Molecular Weight, Buoyant Density, and Composition of Active Subunits of Rat Liver Ribosomes*

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ABSTRACT: Active subunits obtained by dissociation of rat liver ribosomes with urea (Petermann, M. L., and Pavlovec, A. (1971), *Biochemistry 10*, 2770) have been characterized and compared to inactive subunits obtained by EDTA dissociation (Hamilton, M. G., and Ruth, M. E. (1969), *Biochemistry 8*, 851). Molecular weight has been measured by equilibrium centrifugation and composition by chemical analysis and by isodensity equilibrium centrifugation. Both forms of the large subunit had about the same molecular weight, 3 million, and the same protein content, 45%. The active small subunit, however, had a considerably higher molecular weight (1.5 million) and a higher percentage of protein (55%) than the inactive form, which had a molecular weight of 1.2 million and contained 50% protein. The excess mass consisted of "rapidly labeled" RNA and protein; when treated with EDTA, the active form lost this RNA, but since its buoyant density did not increase, apparently very little protein dissociated from the subunit. The active small subunit obtained by dissociation with KCl (Martin, T. E., and Wool, I. G. (1969), *J. Mol. Biol. 43*, 151) was similar in size and composition to the one obtained by the urea method, but seemed to be less stable.

When rat liver ribosomes are dissociated by reducing their magnesium content and exposing them briefly to urea, the subunits can reassociate to monomers and can synthesize polyphenylalanine if given poly(U) as a messenger (Petermann and Pavlovec, 1971). Active subunits are also obtained when ribosomes are dissociated by a high concentration of KCl (Martin and Wool, 1969). In contrast, the subunits obtained when magnesium ions are complexed by EDTA do not reassociate well and are inactive. In this paper we compare the active subunits obtained by the urea method with the EDTA subunits which we have already characterized (Hamilton and Ruth, 1969). Some measurements have also been made on the small subunit obtained by the KCl method.

Materials and Methods

Liver ribosomes from normal adult male rats were isolated and purified by the method of Petermann and Pavlovec (1967). To increase the efficiency of dissociation, monosomes were prepared by an ethanol treatment (Haga *et al.*, 1970). The ribosomes were dissociated by reducing their magnesium content by dialysis and exposing them to 2 M urea for 30 min. After a fast dialysis the subunits were separated by zonal centifugation (Petermann, 1971). To obtain small subunits from KCl-dissociated ribosomes, the method of Martin and Wool (1969) was followed except that 2.5 mM potassium phosphate, pH 7.3, and 1 mM DTT[1] were substituted for Tris-HCl and β-mercaptoethanol. The isolated subunits were fixed with formaldehyde by dialysis for 1 day in the cold against 2 M HCHO (6%)–0.2 mM $MgCl_2$–3 mM potassium phosphate, pH 7.4, and then for a second day or longer against 0.1 M HCHO–0.03 M KCl–0.2 mM $MgCl_2$–0.01 M triethanolamine-HCl, pH 7.2. The techniques of velocity and equilibrium ultracentrifugation and chemical analysis have been described (Hamilton and Ruth, 1969). Additional technical details are given in the figure legends.

Results

Table I summarizes the results of the various measurements.

Molecular Weights. In velocity centrifugation the formaldehyde-fixed small subunit usually appeared monodisperse, but a few samples contained as much as 15% of the dimer, which sediments at 60 S. Experiments with artificial mixtures showed that the dimer contamination was negligible at the speed of centrifugation used in the equilibrium runs. Molecular weight measurements were made on 11 preparations. While 7 of the values were very close to the average, one was much higher, 1.69, and 3 were much lower (1.36, 1.41, 1.42). These results cannot be discarded for any technical reason. Plots of ln c *vs.* r^2 were linear and point-average molecular weights across the cell were constant.

The fixed large subunit also appeared monodisperse in velocity sedimentation, but the addition of 0.1 volume of 0.1 M Na_3EDTA before fixation revealed the presence of 10–15% of small subunits which had dimerized and cosedimented with the large subunit in the initial separation. Since the molecular weight of a dimerized small subunit is 3 million, its presence does not seriously affect the result obtained for the large subunit. One sample which contained only 7% of small-subunit dimers was examined by interference and schlieren optics. The weight-average molecular weight calculated from the interference pattern was 3.19 million (standard error of the slope, 0.01), while the z-average molecular weight calculated from the schlieren pattern was 3.2 million (standard error, 0.06).

Isodensity Equilibrium Centrifugation. Figure 1 shows the band patterns of the fixed small subunit obtained in the analytical ultracentrifuge. In CsCl (Figure 1A) the band was broad, and the buoyant density corresponded to a protein content

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[1] Abbreviation used is: DTT, dithiothreitol.

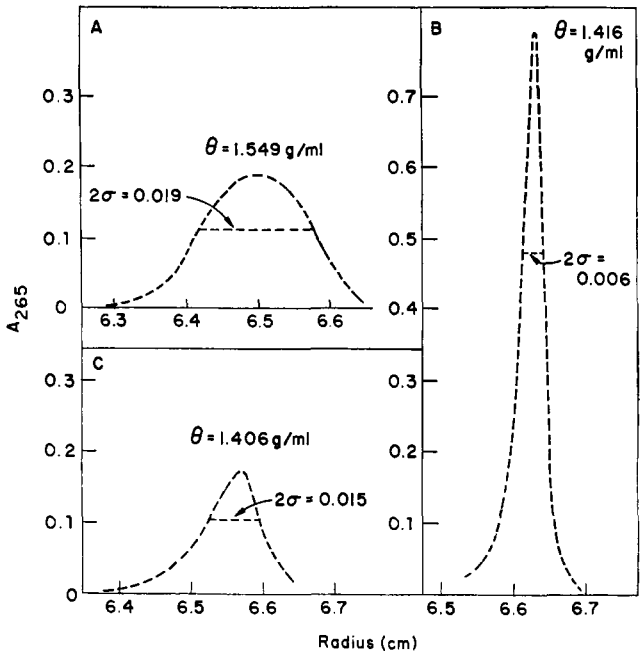

FIGURE 1: Isodensity equilibrium centrifugation of the small subunit in the analytical ultracentrifuge. The subunits were fixed as described in Methods. The photoelectric scanner patterns obtained at 265 mμ have been converted to plots of A_{265} against radius. θ is the buoyant density at the band center, and 2σ is twice the standard deviation of the band in density units. A, CsCl gradient; B, Cs_2SO_4 gradient; C, Cs_2SO_4 gradient; the sample was treated with 0.01 M Na_3EDTA before fixation.

which agreed well with the chemical analysis (see Table I). In Cs_2SO_4, however, the small subunit formed a much narrower band (Figure 1B), and its buoyant density did not correspond to its protein content. After EDTA treatment the band width increased greatly both in CsCl (not shown) and Cs_2SO_4 (Figure 1C).

For the large subunit, the buoyant density in CsCl corresponded approximately to the protein content obtained by chemical analysis (Table I). Unlike the small subunit, however, the large subunit formed a broad band in Cs_2SO_4 as well as in CsCl.

Material Released from the Small Subunit by EDTA. Figure 2A shows the distribution of the small subunit containing ^{3}H-labeled RNA in a preparative CsCl gradient. Most of the radioactivity coincided with the optical density; there was no band at the "informosome" density, 1.40 g/ml. With EDTA treatment (Figure 2B) most of the label was displaced to a higher density, 1.72 g/ml; the subunit density was unchanged. Apparently some ribosomal RNA was labeled.

Properties of the Small Subunit Obtained by Dissociation in KCl. The KCl small subunit showed a great tendency to dimerize. In a buffer, 0.03 M KCl–0.2 mM $MgCl_2$–1 mM potassium phosphate, pH 7.2, where the urea small subunit contained only 10% of dimers, the KCl subunit was 60% dimerized. In one case, after dialysis against 0.01 M KCl, 0.2 mM $MgCl_2$, 1 mM DTT, 1 mM potassium phosphate, pH 7.8, it remained undimerized. A single measurement of its molecular weight gave 1.5 million. It formed a single band in CsCl at a density of 1.549 g/ml, but in Cs_2SO_4 it displayed a complex pattern with components at densities of 1.400, 1.410, and 1.417 g/ml. It was damaged by freezing in 0.88 M KCl and was generally less stable than the urea subunit, readily chang-

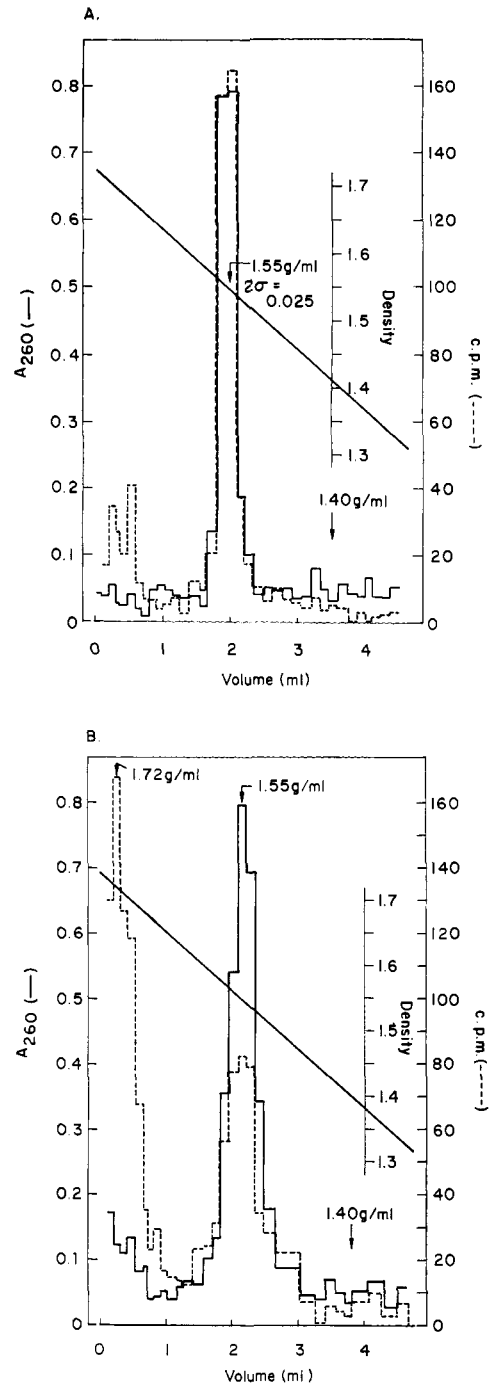

FIGURE 2: Isodensity equilibrium centrifugation in CsCl of the small subunit containing ^{3}H-labeled RNA. Each rat was injected intraperitoneally with 0.15 mCi of [^{3}H]orotic acid 40 min before sacrifice. The ribosomes were dissociated by urea. The isolated small subunits were concentrated by pelleting and after resuspension the solution, containing about 6 A_{260} units, was divided into two parts. One was fixed immediately (see Methods). The other was made 0.01 M in Na_3EDTA, then fixed. After the final dialysis, the samples were transferred to tubes and 2.5 ml of saturated CsCl was added. The final volume was 4.8 ml. Each tube was filled with mineral oil, and centrifuged for 3 days at 20° at 40,000 rpm in a SW 50.1 rotor. Fractions were collected dropwise from the bottom of the tube. The absorbance at 260 mμ was measured and radioactivity was assayed by liquid-scintillation spectrometry. The densities of samples near the top and bottom of the tube were calculated from the refractive indices. A, control; B, after EDTA treatment.

TABLE I: Physical and Chemical Properties of Active Subunits of Rat Liver Ribosomes and the Subunits Obtained by EDTA Treatment.[a,b]

Subunit	Sedimentation coefficient, $s_{20,w}$ (S)	Molecular weight[c] ($\times 10^{-6}$)	Buoyant density in CsCl, θ (g/ml)	Protein Content	
				From $1/\theta$[d] (%)	From Chemical Analysis (%)
Active small	40.9 (0.3, 4)	1.50 (0.03, 11)	1.551 (0.002, 10)	55	54.5 (0.67, 3)
Active large	59.1 (0.5, 3)	3.00 (0.08, 5)	1.614 (0.002, 12)	43	44.7[e]
EDTA small	28.6[f]	1.18 (0.04, 9)	1.593 (0.002, 9)	47	49.5 (2.06, 3)
EDTA large	49.9[f]	3.12 (0.02, 5)	1.602 (0.002, 8)	45	44.7 (0.97, 3)

[a] All measurements except the chemical analyses were made on formaldehyde-fixed samples. The standard error of the mean and the number of determinations are given in parentheses. [b] Data on the EDTA subunits are reproduced from Hamilton and Ruth (1969). [c] Solutions containing about 0.6 A_{260} unit per ml were centrifuged in 2–3 mm columns at 5200 rpm (small subunit) or 3000 rpm (large subunit) for at least 3 days in the cold in an ultracentrifuge equipped with photoelectric scanner–absorption optics. A few samples of the large subunit were also centrifuged in an ultracentrifuge equipped with interference optics at higher concentrations, 2 and 4 A_{260} units per ml. Partial specific volumes of 0.645 for the small subunit and 0.623 for the large subunit were calculated from the chemical composition (see Discussion). The density of the solvent, 0.1 M HCHO–0.03 M KCl–0.2 mM $MgCl_2$–0.01 M triethanolamine-HCl, pH 7.2, was measured in a 3-ml pycnometer at 5°. [d] From a calibration line given in Hamilton (1971). [e] Single determination; the value has been corrected for the presence of 15% of small subunits. [f] $s^0_{20,w}$ from a study of sedimentation coefficient as a function of concentration on unfixed samples in 0.03 M KCl–0.2 mM $MgCl_2$–0.001 M potassium phosphate, pH 7.0.

ing to a 30S form with a buoyant density in CsCl of 1.575 g/ml (50% protein).

Discussion

The homogeneity of ribosomal subunits is difficult to establish. A subunit solution may appear to be monodisperse in sedimentation velocity and yet be contaminated by dimerized or unfolded forms of the other subunit. Monodispersity in sedimentation velocity after the addition of EDTA, which causes the large and small subunits to sediment at different rates, appears to be a good test for such contamination (Petermann and Pavlovec, 1971). By this criterion, our small subunits were homogeneous, although our large subunits were contaminated by dimerized small ones.

In the sedimentation equilibrium method for molecular weight, constancy in M_{app} across the cell is a test of homogeneity. In our experiments that condition was met, but the plots were noisy. For this reason, we have presented replicate values (Table I) rather than detailed mathematical analyses of individual runs. The method requires a value for the partial specific volume, $\bar{v}$. Because we recovered too little material to measure $\bar{v}$ (about 2 mg per zonal separation), we have instead calculated it from the chemical composition and values of 0.53 for RNA and 0.74 for protein. For both the EDTA subunits (Hamilton and Ruth, 1969) and the active subunits, this procedure gives molecular weights which are consistent with the sizes of their RNA molecules. Confidence in the use of a calculated $\bar{v}$ is increased by published data on *Escherichia coli* ribosomes. For ribosomes containing 37% protein, the calculated $\bar{v}$ is 0.646, and the measured value was 0.64 (Tissières *et al.*, 1959). For ribosomes containing 33% protein, the calculated $\bar{v}$ is 0.599, and the measured value was 0.596 (Hill *et al.*, 1969, 1970).

Monodispersity in isodensity equilibrium centrifugation may be another criterion of homogeneity. For example, when a 59S fraction is centrifuged in a CsCl gradient the small-subunit dimers, which contain more protein, form a distinct second band. The two EDTA subunits, on the other hand, are not easy to distinguish, because the difference in protein content is less, and the bands are broad. For both types of subunits, however, the buoyant densities in CsCl are consistent with the chemical composition of the subunits. In Cs_2SO_4 gradients, however, only the EDTA subunits behaved as expected, banding at densities that corresponded to their protein contents, provided that the appropriate calibration curve was used (Hamilton, 1971). For the active forms, however, the protein contents calculated from the buoyant densities in Cs_2SO_4 did not agree with chemical analyses. Moreover, the active small subunit, in contrast to the EDTA subunits and the active large subunit, formed a very narrow band in Cs_2SO_4 (Figure 1B). After EDTA treatment, the subunit formed a broader band (Figure 1C) whose density corresponded to 54.9% protein, in agreement with the result obtained in CsCl (see Figure 2B and below). That is, the same protein content was obtained in both Cs_2SO_4 and CsCl only after EDTA treatment. These effects of Cs_2SO_4 cannot yet be fully explained, but are under study.

An apparent solvated molecular weight can be calculated from the bandwidth (Meselson *et al.*, 1957). In the experiment illustrated in Figure 1, $M_{w,app}$ for the small subunit in CsCl was only about one-third of the true weight, while in Cs_2SO_4 it was twice that value. In some Cs_2SO_4 experiments it was as much as five times the true value, as though the material were on the verge of precipitation. This behavior may reflect a feature of the tertiary structure of the active small subunit which differs from that of the other forms, or simply its higher protein content; the formation of very narrow bands is also characteristic of RNA-free ribosomal proteins (M. G. Hamilton, unpublished observation). In electron micrographs the small subunit has an elongated shape (J. Y. Haga, unpublished observation; Nonomura *et al.*, 1971) and tends to form chain-like arrays (D. Sabatini, personal communication).

It is interesting to compare the active subunits with inactive subunits obtained by EDTA treatment of ribosomes. Data on

the EDTA subunits (Hamilton and Ruth, 1969) are reproduced in Table I. The molecular weights of the two large subunits are very similar. The removal of 5S RNA and a small amount of protein by EDTA treatment (Petermann *et al.*, 1971) does not appreciably affect the size or gross composition. The change in sedimentation coefficient corresponds to a change in shape; electron microscopy of shadow-cast preparations (Haga *et al.*, 1970) showed that the EDTA subunit, 50 S, has much less compact than the 59S subunit. For the small subunit, however, the lower sedimentation coefficient of the 30S form primarily seems to reflect its smaller mass; it has a molecular weight of only 1.2 million (see Table I), 300,000 less than that of the active form, and contains only 50% protein. We calculate that it has lost about 220,000 daltons of protein and 80,000 daltons of RNA.

If the extra mass of the active small subunit were detached by EDTA as a single entity it would sediment at 10 to 15 S. We have not observed a particle of that size in the sedimentation pattern of EDTA-treated ribosomes, although such components have been found in the ribosomes of thyroid (Cartouzou *et al.*, 1968) and reticulocytes (Burny *et al.*, 1969). Recently Pragnell and Arnstein (1970) used EDTA to release a 14S ribonucleoprotein from the small subunit of the reticulocyte ribosome which may be a messenger-RNP.

We have tried to identify the extra material in the 40S subunit. One possibility is messenger RNA. When small subunits containing ^{3}H-labeled RNA were treated with EDTA, then pelleted, 10% of the RNA and two-thirds of the radioactivity remained in the supernatant (M. L. Petermann and A. Pavlovec, unpublished experiments). Since the released RNA was rapidly labeled and of high specific activity, some of it may represent messenger RNA, although the reassociated subunits, active in the presence of poly(U), show no endogenous phenylalanine incorporation. Also, as is shown in Figure 2B, since it banded mainly at a density of 1.72 g/ml, it may have some protein (about 25%) associated with it. It did not resemble the low-density ribonucleoprotein released from polysomes (Olsnes, 1971). The mRNA fragment associated with a single ribosome would be only about 30,000 daltons (Warner *et al.*, 1962). Whether some tRNA is also associated with the 40S subunit is now being examined.

We expected that EDTA treatment of the isolated small subunit would convert it to the same particle we had obtained by EDTA treatment of whole ribosomes. That is, we expected to observe an increase in the buoyant density of the small subunit after treatment with EDTA. In the preparative CsCl experiment, however, the density was unchanged, as if equal amounts of protein and RNA had dissociated, although labeled RNA had been released. Thus, when the subunits were fixed shortly after the addition of EDTA, an intermediate form was obtained. We hope to characterize this form more fully. The 30S form of higher density can, however, be obtained if the EDTA-treated subunit is separated from the released material by sucrose-gradient centrifugation, the procedure used for EDTA-treated whole ribosomes.

The presence of EDTA-dissociable mass on the active small subunit may be a special feature of animal ribosome structure, for when bacterial ribosomes are dissociated with EDTA, the small subunit unfolds with essentially no loss of mass (Gesteland, 1966). The change we observed is less extensive than the deproteinization that results from exposing the small subunit to very high salt concentrations at reduced Mg^{2+} ion concentrations (Reboud *et al.*, 1969). The labile proteins may constitute a group of ribosomal proteins that dissociate more readily than the rest of the "structural" proteins. On the other hand, they may represent nonribosomal factors. In contrast to the "dissociation" factor that has been described for bacterial ribosomes (Subramanian *et al.*, 1968), the extra mass on the liver small subunit behaves like an "association" factor, since its presence is required for reassociation of the subunits to active ribosomes.

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