CYTOCHROME *P*-450 ASSOCIATED WITH FREE POLYSOME FRACTIONS

John A. CRAFT, Michael B. COOPER, Elizabeth A. SHEPHARD and Brian R. RABIN
Department of Biochemistry, University College London, London WC1E6BT, England

Received 24 September 1975

1. Introduction

It has been suggested that constitutive membrane proteins are synthesised on rough endoplasmic reticulum with subsequent translocation, where appropriate, to smooth membranes, [1–4]. However, alternative models may be possible and indeed evidence has been presented suggesting that some membrane proteins may be inserted from a cytoplasmic pool [5–8]. A recent report [9] described the presence of cytochrome *P*-450, a component of the microsomal mixed function oxidase both of completed molecules and in nascent forms, in the free polysome fractions derived from rabbit liver. These molecules were claimed to be associated with a class of free heavy polysomes. We have re-evaluated the experimental basis of this claim, working mainly with rat liver. We conclude that cytochrome *P*-450 in fractions of free hepatic polysomes is associated with membranous contamination and is not present on the ribosomes.

2. Methods

Male and female Sprague–Dawley rats, weighing 200–250 g and male New Zealand rabbits, weighing 2–2.5 kg were used in these studies. Where appropriate rats received a single injection of phenobarbital (40 mg/kg in 0.9% saline) i.p. for four days prior to experiments. All animals were starved for 24 hours prior to experiment.

Smooth endoplasmic reticulum, rough endoplasmic reticulum and free polyribosome fractions were prepared by previously described methods [10]. In some experiments the free polysome fractions were further purified by resuspension in 1.35 M sucrose containing 50 mM Tris–HCl, pH 7.5 at 20°C, 25 mM KCl and 5 mM $MgCl_2$ (TKM buffer). This was overlayered on 2.0 M sucrose–TKM buffer and in turn overlayered with 0.25 M sucrose–TKM buffer. The discontinuous gradient was centrifuged in an angle rotor as before. Samples were collected from both interfaces and polysome pellets resuspended in 0.25 M sucrose–TKM.

Total polysomes were prepared by solubilisation of the membrane in post-mitochondrial supernatant (PMS) by treatment with 1% cholate [11]. PMS was prepared in 0.25 M sucrose-TKM buffer, pH 7.6 at 20°C containing 5 mM EDTA, 1 mM dithiothreitol and 20% glycerol (v/v). Following sonication and solubilisation, PMS was overlayered on 1.7 M sucrose–TKM buffer, pH 7.6, containing 1 mM dithiothreitol and centrifuged at 125 000 g_{max} for 18 h in a swing out rotor.

Enzymes were assayed following the published methods cited:- cytochrome *P*-450 [12], cytochrome b_5 [13], glucose 6-phosphatase [14], aniline hydroxylase [15] NADPH-cytochrome *c*-reductase [16]. Protein was determined by the method of Lowry [17] and RNA by the method of Fleck and Begg [18]. Phospholipid was extracted by the method of Folch [19] and the resulting phosphorous determined by the method of Chen [20].

3. Results and discussion

3.1. *Electron microscopic analysis*

Electron microscopy revealed that both smooth endoplasmic reticulum and rough endoplasmic reticulum fractions consisted of vesicular structures. The rough membrane was well studded with polyribosomes

Table 1
Chemical composition and cytochrome content of microsomal fractions prepared from livers of control and phenobarbital treated male rats

	Free polysomes		Rough membranes		Smooth membranes	
	Control[a]	Treated[b]	Control	Treated	Control	Treated
RNA/protein (mg/mg)	0.73 ± 0.2	0.64 ± 0.04	0.12 ± 0.02	0.19 ± 0.06	0.04 ± 0.01	0.03 ± 0.01
Lipid phosphorus/protein (μg/mg)	1.17 ± 0.15	1.65 ± 0.09	13.2 ± 0.3	14.4 ± 1.35	20.5 ± 2.0	21.9 ± 4.2
Cytochrome *P*-450 nmol/mg protein	0.07 ± 0.01	0.13 ± 0.02	0.63 ± 0.07	1.37 ± 0.12	1.28 ± 0.24	3.01 ± 0.4
nmol/μg L.P.[c]	0.053 ± 0.014	0.091 ± 0.015	0.051 ± 0.007	0.095 ± 0.008	0.066 ± 0.004	0.142 ± 0.023
Cytochrome b_5 nmol/mg protein	<0.012	0.02 ± 0.003	0.23 ± 0.02	0.30 ± 0.02	0.55 ± 0.10	0.59 ± 0.07
nmol/μg L.P.	<0.008	0.013 ± 0.001	0.019 ± 0.002	0.021 ± 0.001	0.029 ± 0.002	0.028 ± 0.003
Cytochrome b_5 cytochrome *P*-450	<0.17	0.14 ± 0.003	0.38 ± 0.04	0.21 ± 0.02	0.43 ± 0.01	0.23 ± 0.04

[a]Data from 5 experiments
[b]Data from 7 experiments
[c]Units are in terms of μg of lipid phosphorus Values are means ± S.D.

Neither fraction was appreciably cross contaminated or contaminated with other organelles. The free polyribosome fractions contained variable contamination by both smooth and rough surfaced vesicles. We found no correlation between the phospholipid content and degree of membranous contamination as judged visibly be E.M. analysis.

3.2. *Chemical and enzymic composition of free polysome fractions*

The chemical composition and cytochrome content of microsomal fractions of control and phenobarbital treated rats are given in table 1.

Although free polysome fractions were characterised by low phospholipid content [21] the presence of cytochrome *P*-450 was clearly demonstrated. In terms of protein, the specific content of cytochrome *P*-450 in this fraction was approximately 10% of that found in rough membrane. However, when the results are expressed in terms of phospholipid phosphorous the specific content is comparable to that found in rough endoplasmic reticulum.

The specific content of cytochrome *P*-450 found in free polysome fractions paralleled that found in membrane fractions after phenobarbital treatment as well as in controls. Phenobarbital increased cytochrome *P*-450 in both membrane and free polysome fractions. Similarly the lower level of membrane cytochrome *P*-450 found in female compared with male animals was reflected in a lower content in the free polysome fractions (Male = 0.056 and 0.81 nmol/mg protein for free polysomes and rough membrane respectively. Female = 0.039 and 0.55 nmoles/mg protein for free polysomes and rough membrane respectively.)

These results strongly suggest that cytochrome *P*-450 found in free polysome fractions is associated with membranous contamination. The free polysome fraction might be expected therefore to contain other microsomal membrane enzymes. Cytochrome b_5 is a particularly good marker in this context since its detection is dependent on the presence of NADH-cytochrome b_5-reductase. In the series of experiments presented in table 1, cytochrome b_5 was only detected in free polysome fractions prepared from phenobarbital treated animals. The ratio of the levels of cytochrome b_5 to cytochrome *P*-450 found in this fraction was significantly less than that found in rough endoplasmic reticulum. This result may indicate that the membranous contamination contained in the free polysome fractions is not typical of bulk smooth or rough endoplasmic reticulum. Evidence has been presented [22,23] that both these fractions may themselves be heterogenous with respect to their enzyme content. Whilst the data of table 1 demonstrates a deficiency of cytochrome b_5 in free polysome fractions this has not been observed in every experiment. In some free polysome preparations from phenobarbital treated animals, the content of cytochrome b_5 was similar to that found in rough endoplasmic reticulum.

In addition to cytochrome *P*-450 and cytochrome b_5 a number of other membrane bound enzymes were also detected in the free polysome fractions prepared from phenobarbital treated rats and some data are presented in table 2. In all experiments the content of these enzymes in terms of protein is low when compared to smooth or rough membranes. However, in terms of phospholipid, the content of some enzymes is similar to that found in the membrane fractions. The two sets of experimental data presented in table 2 indicate the somewhat variable levels of enzyme activ-

Table 2
Enzymatic activities of microsomal fractions prepared from liver of phenobarbital-treated male rats

	Free polysomes		Rough membranes		Smooth membranes	
	Expt.1	Expt.2	Expt.1	Expt.2	Expt.1	Expt.2
Cytochrome *P*-450 nmol/μg L. P.	0.07	0.09	0.09	0.09	0.15	0.16
Cytochrome b_5 nmol/μg L.P.	0.009	0.017	0.021	0.018	0.032	0.031
NADPH-cytochrome *c* reductase OD/min/μg L.P.	0.05	0.09	0.11	0.19	0.12	0.19
Aniline hydroxylase OD $\times 10^{-4}$/min/μg L.P.	1.49	3.00	3.99	2.56	6.16	6.30
Glucose 6-phosphatase μmole phosphate $\times 10^{-2}$/min/μg L.P.	0.74	0.78	0.86	0.84	0.80	0.66
Lipid phosphorus/protein (μg/mg)	2.18	2.06	14.7	14.9	23.8	21.3

Table 3
Enzymic activities of free polysome fractions following purification

	Cytochrome *P*-450 nmol/μg L.P.	Glucose 6-phosphatase μmol phosphate × 10^{-2}/min/μg L.P.	Lipid phosphorus/ protein (μg/mg)
Starting free polysomes	0.078	0.88	2.22
Purified free polysomes	<0.03	<0.02	1.02
Material at 0.25–1.35 M interface	0.075	0.64	–
Material at 1.35–2.0 M interface	0.11	1.13	–

Results are mean of 3 experiments.

ities observed in free polysome fractions in different experiments. Much more consistent values were obtained in smooth and rough endoplasmic reticulum. Thus in some experiments the content of some enzymes in free polysomes appears to be deficient compared to membranes while in other experiments they are identical.

3.3. *Purification of free polysomes*

When free polysomes were subjected to a second buoyant density centrifugation much of the membranous contamination was eliminated, yielding a polysome fraction in which cytochrome *P*-450 and glucose 6-phosphatase could not be detected (table 3). Instead these activities are recovered at the interfacial positions where smooth and rough membranes sediment. Polysomes prepared from detergent treated PMS (see Methods) contained no detectable cytochrome *P*-450 although the cytochrome was still detected in the 0.25 M sucrose layer.

Our results demonstrate that cytochrome *P*-450 found in free polysome fractions obtained from rat liver is probably associated with membranous contamination. However the studies of Ichikawa and Mason [9] were carried out with rabbit liver and there could be species differences. To preclude this possibility we have carried out some experiments with free polysome preparations from rabbits. Cytochrome *P*-450, cytochrome b_5, glucose 6-phosphatase, NADPH-cytochrome *c*-reductase and aniline hydroxylase were all detected in free polysome fractions at levels with respect to phospholipid similar to the membrane fractions. When the rabbit free polysomes were subject to the purification procedures described above, cytochrome *P*-450 and glucose 6-phosphatase sedimented with the interfacial material and were absent in the pelleted polysomes.

Our experiments do not exclude the possibility that cytochrome *P*-450, at levels below the present limits of detection, are present in free polysomes as intermediates in the biosynthesis of this protein. We can however conclude that cytochrome *P*-450 does not occur in free polysomes at the levels previously claimed [9].

Acknowledgements

We thank Ms C. Davie who carried out the electron microscope analysis and acknowledge the financial support of the Cancer Research Campaign and Medical Research Council. Work was carried out in laboratories provided by the Nuffield Foundation.

References

[1] Siekevitz, P. and Palade, G. E. (1967) J. Biol. Chem. 242, 2589.
[2] Kurlyama, Y., Omura, T., Siekevitz, P. and Palade, G. E. (1969) J. Biol. Chem. 244, 2017–2026.
[3] Kuriyama, Y. and Omura, T. (1971) J. Biochem. (Tokyo) 69, 651–658.
[4] Omura, T. and Kuriyama, Y. J. Biochem. (Tokyo) 69, 659–669.
[5] Dehlinger, P. J. and Schimke, R. T. (1971) J. Biol. Chem. 246, 2574–2583.
[6] Dehlinger, P. J. and Schimke, R. T. (1972) J. Biol. Chem. 247, 1257–1264.
[7] Lodish, H. F. and Small, B. (1975) J. Cell Biol. 65, 51–64.
[8] Atkinson, P. H. (1975) J. Biol. Chem. 250, 2123–2134.
[9] Ichikawa, Y. and Mason, H. S. (1974) J. Mol. Biol. 86, 559–575.
[10] Blyth, C. A., Freedman, R. B. and Rabin B. R. (1971) Eur. J. Biochem. 20, 580–586.
[11] Lu, A. Y. H. and Levin, W. (1972) Biochem. Biophys. Res. Commun. 46, 1334–1339.

[12] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379–2385.
[13] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370–2377.
[14] Loekes, A., Siekevitz, P. and Pallade, G. E. (1971) J. Cell Biol. 49, 264–287.
[15] Schenkman, J. B., Remmer, H. and Estabrook, R. W. (1967) Mol. Pharm. 3, 113–123.
[16] Phillips, A. H. and Langdon, R. G. (1962) J. Biol. Chem. 237, 2652–2660.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Fleck, A. and Begg, D. (1965) Biochim. Biophys. Acta 108, 333–339.
[19] Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 326, 487–509.
[20] Chen, P. S., Toribara, T. Y. and Warner, H. (1956) Anal. Chem. 28, 1786.
[21] Murty, C. N. and Hallinan, T. (1969) Biochem. J. 112, 269–274.
[22] Dallner, G., Bergstrand, A. and Nilsson, R. (1968) J. Cell Biol. 38, 257–276.
[23] Glaumann, H. and Dallner, G. (1970) J. Cell Biol. 47, 34–38.