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On the Heterogeneity of Eukaryotic Ribosomal Subunits *in Vitro*

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The circumstances in which monomers and dimers of eukaryotic small and large ribosomal subunits occur were examined by measuring the sedimentation profiles of each subunit with glutaraldehyde fixation. When small ribosomal subunits were prepared from rat liver ribosomes in the medium containing 50 mM Tris-HCl (pH 7.6), 880 mM KCl, 12.5 mM $MgCl_2$, 10 mM mercaptoethanol and 0.1 mM puromycin, about 41.4% of the preparations sedimented as a monomer and the other as a dimer in $T_{10}K_{20}M_5$ (10 mM Tris-HCl, pH 7.6; 80 mM KCl; 5 mM $MgCl_2$) buffer. There were no difference in the monomer content of small ribosomal subunits from free and membrane bound ribosomes from rat liver. Moreover, small ribosomal subunits from the muscle of normal and diabetic rats contained just the same proportion of the monomer and dimer particles when assayed under the same ionic conditions. The proportion of the monomer and the dimer of the small ribosomal subunits changed with change in the concentration of potassium and magnesium at which ribosomal subunits were prepared; the content of the monomer decreased with lowering the ratio of magnesium concentration and was only 10.1% when the preparation was conducted in the medium containing 880 mM KCl and 3 mM $MgCl_2$. The addition of poly U to the small subunit preparations caused the conversion of the dimer into the monomer. Large ribosomal subunits dimerized gradually at low temperature even in high potassium concentration but the dimers of the large subunits dissociated easily into their monomers by incubation at 30°.

The samll and large subunits of eukaryotic ribosomes can occur *in vitro* as either monomers and dimers.[2,3] Wettenhall, Wool and Sherton[4] have reported that about 35% and 65% of the preparations of small subunits of rat liver ribosomes were monomers (40 Sm) and dimers (40 Sd), respectively, and that two species are physically and functionally distinct; the monomer contains more of low molecular weight RNA and less active than the dimer in the binding of aminoacyl-tRNA catalyzed by eukaryotic initiation factor 1(EIF-1) and in the synthesis of polyphenylalanine in the presence of large ribosomal subunits. However, the physiological significance of the heterogeneity of the ribosomal subunits is unknown.

The present study was undertaken to examine the circumstances in which the monomers and dimers of small and large subunits of rat liver ribosomes occur. Since previous studies have paid little attention to the effect of hydrostatic pressure during centrifugation on the distribution of ribosomal subunits, glutaraldehyde fixation was used to measure the distribution. Using this method, effect of the preparative procedures on the occurrence of the monomers and dimers were examined, and, finally, the distributions of small subunits from free and membrane bound ribosomes and also from ribosomes of diabetic animals were compared.

Materials and Methods

Rat liver ribosomal subunits were prepared by suspending ribosomes in Medium A and incubating for 15 min at 37° with 0.1 mM puromycin; the subunits were separated on linear sucrose gradients.[5] The subunit fractions were dialyzed against Medium B and precipitated with 0.2 volume of ethanol. The ethanol-pre-

1) Location: *1-5-8, Hatanodai, Shinagawa-ku, Tokyo.*
2) T.E. Martin, F.S. Rolleston, R.B. Low, and I.G. Wool, *J. Mol. Biol.*, **43**, 135 (1969).
3) Y. Nonomura, G. Blobel, and D. Sabatini, *J. Mol. Biol.*, **60**, 303 (1971).
4) R.E.H. Wettenhall, I.G. Wool, and C.C. Sherton, *Biochemistry*, **12**, 2403 (1973).
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cipitated subunits were suspended in a small volume of Medium B and can be stored for 2 days without deleterious effect such as loss of ribosomal particles on the sucrose gradient. The fixation of the subunits with glutaraldehyde was done by a slight modification of a procedure described before[6] The subunits (4.28 μg of rRNA) were incubated in 100 μl of a solution with various ionic conditions containing 0.29 mg of bovine serum albumin. The albumin was added to prevent loss of the subunits during glutaraldehyde fixation. After incubation the samples were cooled in ice and immediately fixed with 25 μl of 5% glutaraldehyde unless otherwise mentioned. After the fixation, an 80 μl sample was layered onto a 5.2 ml linear 10 to 30% sucrose gradient and centrifuged in a RPS 50 rotor at 45000 rpm for 100 min at 4°. The distribution of ribosomal particles in the gradient was determined with an ISCO density gradient fractionator and ultraviolet analyzer.[5,6] The amount of ribosomal particles was estimated by cutting out and weighing subunit peaks of the optical density tracing. The concentration of ribosomal subunits was taken to be equivalent of 45 μg of rRNA.[7] The recovery of the ribosomal subunits on the gradient was estimated by counting the radioactivity of ribosomal subunits phosphorylated by protein kinase with [γ-^{32}P]ATP as substrate.[8] Phosphorylation of the ribosomal subunits had no effect on the function of the subunits as reported by Eil and Wool.[9]

The media used were 50 mM Tris-HCl (pH 7.6), 880 mM KCl, 12.5 mM $MgCl_2$, 10 mM mercaptoethanol (Medium A); 50 mM Tris-HCl (pH 7.6), 80 mM KCl, 12.5 mM $MgCl_2$, 10 mM mercaptoethanol (Medium B). $T_xK_yM_z$ means the medium which contains x, y and z concentrations in mM of Tris-HCl (pH 7.6), KCl and $MgCl_2$, respectively.

Results and Discussion

Figures 1a and 1b show the sedimentation profiles of small and large ribosomal subunits, respectively, in $T_{10}K_{300}M_3$ medium at 0° without any preincubation at higher temperatures. Throughout the experiments the fixation and subsequent centrifugation were conducted at low temperature to avoid loss of ribosomal particles. As is evident from these curves, small ribosomal subunits sedimented as a monomer with a single symmetrical peak of 40S under these ionic conditions, whereas large ribosomal subunits sedimented as a dimer (60 Sd) having sedimentation coefficient of 90.[2] Moreover, an appreciable number of the large subunits analyzed in that way seemed to be aggregated as judged from the low recovery of the subunits on the gradient. Since the large subunits have a tendency to dimerize at low temperature,[2] the large subunits were preincubated at 30° for 10 min in order to disaggregate dimers and aggregates. After the preincubation the sample was chilled to 0° and fixed immediately. The large subunits thus treated sedimented as a monomer (60 Sm) as shown in Fig. 1c. The amount of dimers of the large subunits increased gradually if the subunits once preincubated at 30° were kept at 0° for 5 min (Fig. 1d) or 1h (Fig. 1e) prior to the glutaraldehyde fixation. It is evident from these results that the incubation of the large subunits at low temperature caused dimerization of 60 Sm particles. In contrast, the distribution of small ribosomal subunits was not affected at all by the incubation at 0°; the small subunits preincubated at 30° for 10 min presented exactly the same pattern as in Fig. 1a. In further experiments, therefore, ribosomal subunits were preincubated at 30° for 10 min, cooled in ice and then fixed immediately. Small and large ribosomal subunits thus analyzed gave more than 95% recovery.

Since the dimerization of ribosomal subunits was known to be conditioned by the concentrations of potassium and magnesium ions,[2-4] the effect of these ionic conditions on the distribution profile was studied with more precision. About 60% of small ribosomal subunits sedimented as a dimer in $T_{10}K_{80}M_5$ medium as shown in Fig. 2a. As can be seen from Figures 2b and 2c, 40Sd particles were converted to the monomer with increasing concentrations of potassium (the concentration of magnesium was kept at 3 mM). Complete dissociation of 40Sd particles into 40Sm was achieved only when the potassium concentration was kept at

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7) I.G. Wool and P. Cavicchi, *Proc. Natl. Acad. Sci.*, **56**, 991 (1966).
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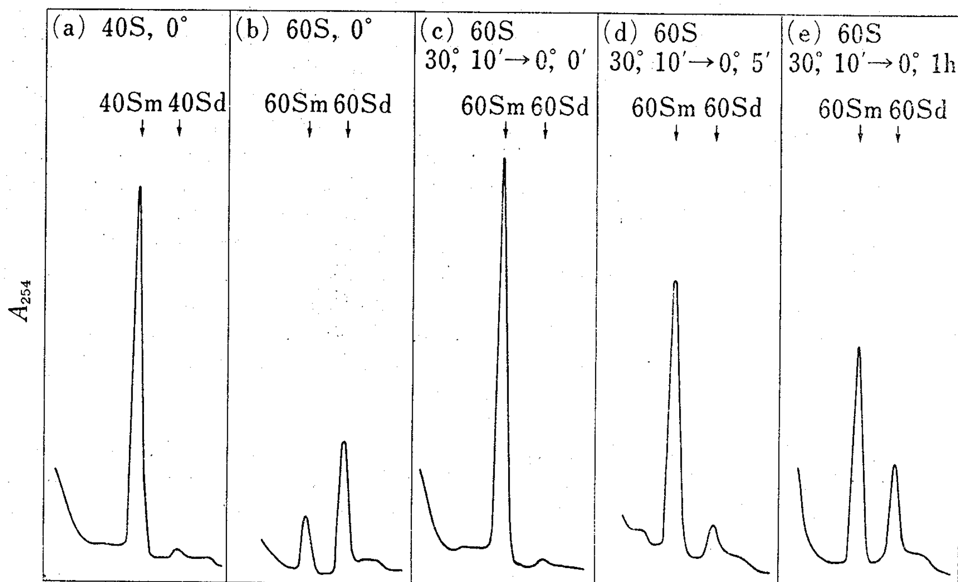

Fig. 1. Effect of the Temperature on Distribution of Small and Large Ribosomal Subunits

Preparations of small(a) and large(b) subunits (4.28 μg of rRNA) were suspended in 100 μl of $T_{10}K_{800}M_3$ medium at 0° and fixed with 25 μl of 5% of glutaraldehyde solution. In (c—e) the large subunits were preincubated in the same medium at 30° for 10 min. After the preincubation, the samples were cooled to 0° for 0 min(c), 5 min(d) or 1h(e) and then fixed with glutaraldehyde. A sample (80 μl), which corresponds to 0.06 optical density at 260 nm, was layered on a 10 to 30% sucrose gradient in $T_{10}K_{800}M_3$ medium. The gradients were centrifuged at 4° for 100 min at 45000 rpm in a RPS 50 rotor and the sedimentation of the particles was determined with and ISCO gradient fractionator and ultraviolet analyzer.

300 mM (Fig. 1a). Large ribosomal subunits did not dimerize in medium containing 80 mM potassium and 5 mM magnesium (Fig. 2d). However, the recovery of the large subunits in this medium was lower when compared with those in higher potassium concentrations (compare Figures 2d and 2e or 2f). The results indicate that the large subunits aggregated in the medium containing low potassium concentration and was sedimented during centrifugation, thereby leading to a decrease in the amount of material contained in the gradient. The recovery of large ribosomal subunits in the sucrose gradient was increased to more than 95% when the potassium concentration was raised to 120 mM (Fig. 2e) or 200 mM (Fig. 2f).

The distribution of the small subunits on the gradient was different if glutaraldehyde fixation was omitted. Without glutaraldehyde fixation the small subunits in $T_{10}K_{120}M_3$ medium sedimented as a single species, whereas an appreciable number of the small subunits in the same medium sedimented as a dimer when fixed with glutaraldehyde (Fig. 3). The possibility that the dimer might be produced artificially by the glutaraldehyde fixation was excluded because no dimerization was observed when the potassium concentration was raised from 120 mM to 300 mM (Fig. 1a). Considering the effect of hydrostatic pressure during centrifugation, it would seem that the 40Sd particles formed in $T_{10}K_{120}M_3$ medium without glutaraldehyde fixation dissociated during the centrifugation.

Although the sample was analyzed immediately after the fixation, there is a possibility that the distribution of the ribosomal subunits might be affected by the reaction between Tris and glutaraldehyde. In fact, the pH of the reaction mixture changed slowly with time due to the reaction. To avoid this effect, HEPES (N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonate) buffer, which has no amino groups reactable with glutaraldehyde, was tested in place of Tris buffer. The distribution of each small and large ribosomal subunits in HEPES buffer was exactly the same as that in Tris buffer if the potassium and magnesium concentrations were the same. However, the recovery of the subunits in the gradient was reduced considerably by an unknown reason (data not shown). Therefore, Tris buffer was preferred to HEPES buffer.

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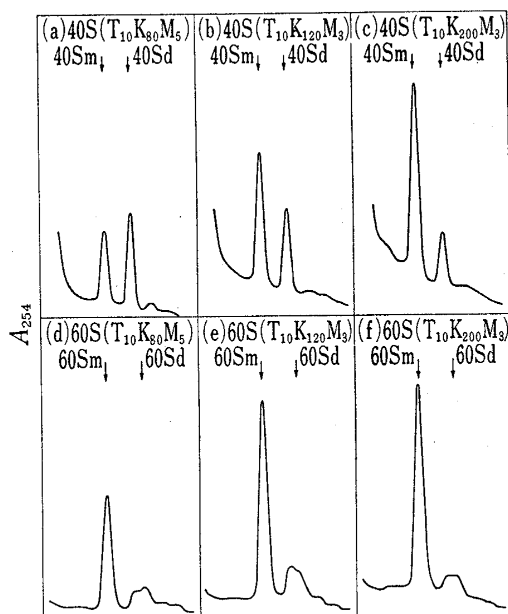

Fig. 2. Effect of the Concentrations of Potassium and Magnesium Ions on the Sedimentation of Small and Large Ribosomal Subunits

Preparations of the small and large subunits were suspended in various media specified. After preincubation at 30° for 10 min, the samples were cooled to 0°, fixed immediately with glutaraldehyde and analyzed as in Fig. 1.

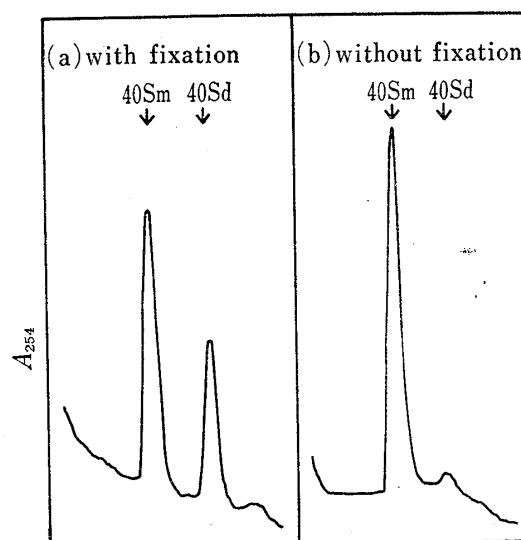

Fig. 3. Effect of Glutaraldehyde Fixation on the Distribution of Small Ribosomal Subunits in Sucrose Gradients

In (a) the small subunits in $T_{10}K_{120}M_3$ medium were fixed with glutaraldehyde and analyzed as in Fig. 2. In (b) the conditions were the same except that glutaraldehyde fixation was omitted.

As reported by Wettenhall, *et al.*,[4] 40Sm species isolated in 80 mM potassium contains more of low molecular weight RNA such as deacylated tRNA than 40Sd. Analysis by gel electrophoresis revealed that the low molecular weight RNA contained fragments of RNA, which are probably derived from mRNA or rRNA, in addition to deacylated tRNA (result not shown). If these low molecular RNA species, either deacylated tRNA or RNA fragments or both, are responsible for the occurrence of the 40Sm and 40Sd particles, removal of the low molecular weight RNA from 40Sm particles or binding them to 40Sd particles would result in the conversion of 40Sm to 40Sd or *vice versa*. However, these low molecular weight RNA species were tightly bound to 40Sm particles and could not be removed by washing 40Sm particles 5 times in 880 mM potassium and 3 mM magnesium or by washing in 3M urea. While the addition of deacylated tRNA to 40Sd particles did not cause disaggregation of the dimer, poly U was effective in causing conversion of 40Sd to 40Sm as shown in Fig. 4. With increasing the amount of poly U, 40Sd particles were converted to 40Sm and 59% conversion was achieved by addition of 100 μg of poly U (Fig. 4). The increase of the base line, especially near the top of the gradient, was due to the presence of poly U in the sample. The disaggregation of the 40Sd particles by poly U was completely prevented by the addition of 0.1 mM aurintricarboxylic acid, which is an inhibitor of the attachment of mRNA to ribosomes. These results support the possibility that low molecular weight RNA species prevent the dimerization of the small subunits by blocking the site at which they interact or by inducing a conformational change unfavorable for dimerization.

The crucial question is whether the existence of dimers and monomers of small ribosomal subunits has any physiological importance or is merely a reflection of the preparative procedures. To examine which is the case, the ribosomal subunits were prepared under various ionic conditions. The proportion of 40Sm and 40Sd particles was changed with change in the concentration of magnesium (or potassium if the magnesium concentration was kept constant) at which the ribosomal subunits were prepared (Table I). When the ribosomal

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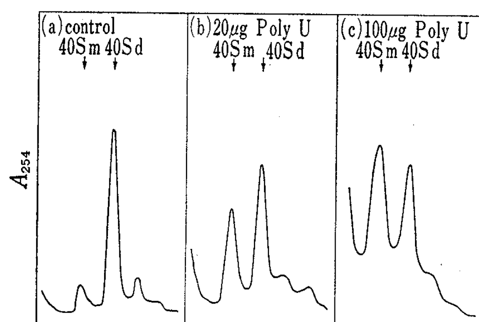

Fig. 4. Effect of Poly U on the Sedimentation of the Small Subunit Dimers

The small ribosomal subunits were separated into 40Sm and 40Sd fractions by centrifugation in sucrose gradient containing 80 mM potassium and 5 mM magnesium. The 40Sd fraction thus obtained was suspended in $T_{10}K_{80}M_5$ medium and mixed with poly U specified. The suspension was incubated for 10 min at 30° and analyzed as in Fig. 2.

Table I. The 40Sm Content of Small Ribosomal Subunits

Concentration of $MgCl_2$	40Sm content
12.5 mM	41.4%
5.0 mM	18.8%
3.0 mM	10.1%

Small ribosomal subunits were prepared with puromycin in media containing 50 mM Tris-HCl (pH 7.6), 880 mM KCl and $MgCl_2$ specified. The small subunit fraction thus obtained was suspended in $T_{10}K_{80}M_5$ medium and analyzed as in Fig. 2.

subunits were prepared in 880 mM potassium and 12.5 mM magnesium, 41.4% of the small subunits sedimented as 40Sm particles in $T_{10}K_{80}M_5$ medium. The 40Sm content decreased with lowering the magnesium concentration and was only 10.1% when preparation was conducted in 3 mM magnesium (the concentration of potassium was kept at 880 mM). It is inferred from these results that some of low molecular weight RNA which are responsible for the occurrence of the two forms of the small subunits were removed by the exposure to high concentration of potassium and low concentration magnesium during the preparative procedures. However, it was impossible to use medium containing magnesium lower than 3 mM, because a fraction of the population of the small subunits decomposed into smaller subparticles having sedimentation coefficient of 32. If the magnesium concentration was raised more than 12.5 mM, the separation of the small and large ribosomal subunits was incomplete. These result indicate that the occurrence of the two forms of the small subunits are at least partly a reflection of the preparative procedures and is not likely to be of physiological significance. This view was further supported by the following findings. There were no difference in the 40Sm content of small ribosomal subunits from free and membrane bound ribosomes from rat liver. Moreover, in spite of the fact that ribosomes from the muscle of diabetic animals are less active than normal in protein synthesis *in vitro*,[10] small ribosomal subunits prepared from normal and diabetic animals contained just the same proportion of 40Sm and 40Sd particles when assayed under the same ionic conditions. It is less likely that the dimer of the large ribosomal subunits can occur *in vivo*, because dimerization of the large subunits could not be observed at 30° even in medium containing low potassium concentration (80 mM).

Finally it needs to be noted that one must be careful of the ionic conditions of the preparative procedures of ribosomal subunits. If ribosomal subunits are prepared in different concentrations of potassium and magnesium, the proportion of 40 Sm and 40Sd will be different, thus resulting in the change in the activities of reconstituted ribosomes. Faust and Matthaei[11] showed a wide variation in the properties of ribosomal subunits from murine myeloma tumors in change with the concentrations of potassium and magnesium at which the ribosomal subunits were prepared.

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