PRESSURE-INDUCED DISSOCIATION OF RIBOSOMES DURING ULTRACENTRIFUGATION

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1. Introduction

Ultracentrifugation is usually regarded as a device of separation or analysis which does not affect the integrity of the macromolecules being studied. It is known, however, that biochemical equilibria are more or less pressure sensitive, depending on the $\Delta\overline{V}$ of reaction [1], and that, on the other hand, hydrostatic pressures of several hundred atmospheres are generated in ultracentrifuge tubes. A striking centrifugation pressure effect was observed for myosin association [2]. Kegeles has given a theoretical treatment of the problem [3]. That these considerations may be relevant for the studies of ribosomes became apparent to me during studies of KCl-treated reticulocyte polysomes.

KCl-treatment according to Miller and Schweet [4] results in the production of ribosomal subunits [5]. The present communication demonstrates that for this effect both high speed centrifugation and KCl are necessary. Liver polysomes behave in the same manner, and it is further shown that under proper ionic conditions, the equilibrium between free liver monosomes and their subunits is affected by the centrifugation process.

2. Materials and methods

Rabbit reticulocyte polysomes were prepared according to Allen and Schweet [6]. They were pelleted from a solution containing approximately 45 mM KCl, 4 mM $MgCl_2$ and 250 mM sucrose, rinsed and resuspended in 250 mM sucrose.

Rat liver polysomes were obtained from male albino rats by the method of Falvey and Staehelin [7], with some modifications. 1.0% Triton X-100 was used instead of deoxycholate. The postmitochondrial supernatant, containing 200 mM sucrose, 100 mM NH_4Cl, 5 mM magnesium acetate ($MgAc_2$) and 1 mM dithiothreitol, was supplemented with 50 mM KCl before addition of the detergent [8]. This and all other solutions used in addition contained 20 mM Tris-HCl pH 7.5. The polysome pellets were resuspended in 100 mM NH_4Cl, 1 mM dithiothreitol, and stored at -70°.

Free, single ribosomes were produced by incubation of liver polysomes under protein synthesis conditions [7]. The ribosomes were pelleted and resuspended as above. Fig. 5A and D reveal a minor contamination with complexed ribosomes.

Complexed ribosomes were obtained by action of crystalline pancreatic ribonuclease (Sigma) on polysomes. 5 mg in 0.5 ml were incubated with 0.05 μg of ribonuclease at 30° for 5 min. The reaction was stopped by chilling and addition of 4 units of ribonuclease inhibitor, prepared and assayed according to Gribnau et al. [9]. The distribution of ribosomal material was determined by sedimentation in exponential sucrose gradients in the SB-60 head of an IEC-B60 centrifuge (reticulocyte ribosomes) or the SW 56 Ti head of the Spinco L2-65B (liver ribosomes). The former gradients were scanned at 260 nm in the IEC-Gilford high resolution gradient analysing system [10], the latter at 254 nm with a Spinco fraction recovery system, connected to a syringe pump, an LKB Uvicord photometer and a Kontron recorder.

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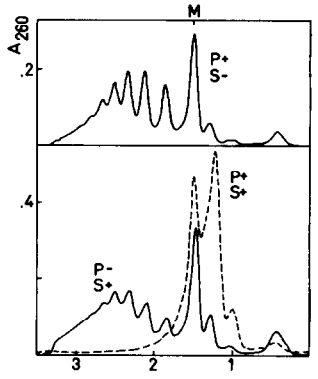

Fig. 1. Sucrose gradient analysis of pelleted (P+) and unpelleted (P−) reticulocyte polysomes, subjected (S+) and unsubjected (S−) to 0.5 M KCl. Enough 4 M KCl was added to polysomes (20 mg/ml) to give a final concentration of 0.5 M. After 15 min, 0.5 ml was transferred to a 2 ml tube and centrifuged for 2 hr at 45,000 rpm [4], while another aliquot was left at +2° for the same time. In the same centrifugation was included a tube of polysomes without KCl. The pellets were redissolved by soaking them in 0.25 M sucrose over night. The preparations were sedimented for 48 min at 60,000 rpm on convex sucrose gradients (3.5 ml in each tube, 2 ml of 0.3 M sucrose per tube in the mixing chamber and 1.4 M sucrose in the reservoir) containing S10/1.5 M marks the monosome position.

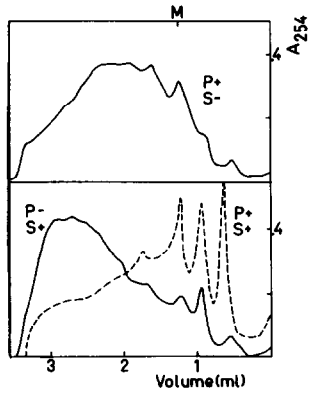

Fig. 2. Sucrose gradient analysis of rat liver polysomes exposed to 0.5 M KCl and/or high speed centrifugation. The treatments and symbols are as for fig. 1, except that the pellets were dissolved in S300/2 containing 0.25 M sucrose, 1 mM dithiothreitol. The preparations were sedimented for 60 min at 56,000 rpm on 3.8 ml concave sucrose gradients of the above composition (2.5 ml 2 M sucrose per tube in the mixing chamber, buffer added from the reservoir).

The combinations of cations used will be referred to as follows: 10 mM KCl, 1.5 mM $MgCl_2$, S10/1.5; 300 mM KCl, 2 mM $MgAc_2$, S300/2; 500 mM KCl, 1 mM $MgAc_2$, S500/1; 150 mM KCl, 1 mM $MgAc_2$, S150/1; 150 mM KCl, 4 mM $MgAc_2$, S150/4; and 100 mM NH_4Cl, 5 mM $MgAc_2$, S100/5.

3. Results

Fig. 1 shows reticulocyte polysomes that have been subjected to combinations of salt (S) and centrifugation (P) treatments. The ionic condition of this gradient centrifugation prevents polysomal dissociation. A previous exposure of the polysomes to high speed centrifugation in the presence of 0.5 M KCl and no added Mg^{2+}, however, causes a dramatic decay to subunits and monosomes. Some dimers of the small subunit also appear on the gradient. The control curves demonstrate that pelleting in the absence of KCl, or incubation with KCl without pelleting gives good polysome profiles. These were not significantly different from those of untreated polysomes.

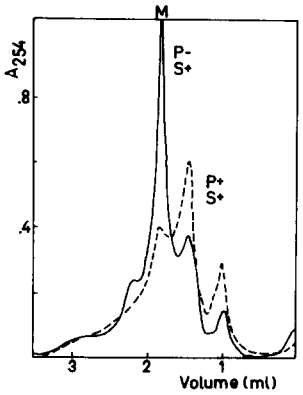

Fig. 3. Sucrose gradient analysis of single, complexed ribosomes exposed to 0.5 M KCl alone or 0.5 M KCl with high speed centrifugation. 240 µl of ribosomes were overlayered with 3.5 ml mineral oil and centrifuged at 56,000 rpm in the SW 56 Ti head from 20 min. The pellet was immediately dissolved in the medium given in fig. 2, and analyzed by centrifugation for 90 min in the concave gradients.

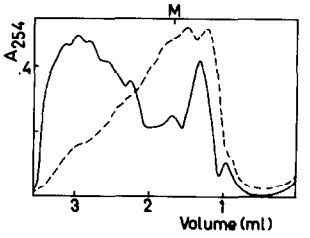

Fig. 4. Polysome breakdown at high K^+, low Mg^{2+} in a sucrose gradient. 200 µl of polysomes were made 0.5 M in KCl and passed through a 0.8 ml column of Sephadex G-25 equilibrated with S500/1 containing 1 mM mercaptoethanol. The preparation was then subjected to gradient centrifugation as in fig. 2, except that one tube contained S500/1 (- - - -).

An analogous experiment for rat liver polysomes is recorded in fig. 2. The K^+ and Mg^{2+} concentrations used in the analytical gradient are known to dissociate free ribosomes but not polysomes [7]. The somewhat lower degree of degradation here compared to the reticulocyte experiment could be due to a difference in the amount of bound Mg^{2+}. Contrary to what has been observed by other workers using chelating agents [11], mild dissociation preferentially releases the small subunit.

The dissociation induced by centrifugation is, as expected, not linked to intact polysomal structures per se. Fig. 3 gives data obtained with single, complexed ribosomes, and demonstrates an extensive transformation of monosomes to subunits. The preponderance of 40S liberation is here not very marked. The same was found for polysomes treated in an identical manner. This may be related to the higher pressure generated by the oil overlay.

It would be desirable to demonstrate on a gradient what is thought to take place during the pelleting. For this purpose polysomes were equilibrated by gel filtration with S500/1. Polysomes are under these conditions slightly labile, as revealed by a subsequent gradient analysis under nondissociating conditions (fig. 4, full line). Gradient centrifugation with the same ionic conditions as in gel filtration shows, however, a much more extensive breakdown.

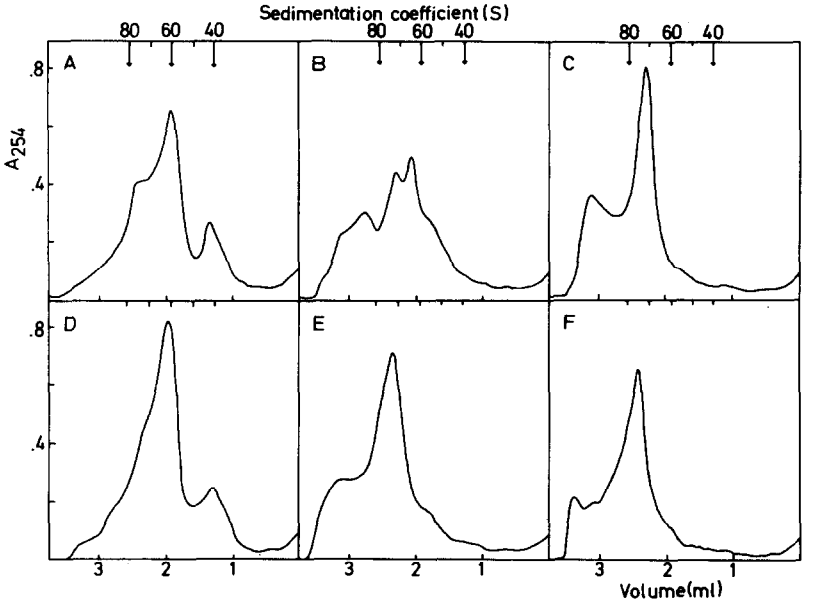

Fig. 5. Dependence of sedimentation profile of free ribosomes on speed of centrifugation. Free ribosomes were sedimented on isokinetic [12] gradients (1 M sucrose in reservoir, 3.7 ml per tube of 0.44 M sucrose in the mixing chamber) at 6° for 75 min at 56,000 rpm (A, B, C) or for 300 min at 28,000 rpm (D, E, F). A and D had S150/1, B and E S150/4, C and F S100/5 in addition to 1 mM mercaptoethanol. Sedimentation constants were assigned based on the position of complexed ribosomes in a separate tube, its S-value assumed to be 80S.

In order to test for possible effects of the centrifugation pressure on the dissociation of free ribosomes, ionic conditions were sought which would give an intermediate profile on high speed zone centrifugation. S150/4 is one such condition (fig. 5B), while S150/1 causes essentially full dissociation, and S100/5 full association [7] (fig. 5A and C). If the dissociation is pressure dependent, centrifugation at lower speed and longer time, keeping $\omega^2 t$ constant, should affect the ribosome distribution more under the first condition than under the two latter. This was indeed found to be the case (fig. 5D, E and F). During the lower speed of centrifugation the free ribosome appeared relatively stable in S150/4. It was furthermore found to sediment with an S-value near 70S, in agreement with an observation of Staehelin and Falvey [13]. At the lower speed and higher $[Mg^{2+}]$ it appears to tighten up, moving now as a 75S particle (fig. 5F). During high speed centrifugation a larger peak and a pronounced shoulder appear behind the 70S peak, probably reflecting the more vigorous breakdown of the ribosome as it moves down the gradient under these conditions, encountering the greater, and rapidly increasing hydrostatic pressures. The same general picture appeared on comparing 6 hr centrifugation at 28,000 rpm with 1½ hr at 56,000 rpm.

4. Discussion

The data presented lead to the conclusion that hydrostatic pressures developed during ultracentrifugation can affect ribosomal stability. This effect appears under ionic conditions which approach the stability limits for complexed, respectively free ribosomes. Polysomes suspended in 0.5 M KCl were found stable, presumably due to Mg^{2+} bound during their preparation. But under the high pressure generated during ultracentrifugation this Mg^{2+} is not sufficient for stability. The analytical ultracentrifuge patterns of Yang et al. [5] actually reveal intermediate stages of breakdown of the polysomes. Zone sedimentation profiles for ribosomes must clearly be interpreted with caution if the ionic conditions do not greatly favour either association or dissociation. Peaks in the profile may not always represent single molecular species. Recentrifugation at a lower speed is then a good test. Analytical ultracentrifugation also suggests itself because of the possibility of several consecutive scans. Until further data of this kind are available, existence of the free mammalian ribosome as a 70S species must be regarded with some reserve. The apparent tightening up at S100/5 could simply mean delayed breakdown of 80S.

$\Delta\bar{V}$ for the formation of *E. coli* ribosomes from subunits as calculated from the mean values of $\bar{v}$ and molecular weights determined by Hill et al. [14] is 140 l/mole. This suggests a pressure sensitivity in the same direction as for liver ribosomes. The real value could be several orders of magnitude lower and still explain phenomena such as those described in this paper. Inserting a $\Delta\bar{V}$ of 0.14 l/mole in the relationship between $K(x)$, the equilibrium constant at distance x from the rotation axis, and $K(x_0)$ for the top of the gradient [3],

$$\ln K(x) = \ln K(x_0) - (\Delta\bar{V}/RT)\,\rho\,(\omega^2/2)(x^2 - x_0^2)$$

one obtains for the SW 56 Ti head at 56,000 rpm a ratio of constants at the top and the bottom of the tube of 10^5. The pressure difference established is 1500 atm. It is not certain, however, that $\bar{v}$ values for ribosomes obtained at 1 atm, even if they could be measured with the required accuracy, would apply at these high pressures. Measurement of equilibria in the centrifuge may indeed be the only way to obtain the relevant $\Delta\bar{V}$ for ribosomal dissociation.

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