DISTRIBUTION OF CONSTITUTIVE ENZYMES AND PHOSPHOLIPIDS IN MICROSOMAL MEMBRANES OF RAT LIVER

Olle NILSSON and Gustav DALLNER

Department of Pathology at Sabbatsberg Hospital, Karolinska Institutet and Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, Stockholm, Sweden

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1. Introduction

The endoplasmic reticulum of liver is responsible for a large number of functions which are mediated by membrane-bound enzymes. The distribution of substrates and products is asymmetric since some of these can be recovere l almost exclusively either in the cytoplasmic or luminal compartments. The uneven distribution of both protein and phospholipid components has been shown in erythrocyte membranes [1–3] and a similar asymmetry of enzymes is also well demonstrated in inner mitochondrial membranes [4]. There are some indications that even in microsomal membranes a few enzymic proteins occur preferentially on the cytoplasmatic surface, like NADPH*–cytochrome *c* reductase and cytochrome b_5 [5], while others, such as nucleoside diphosphatase [6] may be found in inner sites. In this paper the effect of several proteolytic and lipolytic enzymes on intact microsomal vesicles is demonstrated. Using vesicles permeable to macromolecules the inner surface was digested by these enzymes and the chemical constituents analyzed.

* *Abbreviations:* DOC, deoxycholate; PL, phospholipid; AMP and ATP, adenosine mono- and triphosphate; GA, glucuronic acid; G6P, glucose 6-phosphate; IDP, inosine diphosphate, NADH and NADPH, reduced nicotinamide dinucleotide and dinucleotide phosphate; UDP, uridine diphosphate.

2. Materials and methods

Rats starved for 20 h were used. Total microsomal fraction was isolated and washed with 0.15 M Tris–HCl buffer, pH 8.0, in order to remove adsorbed proteins [7]. In order to increase microsomal permeability to macromolecules, microsomes (40 mg protein in 2 ml 0.25 M sucrose) were added under rapid mixing to 9 ml of medium containing 50 mM KCl, 50 mM Tris–HCl, pH 7.5 or 6.5 (trypsin or phospholipase), 0.25 M sucrose and 0.061% sodium deoxycholate [8]. After vaious incubations the suspensions were centrifuged without dilution at 105 000 *g* for 2 h and the pellets were analyzed.

Protein and phospholipid determinations, radioactivity measurements, thin-layer chromatography and measurement of intramicrosomal water space were performed according to previously described methods [9,10]. Enzyme activities and amounts were analyzed as earlier described [9,11].

3. Results and discussion

The chemical and enzymic dissection of microsomal vesicles is demonstrated in table 1. Trypsin treatment removed about 35% of the microsomal protein which also includes ribosomes. The unchanged intramicrosomal water space demonstrated the intactness of the trypsin-treated microsomes. 0.05% DOC in the presence of 50 mM KCl increased microsomal permeability which is apparent from the abolished barrier for high mol. wt dextran. The amount of pro-

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Table 1
Effect of trypsin and detergent on microsomal enzyme activities and amounts

	Treatments			
	None	0.05% DOC	Trypsin	Trypsin + 0.05% DOC
	% of non-treated microsomes			
Protein[a]	16.0	71	66	44
Intramicrosomal water[b]	1.24	25	108	31
NADPH-cyt. *c* red.[c]	0.11	100	12	12
NADH- cyt. *c* red.[c]	1.75	74	35	18
NADH-ferricyanide red.[c]	3.53	75	85	62
Cytochrome b_5[d]	1.98	100	30	5
Cytochrome P-450[d]	1.67	87	59	29
ATPase[e]	0.36	97	81	67
AMPase[e]	0.20	98	95	92
IDPase[e]	1.89	11	95	8
G6Pase[e]	1.05	72	98	12
Esterase[f]	12.6	26	98	24
β-glucuronidase[f]	0.19	29	82	15
UDPGA-transferase[g]	0.018	85	90	76

The values of non-treated microsomes are given as:
[a] mg/g liver (wet weight)
[b] μl/mg dry weight
[c] μmol NADPH or NADH ox/min/mg PL
[d] nmol cytochrome b_5 or *P*-450/mg PL
[e] μmol P_i/min/mg PL
[f] μmol *p*-nitrophenyl liberated/min/mg PL
[g] μmol *p*-nitrophenyl conjugated/min/mg PL.
The substrate used for measurement of esterase activity was *p*-nitrophenyl acetate. In the case of trypsin treatment 50 μg dialyzed trypsin (Boehringer, Mannheim) per mg protein was added to the microsomal suspension containing 50 mM KCl, 50 mM Tris–HCl buffer, pH 7.5 and 0.25 M sucrose, and incubated for 10 min at 30°C in a final vol of 11 ml. At the end of the incubation the reaction was stopped by cooling in ice–water bath. Each value gives the mean of 9–13 experiments.

tein released, 25%, correspond to secretory proteins enclosed into the vesicle lumen, in addition to released ribosomes. An increased solubilization takes place when trypsin digestion is employed in the presence of deoxycholate. Thus, enzymes like trypsin have access to the inner vesicle surface of the microsomes only in the presence of low concentrations of DOC.

Trypsin incubation of microsomes removed or inactivated electron transport enzymes to a varying degree. NADPH–cytochrome *c* reductase was completely solubilized and cytochrome b_5 was liberated to a large extent explaining thereby the inactivation of the NADH–cytochrome *c* reductase system which involves the interaction of the b_5. The NADH–ferricyanide reductase activity itself was affected by trypsin only to a moderate extent. Cytochrome *P*-450 exhibited a different sensitivity with 40% being inactivated by trypsin treatment of the intact microsomes and an additional 20% activity was lost when trypsin gained access to the inner surface.

Both ATPase and AMPase were practically insensitive to proteolytic action on both surfaces, while IDPase already had been released from the vesicles by treatment with 0.05% DOC. The release of IDPase by DOC indicates a loose association of the enzyme with the inner surface. G6Pase was inactivated only by action of trypsin in permeable vesicles indicating association of this enzyme with the inner aspects of the membranes.

Esterase and microsomal β-glucuronidase activities

displayed similar properties as IDPase, i.e. a high degree of solubilization occurred already after increase of permeability by low amount of DOC. UDPGA-transferase, on the other hand, could not be influenced to a larger extent when trypsin was applied from either side of the membrane.

The above experiments with trypsin were repeated using chymotrypsin and protease in a similar manner, that is, proteolysis on both sides of the membrane was maintained until maximal attainable protein release. The amount of protein removed and the pattern of enzyme distribution were very similar to that seen with trypsin digestion.

The microsomal fraction was subjected to treatment with phospholipase A from *Vipera Russeli* and with heat-treated *Naja Naja* snake venom (table 2). A 10 min treatment at 30°C removed 50–65% of the total phospholipids and also made microsomal membranes permeable to high mol. wt dextran. However, incubation in the presence of albumin increased the amount of lipid liberated but left the permeability of intact microsomal membranes unaffected. This demonstrates that albumin binds all the liberated lysocompounds and abolishes their detergent-like action. Incubation of intact vesicles for longer time did not release any more PL. In the presence of 0.05% DOC and in the absence of albumin over 90% of PL is hydrolyzed after 30 min incubation.

Phospholipase A and *Naja Naja* treatment of intact microsomes in the presence of albumin completely liberated phosphatidylethanolamine and phosphatidylserine from the microsomal pellet and at the same time also removed 40% of the membrane-bound phosphatidylcholine. The remaining of this latter lipid together with phosphatidylinositol were hydrolyzed after prolonged incubation when the lipolytic enzymes

Table 2
Effect of phospholipase treatment on microsomal permeability

Treatment	Albumin g in medium	PL mg/g liver	Intramicrosomal H_2O µl/mg dry weight
None	0	6.3	1.19
PLase A	0	3.5	0.54
PLase A	0.5	2.8	1.03
Naja Naja	0	4.0	0.49
Naja Naja	0.5	3.1	1.13

Phospholipase A from *Vipera Russeli* and *Naja Naja* snake venom (Sigma, St. Louis) were heated before use in 50 mM Tris–HCl buffer, pH 6.5 at 90°C for 8 min and the precipitates were separated by centrifugation. The incubation medium in a final vol of 11 ml contained 50 mM KCl, 50 mM Tris–HCl buffer, pH 6.5, phospholipase A (0.5 U/40 mg protein) or *Naja Naja* venom (0.3 mg/40 mg protein) and when indicated 0.5 g bovine serum albumin. Incubation was continued for 10 min at 30°C and terminated by the addition of 10 mM EDTA. Each value gives the mean of 5 experiments.

Table 3
Effect of phospholipase treatment on microsomal phospholipid composition

Phospholipid	Control (cpm)	Incubation PLase A	*Naja Naja*
		% of control radioactivity in pellet	
P-ethanolamine	53 400	16	8
P-serine	1 000	3	18
P-inositol	800	77	84
P-choline	57 100	44	61
Sphingomyelin	700	87	85

For in vivo labeling of microsomal PL, carrier-free isotonic ^{32}P (Radiochemical Centre, Amersham) was injected intraperitoneally (1.2 mCi/100 g body weight) 60 min before decapitation. Lipids were extracted, separated on thin-layer chromatography and the radioactivity in the individual spots was determined as previously [9]. Phospholipase treatments were as described in table 2. P represents phosphatidyl. Each value gives the mean of 9 experiments.

gained access to the luminal compartment. The small quantity as well as the poor degree of labeling of sphingomyelin did not allow for a reliable evaluation of results with this lipid. In table 3 the PL-bound radioactivity remaining in the pellet is given. Chemical measurements of PL gave similar results to those obtained by methods using radioactive PL.

The enzymatic digestion of proteins and lipids in permeable and non-permeable microsomal vesicles give a strong indication that the various components in the endoplasmic reticulum exhibit an asymmetric distribution. There is hardly any doubt that both microsomal enzymes and phospholipids have a well defined and differentiated distribution in the vertical plane of the membrane. After trypsin treatment, the microsomal enzymes could be divided into three groups, in the first, solubilization or inactivation occurred by treatment of the intact vesicles; in the second, enzymes were influenced after introduction of trypsin into the vesicle lumen; and in the third group the enzymes were not affected by proteolysis. Phospholipids exhibited an asymmetric distribution with ethanolamine and serine containing lipids being found on the cytoplasmic surface, with phosphatidylinositol at the luminal side, and lecithin being more evenly distributed. The possible asymmetric distribution may serve a functional purpose which is determined by the availability of substrate or deposition of product. At present we have no explanation as to why microsomal phospholipids display asymmetry. However, the reason for the lipid asymmetry might be related to either enzyme associated functions for lipid requiring enzymes, specific localization of the biosynthetic pathway or the necessity of a homogeneity in the lateral plane in order to facilitate the lateral movement of enzyme proteins.

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