DETERMINING THE AFFINITY IN VITRO OF HEPATIC RIBOSOMAL SUBUNITS FOR DERIVATIVES OF THE ROUGH ENDOPLASMIC RETICULUM

T. Ekren*, T.,. Shires**, and H.C. Pitot

McArdle Laboratory for Cancer Research, The University of Wisconsin Medical School, Madison, Wisconsin 53706

Received July 19, 1973

Summary. By a method employing centripetal floatation of membranes away from sedimenting particles, the binding capacity of pyrophosphate/citrate-treated rough membranes at 3° for large ribosomal subunits has been found to be greater than for small subunits. Accurate comparison of the membrane affinity for the 2 subunits is apparently precluded however by greater instability of the small subunit, even in the presence of ribonuclease inhibitor.

Accurate measurement of the interaction in vitro of ribosomal subunits with biomembranes relies on the development of methods for quantitative separation of those particle populations which attach to membranes from those which do not attach. Established methods for this separation have employed high speed centrifugation of a membrane-polysome mixture in sucrose (d=1.062, 5°) overlying a layer of 1.3031 g/ml (1,2). The denser layer of sucrose permitted the pelleting of unbound polysomes, while membranes together with their attached polysomes were caught at the junction of the two sucrose densities. In this type of sedimentation separation system, it was difficult to clear unbound ribosomal subunits from above the dense sucrose and, therefore, difficult to distinguish between subunits attached and unattached to membranes. In the absence of membranes, as high as 15-20% of the total subunit radioactivity remained in

* Present Address: Biol. Chem. Lab., Dept. Surgery, Kantonsspital, Basal, Switzerland.

** Present Address: The Toxicology Center, Department of Pharmacology and Pathology, The University of Iowa Medical School, Iowa City, Iowa, 52242 USA.

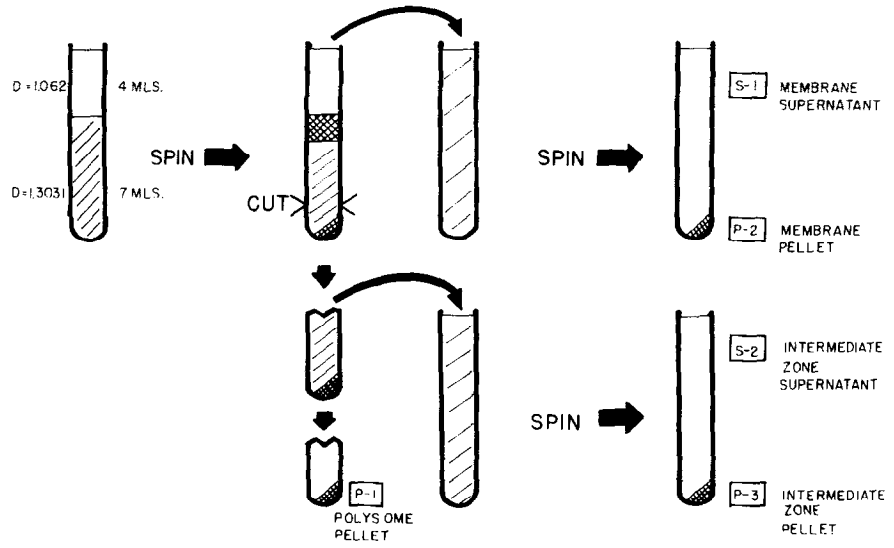

Fig. 1. Scheme for Floatation-analysis of polysome/subunit interaction with microsomal membranes. Measurement of the association in vitro between previously isolated polysomes or ribosomal subunits and membranes is initiated by their mixture in the presence of 0.5 mls of freshly prepared postmicrosomal supernatant at 0-3° (1,2) in a final volume of 1.9 mls 0.44 M sucrose-TKM (50 mM Tris, pH 7.2 at 0°, 0.1 mM Mg^{2} and 25 mM KCl). The density of this mixture is adjusted upward to d=1.3031 (5°) by addition of sucrose, with a final volume of 7 mls. Then, in a polyallomer tube, the mixture is overlaid with 4 mls of fresh 0.44 M sucrose-TKM. The tubes are spun 24-36 hrs at 226,000g_{max}. After centrifugation, they are cut 2 cm from the bottom (at the point of the lower 3 mls of the tube), the contents above the cut (including any membranes present in the initial mixture) being decanted into a clean tube, and the sucrose below the cut into another. Both these tubes are spun at 226,000g_{max} for 3 hours and their pellets (P-2 and P-3) and supernatant fractions (S-1 and S-2) are collected for scintillation counting. One ml of each supernatant fraction (vol≈11.5 mls) was added directly to the premixed cocktail "Scintisol" (Isolabs), while the pellets were first dissolved in formic acid and then added to the cocktail. The extent of interaction of a subunit or polysome population was defined as the percent of the total recovered radioactivity that is membrane associated:

$$\beta' = \frac{(P_2)(C)}{P_1+P_2+P_3+S_1+S_2}$$

Values for P_1 . . . P_3 and S_1 . . . S_2 are the DPM from fractions P-1 . . . P-3 and S-1 . . . S-2 respectively, for a particular tube. Values for C are obtained from the P-2 ("membrane") fraction of a control tube which contains the same amount of labeled particles (and the same total radioactivity) as those experimental tubes containing both particles and membranes. The control tubes contain no membranes. One control tube is centrifuged in each rotor, and that tube alone provides the value for C for the calculation of β' for the other tube of the rotor. C is required because a small yet measureable amount of radioactivity may not be cleared from the P-2 fraction (viz Table 1).

this region at the end of centrifugation periods that were sufficient to clear all but a trace of radioactivity using labeled polysomes.

As a result of these difficulties, a system more efficient in the separation of membranes and ribosomes, subunits and polysomes was developed. Tritium-labeled subunits (or polysomes) and membranes are mixed and the density of the mixture adjusted upwards (Fig. 1). After centrifugation, the contents of each tube is apportioned into 5 fractions as shown in Fig. 1. Cutting 2 cm from the bottom of the tube divides the gradient into an upper membrane-containing zone and a zone intermediate between membranes and the pellet of non-interacting material (P-1) at the bottom of the tube. The contents of each zone is then divided into 2 fractions by further centrifugation into sedimentable (P-2 and P-3) and supernatant fractions (S-1 and S-2).

Table 1a shows an example of the distribution attained with hepatic polysomes isolated from rats injected 18 hrs earlier with ^{3}H-orotic acid. No membrane was present with the polysomes. Almost 93% of the total radioactivity was recovered from the polysome pellet. If polysomes were mixed with rough membranes that are conditioned for polysome acceptance with pyrophosphate-citrate (1), nearly 43% of the radioactivity from the polysomes is distributed to the P-2 ("membrane") fraction (Table 1b). The percent radioactivity in the supernatant fractions (S-1 and S-2) and in the P-3 fraction remain nearly the same as in experiment 1a.

Table 1c and 1e show the distribution of radioactivity from large and small subunits in the absence of membranes. Radioactivity from both subunits was cleared from the P-2 ("membrane") fraction. Unlike the experiments with polysomes (Table 1a), a high percentage of subunit radioactivity appeared in the P-3 ("intermediate") fraction: 22.7% and 18.7% for the small and large subunits, respectively. In addition, there was a tendency for greater amounts

Table 1

Distribution of Radioactivity in Fractions.

Percent Total Recovered DPM*

	Polysome pellet (P-1)	Membrane pellet (P-2	"Membrane"zone supernatant (S-1)	Intermediate zone pellet (P-3)	Intermediate zone supernatant (S-2)
a) ^{3}H-Polysomes (no membranes)	92.8	0.7	4.0	1.0	1.5
b) Polysomes + R pyro**	45.2	42.9	4.6	4.3	3.0
c) ^{3}H-Small SU (no membranes)	52.1	5.6	3.3	22.7	10.3
d) Small SU + R pyro**	58.5	8.3	4.7	18.9	9.6
e) ^{3}H-large SU (no membranes)	66.1	6.9	2.2	18.7	6.1
f) Large SU + R pyro**	41.3	43.2	2.3	11.0	2.2

*Experiments carried out as described in Fig. 1. Tritium-labeled polysomes were made as previously described (2). Large and small subunits (su) were made with 500 mM KCl and puromycin (6). All tubes contained 100 µg of polysomes or ribosomal subunits, 0.5 ml of liver postmicrosomal supernatant (1,2). and 1 mg of membranes unless otherwise indicated.

**R pyro is a preparation of rough microsomal membranes (1,2) from which the endogenous polysome population has been removed by treatment with 50 mM pyrophosphate and 50 mM citrate, described in reference 1.

Table II

Average % Interaction (β') of Ribosomal Subunits with Membranes*

	% Total Recovered DPM on Membranes (β')	% Recovered DPM in Supernatants (S-1 + S-2)
Large Subunits		
"KCl-Puro" Preps	18.8	9.0 9.7
"Energy"	20.2	7.4 5.9
Small Subunits		
"KCl-Puro Preps	3.5	10.3 9.5
"Energy" Preps	2.4	18.9 19.0

*"KCl-Puro" subunits and "energy" subunits were prepared as described in references 6 and 7, respectively. Membrane preparations, the relative concentrations of interactants, and the analytical methods are the same as described in Fig. 1 and Table I.

of radioactivity to appear in the soluble fractions (S-1 and S-2), especially in the case of the small subunit. The high percentages of radioactivity in supernatant fractions has not been reported by other investigators of subunit interaction (3,4).

The extent of interaction of large and small subunits with membranes is shown in Table II. Two preparations of subunits were examined: subunits produced by incubation of polysomes with puromycin in the presence of high KCl concentrations and those produced by incubation with puromycin in the presence of an amino acid incorporating system. The results, expressed as an average of the β' values obtained (viz Fig. 1), indicates very little interaction by small subunits. However, about 1/5 of the large ribosomal subunits, prepared by either method, became attached to the membranes.

Recent interest has focused on the relative affinity of membranes for large compared with small subunits in hope of gaining some insight into the formation of rough endoplasmic reticulum in vivo (3,4). Two aspects of the data shown in Table I and II make critical comparison difficult. First, supernatant fractions for small subunits (particularly those made with the energy system) account, on the average, for almost 2X as much of the radioactivity as they do with the large subunits (Table II). Second, several control tubes (e.g Table 1c) containing subunits prepared by the high KCl method also had very high supernatant activities. The presence of such supernatant radioactivity has not been previously considered (3,4). Its source, whether from attached or unattached populations, is not known. Its occurrence is not prevented by the presence of postmicrosomal supernatant (containing ribonuclease inhibitor - 1,2) in the initial binding mixture, nor is it restricted only to individual preparations made by either method of subunit production. Studies like those shown in Tables I and II underline the need to monitor

continuously in these experiments for non-sedimentable radioactive components, even when ribonuclease inhibitors are employed. Clearly, if the primary source of soluble radioactivity is either the membrane-attached subunits or subunits which might have attached to the membranes, estimates of interaction based on distribution of labeled ribosomal RNAs would be compromised no matter how calculation of interaction was made.

However, it may be seen that analysis of binding by the floatation technique has a number of advantages. A larger number of samples may be examined than was previously possible (1,2). Trapping of particles by membranes (a process distinguishable from binding in that non-adsorbed particles are caught in membrane-containing regions of gradients and not allowed to move centrifugally) does not seem to occur with the floatation method (Ekren et al., unpublished). Trapping has been a widespread problem with other analytical procedures (1,2). The floatation technique also allows simultaneous study of the binding of different polysome populations derived from the same or different polysome preparations to membranes derived from a single preparation, thus extending the range of affinity comparisons possible in a single experiment. This latter advantage has been exploited in comparing the binding of polysomes isolated from different tissues where the compared populations were stable contributing little radioactivity to supernatant fractions (5).

This work was supported by grants from the National Cancer Institute, The National Institute of General Medical Sciences, and the American Cancer Society.

References

1. W.L. Ragland, T.K. Shires, and H.C. Pitot, Biochem. J. 121, 271 (1971).
2. T.K. Shires, L. Narurkar, and H.C. Pitot, Biochem. J. 125, 67 (1971).
3. F.S. Rolleston, Biochem. J. 129, 721 (1972).
4. T. Scott-Burden, and A.O. Hawtrey, Hoppe-Seyler's Z. Physiol. Chem. 352, 575 (1971).
5. H.C. Pitot, and T.K. Shires, Fed. Proc. 32, 76 (1973).
6. G. Blobel, and D.D. Sabatini, Proc. Nat. Acad. Sci., USA 68, 390 (1971).
7. G.R. Lawford, Biochem. Biophys. Res. Comm. 37, 143 (1969).