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Spermidine inhibits transient and stable ribosome subunit dissociation

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Abstract **Recent light-scattering experiments and sucrose density gradient centrifugational 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.**

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 NH_4Cl, 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 NH_4Cl, 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 38% sucrose solutions in buffer identical to buffer A except that it contained only 30 mM NH_4Cl and centrifuged for 8 h at 35000 rpm in a BECKMAN 45 Ti rotor at 4 °C. The crude ribosome pellet was then suspended in buffer B and laid on a 20% sucrose solution 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 with 436 nm of wavelength. To study the ribosomal dissociation, two mixtures, namely, ribosome-mix and factor-mix, were prepared. Ribosome-mix contained 1.6 μM of ribosome in buffer C while factor-mix contained the factors indicated in the figure legends in PURE buffer with or without spermidine (see figure legend). Prior to combining these 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 NH_4Cl, 7 mM β-mercaptoethanol) with or without 2 mM spermidine (see figure legend) followed by ultra centrifugation 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 OD_{254} 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 of spermidine it took at least 540 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,

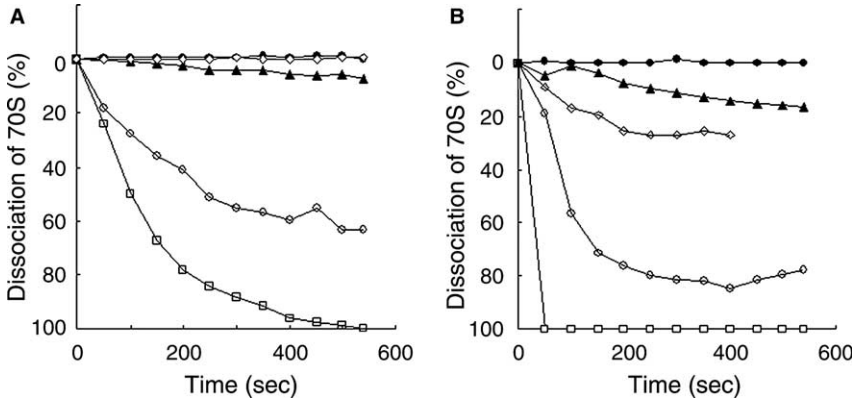

Fig. 1. Transient and stable dissociation of 70S is observed but is greatly affected by the presence of spermidine. Washed ribosomes (0.16 μM) and the factors RRF (20 μM), EF-G (20 μM) and IF3 (4.5 μM) were mixed manually and incubated at 30 °C in the presence (A) or absence (B) of 2 mM spermidine. The rate with which 70S dissociates was measured by light-scattering experiments, as described in Section 2. Closed circle, without factors; open square, without factors but with lower magnesium concentrations (0.6 mM); closed triangle, with IF3; open diamond, with RRF and EF-G; open circle, with RRF, EF-G and IF3. The decrease of intensity was converted to the dissociation rate (%) based on the 100% dissociation observed in the absence of spermidine. Shown is one example of several experiments performed, all with similar results.