SITE OF SYNTHESIS OF RAT LIVER NADH–CYTOCHROME b_5 REDUCTASE, AN INTEGRAL MEMBRANE PROTEIN

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1. Introduction

Studies in cell-free systems have given valuable information concerning site of synthesis of individual proteins, existence of precursors and mechanisms of post-translational modifications. Particular interest has been devoted to the biosynthesis of membrane proteins, many of which have been shown to be synthesized on membrane-bound polyribosomes [1–6]. For some transmembrane proteins, i.e., vesicular stomatitis virus glycoprotein (G protein) [1], Sindbis virus envelope protein [2] and $H2D^d$ histocompatibility antigen of mouse lymphocytes [4], it has been demonstrated that insertion into the endoplasmic reticulum membrane occurs cotranslationally, by a mechanism reminiscent of the one responsible for the transmembrane transfer of secretory proteins [7,8]. On the other hand, the microsomal integral membrane protein cytochrome b_5, which is probably restricted to the cytoplasmic half of the lipid bilayer [9], has recently been shown to be synthesized on free polyribosomes [5,10].

NADH–cytochrome b_5 is an integral membrane protein with a disposition in the bilayer probably similar to cytochrome b_5 [9,11]. Our studies with rat liver, have shown that, in addition to its localization in ER and outer mitochondrial membranes, this enzyme is also present in Golgi membranes [12,13], that the activity in its different locations is most probably due to the same protein molecule [12,14], and that the newly synthesized molecules are inserted independently into the different membranes [15].

Abbreviations: DOC, Na^+ deoxycholate; EDTA, ethylenediaminetetracetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, Na^+ dodecyl sulfate

The latter finding is consistent with the possibility that this protein is synthesized by free polyribosomes. Here we confirm this hypothesis by analyzing the products of rat liver free and bound polyribosomes in a cell-free system.

2. Materials and methods

2.1. *Materials*

The following chemicals were purchased from the indicated sources: [^{35}S]methionine (spec. act. 700–1100 Ci/mmol), the Radiochemical Centre (Amersham); SDS, micrococcal nuclease, Lubrol PX, Triton X-100 and all reagents for cell-free aminoacid incorporation, Sigma Chemical Co. (St Louis, MO); acrylamide, bisacrylamide and TEMED, Eastman (Rochester, NY); Trasylol, Bayer (Leverkusen); low molecular weight standards, Biorad (Milan); protein A–Sepharose CL-4B and CNBr activated Sepharose 4B, Pharmacia (Uppsala); hog pancreas amylase, Boehringer (Mannheim).

Male Sprague Dawley rats (150–200 g) were used for the preparation of free and bound polyribosomes and of NADH–cytochrome b_5 reductase.

2.2. *Production of antireductase antisera and purification of antibodies*

Antisera against lysosome-solubilized reductase, purified by the method in [16], were raised in rabbits as in [12]. Antireductase antibodies were purified from the antisera by affinity chromatography, using a highly purified lysosome-solubilized reductase preparation conjugated to Sepharose 4B [14]. The resulting antibody preparation yielded only one precipitation arc, when challenged with lubrol-solubilized rat liver

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microsomes in the crossed immunoelectrophoresis system of Laurell [14,17].

2.3. *Preparation of detergent-solubilized NADH–cytochrome* b_5 *reductase*

The procedure used to obtain detergent-solubilized reductase (which contains both the hydrophylic and the hydrophobic domains of the molecule [18]), is detailed in [14]. Briefly, a γ-globulin preparation containing antireductase antibodies was immobilized to CNBr activated Sepharose 4B. Rat liver microsomes were solubilized with 0.5% DOC + 2% Triton X-100 and passed through the column. After replacement of DOC and Triton X-100 with 0.2% lubrol, the immunoadsorbed proteins were eluted with 0.2 M glycine–HCl (pH 2.2) + 2.0% lubrol. The eluted proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The major band of the electrophoretic pattern, which migrates slightly behind lysosome-solubilized NADH–cytochrome b_5 reductase, was identified as the detergent-solubilized form of the enzyme by two-dimensional analysis of the peptic peptides [14].

2.4. *Cell-free amino acid incorporation and immunoprecipitation of NADH–cytochrome* b_5 *reductase synthesized in vitro*

Free and detergent-solubilized bound polyribosomes, separated by the method in [19] from γ-amylase-treated homogenates of non-starved rats [20], were used for cell-free amino acid incorporation. The latter was carried out in a rabbit reticulocyte lysate treated with micrococcal nuclease according to [21] and desalted by passage through a Sephadex G-15 column. Incorporation was allowed to proceed at 29°C for the times indicated in the figure legends and the radioactivity incorporated into protein was determined as in [22]. To immunoprecipitate NADH–cytochrome b_5 reductase synthesized in vitro, incubated amino acid incorporation mixtures were supplemented with 1/9th vol. solution containing 1.2 M NaCl, 20% Triton X-100, 1000 kIU/ml Trasylol, 50 mM EDTA, 0.4 M Tris–HCl (pH 7.5), 0.4 mM cold methionine. Antireductase antibodies were then added (5 μg/100 μl incubation mixture). After standing overnight at 4°C, the mixtures were incubated with protein A–Sepharose Cl-4B beads (3 mg/100 μl incubation mixture) for 1 h at room temperature. The beads were collected by centrifugation in a Beckman microfuge, washed 3 times with a buffer containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 100 kIU/ml Trasylol, 1 μl/ml methionine, and then once with the same buffer lacking Triton X-100.

Aliquots of incubated amino acid incorporation mixtures or immunoadsorbed proteins bound to protein A–Sepharose Cl-4B beads were solubilized by boiling for 2 min in the presence of SDS (3%) and dithiothreitol (7 mM), alkylated with a 10-fold excess of iodoacetamide and electrophoresed on 1 mm thick 10% polyacrylamide slab gels [23]. After staining with Coomassie brilliant blue, gels were processed for fluorography [24].

3. Results and discussion

Fig.1 shows the time course of cell-free amino acid incorporation into proteins by rat liver free and detergent solubilized bound polyribosomes. Both classes of polyribosomes stimulated incorporation >200-fold over controls, incubated without added polyribosomes. Since the efficiency of free and bound polyribosomes was approximately the same, meaningful comparisons between their translation products could be made.

As can be seen from fig.2, free and bound polyribosomes directed the synthesis of distinctly different products. In particular, a band of app. mol. wt

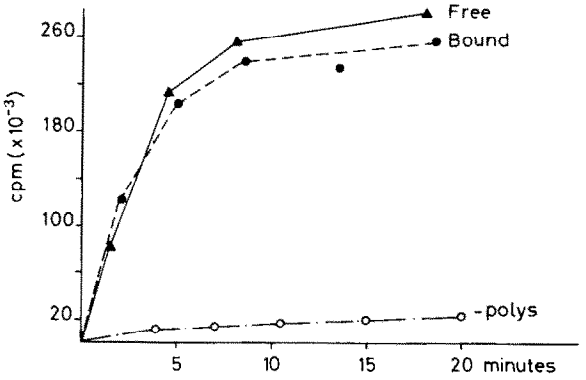

Fig.1. Time course of amino acid incorporation in a nuclease-treated reticulocyte lysate programmed with free or detergent-solubilized rat liver polyribosomes. Each 100 μl reaction mixture [25] contained 25 μCi [^{35}S]methionine and 1.7 A_{260} units of free (▲——▲) or bound (●– – –●) polyribosomes. A control sample was incubated without added polyribosomes (○—·—○). At the times indicated, 2 μl aliquots were removed and hot acid-insoluble radioactivity was determined.

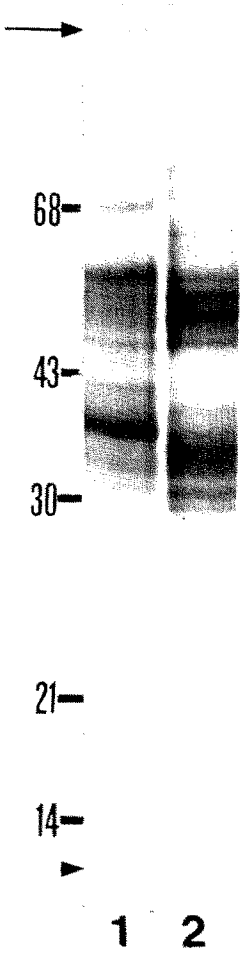

Fig.2. SDS–PAGE analysis of products synthesized by free and bound polyribosomes. After 30 min incubation, 2 μl aliquots of the mixtures containing detergent solubilized bound (lane 1) or free (lane 2) polyribosomes were analyzed on a 10% slab gel, which was then processed for fluorography. The following ^{125}I-labeled marker proteins were used (mol. wt): bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (21 000), lyzozyme (14 300). Arrow and arrowhead indicate origin and front of the gel, respectively.

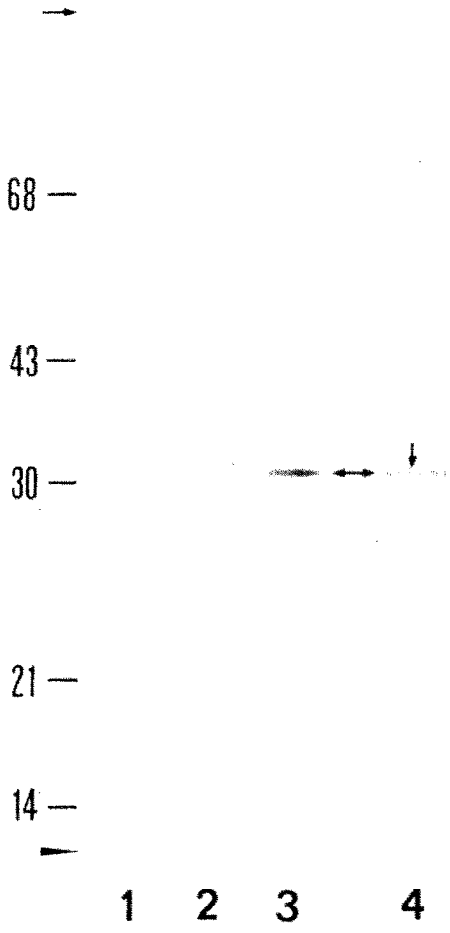

Fig.3. SDS–PAGE analysis of in vitro synthesized proteins immunoadsorbed to protein A–Sepharose Cl-4B with antireductase antibodies. After 30 min incubation, 110 μl of the mixtures programmed with detergent-solubilized bound (lane 1) or free (lanes 2 and 3) polyribosomes were immunoadsorbed onto protein A–Sepharose beads with antireductase antibodies, as in section 2. The immunoadsorbed proteins were analyzed on a 10% slab gel, which was then processed for fluorography. The sample analyzed in lane 2 was supplemented with 25 μg purified lysosome-solubilized NADH–cytochrome b_5 reductase before addition of antireductase antibodies. Lane 4 shows the Coomassie brilliant blue staining pattern of a preparation enriched in detergent-solubilized NADH–cytochrome b_5 reductase, indicated by the vertical arrow (see section 2.2). Molecular weight markers are as in fig.2. Horizontal arrow and arrowhead indicate origin and front of the gel, respectively.

68 000, comigrating with bovine serum albumin, was present exclusively among the products synthesized by bound polyribosomes.

As shown in fig.3, SDS-PAGE analysis of in vitro synthesized proteins, immunoadsorbed to protein A–Sepharose Cl-4B beads with antireductase antibodies, revealed a major band among the products of free polyribosomes (lande 3) which exactly comigrated with

Table 1
Quantitation of immunoprecipitated products synthesized by free and membrane-bound polysomes

	Immunoprecipitated protein	cpm in gel band/100 μl incorporation mixture	
		Free polyribosomes	Bound polyribosomes
Expt I	NADH–cytochrome b_5 reductase	7143	1121
	Albumin	22 466	256 567
Expt II	NADH–cytochrome b_5 reductase	10 169	2644

The regions of the dried fluorographed SDS–polyacrylamide slab gels containing the immunoadsorbed proteins were excised, immersed in toluene + PPO–POPOP and then counted. Data are corrected for background which was ~50 cpm

detergent-solubilized microsomal NADH–cytochrome b_5 reductase (lane 4). No such major band was apparent among the products of bound polyribosomes (lane 1). The band comigrating with NADH–cytochrome b_5 reductase did contain the enzyme, since its intensity was diminished when competing, non-radioactive lysosome-solubilized reductase was added to the incubation mixtures before addition of anti-reductase antibodies (lane 2).

In order to obtain a quantitative estimate of the capacities for reductase synthesis of free and bound polyribosomes, the reductase band from the fluorographed gels was cut out and counted in a scintillation counter. As can be seen from table 1, 4–7-fold more reductase was synthesized by free than by bound polyribosomes. As a check of the specificity of the results obtained with NADH–cytochrome b_5 reductase, in 1 expt, an aliquot of incubation mixture was treated with anti-rat albumin antibodies, and the resulting immunoadsorbed proteins analyzed by SDS–PAGE. As expected, bound polyribosomes synthesized much more (>10-times) albumin than free polyribosomes.

In conclusion, these results demonstrate that NADH–cytochrome b_5 reductase is synthesized mainly on free polyribosomes. On the basis of mobilities on SDS–polyacrylamide gels, the primary translation product appears to be identical to the mature enzyme. However, further work is required to exclude the existence of a precursor form of this protein.

As indicated by the data of table 1, some reductase also appeared to be synthesized by bound polyribosomes. The presence of reductase among the products of the bound polyribosome fraction could be simply due to contaminating free polyribosomes. However, the degree of purification of the two polyribosome fractions we obtained seems quite good, as indicated by the striking difference among their in vitro products (fig.2), clearly demonstrated in the case of albumin (table 1). It is possible that some reductase-synthesizing polyribosomes are associated with microsomal membranes because of an interaction of nascent reductase with membranes, before termination of its synthesis. The membrane binding segment of NADH–cytochrome b_5 reductase is located towards the C-terminus of the molecule [11], and this might explain why a large fraction of the reductase-synthesizing polyribosomes are free in the cytosol.

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