THE TEMPLATE BINDING AND SELF-ASSEMBLY IN THE RECONSTITUTION OF MICROSOMAL REDOX CHAINS: ELECTRON CARRIERS WHICH DO AND DO NOT BIND WITH MICROSOMAL MEMBRANES

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1. Introduction

Three methods were used for reconstitution of the microsomal membrane redox chains. The first involves no template being introduced into the system to be reconstituted. Reconstitution proceeds by means of the self-assembly of the solubilized membrane proteins and lipids. The second method is based on membrane proteins being bound with egg lecithin liposomes which are used as non-specific templates. The third method is when solubilized complexes bind to the membranes from which they were isolated [1]. In this case the latter are in fact specific templates. Reconstitution was carried out from the membrane proteins and lipids solubilized by sodium cholate.

2. Methods

Male rats (200-250 g) were used in this work. The microsomal fraction was obtained as described previously [2]. The isolation media contained 1.15% KCl with 0.2 mM EDTA and 1 mM dithiothreitol. The microsomes were suspended in 100 mM Tris–HCL, pH 7.4, containing 0.2 mM EDTA and 1 mM dithiothreitol (TED solution).

The following parameters in the microsomes and reconstituted membranes were determined: the content of protein [3]; cytochromes b_5 and P 450 [4]; the activity of NADPH- and NADH-specific reductases [5]; the activity of NADPH- and NADH-dependent *p*-hydroxylases of aniline [6] and *N*-demethylases of dimethylaniline (DMA) [7] and activity of inosine diphosphatase [8]. Egg lecithin liposomes were prepared as described by Bangham [9].

It was established by treating the microsomal fraction (fraction 1) with different concentrations of the detergent that 0.15% sodium cholate ruptures vesicles but does not solubilize the carriers [8]. Ruptured microsomal membranes isolated by centrifugation at 150 000 *g* for 90 min close up to form vesicles which tentatively called the 'ghosts' of microsomal vesicles, which are used as specific template. The supernatant obtained by resedimentation of the 'ghost' fraction treated with 4% sodium cholate, represents a fraction containing in a solubilized state, membrane proteins and lipids and is tentatively called 'solubilizate'. To remove from the 'solubilizate' 4% sodium cholate, which, when used in such a high concentration, dissolves microsomal membranes, the 'solubilizate' was dialyzed against a solution containing 20 mM Tris–HCl, pH 7.4, 0.2 mM EDTA, 1 mM dithiothreitol and 0.1% sodium cholate for 22 hr at 4°C. Addition of 0.1% sodium cholate to this media is necessary to prevent self-assembly. The dialyzed 'solubilizate' (fraction 2) was used as starting material for reconstitution of the microsomal redox chains. To study the reconstitution of microsomal redox chains, four dialysis systems were used. Dialysis was performed against a solution, containing 20 mM Tris–HCl, pH 7.4, 0.2 mM EDTA and 0.2 mM dithiothreitol for 22 hr at 4°C. The first incubation medium contained 6 ml (15 mg/ml protein) of 'ghosts' and 55 ml (2 mg/ml protein) of 'solubilizate' (template binding). The second system contained 6 ml of 'ghosts' and 55 ml TED (control mixture). The third contained

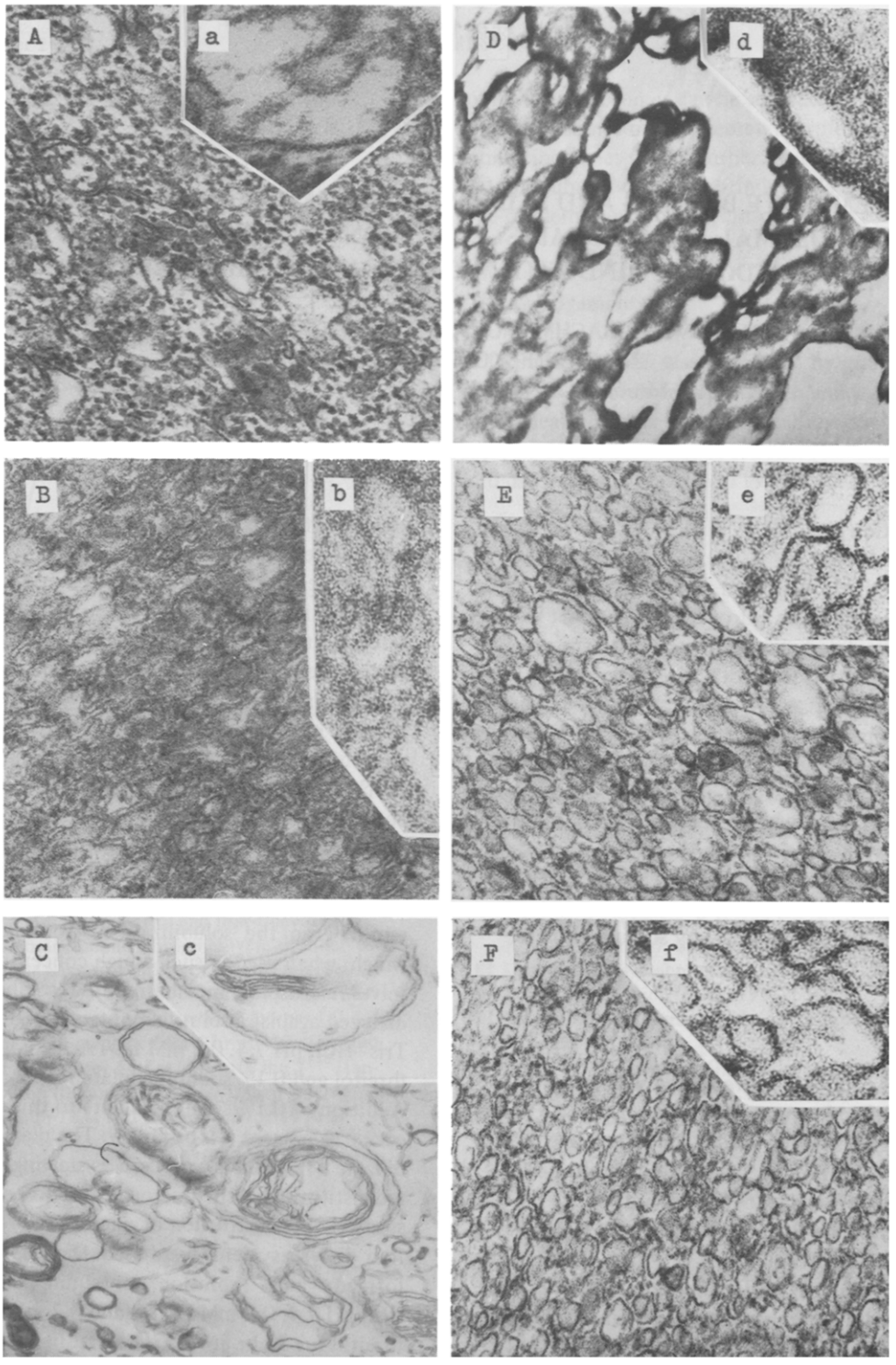

Fig. 1. The electron micrographs of microsomes and reconstituted membranes. The electron micrographs are made from thin sections of Araldite-embedded pellets fixed by OsO_4. The sections were stained with lead citrate and uranyl acetate and examined in the Hitachi-11E electron microscope. The pellet of microsomal fraction × 70 000 (A) and × 180 000 (a); 'ghosts' of microsomal vesicles × 70 000 (B) and × 180 000 (b); liposomes × 22 000 (C) and × 70 000 (c); the pellet of 'solubilizate' (self-assembly) × 70 000 (D) and × 180 000 (d); 'ghosts + solubilizate' (the binding with specific template) × 70 000 (E) and × 180 000 (e); liposomes + 'solubilizate' (the binding with non-specific template) × 70 000 (F) and × 180 000 (f).

Table 1
The content of cytochromes b_5, P 450 and P 420 in reconstituted fractions.

Fraction	Cytochrome b_5, reduced by $Na_2S_2O_4$		Cytochrome b_5, reduced by NADH		Cytochrome P 450		Cytochrome P 420	
	a	b	a	b	a	b	a	b
1	0.45	–	0.28	–	0.57	–	–	–
2	0.53	58.3	0.41	45.1	0.25	27.5	0.59	64.9
3	0.81	63.0	0.54	43.0	0.74	57.6	0.18	14.0
4	0.60	29.5	0.40	19.4	0.86	41.8	–	–
5	0.71	28.6	0.56	22.4	0.42	16.8	0.42	16.8
6	0.64	25.1	0.50	19.1	0.39	14.9	0.36	13.7

The content of cytochromes b_5, P 450 and P 420 is calculated as nmoles · mg^{-1} (a) and nmoles in total fraction (b).

55 ml of 'solubilizate' and 6 ml TED (self-assembly). The fourth contained 55 ml of 'solubilizate' and 6 ml suspension (3 mg/ml lecithin) of liposomes (liposomes binding). The ratio of the dialyzate to the dialyzing solution was 1 : 70 (v/v). After dialysis the experimental material was sedimented at 150 000 g for 90 min. The pellets obtained: 'ghosts'+'solubilizate' (fraction 3), 'ghosts' (fraction 4), the pellet of 'solubilizate' (fraction 5) and liposomes + 'solubilizate' (fraction 6) were suspended in the TED solution and used for analysis.

3. Results and discussion

Fig. 1. presents the data on the electron microscope analysis of the fractions. It is seen that the fraction of 'ghosts' contains closed microsomal vesicles, which have no ribosomes and are poorly stained (fig. 1B,b). The membrane structures which are reconstituted from the componets of the 'solubilizate' form a network consisting of fused vesicles (fig. 1D,d). Such structures do not form if the reconstitution is performed from the 'solubilizate' using the 'ghosts' and liposomes as specific and non-specific templates. In these cases the distinctly stained vesicles of microsomes with bilayer membrane are formed (fig. 1E,e and 1F,f). The fraction of the liposomes contained large vesicles contoured with some poorly stained membranes (fig. 1C,c). Addition of the 'solubilizate' to this fraction gives rise to little vesicles covered with bilayer membrane which is well stained.

Reconstituted membranes contain more cytochrome b_5 than the starting microsomes (table 1). The content of cytochrome b_5 is the highest in the membranes obtained as a result of binding of the carrier with the 'ghosts' of microsomal vesicles (fraction 3). With liposomes the binding is much worse (fraction 6). The specific content of the carrier bound with liposomes is lower than in the fraction of membranes obtained by self-assembly. Cytochrome P 450, in contrast to cytochrome b_5, is not incorporated into 'ghosts' of the microsomal membranes (table 1) At the same time, the reactivation of the carrier is very distinct in the processes of self-assembly and binding with liposomes. The ratio of cytochromes P 420 to P 450 equal to 2.3 in 'solubilizate' (fraction 2) decreases in fraction 5 (the pellet of 'solubilizate') and

Table 2
The activity of the NADH- and NADPH-specific reductases in reconstituted fractions.

Electron acceptor	Electron donor		Fraction					
			1	2	3	4	5	6
Cytochrome c	NADH	a	1940	3620	3890	1980	3940	4150
		b		398000	303000	96200	158000	168000
	NADPH	a	190	129	216	163	218	217
		b		14200	16800	7920	8720	8290
Ferricyanide	NADH	a	6600	7430	11600	7440	11400	13800
		b		817000	905000	361000	456000	528000
	NADPH	a	154	142	206	155	201	210
		b		15600	16000	7530	8040	8020

The activity of reductases is calculated as nmoles · mg^{-1} · min^{-1} (a) and nmoles · min^{-1} in total fraction (b).

Table 3
The hydroxylase activities in reconstituted fractions.

Type of hydroxylation		Fraction				
		1	3	4	5	6
NADPH-dependent *N*-demethylation of DMA	a	10.7	6.07	9.64	2.14	1.69
	b		513	489	90	67
NADPH-dependent *p*-hydroxylation of aniline	a	0.64	0.40	0.70	0.070	0.065
	b		32.8	33.8	2.9	2.6
NADH-dependent *N*-demethylation of DMA	a	0.88	0.81	0.92	0.61	0.49
	b		69.6	47.3	25.6	20.4
NADH-dependent *p*-hydroxylation of aniline	a	0.06	0.076	0.10	0.03	0.029
	b		6.28	4.76	1.18	1.11

The activity of hydroxylases is calculated as nmoles $\cdot mg^{-1} \cdot min^{-1}$ (a) and nmoles $\cdot min^{-1}$ in total fraction (b).

in fraction 6 (liposomes + 'solubilizate') to 1. The specific activity of NADH- and NADPH-specific reductases in fractions 3,5,6 is much higher than that in fractions 1 and 4 (table 2). However, a calculation of the total activity of reductases in the reconstituted fractions has shown that it is only with NADH-specific flavoprotein that additional binding takes place. The total activity of this enzyme in 'ghosts+solubilizate' (fraction 3) is much higher than the sum of its activities in fractions 4 ('ghosts') and 5 (the pellet of 'solubilizate').

No complete reconstitution of the activity of the hydroxylation system was observed in any case of incorporation and self-assembly (table 3). From 10 to 20% of the initial activity of the NADPH-dependent *p*-hydroxylation of aniline and *N*-demethylation of DMA was reconstituted by self-assembly. In the case of NADH-dependent *p*-hydroxylation of aniline, in self-assembly about 25% of the activity was restored, and with *N*-demethylation, the activity was much higher – about 55% from the initial level. The incorporation of the carriers into the membranes of the microsomal vesicles, 'ghosts', was not accompanied by reconstitution of the hydroxylation systems. This may be explained by the fact that the terminal component of all the hydroxylation systems – cytochrome *P* 450 – does not incorporate into 'ghosts'.

The results obtained testify to the fact that cytochrome b_5 and NADH-specific flavoprotein bind with the 'ghosts' of microsomal membranes. No binding of cytochrome *P* 450 and NADPH-specific flavoprotein with the 'ghosts' of the microsomal membranes was observed. At the same time the self-assembly of membranes and incorporation of the carriers into liposomes caused reactivation of cytochrome *P* 420. The ratio of cytochromes *P* 420 to *P* 450 changed from 2.3 in the starting 'solubilizate' to 1 in the membranes reconstituted therefrom.

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