Spermidine inhibits transient and stable ribosome subunit dissociation

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Received 14 November 2005; revised 4 January 2006; accepted 12 January 2006

Available online 20 January 2006

Edited by Lev Kisselev

Abstract Recent light-scattering experiments and sucrose density gradient centrifugal analyses suggested that the 70S ribosome undergoes RRF- and EF-G-triggered transient subunit dissociation that is followed by IF3-induced stable dissociation. However, the experimental conditions did not include the ubiquitous cellular polyamine spermidine, which is required for efficient translation. We found that when spermidine was present, the transient dissociation was inhibited. Moreover, the published experiments used ribosome concentrations that were far lower than the physiological concentration. We found that when spermidine and higher ribosome concentrations were included in the experimental conditions, only very limited stable subunit dissociation was observed. These results suggest that neither transient nor stable dissociation occurs under physiological conditions applied here.

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Keywords: Transient dissociation; Stable dissociation; Ribosome; Spermidine; Light-scattering; Sucrose density gradient centrifugation

1. Introduction

The bacterial ribosome consists of two subunits, the 50S and 30S subunits. In physiological conditions, most of the free ribosomes that are not participating in the translational process exist as the 70S form [1]. While the bacterial translational process is now generally well understood, some questions still remain about the post-termination process. Specifically, it is not clear whether the subunit dissociation of the 70S particle that is involved in these processes depends on the initiation factors IF1 and IF3 [2,3]. Recently, quench-flow [4] and light-scattering [5] experiments suggested the existence of another process of ribosomal dissociation named “transient dissociation” that is triggered by ribosome recycling factor (RRF) and GTP hydrolysis driven by elongation factor-G (EF-G) [4]. It was also proposed that after this putative transient dissociation, IF3 binds to the dissociated 30S subunit, thereby preventing subunit reassociation. This IF3-induced stabilization is called “stable dissociation”. Its discovery suggested IF3 acts as an anti-association factor [6,7] rather than as the dissociation factor [8,9].

Apart from such factor-dependent ribosomal dissociation, it is also known that ribosomal subunit dissociation and association is greatly affected by the ionic conditions, such as magnesium ions, monovalent ions and polyamines (spermine, spermidine and putrescine) [10,11]. In particular, the polyamines, which are organic cations, are present at millimolar concentrations in living organisms and form complexes with RNA [12]. In particular, spermidine plays a crucial role in the cell-free translation system [13], as it is required for the efficient translation reaction and shifts the equilibrium of association between 70S and subunits toward association [14]. The model of transient dissociation described above is based on in vitro quench-flow [4] and light-scattering [5] experiments using in vitro reactions that lack polyamines. Given the significant role played by spermidine in cell-free translation, we asked whether the model of transient/stable dissociation actually reflects the in vivo process. To address this question, we evaluated the effect of spermidine on the transient and stable subunit dissociation detected by light-scattering experiments and sucrose density gradient centrifugation (SDG), respectively.

2. Materials and methods

2.1. Buffers and factors

Buffer A consists of 10 mM Mg(OAc)₂, 100 mM NH₄Cl, 3 µg/ml of RNase-free DNase I, 7 mM β-mercaptoethanol and 20 mM HEPES–KOH, pH 7.6, while buffer B contains 10 mM Mg(OAc)₂, 500 mM NH₄Cl, 7 mM β-mercaptoethanol and 20 mM HEPES–KOH, pH 7.6. Buffer C contains 6 mM Mg(OAc)₂, 30 mM KCl, 7 mM β-mercaptoethanol and 20 mM HEPES–KOH, pH 7.6, while PURE buffer consists of 6 mM Mg(OAc)₂, 150 mM potassium glutamate, 30 mM K(OAc), 2 mM spermidine and 1 mM DTT, pH 7.6. RRF and EF-G was prepared as described by Shimizu et al. [15] and native IF3 without a tag was prepared as described by Udagawa et al. [16] with slight modifications.

2.2. Ribosome preparation

Escherichia coli A19 cells (200 g) were disrupted by a French press (10000–15000 psi) in 200 ml of buffer A. After removing the cell debris, the lysate was centrifuged for 45 min at 19600 rpm. The supernatant was laid on a sucrose cushion and centrifuged for 8 h at 35000 rpm in a BECKMAN 45 Ti rotor at 4 °C. The crude ribosome pellet was then resuspended in buffer B and layered on a 20% sucrose cushion in buffer B and centrifuged for 4 h at 55000 rpm in a BECKMAN TLA100.3 rotor (crude ribosomal wash). This washing procedure was repeated, after which the salt-washed crude ribosome pellet was resuspended in buffer C. The tight-coupled 70S ribosome was purified from the crude ribosome as described by Shimizu et al. [15]. Before

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using the washed 70S, the removal of RRF, EF-G and IF3 from the ribosome fraction was verified by Western blot analysis (data not shown).

2.3. Light-scattering assay
Light-scattering assays were performed with a FP-6500 Spectrofluorometer (JASCO). All experiments were performed at 30 °C and the 90° scattering light against the 436 nm of the incoming ray was monitored at 100% dissociation was determined by measuring the intensity when the magnesium concentration in the scattered light corresponding to 100% dissociation was determined (Fig. 1). Prior to combining the two mixtures, dust particles and aggregations were spun down for 3 min at 14000 rpm. Subsequently, the ribosome-mix (15 μl) and factor-mix (135 μl), which had been preincubated separately at 30 °C, were mixed manually, and the intensity of scattered light was monitored from 40 seconds after mixing. The intensity of the scattered light corresponding to 100% dissociation was determined by measuring the intensity when the magnesium concentration in the spermidine-free PURE buffer was lowered to 0.6 mM.

2.4. Sucrose density gradient assay
Reaction mixtures in PURE buffer with or without 2 mM spermidine as described in the figure legends were incubated for 20 min at 37 °C and then halted on ice. The reaction mixtures were loaded onto a 10–30% sucrose gradient in buffer D (20 mM HEPES–KOH, pH 7.6, 6 mM Mg(OAc)₂, 30 mM NH₄Cl, 7 mM β-mercaptoethanol) with or without 2 mM spermidine (see figure legend) followed by ultracentrifugation in a SW41 rotor (BECKMAN) for 6 h at 35 K rpm. The distribution of 70S and its dissociated subunits in SDG was monitored at OD₂₅₄ by Bio-Mini UV Monitor AC-5200L (ATTO).

3. Results
3.1. Minimal transient dissociation is observed in the spermidine-containing PURE buffer
We first used the light-scattering assay to examine the transient dissociation in PURE buffer as described in Section 2. PURE buffer is the simplified polymix buffer [17] that contains ions and polyamines at concentrations comparable with those observed in vivo and that has been optimized for the cell-free translation system called the PURESYSTEM [15,16,18]. Inducing spontaneous dissociation of subunits by lowering magnesium concentrations from 6 mM to 0.6 mM, in the absence of spermidine, it was too fast to trace the rate of ribosomal dissociation (Fig. 1B), while in the presence of 2 mM spermidine it took at least 300 s for complete dissociation of subunits (Fig. 1A). As shown in Fig. 1B, when spermidine was absent, we detected transient 70S dissociation in the presence of 20 μM each of RRF and EF-G, which is estimated to be their physiological concentrations. About 30% of the ribosomes dissociated at 400 s in the presence of RRF and EF-G. In the presence of IF3 alone at 4.5 μM, which is close to its estimated concentration in the cell, slight 70S dissociation (20%) was observed. When 20 μM RRF, 20 μM EF-G, and 4.5 μM IF3 were all present, substantial 70S dissociation was observed, as about 80% of the ribosomes had dissociated by 10 min in the presence of these factors. However, when 2 mM spermidine was present (Fig. 1A), the dissociation induced by RRF, EF-G, and IF3 somewhat reduced to about 60% of the ribosomes, IF3 induced even less dissociation (8%), and significantly, no transient 70S dissociation was detected in the presence of RRF and EF-G alone. Thus, the transient dissociation of 70S induced by RRF and EF-G does not proceed in reaction conditions containing the concentration of spermidine that is optimal for translation and that is present in vivo.

3.2. Transient dissociation is inhibited by spermidine
We next examined the effect of spermidine on the transient dissociation of 70S more carefully. As shown in Fig. 1B, in the absence of spermidine, 30% of the ribosomes were split by 20 μM each of RRF and EF-G. When we assessed the effect of various spermidine concentrations on transient ribosome disassembly, we found that 10% and 0% of the ribosomes were split when 1 and 2 mM spermidine were present, respectively (Fig. 2). The fact that physiological concentrations of spermidine inhibit the transient dissociation of 70S suggests that such transient subunit dissociation does not occur in the cell.

3.3. Stable dissociation depends profoundly on ribosomal concentrations
Fig. 1 shows that when IF3, RRF and EF-G were all present, 70S was markedly split into its subunits, while IF3 on its own or RRF plus EF-G had only minimal effects. Thus,
it appears that IF3 effects the stable dissociation of 70S in concert with RRF and EF-G. This was observed regardless of whether spermidine was present or not. This stable dissociation is in good agreement with the report by Peske et al. [4]. However, it should be noted that the concentrations of ribosome used in our light-scattering experiments (0.16 μM) and the experiments by Peske et al. [4] (0.05–0.1 μM) are over 100-fold lower than the physiological concentration of ribosome (20 μM). Consequently, we next assessed the dissociation of 70S by IF3, RRF and EF-G using a wide range of ribosome concentrations.

Since light-scattering experiments have a technical limitation with regard to using higher substrate concentrations, we examined the effect of various concentrations of ribosome on the stable dissociation of 70S in the presence and absence of spermidine by SDG analysis. Notably, in the absence of spermidine and other factors, the 70S peak in SDG analysis became broader than the peak in the presence of 2 mM spermidine (compare A and G in Fig. 3). This reveals that, 70S physically splits into subunits at detectable levels by centrifugal force and/or frictional force during SDG in the absence of spermidine (Fig. 3G). When IF3 was added in the absence of spermidine, stable splitting of 70S was observed (Fig. 3H). Total stable dissociation in the absence of spermidine was also triggered by RRF, EF-G and IF3 (Fig. 3J). Notably, although the reaction duration was sufficient for the dissociation of 0.16 μM 70S that was triggered by RRF, EF-G and IF3, the dissociation rate decreased when higher 70S concentrations (0.6 and 1.2 μM) were used in the reaction mixture (compare J–L of Fig. 3).

In the presence of 2 mM spermidine, the 70S peak is sharp. Moreover, only slight IF3-induced dissociation of 70S was detected; this in agreement with the light-scattering experiment shown in Fig. 1A. When spermidine was present, the stable subunit dissociation caused by IF3, RRF and EF-G was observed when 0.16 μM 70S was used, although the dissociation was less complete than when spermidine was absent. However, at higher concentrations of ribosome, namely, 0.6 μM (Fig. 3E) and 1.2 μM (Fig. 3F), very little stable dissociation was observed. Note that 1.2 μM ribosome is still only about one-tenth of the physiological ribosome concentration.

**4. Discussion**

The most important consideration when performing in vitro experiments is to determine whether the obtained data properly reflect the in vivo situation. To this end, it is necessary to compare the reaction conditions with the physiological conditions and to try as best possible to emulate the latter. Two recent studies by Peske et al. [4] and Hirokawa et al. [5] involving quench-flow and light-scattering experiments led to the hypothesis that RRF and EF-G trigger the transient dissociation of 70S, and that this dissociation is stabilized by IF3. However, these experiments were performed without the ubiquitous cellular polyamine spermidine [12], which plays crucial roles in maintaining cell viability [19]. According to Davis [20], the lysate from _E. coli_ contains a high level of polysome (60%) and the concentration of free ribosome in _E. coli_ cell is estimated to be around 8 μM. Thus, the ribosome concentrations in the experiments by Peske et al. (0.05–0.1 μM) or...
Hirokawa et al. (0.07 \mu M) are extremely lower than estimated physiological concentration. Thus, we repeated the light-scattering and SDG experiments of Peske et al. [4] and Hirokawa et al. [5] using buffer conditions that better approximate the physiological situation. However, when physiologically appropriate conditions were used, we did not observe either the putative RRF- and EF-G-induced transient dissociation of 70S or the putative stabilization of this dissociation by IF3.

We found that the putative RRF- and EF-G-induced transient dissociation of 70S that was observed by Peske et al. [4] and Hirokawa et al. [5] is actually due to the lack of spermidine. The broad peak of 70S in the SDG profile that was observed in the absence of spermidine in Fig. 3G shows the lability of 70S when spermidine is not present. This lability is not detectable by the quench-flow analyses by Peske et al. [4] or light-scattering experiment by Hirokawa [5] under single reaction condition. However comparing spermidine concentration in Fig. 1A (2 mM) and Fig. 1B (0 mM) by lowering concentration of magnesium ion from 6 to 0.6 mM, the fast subunit dissociation in the absence of spermidine obviously suggests that spermidine strengthens the physical interaction of subunits. This supports the notion that different pockets in rRNA bind the polyamine and the metal ions to stabilize the secondary structure of rRNA [21,22]. Cryo-EM data shows that 30S and 50S subunit assembly is maintained by bridges largely derived from the rRNAs [23]. Thus, in the absence of spermidine, the ribosome becomes labile because the intensity of the subunit interactions is lowered; this causes the transient dissociation apparently induced by RRF and EF-G. Supporting this is that the transient dissociation putatively induced by RRF and EF-G is inhibited in a dose-dependent manner by adding spermidine (Fig. 2). It is true that RRF and EF-G seems to be able to induce the transient dissociation of 70S (Fig. 1B), as proposed by Peske et al. [4] and Hirokawa et al. [5], but our experimental results using more physiologically representative conditions (Figs. 1A and 3C) suggest this activity might not be physiologically relevant.

In the presence of 2 mM spermidine, IF3 poorly splits 70S into its subunits. This suggests IF3 in itself has only a very weak dissociative function, unlike what was predicted earlier [8,9]. This very weak dissociative function is slightly enhanced in the absence of spermidine, probably because of the weakened subunit interaction resulting from the absence of spermidine. As mentioned above, RRF and EF-G could not split 70S at all in the presence of 2 mM of spermidine. However, when IF3 was also present, partial dissociation of 70S was observed (Figs. 1A and 3D). This suggests that IF3 must act with RRF and EF-G at the same time because after GTP hydrolysis of EF-G at the recycling step, RRF and EF-G immediately leave the ribosome [24,25]. RRF binding site on the ribosome is near A/P site [26–28], while the binding site of IF3 is near the P and E sites of the ribosome [29,30] hence IF3 binding site does not overlap the RRF and EF-G binding sites. And two reports have shown that IF3 can bind to 70S and loosen its structure without splitting it into its subunits [31,32]. Thus, we suspect that the substrate for the stable dissociation is the 70S-IF3 complex.

In cell-free translation systems, polyamines, especially spermidine, enhance the translational efficiency and accuracy [17,33]. For example, spermidine is required in our PURESYSTEM [15,16,18]; when it is present at its optimal concentration (around 2 mM), the translational efficiency is 10 times higher than that in the absence of spermidine (data not shown). Cryo-EM analysis has revealed that 2 mM spermidine aids the precise localization of tRNA on the ribosome, which indicates spermidine stabilizes the correct ribosomal structure [34]. Since Peske et al. [4] and Hirokawa et al. [5] observed transient dissociation using conditions that lacked spermidine, and also did not show the efficiency of the translation process they employed by using a natural mRNA, it is not clear whether the transient dissociation they detected truly reflects in vivo reactions.

While the SDG technique cannot detect the transient dissociation of 70S (Fig. 3C), it may well be suitable for estimating transient dissociation indirectly by observing the stable dissociation, since, once 70S is split into its subunits by IF3, the resulting 30S-IF3 complex is very stable and inhibits the reassociation of 50S subunit [7]. Zavialov et al. [35] and Kaji’s group [5] recently reported the stable dissociation of 0.33 \mu M termination complex or 0.07 \mu M 70S, respectively, in polyimix buffer [17] that contains 1 mM of spermidine and 8 mM of putrescine. These studies also suggested 70S is subject to transient and stable dissociation mediated by RRF, EF-G and IF3. However, the estimated physiological concentration of free ribosomes in E. coli is about 8 \mu M whereas the concentration of ribosome that the others and we used in SDG analyses is 0.07–0.33 and 0.06–1.2 \mu M, respectively. We found that the stable dissociation of 70S is reduced in high concentrations of ribosome regardless of spermidine (Fig. 3). Thus, if we take the physiological concentration of ribosome into account, it became apparent that RRF+EF-G+IF3-triggered stable dissociation of 70S would occur only very infrequently. These observations suggest that the first round of the translation initiation pathway does not involve the stable dissociation of 70S via its initial transient dissociation.

Our reaction conditions were based on the polyimix buffer that nearly completely reflects in vivo conditions [17] and that leads to efficient translation (data not shown). Moreover, while our light-scattering experiment was not performed using a stopped-flow technique, our SDG experiments quantitatively reinforce the light-scattering data we obtained. One might argue that the ribosomes, which we used in this study, are washed ribosomes containing tRNAs and mRNA fragments and thus not being authentic termination complexes. However, we note that the complexes used by Peske et al. [4] did not contain a stop codon at the A site and thus are not termination complexes.

Our results demonstrate that more physiological conditions concerning buffer components, in particular spermidine and ribosome concentration are important to draw more reliable picture of post-termination process. Our results further suggest that the experimental data contributing to the transient dissociation in the presence of RRF, EF-G and IF3 are questionable due to the experiments under unphysiological condition and the transient dissociation model thus would be reconsidered.

Acknowledgements: We thank Dr. Hideki Taguchi (Tokyo University) for technical advice regarding the light-scattering experiments. This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan.

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