THE BIOSYNTHESIS OF CYTOCHROME P450 BY ROUGH ENDOPLASMIC RETICULUM IN VITRO

A significant proportion of newly-biosynthesised cytochrome P450 is resistant to proteolytic digestion in intact vesicles

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1. Introduction

Membrane proteins synthesised on polysomes of the rough endoplasmic reticulum are thought to be directly inserted into the membrane at the site of synthesis [1–6]. However, we have recently demonstrated that a part of the newly-biosynthesised cytochrome P450, a major membrane protein, can be found inside vesicles derived from the rough endoplasmic reticulum after protein synthesis in vitro [7,8]. Newly-biosynthesised cytochrome P450 has also been located inside vesciles derived from rough and smooth endoplasmic reticulum after incorporation of radiolabelled amino acids in situ by perfusion of the liver [8]. In these studies intravesicular content is defined as the material released from reticular fractions by treatment with a low concentration of deoxycholate (0.05% w/v). Under the experimental conditions employed in these studies this level of detergent does not solubilise any of the spectrally-detectable cytochrome P450 [7–9]. However, the possibility cannot be entirely eliminated that the newly-biosynthesised intravesicular cytochrome P450 is a minor component which becomes highly radiolabelled during protein synthesis and is released from the membrane by low concentrations of deoxycholate.

Intact microsomal vesicles are impermeable to macromolecules and thus treatment with proteases affects only those susceptible bonds which are present in proteins exposed on the outer membrane surface [10]. Treatment of vesicles with deoxycholate (0.05% w/v) removes the permeability barrier and permits macromolecules to penetrate inside where they may interact with components in the luminal space and on the inner membrane surface [9,11,12]. In this study we show that a substantial proportion of the newly-biosynthesised, cytochrome P450, released by deoxycholate, is resistant to tryptic digestion of intact vesicles and therefore probably derives from the intravesicular compartment.

2. Methods

The rats used in these experiments were kept and treated with phenobarbital as in [7]. Rough endoplasmic reticulum was prepared from the livers of these animals [13].

Cytochrome P450 was purified from the livers of phenobarbital-treated rats [8] and used to raise antibodies in New Zealand white rabbits [7,8]. The antibodies obtained are specific for cytochrome P450 as judged by Ouchterlony analysis, ability to precipitate spectrally-detectable cytochrome P450 from solubilised membrane and electrophoretic analysis of immunoprecipitates [7,8]. Radiolabelled polypeptides immunoprecipitated from subreticular fractions after protein synthesis in vitro have the same electrophoretic mobility as the cytochrome P450 used as antigen to prepare the antibodies [8].

Protein synthesis in vitro was carried out as in [7] but using ^{14}C-labelled amino acid hydrolysate

(56 mCi/matom) 4 μCi/ml. Incubations were supplemented with a non-radioactive mixture of those amino acids not present in the hydrolysate.

After protein synthesis in vitro the particulate fraction was collected by centrifugation at 105 000 × *g* for 1 h at 4°C. The resultant pellet was resuspended to 2.6 mg membrane protein/ml in sodium pyrophosphate (0.1 M)/EDTA (10 mM) solution adjusted to pH 7.5 with HCl. The protein concentration given here and later is based on the initial quantity of membrane used. After 30 min incubation at 0°C the membrane was recovered by centrifugation as before. The membrane pellet was resuspended to 1.8 mg protein/ml in a solution containing Tris–HCl (50 mM, pH 7.5), KCl (50 mM) and $MgCl_2$ (5 mM) (solution A). Aliquots of the resuspended membrane were either incubated without further additions (control treatment), or incubated with 50 μg trypsin/mg microsomal protein (trypsin treatment) or incubated with trypsin (50 μg/mg microsomal protein) followed by deoxycholate to give final conc. 0.05%, w/v (trypsin and deoxycholate treatment). The tubes were incubated for 30 min at 30°C and proteolysis terminated by the addition of a soyabean trypsin inhibitor (2 μg/μg trypsin) followed by transfer to a bath at 0°C. The particulate fraction was recovered by centrifugation as above. Aliquots of the resultant supernatants (S3) were retained for determination of radioactivity incorporated into material precipitated by trichloracetic acid. The particulate fractions were resuspended to 1.8 mg membrane protein/ml in solution A. Soyabean trypsin inhibitor was added as before. Deoxycholate was added to 0.05% w/v and the membranes incubated for 30 min at 0°C. Particulate fractions (P4) were recovered by centrifugation as before. Aliquots of the resultant supernatants (S4) were used for the determination of incorporated radioactivity either by immunoprecipitation or precipitation with trichloroacetic acid. The residual membrane pellets (P4) were resuspended to 10 mg protein/ml in a solution containing sucrose (0.25 M), glycerol (20%, w/v) EDTA (1 mM) and sodium phosphate (100 mM, pH 7.7) (solution B). An aliquot of the resuspended membranes was used for the determination of incorporated radioactivity in material precipitated by trichloracetic acid. Where immunoprecipitation was required an aliquot of the resuspended membranes was solubilised by the addition of the appropriate volumes of a solution of sodium cholate dissolved in solution B (10%, w/v) to give final conc. 3 mg/mg membrane protein. The mixture was incubated for 30 min at 0°C and non-solubilised material removed by centrifugation as above.

The determination of incorporated radioactivity in material precipitated by trichloroacetic acid and in immunoprecipitates have been detailed in [7]. Other methods were as in [7].

3. Results and discussion

The radiolabelled amino acids incorporated in vitro into polypeptides in rough endoplasmic reticulum partition into various reticular subfractions. Newly-biosynthesised cytochrome P450 can be detected and quantified in these subfractions. Table 1 shows the effect of trypsin treatment on the distribution of radioactive polypeptides incorporated into subfractions of rough endoplasmic reticulum. In these experiments rough endoplasmic reticulum was incubated in a protein synthesis system in the presence of ^{14}C-labelled amino acids derived from a protein hydrolysate. Polysomes were removed by treatment with pyrophosphate/EDTA prior to treatment with trypsin either in the presence or absence of deoxycholate. Trypsin treatment in the absence of deoxycholate resulted in the loss of $> 50\%$ of the incorporated radioactivity compared to untreated membrane. The loss in radioactivity is largely from the membrane (P4) although a small but significant loss from the vesicle content (S4) is observed probably due to breakage of some vesicles under the conditions of trypsin treatment. Other reports have also detailed a partial susceptibility of vesicle content to tryptic digestion [14]. The protective effect of membrane vesicles on vesicle content can clearly be seen when the data is examined for trypsin treatment in the presence of deoxycholate. Under these conditions $> 80\%$ of incorporated radioactivity is lost, the radioactivity being greatly reduced in each subfraction. Thus ~40% of radioactivity present in vesicles after in vitro protein synthesis is resistant to trypsin digestion either in the vesicular space or on the luminal membrane surface. Only a small portion of the incorporated radioactivity is resistant to digestion if there is access to both sides of the membrane. It cannot be deter-

Table 1
Susceptibility of newly-biosynthesised proteins precipitated by trichloroacetic acid to trypsin digestion in the presence and absence of deoxycholate

Reticular subfraction	% of radioactivity precipitated by trichloroacetic acid		
	Control	Trypsin	Trypsin and deoxycholate
S3	4 ± 1	4 ± 1	6 ± 2
S4	32 ± 2	20 ± 2	3 ± 2
P4	58 ± 3	21 ± 1	8 ± 1
Total recovered	93 ± 3	44 ± 3	18 ± 4

Protein synthesis in vitro was carried out for 1 h in the presence of ^{14}C-labelled amino acids. Membrane-bound polysomes were removed by treatment with EDTA/pyrophosphate as described. The membrane thus obtained was resuspended in solution A and either untreated (control column) treated with trypsin in the absence (trypsin column) or presence (trypsin and deoxycholate) of 0.05% deoxycholate as in section 2. Trypsin digestion was terminated with soyabean trypsin inhibitor and membranes recovered by centrifugation. Radioactivity precipitated by trichloroacetic acid was determined in the resulting supernatants (S3). The membranes were resuspended in solution A and treated with deoxycholate (final conc. 0.05% w/v). The residual membrane (P4) was recovered by centrifugation and trichloroacetic acid precipitable radioactivity determined in this fraction and the corresponding supernatant (S4). The results are expressed as the % of radioactivity recovered in each subfraction based on the amount of radioactivity in the starting control stripped membrane which ranged from 23 000–42 000 cpm/mg protein

Table 2
Effect of trypsin treatment of rough endoplasmic reticulum after protein synthesis in vitro: Recovery of immunoprecipitable radioactivity in reticular subfractions

Reticular subfraction	% of immunoprecipitable radioactivity in subfraction			
	Control treatment		Trypsin treatment	
	Antibody to cyt. P450	Antibody to serum proteins	Antibody to cyt. P450	Antibody to serum proteins
S4	20 ± 2	78 ± 1	12 ± 1	51 ± 3
	(22 ± 5)	(38 ± 2)	(19 ± 2)	(40 ± 3)
P4	81 ± 1	21 ± 1	24 ± 2	23 ± 1
	(62 ± 7)	(12)	(48 ± 4)	(16)
Total	100	100	36	74

Protein synthesis in vitro was carried out in the presence of ^{14}C-labelled amino acids. Membrane-bound polysomes were removed by treatment with EDTA/pyrophosphate and the resulting membrane resuspended in buffer A. This membrane was incubated in the absence (control) or presence of trypsin. Membrane was recovered by centrifugation and used to obtain the fraction released by deoxycholate (S4) and the residual membrane fraction (P4). (See section 2 and the legend to fig.1.) Aliquots of each fraction were subjected to immunoprecipitation as in section 2 using an antibody to cytochrome P450 or to rat serum proteins. The data show the % of immunoprecipitable radioactivity in each subfraction compared to the total recovered (S4 + P4) in the control membrane. This ranged from 4300–8900 cpm/mg protein using antibody to cytochrome P450 and 5000–7200 cpm/mg protein using antibody to serum proteins. Also included, in parentheses, is the % of immunoprecipitable radioactivity compared to the trichloroacetic acid precipitable radioactivity found in each subfraction

mined whether those membrane proteins resistant to digestion in the presence of deoxycholate are inherently resistant to the action of trypsin or whether they are buried in the membrane and are thus inaccessible.

The nature of the radiolabelled proteins present in the reticular subfractions after trypsin treatment has been investigated by quantitative immunoprecipitation. The results are shown in table 2 which compares the immunoprecipitable radioactivity found in each subfraction compared to the total immunoprecipitable radioactivity recovered in the control membrane. Following trypsin treatment, a significant proportion of the radioactivity found in the fraction released by deoxycholate is cytochrome P450. The amount of immunoprecipitable radioactivity attributable to cytochrome P450 recovered in this subfraction after trypsin treatment is reduced compared to control membrane. This is consistent with loss due to vesicular damage since the amount of radioactivity precipitated by antibody to serum proteins present in the vesicle content is also reduced. The fractional loss is identical within the experimental errors for serum proteins and cytochrome P450 (table 2). When the radioactivity immunoprecipitated from vesicle content by both antibody types is compared to the corresponding trichloroacetic acid-precipitable radioactivity the results are again identical for control and trypsin-treated membrane (table 2, figures in parentheses).

Following trypsin treatment the amount of radioactivity which is present in the residual membrane fraction and which is precipitated by antibody to cytochrome P450 is greatly reduced. When the vesicle content was released in the presence of trypsin it contained $< 0.1\%$ of the radioactivity immunoprecipitable by antibody to cytochrome P450 compared to the control treatment. The residual membrane also contained insignificant amounts of immunoprecipitable radioactivity after a combined treatment with deoxycholate and trypsin.

Cytochrome P450 exists in multiple forms characterised by different spectral properties [15,16], different catalytic specificities [16,17], and by different molecular weights [16,17]. Topographical analysis of reticular membranes [9] has indicated that spectrally-detectable cytochrome P450 is distributed on both faces of the membrane bilayer as well as in the membrane core. This complex distribution is unusual for membrane proteins which are usually specifically and asymmetrically distributed in the transverse plane of the membrane [9,18,19]. It may well be that individual variants of cytochrome P450 are also distributed specifically and asymmetrically across the membrane. The results of this study are highly relevant to this possibility since the topological variants of the cytochrome are probably synthesised by different routes. Thus the major part of the radioactivity incorporated into membrane-associated cytochrome P450 is accessible to trypsin. This may represent a topological variant inserted directly into the membrane, since the N-terminal amino acid sequence of one electrophoretic variant of cytochrome P450 has been shown to be homologous to the signal sequence of various pancreatic preproproteins [20]. This form of the cytochrome may be anchored directly into the membrane in the course of biosynthesis via its non-polar 'signal sequence'. Our data clearly show that one or more variants of the cytochrome are translocated across the membrane in the course of biosynthesis, like export proteins. It is reasonable to suggest that these newly-biosynthesised intravesicular molecules eventually are inserted into the luminal membrane surface after transport through other components of the endoplasmic reticulum [8]. The results indicate that the radioactivity in the vesicle content, immunoprecipitable by antibody to cytochrome P450 does not arise by detergent displacement from a site on the exterior membrane face.

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References

[1] Dallner, G., Siekevitz, P. and Palade, G. E. (1966) J. Cell Biol. 30, 97–117.
[2] Murat, A., Siekevitz, P. and Palade, G. E. (1967) J. Biol. Chem. 242, 2389–2396.

[3] Kuriyama, Y. and Omura, T. (1971) J. Biochem. (Tokyo) 69, 651–658.
[4] Omura, T. and Kuriyama, Y. (1971) J. Biochem. (Tokyo) 69, 659–669.
[5] Harano, T. and Omura, T. (1978) J. Biochem. (Tokyo) 82, 1541–1549.
[6] Harano, T. and Omura, T. (1978) J. Biochem. (Tokyo) 82, 1551–1557.
[7] Craft, J. A., Cooper, M. B. and Rabin, B. R. (1978) FEBS Lett. 88, 62–66.
[8] Craft, J. A., Cooper, M. B., Rees, D. E., Estall, M. and Rabin, B. R. (1979) submitted.
[9] Nilsson, O. S. and Dalner, G. (1977) J. Cell Biol. 72, 568–583.
[10] Ito, A. and Sato, R. (1969) J. Cell Biol. 40, 179–189.
[11] Kreibich, G., Hubbard, A. L. and Sabatini, D. D. (1974) J. Cell Biol. 60, 616–627.
[12] Welton, A. F. and Aust, S. D. (1974) Biochim. Biophys. Acta 373, 197–210.
[13] Blyth, C. A., Freedman, R. B. and Rabin, B. R. (1971) Eur. J. Biochem. 20, 580–586.
[14] Warren, G. and Dobberstein, B. (1978) Nature 273, 569–571.
[15] Ryan, D., Lu, A. Y. H., West, S. and Levin, W. (1975) J. Biol. Chem. 250, 2157–2163.
[16] Haugen, D. A., Van der Hoeven, T. A. and Coon, M. J. (1975) J. Biol. Chem. 250, 3567–3570.
[17] Ryan, D., Lu, A. Y. H., Kawalek, J., West, S. B. and Levin, W. (1975) Biochem. Biophys. Res. Commun. 64, 1134–1141.
[18] Rothman, J. E. and Lenard, J. (1977) Science 195, 743–753.
[19] DePierre, J. W. and Ernster, L. (1977) Ann. Rev. Biochem. 46, 201–262.
[20] Haugen, D. A., Armes, L. G., Yasunobu, K. T. and Coon, M. J. (1977) Biochem. Biophys. Res. Commun. 77, 967–973.