THE BIOSYNTHESIS OF CYTOCHROME *P*-450 IN VITRO

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1. Introduction

The role of the rough endoplasmic reticulum in the biosynthesis of proteins exported from the cell and intrinsic membrane proteins is well known [1]. Exported proteins are extruded through the membrane into the intracisternal space where post-translational modifications can occur in the rough membranes or other components of the endoplasmic reticulum [2]. It has been suggested that some membrane proteins are inserted directly into the membrane in the course of biosynthesis [3–6]. However, recent work suggests that the pathway of biosynthesis of membrane glycoproteins is similar to that of exported proteins and involves intracisternal passage to the Golgi apparatus [7,8]. Since highly purified cytochrome *P*-450 contains carbohydrate [9], and is probably a glycoprotein, we have investigated the possibility that this membrane protein passes into the intravesicular space in the course of biosynthesis in vitro in vesicles derived from rough endoplasmic reticulum.

2. Methods

Male Sprague-Dawley rats, 200–250 g, permitted food and water ad libitum, were used in this study. Animals received a single i.p. injection of phenobarbital (40 mg/kg in 0.9%, w/v, NaCl) on each of 4 days prior to sacrifice on day 5. Rough endoplasmic reticulum was prepared as in [10] by centrifugation of post-mitochondrial supernatant on a discontinuous sucrose gradient in a fixed angle rotor at 105 000 × g_{av} for 3.5 h.

Antibodies were raised to cytochrome *P*-450 and to rat serum proteins, in New Zealand White rabbits. The IgG fractions were isolated from serum as in [11]. A control IgG fraction was prepared by injecting a rabbit with 50% Freund's complete adjuvant. Ouchterlony double diffusion analysis revealed that this control antibody preparation did not react with either cytochrome *P*-450 or rat serum.

Protein synthesis in rough membranes in vitro was done as in [12] with the exceptions that the incubation contained [^{14}C]leucine 4 μCi/ml, 0.5 mM 2-mercaptoethanol instead of dithiothreitol, and was performed at 20°C.

After amino acid incorporation the particulate fraction was collected by centrifugation at 105 000 × g for 1 h at 4°C. The resultant supernatant (S1) contains newly biosynthesized polypeptides released from the membrane. This fraction was then dialysed against 50 mM sodium phosphate, pH 7.7, to remove 2-mercaptoethanol. In order to release bound polysomes the pellet was resuspended in sodium pyrophosphate (0.1 M), ethylene diamine tetraacetic acid (EDTA) (1 mM) adjusted to pH 7.5 with HCl, and incubated for 30 min at 0°C. The membrane fraction was collected as above and the supernatant fraction (S2) retained. The pellet was resuspended to protein conc. 1.8 mg/ml in solution containing 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl (buffer A). Vesicular contents (S3) and particulate fraction (P3) were then obtained from this material as in [13] which involves addition of 1% (w/v) deoxycholate to final conc. 0.05%. Alternatively the vesicle content was obtained by resuspending the pellet resulting from pyrophosphate/EDTA treatment in double distilled water and incubated at 30°C for 15 min as in [14]. The pellets obtained from both procedures were resuspended in a buffer containing 0.25 M

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sucrose, 20% glycerol, 1 mM EDTA, 0.01 M sodium phosphate, pH 7.7. For investigation by quantitative immunoprecipitation the membrane fraction was solubilized by the addition of 1.5 mg cholic acid/mg protein. Following incubation for 45 min at 0°C, any remaining nonsolubilized material was removed by centrifugation at 105 000 × *g* at 4°C for 1 h.

The incorporated radioactivity present in each sub-membrane fraction was determined by precipitation with trichloroacetic acid, final conc. 10%. The precipitates were collected onto glass fibre discs, dried and counted in a scintillation fluid containing 5 g/l PPO in toluene.

Immunoprecipitations were carried out by reacting the antigen containing material, for 24 h, with an excess of antibody in 50 mM sodium phosphate, pH 7.7, at 4°C. When precipitation of proteins synthesized in vitro was required, non-radioactive carrier antigens (200 μg) were added in the incubations to facilitate recovery of the precipitates. The resultant immunoprecipitates were washed prior to further acid precipitation with 10% trichloroacetic acid, collected onto glass fibre filters and radioactivity determined as before. Protein was determined as in [15], RNA [16], phospholipid [17,18], and cytochrome *P*-450 [19].

3. Results and discussion

Cytochrome *P*-450 was purified from microsomes prepared from the livers of phenobarbital-treated rats as in [20]. The preparation had spec. act. 19 nmol/mg protein, contained no detectable cytochrome b_5 or NADPH cytochrome *c* reductase, and could be resolved, electrophoretically into 3 bands. These were mol. wt 49 750 (57%), 46 300 (28%), and 43 500 (16%); the relative quantities of each are indicated in parenthesis. It can be deduced from the specific activity, molecular weight and stoichiometry that each band represents at least one form of cytochrome *P*-450. It should be noted that in the preparation [20] only one polypeptide band was found. However, immature rats were used and their product was analysed by a different electrophoretic system.

Antibodies prepared from rabbits immunized with cytochrome *P*-450 preparation react with antigens present in cholate-solubilized reticular membranes.

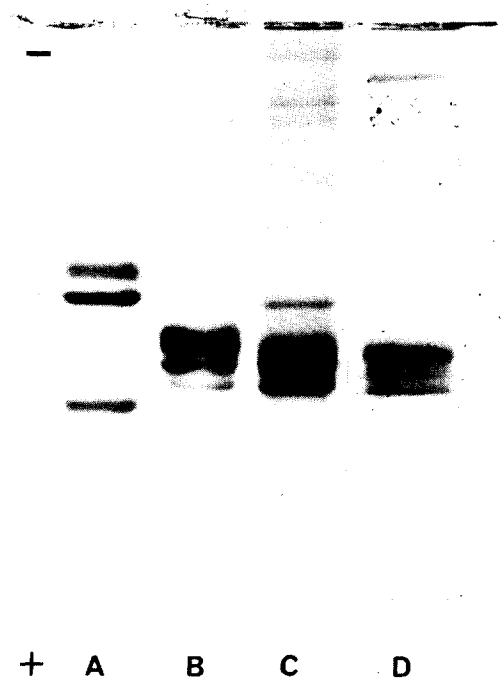

Fig.1. SDS–Polyacrylamide gel electrophoresis, under non-reducing conditions, of (A) standard proteins, bovine serum albumin, pyruvate kinase, alcohol dehydrogenase and ovalbumin (2 μg each), (B) purified cytochrome *P*-450 (15 μg), (C) smooth endoplasmic reticulum (30 μg), (D) immunoprecipitate formed by reaction of smooth endoplasmic and antibody to cytochrome *P*-450 at a loading equivalent 15 μg total membrane protein. Separation was accomplished on a 10% gel as in [21].

When the resulting precipitate is dissolved and electrophoresed under non-reducing conditions, 3 bands are observed having the same mobilities as those in the original antigen preparation (fig.1). The bands seen at high molecular weight do not correspond to any band in the antigen and thus are derived from the precipitating IgG molecules [22].

Vesicles of RER have been subfractionated by sequential procedures. Initially the membrane was treated with pyrophosphate/EDTA, resulting in the detachment of at least 80% membrane-associated RNA. Degranulated membrane thus obtained was separated into vesicle contents and residual membrane components. This was accomplished by either treatment with 0.05% deoxycholate [13] or by water

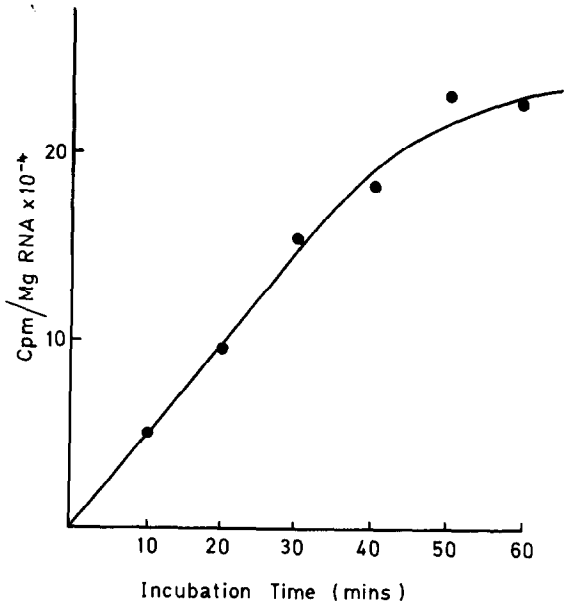

Fig.2. Kinetics of incorporation in vitro of [^{14}C]leucine into trichloroacetic acid-precipitable material by rough endoplasmic reticulum.

thermal treatment [14]. The released fraction (assumed vesicle contents) obtained by both methods was found to contain a mixture of polypeptides distinct from the residual membrane fractions as adjudged by electrophoretic analysis. Neither treatment was found to release membrane phospholipid or cytochrome *P*-450. However the water thermal treatment causes the release of a small part of the residual bound RNA present in the degranulated membrane.

Rough vesicles incorporate [^{14}C]leucine in vitro (fig.2), into polypeptides which can be recovered in subreticular fractions prepared as above. The distribution of the radiolabelled polypeptides produced after 1 h in vitro protein synthesis are shown in table 1.

The effectiveness of the various treatments was checked by a number of control incubations in which pelleted material was resuspended in buffer containing 0.25 M sucrose, 50 mM Tris–HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$ instead of the appropriate treatment buffer. After recovery of the membrane by centrifugation less then 5% of the incorporated radioactivity could be detected in the supernatants. When pyrophosphate-treated material was resuspended in buffer A and incubated in the absence of deoxycholate, again less than 5% of the radioactivity was found in the supernatant after harvesting the membrane.

The nature of the radiolabelled polypeptides in each subreticular fraction was assessed by immunoprecipitation using antibody to either cytochrome *P*-450 or rat serum proteins (table 2). A high percentage of the radioactivity released from the vesicles by deoxycholate or water-thermal treatment was

Table 1
Recovery of trichloroacetic acid-precipitable radioactivity in subfractions of rough endoplasmic reticulum after in vitro incorporation of [^{14}C]leucine

Subreticular fraction	^{14}C Label recovered	
[^{14}C]Polypeptides not associated with RER (S1)	0.05 ± 0.02	
[^{14}C]Polypeptides released by pyrophosphate/EDTA treatment (S2)	0.22 ± 0.03	
	Deoxycholate treatment	Water thermal treatment
Intravesicular [^{14}C]polypeptides (S3)	0.23 ± 0.03	0.10 ± 0.05
[^{14}C]Polypeptides associated with intrinsic, membrane protein (P3)	0.48 ± 0.08	0.45 ± 0.02
Total incorporated ^{14}C recovered	0.95 ± 0.08	0.92 ± 0.08

Protein synthesis in vitro was carried out for 1 h and subreticular fractions prepared (see section 2). The incorporated radioactivity was determined by trichloroacetic acid preparation and the amount of ^{14}C found in each fraction is expressed as a fraction of the total incorporation. Results show means ± SD from 4 experiments

Table 2
Recovery of immunoprecipitable radioactivity in subfractions of rough endoplasmic reticulum after in vitro incorporation of [^{14}C]leucine

Subreticular fraction	^{14}C precipitated by antibody / ^{14}C precipitated by TCA in fraction			
	Antibody to rat serum proteins		Antibody to cytochrome *P*-450	
S1	0.08 ± 0.04 (0.03)		0.07 ± 0.01 (0.05)	
S2	0.19 ± 0.01 (0.13)		0.27 ± 0.05 (0.20)	
	Deoxycholate treatment	Water thermal treatment	Deoxycholate treatment	Water thermal treatment
S3	0.48 ± 0.07 (0.39)	0.27 ± 0.02 (0.34)	0.21 ± 0.05 (0.25)	0.21 ± 0.04 (0.23)
P3	0.15 ± 0.02 (0.45)	0.16 ± 0.03 (0.49)	0.19 ± 0.03 (0.49)	0.34 ± 0.04 (0.49)

Protein synthesis in vitro was carried out for 1 h. Subreticular fractionation, immunoprecipitation and trichloroacetic acid precipitation were carried out as section 2. Radioactivity present in immunoprecipitates has been corrected for non-specific precipitation. This was determined in incubations in which specific antibodies were replaced with IgG, prepared from control rabbits, followed by precipitation with antibody to rabbit IgG produced in the goat. The identity of each fraction is as described in table 1. The figures in parenthesis show the fraction of immunoprecipitable radioactivity in each fraction compared to total immunoprecipitable radioactivity. Results show means ± SD from 4 experiments

precipitated by antibody to rat serum as in [23,24]. However, as can be seen from table 2, as much as 20% of the radioactivity precipitated by trichloroacetic acid in this fraction, is incorporated into cytochrome *P*-450.

The finding that newly synthesized cytochrome *P*-450 can be detected in the vesicle content indicates that the path of biosynthesis of this intrinsic membrane protein may be initially similar to that reported for export proteins. Nascent cytochrome *P*-450 appears to be extruded into the intracisternal space prior to insertion at its ultimate membrane locus. Isolated cytochrome *P*-450 can be reinserted in membranes depleted of the enzyme to produce a catalytically active complex. Our findings suggest that at least some of the newly synthesized cytochrome *P*-450 polypeptide may require post-translational modification before insertion into the membrane.

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