PROTEIN-SYNTHESISING ACTIVITY OF FREE AND MEMBRANE-BOUND RIBOSOMES IN VITRO AND THEIR DIFFERENTIAL SENSITIVITY TO PROTEIN SYNTHESIS INHIBITORS

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1. Introduction

Membrane-bound ribosomes of rat liver were found to be more sensitive to various protein synthesis inhibitors introduced in vivo than free ribosomes [1]. Also in cell-free protein synthesis, the attached ribosomes were shown to behave in some aspects differently from the free ones [2–5], indicating a close relationship of the bound ribosome with the membrane.

These studies describe the behaviour of free and membrane-bound ribosomes in a cell-free system towards inhibitors of some of the steps in protein synthesis. Membrane-bound ribosomes were generally found to be more sensitive towards these inhibitors, especially to cycloheximide. After solubilisation of membranes by Triton X-100, this greater sensitivity to the inhibitors was lost.

2. Methods

The fractions were prepared from the livers of male albino rats weighing approx. 150 g, which were starved for 16–18 hr before an experiment. The livers were homogenised (0.007″ cleavage) in 2 vol of 0.25 M sucrose in TKM buffer (50 mM Tris–HCl, pH 7.6 at 20°C; 25 mM KCl; 5 mM magnesium acetate) containing 1 mM dithiothreitol. The post-mitochondrial supernatant was prepared by centrifuging the homogenate for 10 min at 10 000 *g*. It was then fractionated using a discontinuous sucrose gradient consisting of 1.35 M sucrose and 2.0 M sucrose in TKM buffer [6].

The cell sap fraction was passed through a column (1.8 × 50 cm) of Sephadex G25 equilibrated with 0.25 M sucrose–1 mM dithiothreitol in TKM buffer. Membrane-bound ribosomes were collected from the 1.35–2.0 M sucrose interface, homogenised in glass apparatus with 2.5 vol of 2 M sucrose in TKM buffer and 1/10 of the volume of the cell sap, overlaid with 0.25 M sucrose/TKM and centrifuged for 1 hr at 135 000 *g*. The floated membrane-bound ribosomes were collected and homogenised. The RNA/protein ratio for this fraction was 0.15–0.21. The free ribosomal fraction was prepared as follows. The 2 M sucrose layer, after removal of rough membranes, was diluted with 3 vol of TKM buffer and centrifuged for 1 hr at 135 000 *g*. The pellet thus obtained consists of the total population of free ribosomes.

The ribosomal samples (0.1 ml; 50–100 μg of RNA) were incubated at 37°C for 30 min in a final volume of 0.5 ml containing the following: 0.1 ml of Sephadex G25-treated cell sap (approx. 2.3 mg of protein); 2 mM ATP; 0.25 mM GTP; 5 mM creatine-phosphate; 25 μg of creatine-kinase; 0.105 μCi of [^{14}C]-L-valine (280 mCi/mmole); 50 mM Tris–HCl, pH 7.6 at 20°C; 75 mM KCl; 5 mM magnesium acetate and 3 mM dithiothreitol. Inhibitors were dissolved in TKM buffer and added at concentrations indicated. In some experiments, Sephadex G25-treated post-mitochondrial supernatant (0.2 ml) was incubated as above instead of the ribosomes and the cell sap. Where indicated, a solubilisation of membranes by Triton X-100 was performed as follows: 11% (w/v) Triton X-100 in TKM buffer was added to subcellular fractions to a final concentration of 1% and after approximately 2 hr on ice, the treated fractions were added to cell-free incubations so that the resulting concentration of Triton X-100 in the system was 0.2%. The untreated

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control fractions were diluted with the same amount of TKM buffer. Incorporation of radioactivity into protein was assayed by the method of Mans and Novelli [7].

Density-gradient analysis was carried out on free and membrane-bound ribosomes which were prepared as described above, except that membrane-bound fraction was not floated. Both fractions were treated with Triton X-100 (final conc. 1.3%) in the presence of cell sap and 150 mM KCl [8] and the detergent treated ribosomes were pelletted by centrifugation for 1 hr at 135 000 *g*. Ribosomal profiles were analysed by layering the samples (about 30 mg of ribosomes) over a linear density gradient, consisting of sucrose (20–40%, w/v) in TKM buffer. Centrifugation was carried out for 7 hr at 25 000 rpm in an aluminium zonal rotor B-29 of a MSE SS-75 ultracentrifuge. The E_{260}, density and viscosity were continuously monitored, the data were collected on a punched tape and the S values were calculated using a computer as described by Ridge [9].

3. Results and discussion

Relative protein-synthesising activity of free and membrane-bound ribosomes in cell-free system has been shown to be dependent on methods used for preparation of subcellular fractions [10,11]. Under the conditions described above, membrane-bound ribosomes were obtained whose activity, one the basis of RNA content was significantly higher than that of free ribosomes (table 1). The same conditions were used for cell-free incubations of free and bound ribosomes (see Methods), since only small differences were found to exist in their requirements: relatively higher magnesium concentration (9 mM) and lower dithiothreitol concentration (2 mM) were optimal for free ribosomes than those for bound ribosomes (5 mM and 4 mM respectively). The optimal concentration of K^+ ions was 75 mM for both fractions. The Mg^{2+} concentration used in our system (5 mM) was only slightly suboptimal for free ribosomes, causing about 10% decrease in the level of amino acid incorporation. Higher protein-synthesising activity of membrane-bound ribosomes seemed to be dependent on the integrity of the membranes rather than on inherent properties of the ribosomes. After the membranes had been solubilised by Triton X-100, specific activity of the ribosomes decreased almost to the level of free ribosomes. Free ribosomes, however, were inhibited by Triton X-100 to much smaller extent.

The analysis of polyribosome profiles (fig. 1) shows that the difference in ribosomal activity was not due to a larger extent of free polysomes breakdown. Relatively undegraded profiles were obtained with both fractions. The computer calculated S values were in good agreement with other published figures and no differences were found between membrane-bound and free ribosomal population.

The following inhibitors of protein synthesis were tested in a cell-free system for their ability to differentially inhibit amino acid incorporation by free and bound ribosomes: cycloheximide, aurin tricarboxylic acid, sodium fluoride, and homopolynucleotide poly I as initiation inhibitors [12–15], and fusidic acid as a translocation inhibitor [16]. However, it must be realised that the effects of inhibitors are seldom entirely specific and often are concentration-dependent.

Fig. 2 shows the response of free and membrane-bound ribosomes to increasing concentrations of the inhibitors described above. It can be seen that in each case membrane-bound ribosomes tend to be more sensitive to inhibitors than the free ones; the greatest differential, however, was obtained with cycloheximide. The cause of the stimulatory effect of aurin tricarboxylic acid at low concentrations is not understood but could be due to impurities present in the reagent.

Both ribosomal fractions were prepared by centrifugation through 2 M sucrose layer (sedimentation of free ribosomes and floating of membrane-bound ribosomes) to eliminate any selective removal of initiation factors by concentrated sucrose. Still the results of the experiments seem to indicate relatively greater ability of membrane-bound ribosomes to initiate protein synthesis (higher sensitivity to poly I, low concentration of cycloheximide and to sodium fluoride – i.e. initiation inhibitors). However, the extent of inhibition (especially by poly I) was not very large with either fraction, suggesting that isolated free and membrane-bound ribosomes have relatively low ability to initiate protein synthesis in a cell-free system. Post-mitochondrial supernatant was found to be more sensitive to poly I and to cycloheximide than fractionated ribosomes and thus seems to be more efficient in the initiation. However, the inhibitors cannot be used as the only measure of initiation and

Table 1
Protein-synthesising activity of membrane-bound and free ribosomes and of a post-mitochondrial supernatant from rat liver and the effect of Triton X-100 treatment

	Membrane-bound ribosomes			Free ribosomes		Post-mitochondrial supernatant	
	RNA/prot.	cpm/mg RNA	cpm/mg RNA after Triton X-100 treatment	cpm/mg RNA	cpm/mg RNA after Triton X-100 treatment	Before Triton X-100 treatment cpm/mg RNA	After Triton X-100 treatment cpm/mg RNA
Experiment 1	0.156	101200 (32)	–	67000 (80)	–	129 660 (50)	86 620 (50)
2	0.210	79800 (94)	–	56000 (84)	–	127 800 (40)	105 000 (40)
3	0.145	120000 (42)	45400 (42)	35700 (64)	30400 (69)	143 000 (96)	119 000 (96)
4	0.182	127000 (45)	91500 (45)	74000 (43)	66000 (43)	159 740 (47)	140 180 (47)

The fractions were prepared and their ability to incorporate [^{14}C]valine was tested as described in Methods. Triton X-100 was added to ribosomes or to a post-mitochondrial supernatant to a final concentration of 1%. Final concentration of Triton X-100 in a cell-free system was 0.2%. The numbers in parenthesis represent the amount of RNA (μg) incubated in 0.5 ml.

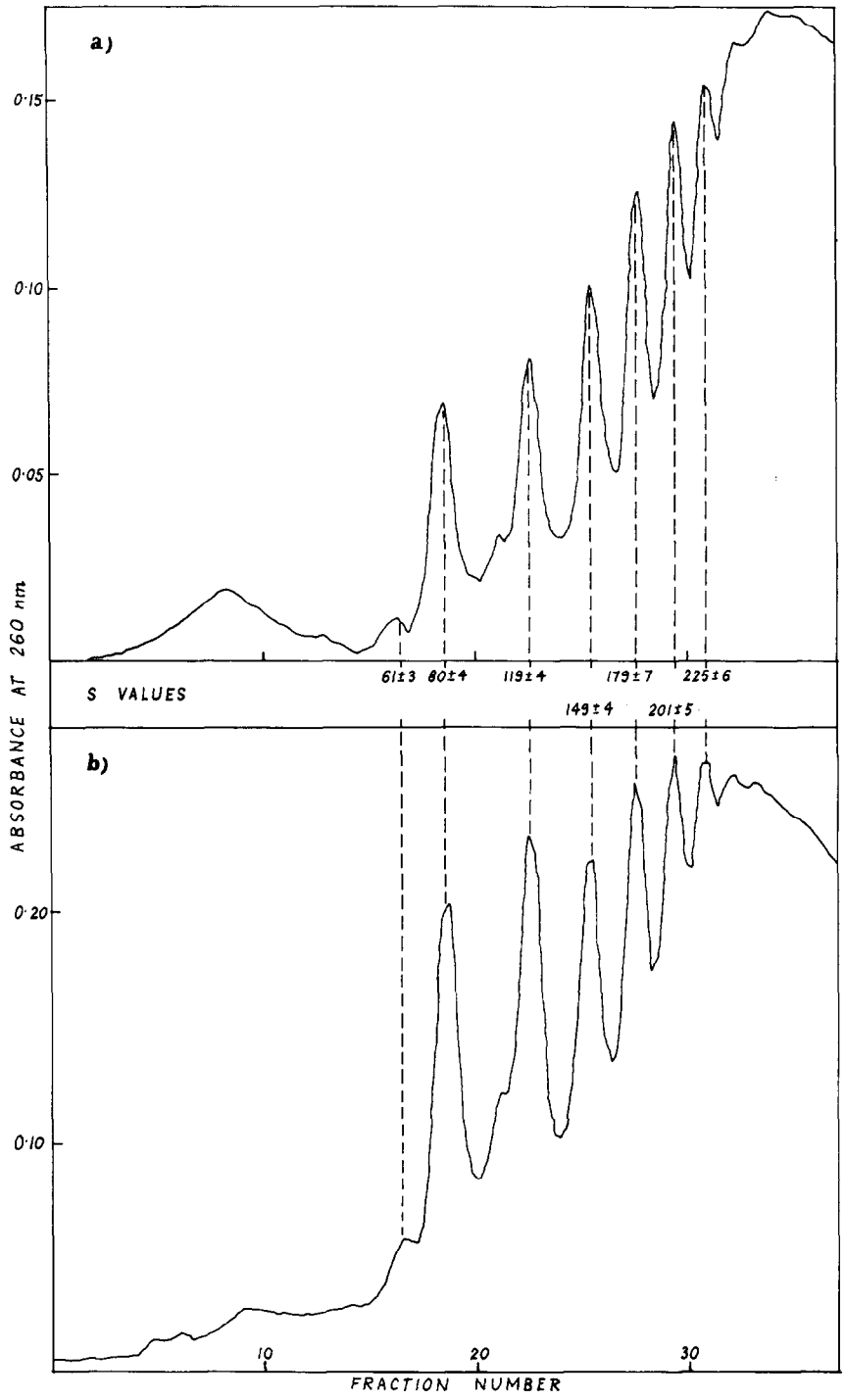

Fig. 1. Sucrose density gradients of free (a) and membrane-bound (b) polyribosomes. For experimental details see Methods. The figures represent mean S values ± S.D. of 4 separate determinations.

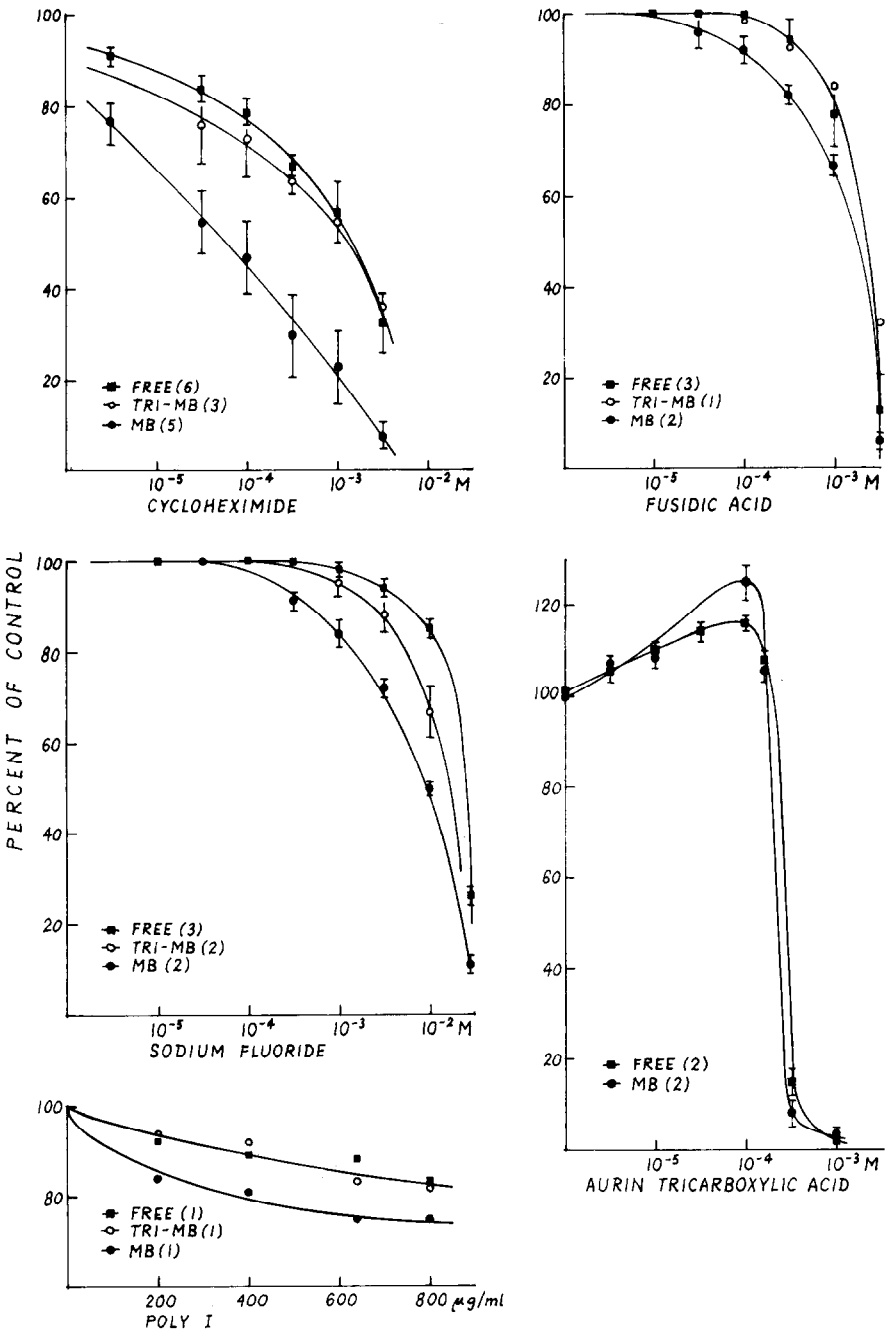

Fig. 2. The effect of inhibitors on [^{14}C]valine incorporation by free (■–■), membrane-bound (●–●) and Triton X-100-treated membrane-bound (○–○) ribosomes. The incubations were performed as described in Methods at 37°C for 30 min except for these with poly I, which were at 37°C for 10 min. Each value represents the mean ± S.D. of separate determinations and the number of experiments is shown in parenthesis. Usual control specific radioactivities and the effect of Triton X-100 treatment are shown in table 1.

more direct methods would be needed.

Since fusidic acid is a steroid and the latter were shown to be involved in the interaction of free ribosomes with membranes [17] and may also be present on the ribosomes [18], the possible effect of preincubation of free and membrane-bound ribosomes with testosterone or oestradiol on the extent of inhibition by fusidic acid was investigated. The preincubation mixtures contained ribosomes (0.47 mg RNA/ml), cell sap (12 mg protein/ml) and steroids (5×10^{-5} M) and were kept on ice for 3 hr. The steroid-treated ribosomes were inhibited by fusidic acid to the same degree as the untreated ones. The actual presence of steroids (2×10^{-5} M) in a cell-free system did not have any effect on the amino acid incorporation by any fraction.

It has been reported that in concentrations up to 1% (w/v), a presence of a non-ionic detergent in a cell-free system from rabbit reticulocytes [19] or from rabbit liver [20] does not interfere with a protein synthesis. Thus, it may be possible to study the behaviour of membrane-bound ribosomes and of a post-mitochondrial supernatant before and after their membranes were solubilised by Triton X-100. The subcellular fractions were treated with 1% Triton X-100 and then added to a cell-free system so that a final concentration of Triton X-100 was 0.2%. As shown in table 1, Triton X-100 did, in these studies, interfere with the amino acid incorporation: the specific activity of membrane-bound ribosomes was inhibited by 30–60%, that of a post-mitochondrial supernatant by 10–30% and that of free ribosomes by 10–15%. Treatment of rough membranes with Triton X-100 produces ribosomes which show a pattern of inhibition similar to free ribosomes (fig. 2). Triton X-100 treatment of free ribosomes, however, did not effect their response to inhibitors. Thus, also the higher sensitivity of membrane-bound ribosomes to the inhibitors, seems to be dependent on the integrity of the membranes.

Similarly to membrane-bound ribosomes, Triton X-100 treatment of the post-mitochondrial supernatant resulted in a decreased sensitivity to the inhibitors, though the differences were less marked than in the case of fractionated membrane-bound ribosomes. This may be explained by the presence of a mixed population of ribosomes in this fraction.

These findings are in good agreement with the previous report of Glazer and Sartorelli [1] who found similar differences in the sensitivities of free and bound ribosomes to protein synthesis inhibitors employed in vivo.

The association of ribosomes with membranes can result in the alteration of ribosome properties. After attachment to membranes, free ribosomes were shown to increase their resistance to pancreatic ribonuclease and EDTA [2] as well as to increase their requirements for sulphydryl compounds [3] and to decrease their divalent cation requirements [4] in cell-free protein synthesis. Towers and his colleagues [5] showed that temperature-induced phase changes in membranes can influence protein-synthesising activity of membrane-bound ribosomes. In our studies, similar close relationships between membranes and the attached ribosomes is indicated, though the nature of this interdependence is not clear.

The differential sensitivity of free and bound ribosomes to inhibitors of cell-free protein synthesis may be used as one of the criteria for comparing rough membranes with membrane-ribosomal complexes produced in vitro.

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