SYNTHESIS OF LIVER FERRITIN ON FREE AND MEMBRANE-BOUND POLYRIBOSOMES OF DIFFERENT SIZES

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1. Introduction

Much evidence supports the thesis that secreted proteins are made on membrane-attached ribosomes, whereas proteins retained within cells are generally synthesized by ribosomes lying free in the cytoplasm [1]. Ferritin, a retained protein found in many tissues, consists of an outer shell of apoferritin subunits surrounding a core of iron salts. Studies in our laboratory [2] and by Redman [3] agree in showing preferential synthesis of apoferritin by the free ribosomes of rat liver; after incubation with labeled amino acids, free ribosomes show many more nascent chains of apoferritin than total or membrane-attached ribosomes, whereas the reverse is true for albumin. Nevertheless, Redman observed that, a few minutes after intact rats were injected with [^{14}C]leucine, only 75% of the radioactivity in the nascent apoferritin of the liver was recovered in the free ribosome population, the remaining 25% being associated with membrane-bound ribosomes. Contamination with free ribosomes could account for about 10% of this. Puro and Richter [4] also used in vivo injection of labeled leucine and found that label was incorporated into nascent liver apoferritin about 20–30% as well by microsomal ribosomes as by free ribosomes. Contamination of their microsome fraction with free ribosomes did not exceed 4–8%. While these studies indicate preferential synthesis of ferritin on free liver ribosomes, they leave doubts about these as the exclusive site of synthesis.

The size of polysomes synthesizing a given protein depends on the number of amino acid residues for which the messenger codes. Although horse spleen apoferritin appears to have 24 identical subunits with a mol. wt. of 18 500, we [5] have recently observed by gel electrophoresis that rat liver ferritin contains two types of subunits with molecular weights of 18 500 and 12 000 respectively. We have therefore examined the free and membrane-bound ribosomes of rat liver for the presence of polysomes consistent with synthesis of these two subunits by reacting each population of ribosomes with ferritin antibody coupled to ^{125}I. On free polysomes, the antibody was bound by trimers and tetramers, consistent with a peptide, having a mol. wt. of 12 000, whereas the ferritin antibody attached to pentamers in the membrane-bound ribosome population, consistent with a product of 17 000 molecular weight. We conclude that free and membrane-bound ribosomes contribute different subunits to liver ferritin.

2. Materials and methods

^{125}Iodine (sodium salt, carrier free) was obtained from New England Nuclear. Lactoperoxidase was purchased from Calbiochem., and heparin from Nutritional Biochemicals. Other chemicals were reagent grade. Rabbit antiserum against horse spleen ferritin was generously supplied by Dr. Linder (M.I.T.). Most of the media were made up in TKM buffer (50 mM Tris–HCl, pH 7.8; 25 mM KCl; 5 mM $MgCl_2$) containing heparin to inhibit ribonuclease.

2.1. *Preparation of bound and free polysomes*

Male albino rats (about 150 g) were injected intraperitoneally with ferric ammonium citrate (450 μg Fe/100 g body weight) daily for four days. Control

rats received saline. After the third day, the animals were fasted overnight and sacrificed 5 hr after the final iron injection. The livers were pooled, homogenized in 0.375 M sucrose in TKM buffer and the post-mitochondrial supernatant (PMS) was prepared [6]. The PMS was layered over a discontinuous gradient (0.5 M over 2 M sucrose in TKM buffer containing 100 mg/ml heparin) and the two ribosome populations were harvested by centrifuging for 16 hr at 105 000 *g* using Rotor Ti 50. The pellet of free ribosomes was resuspended and centrifuged twice at 105 000 *g* in Rotor Ti 50 for 12 hr through a discontinuous gradient having an additional cushion of 1 ml of 2.5 M sucrose below the 2 M and 0.5 M sucrose to keep endogenous ferritin in suspension while the polyribosomes pelleted. In these sucrose layers, the KCl of TKM was replaced by 0.5 M NH_4Cl to dislodge proteins (including ferritin) from the ribosome surface. The bound ribosomes sedimenting at the interface between the 0.5 and 2 M sucrose were treated with 1% deoxycholate, then dialyzed against three changes of 0.14 M sucrose in TKM buffer containing 100 mg heparin/ml and 1 mM dithiothreitol for 6 hr, and finally purified as for the free ribosomes by centrifuging twice through the discontinuous gradient containing 2.5 M sucrose below 2 M and 0.5 M sucrose.

2.2. *Preparation of ^{125}I-labeled ferritin antibody*

Purification and iodination of antiferritin gamma-globulin was carried out as described by Palacios et al. [7]. The gamma-globulin fraction had a final concentration of 5.8 mg/ml and the specific activity was 5×10^5 cpm/mg in Triton–toluene (1:2) scintillation mixture. The binding of the [^{125}I]gamma-globulin to nascent apoferritin was carried out either by reacting with the PMS and subsequent isolation of free and bound ribosomes, or by direct addition of antibody to the isolated ribosome fractions, as detailed in the figure legends. Radioactivity adhering to the ribosomes was counted in presence of Triton–toluene (1:2) scintillator, or solubilized in NCS and counted in the toluene scintillator. In Expt. 2, the isolated ribosomes were pretreated with the gamma-globulin fraction (5 mg protein/ml) purified, as described above, from rabbits not exposed to ferritin.

2.3. *Protein and ribosome estimation*

Protein was estimated by the method of Lowry [8] using bovine serum albumin as standard. Ribosome concentrations were calculated from ultraviolet absorption at 260 nm assuming $A^{1\%}_{1\,cm}$ to be 150 absorbance units and ribosomes to have a mol. wt. 4.1×10^6 [9].

3. Results

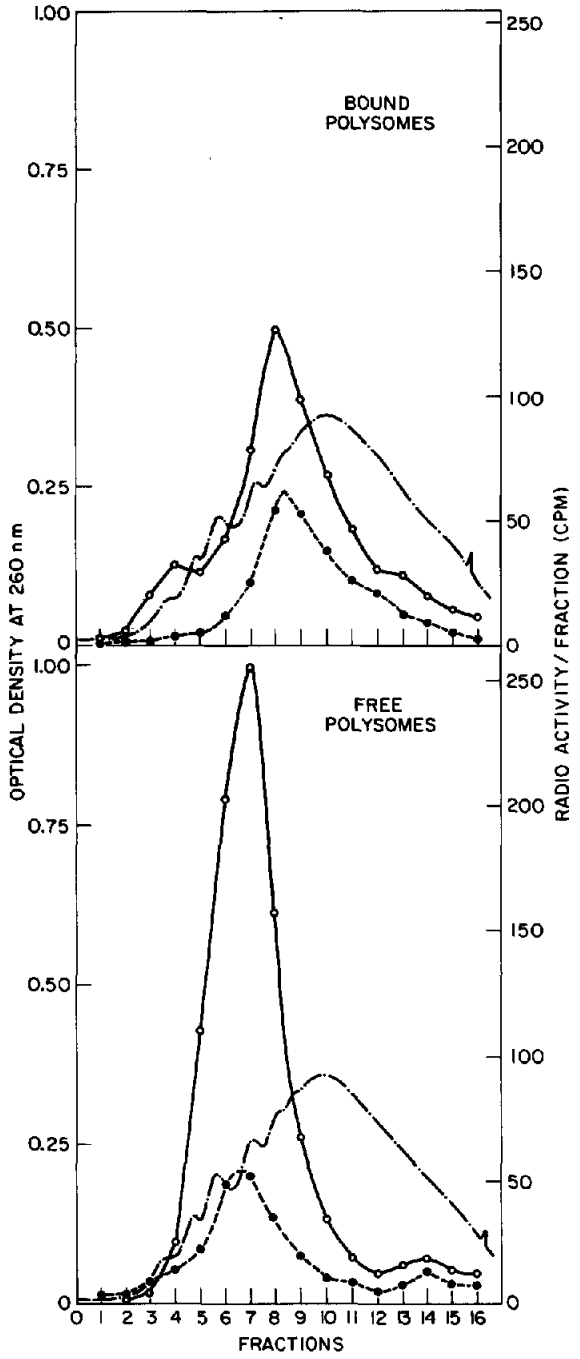
In the first study, the post-mitochondrial supernatant fraction (PMS) was prepared from untreated rats and from iron-injected rats and was reacted with [^{125}I]antiferritin. The free and bound polysomes were then harvested. Fig. 1 shows that [^{125}I]antiferritin was recovered from both the membrane-bound and the free ribosomes, but at different regions of the polysome profiles. Antiferritin bound maximally to the trimer–tetramer region of the free polysomes and to the pentamer region of the bound polysomes. These would correspond to messengers making polypeptides of about 12 000 and 17 000 mol. wt. respectively. Iron pretreatment caused antiferritin binding to the polysomes to increase on both the free and membrane-bound ribosomes. However, this effect was greater for the free ribosomes, so that the ratio of counts free/bound rose from 1.1 (untreated) to 2.4 (treated).

A second study was made in which the free and membrane/bound ribosomes were first separated and were then reacted with the [^{125}I]antiferritin preparation. A fixed amount of antibody was reacted with different amounts of each ribosome type from control and from iron-treated rats, excess antibody being then removed before counting. Fig. 2 shows that binding of ^{125}I was linearly proportional to the amount of ribosomes added in all cases. In untreated rats, the ratio of ^{125}I bound to the free ribosomes relative to bound ribosomes was 1.2, whereas iron-treated rats showed a ratio of 3.0, thus agreeing with the first experiment in showing a preferential increase in nascent apoferritin chains on the free ribosomes as a result of iron treatment.

4. Discussion

These experiments suggest that ferritin peptides of different molecular weight are made by free and

membrane-bound ribosomes in the liver. The possibility that the bound ribosomes are seriously contaminated by free ribosomes seems excluded because the antibody binds to polysomes of different sizes (fig. 1)



and also because the amount bound to the membrane-attached ribosomes is as great as for free ribosomes in control animals (fig. 2) but is differentially stimulated by iron. Although ^{125}I-labeled antiferritin globulin would bind to preformed ferritin contaminating such preparations, this source of error seems unlikely for the following reasons: (a) The two ribosome populations were extensively purified; (b) addition of antibody to the PMS (fig. 1) or to the isolated ribosomes (fig. 2) gave concordant results; (c) ferritin antibody was not attached to the monosome area at the top of the gradient where ferritin is usually found; (d) the various ribosome preparations did not absorb at 320 nm, as ferritin would; (e) finally, a rough calculation of the proportion of apoferritin chains based on the amount of antibody bound suggests that 2% of the free ribosomes of the control rats were making apoferritin, an estimate in agreement with the results of Redman [3] and of Puro and Richter [4] who used labeling of the nascent chains in vitro with amino acids as the criterion.

Synthesis of the peptide chains of a protein at two different locations is not unknown. Ragnotti et al. [10] found that the free and membrane-bound ribosomes of rat liver both carry nascent chains of NADP cytochrome reductase, but Omura and Kuriyama [11] concluded from pulse–dose studies with [^{14}C]leucine in whole animals that this enzyme is made only on membrane-bound ribosomes. A more analogous case is the synthesis of some mitochondrial proteins whose subunits are partly made on cytoplasmic ribosomes and partly on mitochondrial ribosomes [1]. Our data suggest that different subunits of liver ferritin are made on free and membrane-bound cytoplasmic ribosomes. It is claimed by Tanaka and Ogata [12] that membrane-

Fig. 1. Binding of ^{125}I-labeled antiferritin to free and membrane-bound polyribosomes. The livers of 6 control and 6 iron-treated rats were used. The [^{125}I]antiferritin was added to the post-mitochondrial supernatant (PMS) fraction (1 ml antiserum to 4 ml PMS) and reacted for 1 hr at 4°C. The treated PMS was centrifuged to yield purified free and membrane-bound ribosomes (see materials and methods) which were resolved according to polysome sizes on a 10–50% linear gradient of sucrose in TKM buffer by spinning for 70 min at 100 000 *g* in the SW 50 rotor of the Spinco centrifuge. The gradient was monitored at 260 nm for the profile (.-.-.). Fractions of 8 drops were counted in Triton–toluene (1:2) scintillation mixture. The amount of radioactivity (cpm/fraction) is shown for untreated (●—●—●) and iron-treated (●–o–o) rats.

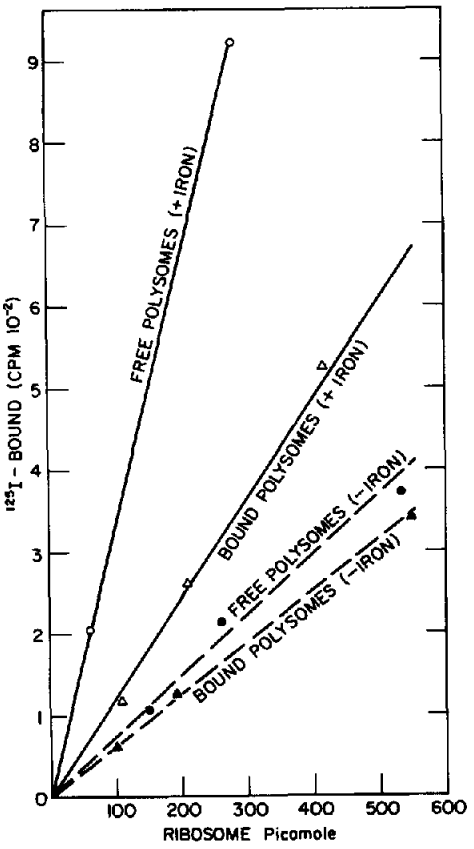


Fig. 2. Binding of ^{125}I-labeled antiferritin to different amounts of ribosomes from control and iron-treated rats. The free and membrane-bound ribosomes were first harvested and purified from 6 control and 6 iron-treated rats. To prevent non-specific binding 0.2 ml of purified gamma-globulin from prepared untreated rabbits was added per 1.0 A_{260} units of ribosomes, and incubated for 1 hr at 4°C. The ribosomes were then reacted for 1 hr at 0°C with the [^{125}I]antiferritin preparation (0.2 ml to 1.0 A_{260}), then layered over 0.5 M sucrose in TKM and centrifuged for 24 hr at 160 000 g in a SW 50 rotor. The pellet was dissolved in 0.5 ml NCS and counted in a toluene-based scintillator.

bound liver ribosomes can be separated into tightly-bound ribosomes making secreted proteins and loosely-bound ribosomes making other proteins. It is possible that one subunit of apoferritin is made by the latter type of membrane-bound ribosomes.

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