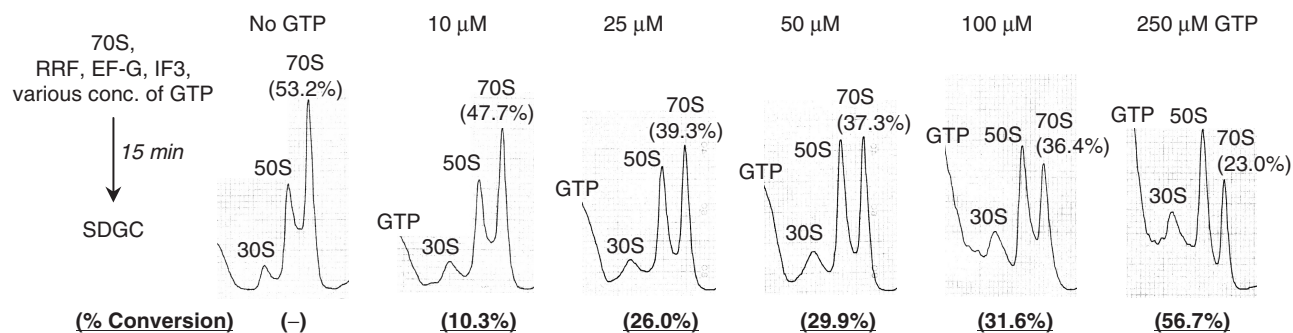
### **Exhaustion of GTP caused re-association of subunits: evidence for transient ribosomal splitting by RRF and EF-G in the absence of IF3**

In our preceding communication (23), we presented evidence that GTP, but not the nonhydrolyzable analog (GMPPCP), functioned for the disassembly of post-termination complexes. This is in contrast to the conventional translocation reaction where the nonhydrolyzable analog of GTP functioned (44), though slowly (45). It was therefore of interest to examine what happened to the splitting reaction when GTP was exhausted. For this purpose, in Figure 1, we estimated the minimal concentration of GTP that was sufficient for the splitting of 70S ribosomes under our experimental conditions.

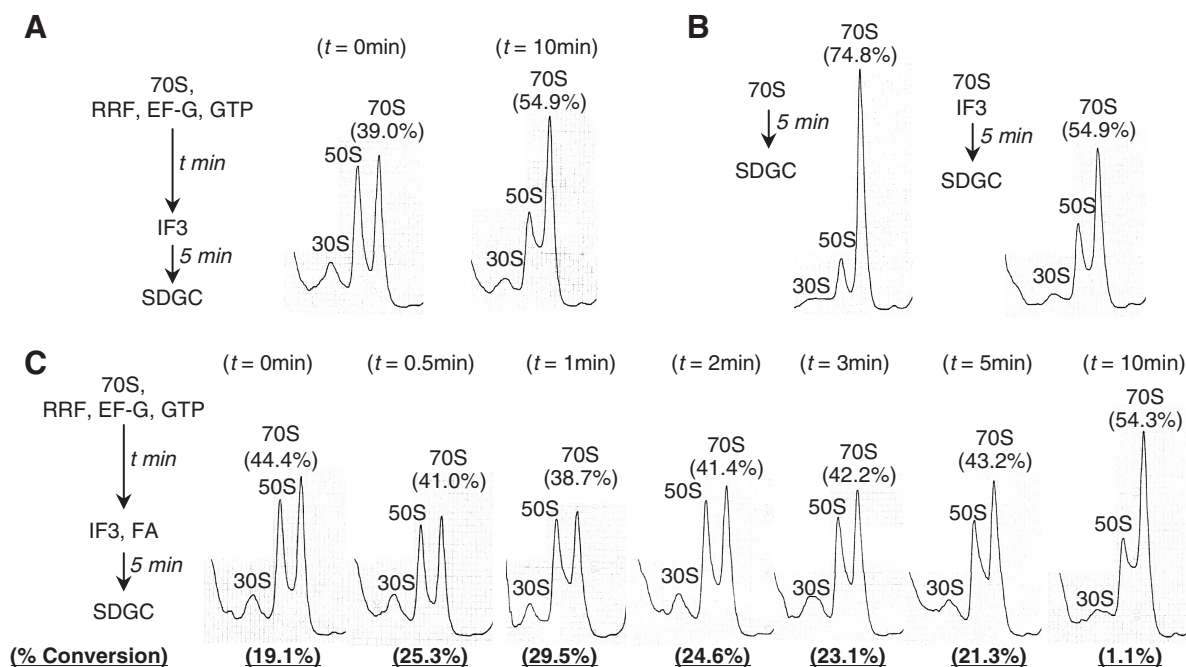
Most of the experimental systems we used in this paper, as shown in Figure 1, were to observe the splitting of vacant ribosomes by EF-G, RRF and GTP in the presence of IF3 followed by sucrose density gradient centrifugation (SDGC) to measure the amount of 70S, 50S and 30S ribosomes. The SDGC was an analysis method and had nothing to do with the splitting reaction. When IF3 was added, the split subunits were prevented from re-associating back to 70S ribosomes and the reaction was completely finished before the reaction mixture was placed on the sucrose gradient for analysis.

In the experiment described in Figure 1, the 70S ribosome preparation was incubated with RRF, EF-G and IF3 with various concentrations of GTP (0–250  $\mu$ M) for 15 min and the sedimentation patterns of ribosomes were analyzed by SDGC. The result showed that more subunits were detected with the increasing concentrations of GTP in a dose-dependent manner. In the presence of 50  $\mu$ M GTP, about 30% of 70S ribosomes were split into their subunits (the percentage of conversion values are indicated below each profile). We concluded that the extent of splitting was dependent on the amount of GTP.

We then examined the effect of the depletion of GTP during the ribosomal splitting by RRF and EF-G. As shown in the left profile of Figure 2A, after the 70S ribosome preparation was incubated for 5 min with RRF, EF-G, IF3 and 50  $\mu$ M GTP, 39% of the total ribosomes were 70S ribosomes. In the ribosome preparation used, 74.8% were 70S ribosomes (see Figure 2B, left profile). We therefore concluded that significant splitting was observed with 50  $\mu$ M GTP. On the other hand, when 70S ribosomes were incubated for 10 min without IF3, which was added at the end of the 10-min incubation, more 70S ribosomes (54.9%) were observed. Since the same amount of 70S ribosomes were observed upon incubation with IF3 only (Figure 2B, right profile) we concluded that all subunits resulted from spitting of 70S ribosomes by RRF and EF-G/GTP re-associated back to 70S ribosomes during the 10-min incubation without IF3, due to the exhaustion of 50  $\mu$ M GTP.



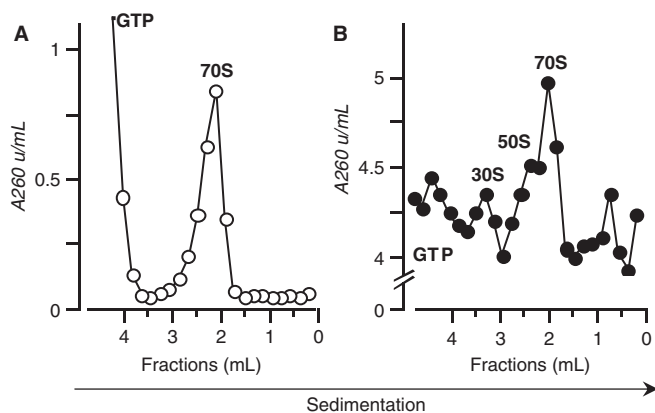
**Figure 1.** Effect of various concentrations of GTP on the extent of ribosomal splitting by RRF, EF-G and IF3. The 70S ribosome preparation (0.05 μM) was incubated with RRF (5 μM), EF-G (5 μM) and native IF3 (1 μM) with various concentrations of GTP at 30°C for 15 min, and the ribosomal sedimentation patterns were analyzed as described in Materials and methods section. Sedimentation was from left to right. The numbers above the 70S ribosome peaks indicate the percentages of 70S ribosomes of the total quantity of ribosomes. The numbers at the bottom of each sedimentation pattern indicate the percentage of conversions of 70S ribosomes by RRF, EF-G and IF3 calculated using the following equation: % conversion =  $100 \times (53.2 - \text{percentage of 70S ribosomes indicated above the peak})/53.2$ ; 53.2 represents the percentage of 70S ribosomes without GTP (left profile).



**Figure 2.** Upon GTP exhaustion, ribosomal subunits re-associated: evidence for transient nature of the splitting of 70S ribosomes by RRF and EF-G. The numbers above the profiles are percentages of 70S ribosomes as in Figure 1. (A) Fifty micromolar GTP were exhausted within 10 minutes. Left panel: the 70S ribosome preparation (0.05 μM) was incubated for 5 min with RRF (5 μM), EF-G (5 μM), GTP (50 μM) and native IF3 (1 μM) simultaneously. Right panel: the same ribosomes (0.05 μM) were incubated for 10 min with RRF (5 μM), EF-G (5 μM) and GTP (50 μM) without IF3, and then native IF3 (1 μM) was added and incubated for an additional 5 min. Note that there was no effect of RRF and EF-G due to the exhaustion of GTP during the 10-min incubation without IF3 (54.9% of 70S ribosomes were present, which is the same amount as with IF3 alone. See Figure 2B, right profile). (B) Control of (A). Left panel: the 70S ribosome preparation (0.05 μM) alone was incubated for 5 min. Right panel: the 70S ribosome preparation was incubated for 5 min with native IF3 (1 μM). (C) Time course of 70S splitting by RRF and EF-G with 50 μM GTP. The 70S ribosome preparation (0.05 μM) was incubated for various periods (0 to 10 min) with RRF (5 μM), EF-G (5 μM) and GTP (50 μM), then with native IF3 (1 μM) and fusidic acid (FA, 200 μM) for an additional 5 min. Ribosomal sedimentation patterns were analyzed as in Figure 1. Next to the sedimentation patterns, experimental designs are described in a schematic style. The *t*-values in parenthesis above each profile indicate the time periods for the incubation with RRF, EF-G and GTP without IF3. % conversion =  $100 \times (54.9 - \text{percentage of 70S ribosomes indicated above the peak})/54.9$ ; 54.9 represents percentage of 70S ribosomes with IF3 only (Figure 2B, right profile).

To find out when the added GTP was exhausted in the above experiment, the experiment shown in Figure 2C was performed. In this experiment, after the 70S ribosome preparation was incubated with RRF, EF-G and GTP, for various periods (0–10 min), IF3 and fusidic acid were added. Fusidic acid inhibits the splitting of

post-termination complexes and 70S ribosomes into subunits (23), while IF3 inhibits the association of the separated subunits (46). Therefore, the addition of these two components ‘froze’ the splitting reaction at various time intervals from the onset of the energy-dependent ribosomal splitting. The splitting reaction proceeded up to 1 min



**Figure 3.** Supplying GTP and factors during SDGC revealed the transient splitting of 70S ribosomes by SDGC. (A) The 70S ribosome preparation (0.05  $\mu$ M) was incubated with RRF (1  $\mu$ M), EF-G (1  $\mu$ M) and GTP (0.36 mM) 'without' IF3 at 30°C for 10 min and the sedimentation behavior of ribosomes were analyzed with regular SDGC as described in Materials and methods section. Sedimentation was from left to right. (B) The splitting reaction and the SDGC analysis conditions were the same as (A), except that the SDGC buffer contained RRF (1  $\mu$ M), EF-G (1  $\mu$ M) and GTP (0.36 mM) to keep the separated units apart during the centrifugation. The background absorbance was about 4.4 A<sub>260</sub> units mostly due to added GTP in the SDGC buffer.

because the amount of 70S ribosomes was the lowest (38.7%) at 1 min after the onset of the reaction. Longer incubation with RRF and EF-G resulted in the increase of 70S ribosomes to the final value of 54.3% at the 10-min incubation time (Figure 2C, the extreme right panel). This value was almost identical to that with IF3 alone (Figure 2B, right panel). This was the background value of these experiments because IF3 alone split the 70S ribosomes to some extent. Taken together, we concluded that the GTP depletion caused the re-association of the ribosomal subunits indicating that the energy-dependent ribosomal splitting by RRF and EF-G was transient.

#### Detection of transiently split subunits by SDGC

We postulated that the splitting of 70S ribosomes by RRF and EF-G could not be observed by SDGC unless IF3 was added to the reaction mixture. This was because, as described above, the reaction was transient and the split subunits would associate back to 70S ribosomes unless IF3 bound to the 30S subunits to stop the re-association (23). To prove this postulate further, the experiment described in Figure 3 was performed. This experiment was done 'without IF3' to demonstrate that one could observe the split subunits by SDGC 'even in the absence of IF3' if we created special analytical conditions where EF-G/GTP and RRF were constantly present during the centrifugation analysis.

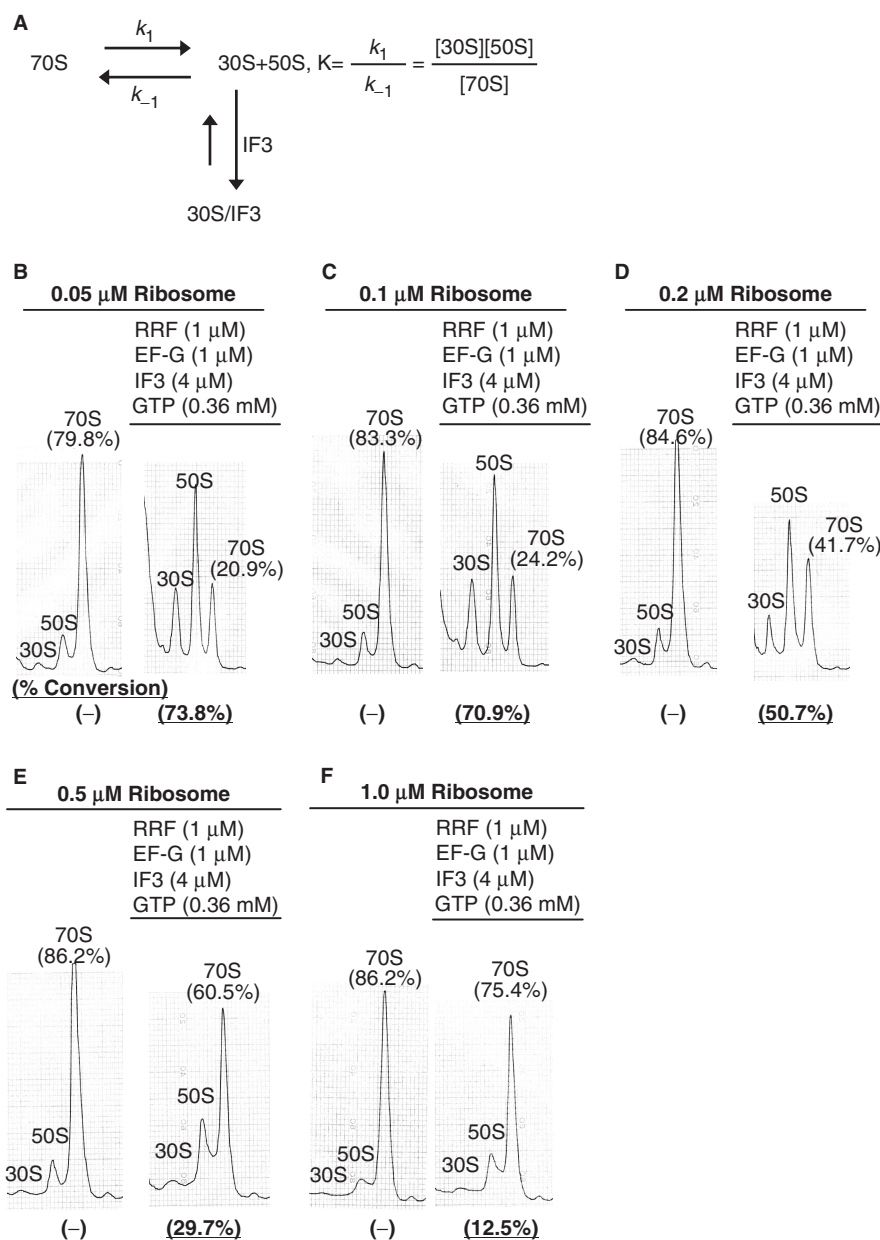
In the experiment described in Figure 3, the 70S-ribosome preparation was incubated with RRF, EF-G and GTP but 'without IF3'. In Figure 3A, the reaction mixture was placed on regular SDGC containing no RRF, EF-G and GTP, centrifuged and sedimentation behavior of ribosome was examined. Clearly, no splitting of the 70S ribosome was observed, despite the splitting must have occurred in the reaction mixture as shown in

Figures 1 and 2. In contrast, in Figure 3B, an identical reaction mixture was layered on the sucrose gradient containing RRF, EF-G and GTP so that the splitting reaction constantly took place during the time the ribosome sedimented through the sucrose gradient. It is clear from this figure that 'even without IF3', we were able to observe the splitting of 70S ribosome. The reason why the subunits were not well separated was probably because splitting and re-association were constantly occurring during the centrifugation.

#### The inhibitory effect of increased 70S ribosome concentration on its splitting was overcome by raising RRF, EF-G and GTP concentrations

Under physiological conditions, RRF and EF-G must split 20  $\mu$ M 70S ribosomes (47) during the lag phase. The 70S ribosomes and subunits are in equilibrium as shown in Figure 4A. The extent of 70S ribosomes dissociation into their subunits (50S and 30S) is determined by the equilibrium constant,  $K$ . Although the equilibrium constant is influenced by the ionic conditions, in a given milieu, an increase in the concentration of 70S ribosomes would result in an increase in the concentration of the subunits. However, as a single 70S ribosome gives rise to the two subunits (50S and 30S), the increase in the subunits is governed by the product of their concentrations. Hence, even though an increase in the concentration of 70S ribosomes would, at equilibrium, lead to an increase in the absolute concentration of the dissociated subunits, in terms of their overall percent fractions (with respect to the total ribosomes), it would be seen as a decrease in the abundance of the subunits. Since EF-G and RRF function to facilitate the reaction to the right, more EF-G, RRF and GTP are expected to be needed when 70S ribosomes are increased. As shown in Figure 4B through F, with constant concentration of factors and GTP, a gradual increase of 70S ribosomes resulted in persistently less subunit dissociation as predicted from the equation shown in Figure 4A. With 1  $\mu$ M 70S ribosomes, practically no splitting was observed with 0.36 mM GTP, 1  $\mu$ M EF-G and RRF. It would have been ideal if we could use 20  $\mu$ M ribosome (physiological concentration), but such an experiment is technically impossible.

The experiments described in Figure 5 show that the increase of GTP and the factors could overcome the effect of increased ribosome concentration. In Figure 5A, GTP was increased to 2 mM, but only 15.6% of 1  $\mu$ M 70S ribosomes were split. On the other hand, in Figure 5B, both GTP (up to 2 mM) and factors (20  $\mu$ M each) were increased. As noted from this figure, about 60% of the 70S ribosomes were converted to subunits. Figure 5C was conducted as a control experiment showing the background splitting with IF3 and GTP only. Since IF3 alone splits about 20% of 70S ribosomes, we concluded that about 40% of 1  $\mu$ M 70S ribosome was converted to subunits by 20  $\mu$ M RRF and EF-G in the presence of 2 mM GTP. The physiological concentrations of EF-G (34,35) and RRF (36) are about 20  $\mu$ M each, and IF3 is about 4  $\mu$ M (35).



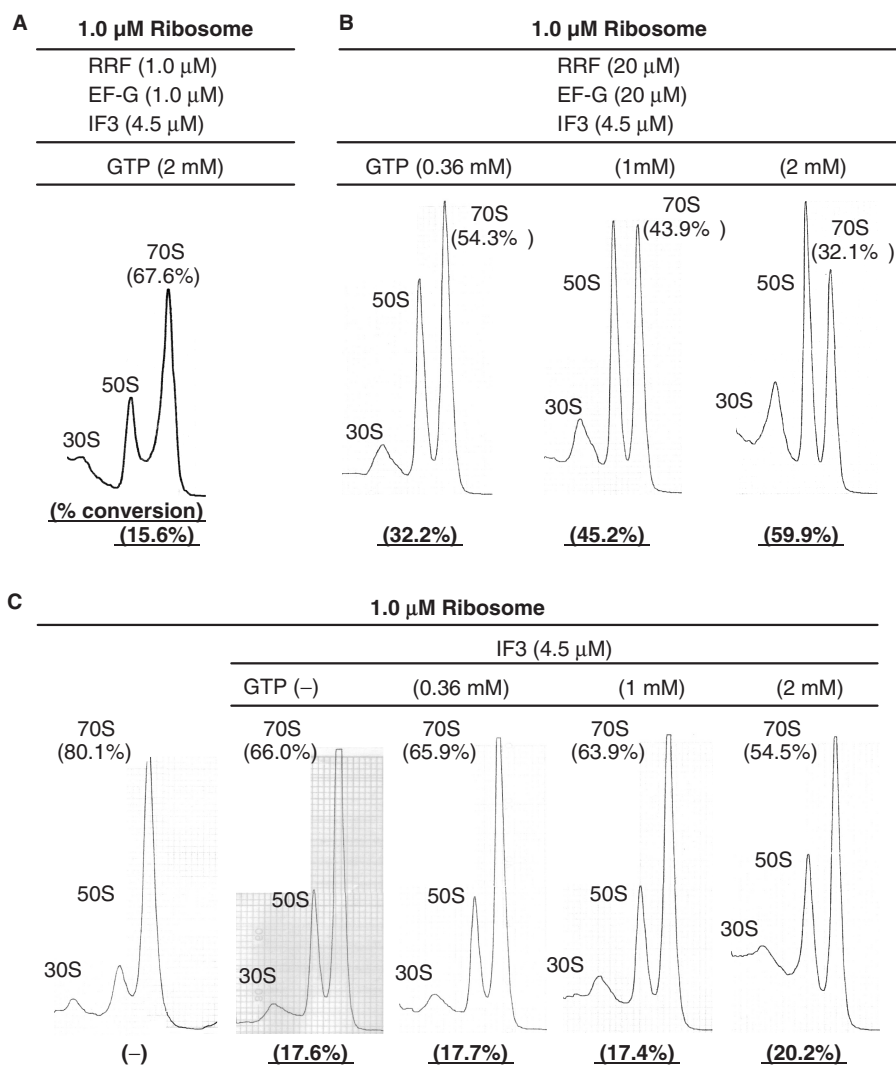
**Figure 4.** Decrease of the extent of RRF/EF-G-dependent splitting due to increase of the 70S ribosomes. (A) Equilibrium between the 70S ribosomes and dissociated subunits.  $K$ ,  $k_1$  and  $k_{-1}$  are equilibrium, forward rate, and reverse rate constants, respectively. [70S], [50S] and [30S] indicate the concentrations of the ribosomes and subunits, respectively. When [70S] increases, the subunits concentrations increase accordingly. The numerator, which is the multiple of both subunits concentrations, increases more than [70S] (denominator), thus driving the equilibrium to the left. IF3 binds to the 30S subunit and prevents reassociation. (B–F) Ribosomes as indicated were incubated alone or with RRF (1  $\mu$ M), EF-G (1  $\mu$ M), His-IF3 (4  $\mu$ M) and GTP (0.36 mM) in buffer R at 30°C for 20 min. Ribosomal sedimentation patterns were analyzed as described in Materials and methods section. The percentages of 70S ribosomes of the total ribosomes are indicated above each profile. The 70S ribosomes split are represented by % conversion =  $100 \times (\text{percentage of 70S alone} - \text{percentage of 70S with factors}) / \text{percentage of 70S alone}$ . The % conversions are indicated below the 70S peaks. It was noted that percentage of 70S ribosomes [left profiles of (B) through (F)] increased as the concentration of 70S ribosomes increased.

#### Kinetics of energy-dependent ribosomal splitting by RRF and EF-G

In the preceding section, we showed that both GTP and EF-G/RRF had to be raised to dissociate 1  $\mu$ M ribosome. It is therefore important to examine the kinetic constants of the EF-G/RRF reaction to find out if the splitting reaction can take place *in vivo*. To obtain the kinetic constant, the reaction should be followed in real time.

In the preceding publication, we showed that the real-time 70S ribosome splitting can be followed by observing the decrease of light scattering caused by ribosomes (22,23,48). This is based on the principle that 70S ribosomes, due to their larger size, scatter more light than the subunits.

In the experiment shown in Figure 6, the amount of the 70S ribosome preparation (0.15  $\mu$ M), RRF (5  $\mu$ M) and GTP (0.5 mM) was kept constant and various amounts



**Figure 5.** Decrease of the RRF/EF-G-dependent splitting due to the increase of 70S ribosomes was overcome by the increase of factors and GTP. The numbers below and above the profiles are conversions and percentages of 70S ribosomes, respectively. (A) The increase of GTP alone did not overcome the effect of increased 70S ribosomes (1 μM). (B) The increase of both GTP and factors overcame the effect of increased ribosomes. The reaction conditions were identical to (A) except that the amounts of factors were increased 20-fold. (C) Controls of (A) and (B). It shows the effect of IF3 and various concentrations of GTP on 70S ribosomes. 70S ribosome split was represented by % conversion =  $100 \times (80.1 - \text{percentage of 70S with factors}) / 80.1$ ; 80.1 represents percentage of 70S ribosomes alone.

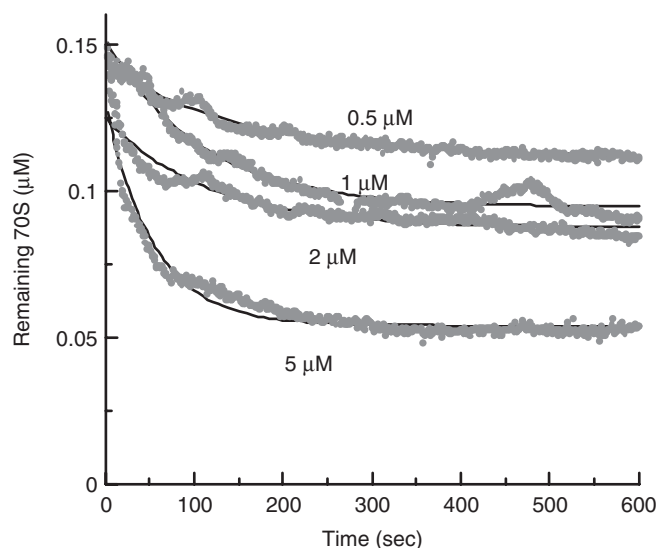
of EF-G (0.5–5 μM) were added to the reaction mixture. The data showed that both the rate and the steady-state level of the splitting were affected by the amount of added EF-G. This explains the data presented in Figure 5B showing that increased RRF, EF-G and GTP overcame the negative effect of increased 70S ribosomes. Most of the data were in line with the single exponential curves (solid lines). From the rates obtained with single exponential fittings as shown in Figure 6, the Michaelis–Menten constant ( $K_m$ ) and  $k_{cat}$  were estimated to be 0.59 μM and 0.35/min, respectively. The  $K_m$  value for the EF-G/GTP for the splitting of vacant 70S ribosome is higher than that for the translocation [0.25 μM (49) or 0.07 μM (50)]. This is understandable from the observation that EF-G/ribosome-dependent GTPase is inhibited by RRF (27), and the presence of RRF on the ribosome makes it harder for EF-G to bind to the ribosome (39). It should be mentioned that the ratio (ribosome/factor) was not that

of *in vivo* conditions, but this did not matter for determination of the kinetic constants of EF-G/RRF. Considering *in vivo* concentration of ribosome and factors, the  $k_{cat}$  value of EF-G/RRF as calculated from Figure 6 suggest that the EF-G/RRF-dependent splitting of vacant 70S ribosomes must be sufficient to split ribosomes *in vivo* during the lag phase.

It should be mentioned that the rate of splitting as shown in Figure 6 is strictly determined by the amount of EF-G and RRF. As shown previously, the addition of IF3 did not influence the rate at all while the final level of the splitting was very much dependent on the amount of IF3 (23). This fact was recently confirmed (24).

#### Spd at 2 mM but not at 0.3 mM completely inhibited the splitting of 70S ribosomes

Recently, Umekage and Ueda (32) showed that, in the presence of 2 mM Spd, 0.6–1.2 μM ribosomes were not



**Figure 6.** Kinetic studies on the splitting of 70S ribosomes by EF-G and RRF without IF3: decrease of ribosomal light scattering due to the splitting of the ribosomes. The 70S ribosome preparation (0.15  $\mu\text{M}$ ) was incubated with 5  $\mu\text{M}$  RRF, 0.5 mM GTP and various concentrations of EF-G (as indicated) as described in Materials and methods section. The change of the light scattering relative to the initial value at 20 s was measured against the incubation time and expressed as the amount of remaining 70S ribosomes in micromolar. Data were fit to single- (solid lines) exponential decay curves. The control value without EF-G gave about 10% of 70S ribosomes dissociation (0.135  $\mu\text{M}$  remaining 70S ribosomes) in 6 min by RRF alone (11) under similar conditions.

split by 20  $\mu\text{M}$  RRF and EF-G (physiological concentration) even with 2 mM GTP (51). On this basis, they suggested that, splitting of 70S ribosomes by RRF and EF-G may be an *in vitro* artifact and the splitting may not occur, or it involves some other mechanisms *in vivo*. In Figure 7A, we confirmed the results presented by Umekage and Ueda indicating that under the conditions similar to theirs we do not observe significant splitting of 70S ribosomes by RRF and EF-G (right three profiles).

However, in the presence of physiological polyamine concentrations [Spd 0.3 mM and Ptc 15 mM, (37,38)], RRF, EF-G and IF3 split significant amount of 70S ribosomes (Figure 7A, left three profiles). Figure 7B shows that under these conditions, as the concentration of GTP increased, decreasing amount of 70S ribosome was observed, indicating that GTP concentration was critical. Figure 7C shows the control indicating that slight dissociation of 70S ribosomes (6.7%) occurred with 2 mM GTP and IF3 only. We concluded that the major reason why Umekage and Ueda were not able to split 70S ribosomes is their unusually high concentration of Spd (more than six times higher than the natural concentration).

#### Spd at 2 mM completely inhibited the anti-association activity of IF3

In addition to its main activity to facilitate binding of mRNA to the 30S subunits (20), IF3 is also known as an anti-association factor (46), meaning that it inhibits association of split subunits. The latter activity is crucial for the disassembly of the post-termination complex (23).

In the experiment described in Figure 8, we showed that 2 mM Spd inhibited the anti-association activity of IF3. This must be one of the reasons why Umekage and Ueda did not observe the splitting of 70S ribosomes by RRF, EF-G and IF3 in the presence of 2 mM Spd.

When 70S ribosomes were exposed to 1 mM  $\text{Mg}^{2+}$ , all of them were split into subunits as shown in profile 1 of Figure 8. However, when the split subunits were exposed to 6 mM  $\text{Mg}^{2+}$ , 57.5% of the subunits were re-associated to form 70S ribosomes (profile 2). This re-association of subunits was mostly prevented in the presence of 4.5  $\mu\text{M}$  IF3 (profile 4), indicating the anti-association activity of IF3. When the identical experiment was performed in the presence of 2 mM Spd, the anti-association activity of IF3 was inhibited and re-associated 70S ribosomes appeared even with IF3 (compare profiles 4 with 5). Profile 4 shows that Spd has strong association activity suggesting that this is one of the reasons why it has the inhibitory activity on the anti-association activity of IF3.

Physiological concentration (0.3 mM) of Spd had only slight inhibitory activity on IF3 activity (profile 6). Even in combination with 15 mM Ptc, 0.3 mM Spd still permitted the anti-association activity of IF3 to a large extent (profile 7).

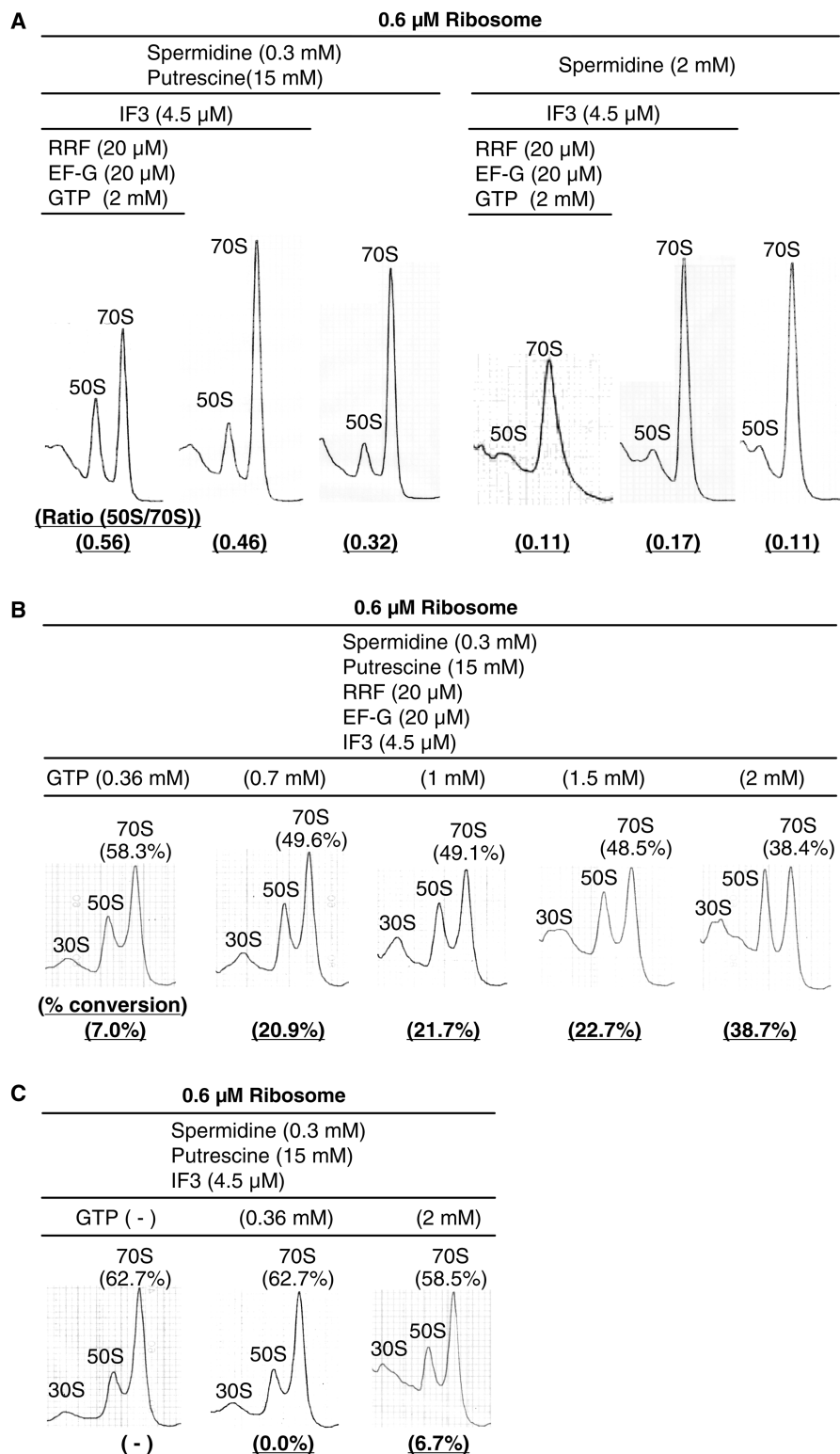
## DISCUSSION

### The transient nature of RRF/EF-G-dependent splitting of 70S ribosomes

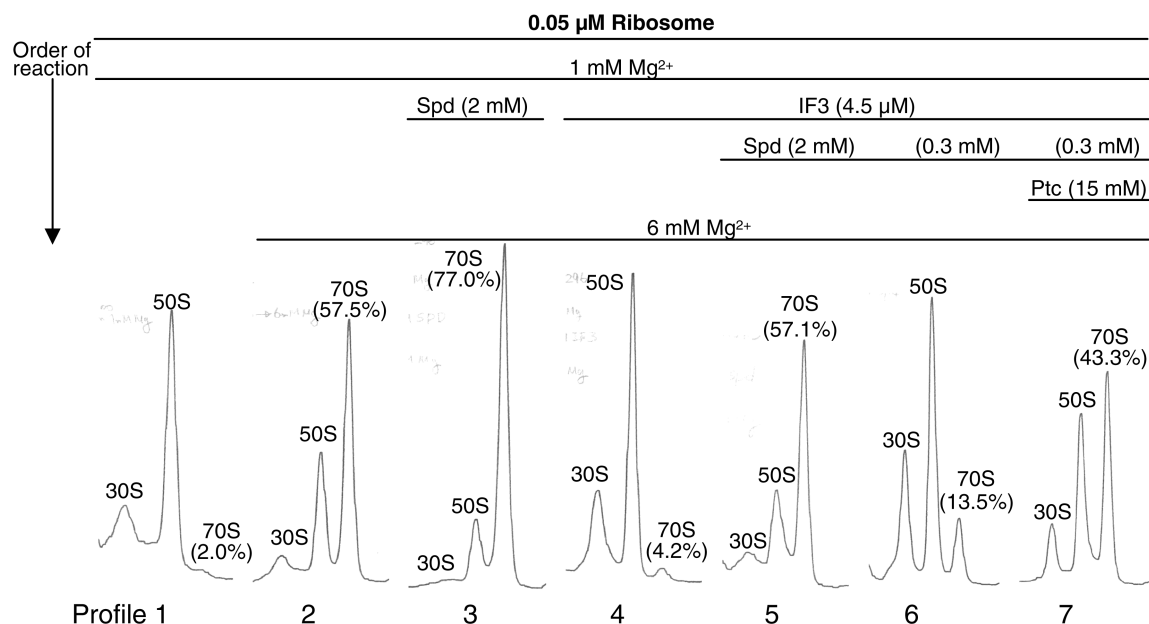
As mentioned briefly in the introduction, the splitting of vacant 70S ribosomes is a process, which must take place when bacteria adjust for the shift-up environmental conditions (the lag phase). In general, under poor nutritional conditions or adverse situations such as low or high culture temperature, bacterial ribosomes take the form of 70S ribosomes or 100S ribosomes (31), rather than polysomes. Upon shift-up, bacteria must utilize these 70S ribosomes to increase protein synthesis. In the normal growth phase, the post-termination complexes must also be split. In this paper, we establish that the splitting of vacant 70S ribosomes catalyzed by RRF and EF-G/GTP is transient. Previously, we and others could not observe the splitting by SDGC technique 'unless IF3 was added' (23,27). In contrast, one can observe the splitting 'without IF3' with the light scattering method (23) or with the subunits exchange method (27). These observations are consistent with the notion that the splitting by RRF and EF-G is transient, and the split subunits have to be stabilized by IF3 to be observed with SDGC. Since the decrease of ribosomal light scattering is an indirect method and is not observing the actual subunits' formation, there has been no direct evidence for the hypothesis that RRF and EF-G splits 70S ribosomes transiently in the *in vitro* system.

With the use of a limited amount of GTP, IF3 [inhibitor of the reassociation of subunits (46)] and fusidic acid [inhibitor of the splitting of post-termination complexes into subunits (23)], we showed that the formation of subunits by RRF and EF-G was transient (Figures 1 and 2) and a constant supply of GTP was necessary to keep them apart





**Figure 7.** Splitting of 70S ribosomes in the presence of polyamines. **(A)** Ribosomes were split in physiological concentrations of polyamines, but not in 2 mM spermidine (Spd). The 70S ribosome preparation (0.6  $\mu$ M) was incubated in the presence of polyamines and sedimented through the sucrose gradient containing polyamines as indicated in the figure. The 30S peak was not clearly visible due to the presence of polyamines in the gradient. The ratio of 50S subunits to 70S ribosomes are shown below the 50S peaks. **(B)** The effect of various concentrations of GTP on the splitting of 70S ribosomes in the presence of physiological concentration of polyamines. Ribosomes were incubated, as in (A), in buffer containing physiological concentrations of polyamines, GTP, RRF and EF-G and sedimented through sucrose gradient in buffer R containing no polyamine. **(C)** Control of (B). The effect of IF3 without RRF/EF-G. The numbers below and above the profiles in (B) and (C) are conversion and percentages of 70S ribosomes, respectively. The 70S ribosome split was represented by % conversion =  $100 \times (62.7 - \text{percentage of 70S in P buffer with factors and GTP}) / 62.7$ ; 62.7 represents percentage of 70S ribosomes in P buffer with 4.5  $\mu$ M IF3 (left profile).



**Figure 8.** Spermidine (2 mM) inhibited anti-association activity of IF3. The 70S ribosome preparation was split into subunits in the low magnesium buffer (profile 1). Subunits thus prepared were mixed with Mg-acetate to the final concentration of 6 mM  $Mg^{2+}$  and incubated in the absence (profile 2) or presence (profile 4) of IF3. Profile 3 indicates subunits exposed to 2 mM Spd in addition to 6 mM  $Mg^{2+}$ . Profile 5 shows the ribosomes after the subunits were exposed to IF3 followed by the addition of 2 mM spermidine and  $Mg^{2+}$ . Profiles 6 and 7 are identical experiments to profile 5 except that the polyamine concentrations were changed as indicated. Ribosomal sedimentation profiles were analyzed as described in Materials and methods section. The percentages of 70S ribosomes are indicated above the 70S peak in each profile.

unless IF3 was added. Direct evidence with the use of SDGC for the subunits formation by RRF and EF-G without IF3 was obtained by inclusion of the reaction components in the sucrose gradient (Figure 3).

#### Constant GTP requirement for the splitting of 70S ribosomes

The GTP requirement for the EF-G/RRF-dependent splitting of 70S ribosome was different from that for the EF-G-dependent translocation in two aspects; first, the splitting required a constant supply of GTP to keep the subunits separate (Figures 1–3). In contrast, the results of translocation was irreversible when the E-site was empty (52,53). Second, a single round of translocation took place with nonhydrolyzable GTP analog (44), though slowly (45), while a single round of splitting, even in the presence of IF3 was strictly dependent on GTP but not on the nonhydrolyzable analog (23,27,28). Perhaps the inability of GMPPCP to cause the splitting may be because, in the presence of GMPPCP, the EF-G/GMPPCP complex remains on the ribosome (39,54). The bound EF-G/GMPPCP may function to bridge two subunits together, resulting in the inability of GMPPCP to catalyze the splitting. This possibility was strengthened by our recent observation that yeast 80S ribosomes were stabilized by eEF2 (eukaryotic homolog of EF-G) and GMPPCP (Demeshkina, N., Hirokawa, G., Kaji, A. and Kaji, H., unpublished data).

When GTP was limiting, as shown in Figures 1 and 2, only a limited amount of splitting took place because GTP was exhausted in the reaction mixtures. It was unlikely that the need for 2 mM GTP concentration for the splitting (Figure 5) was to stimulate the rate of the reaction because  $K_d$  value of GTP for EF-G was about 20  $\mu$ M (55).

The requirement for 2 mM GTP to split 1  $\mu$ M ribosome must therefore be due to the actual need for the energy to split with this amount of ribosome.

#### Does the RRF/EF-G-dependent splitting of the 70S ribosome occur *in vivo*?

The time course of the splitting of the 70S ribosomes shown in Figures 2C and 6 is in agreement with our original and other time-course studies on the disassembly of the post-termination complex (27,56). However, the velocity of the splitting as shown in Figure 6 is much slower than that reported recently with a similar light scattering method (24). Splitting of post-termination complexes observed through the FRET change of fluorescence-labeled ribosomes with mRNA (28) was also much faster than these time courses. This may be partly because it deals with post-termination complexes and partly because the FRET change may include internal structural change before the actual physical separation of the subunits (57). We observed that 5  $\mu$ M EF-G split 0.1  $\mu$ M ribosome in about 2 min (Figure 6). This means that it will take 100 min to split 20  $\mu$ M ribosomes for 20  $\mu$ M EF-G. Since the lag phase of *E. coli* is about 180 min under our experimental conditions (58), this rate is sufficient to split most of the vacant 70S ribosomes during the lag phase. The data shown in Figures 4 and 5 indicate that the shift of equilibrium due to the increase of the 70S ribosomes concentration was overcome by physiological concentrations of GTP (2 mM) and RRF/EF-G (20  $\mu$ M each). This gives further support for the concept that RRF- and EF-G/GTP-dependent splitting of vacant 70S ribosomes takes place *in vivo*. In a recent paper, Pavlov *et al.* suggested that splitting of stored ribosomes at lag phase may be carried

out by IF1 and IF3 as originally shown by Godefroy-Colburn *et al.* (22) but the rate (24) and extent (23) of the vacant ribosome splitting by RRF, EF-G and IF3 were more than those by IF1 and IF3 suggesting the major role of RRF for activation of the dormant ribosomes.

However, Umekage and Ueda (32) recently suggested that the splitting of 70S ribosomes by RRF and EF-G may not take place *in vivo*. This was based on the lack of splitting of 0.6–1.2  $\mu\text{M}$  ribosomes by physiological concentration of EF-G/RRF using the modified PURE buffer containing 2 mM Spd. In contrast, as shown in Figure 7, in the presence of EF-G, RRF (20  $\mu\text{M}$  each), 2 mM GTP, 0.3 mM Spd and 15 mM ptc, 0.6  $\mu\text{M}$  ribosomes were dissociated. The concentrations of the aforementioned components were those of *in vivo* (34–38,51). The concentration of polyamine in the original PURE buffer is 1 mM Spd, 8 mM ptc (42) and the polymix buffer designed to obtain maximum *in vitro* protein synthesis (33) contained 8 mM ptc and 1 mM Spd. Therefore, the Spd concentration used by Umekage and Ueda is higher not only than the physiological concentration of Spd but also than that of those buffers used by other laboratories. It appears, therefore, that the reason why Umekage and Ueda were not able to observe splitting of 70S ribosomes was the use of an unusually high concentration of Spd (2 mM).

It has been shown that paromomycin, which inhibited the RRF activity (59) strongly inhibited the anti-association activity of IF3 (60) by binding to helix 69 of the 50S subunits (12,61). It appeared that 2 mM Spd had similar inhibitory effects on the anti-association activity of IF3. This explains the lack of splitting by RRF and EF-G in the presence of 2 mM Spd even in the presence of IF3. We conclude that the RRF/EF-G-dependent splitting occurs *in vivo*.

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