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LOCALIZATION OF PROTEIN-BOUND CARBOHYDRATE RESIDUES ON THE CYTOPLASMIC SURFACE OF ROUGH AND SMOOTH MICROSOMES AND GOLGI VESICLES FROM RAT LIVER

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Summary

Rough and smooth microsomes and Golgi membranes isolated from rat liver were treated with proteolytic enzymes under conditions which removed 30–40% of the surface proteins without seriously disrupting the membrane structure. This treatment also removed 40—60% of protein-bound mannose, galactose and glucosamine. When protease treatment was combined with neuraminidase treatment, 80% of the sialic acid was removed from intact rough microsomal and Golgi vesicles and about half of the sialic acid of smooth microsomes was solubilized. It appears that half, or probably more, of the membrane glycoproteins are associated with the cytoplasmic surface of these membranes.

During recent years investigations based on proteolysis, antibodies, ^{125}I-labeling, substrate permeability, latency and inhibitors have led to the conclusion that enzyme proteins are asymmetrically distributed in the transverse plane of the microsomal membrane [1, 2]. In the case of glycoproteins it has been established that most, or all, are attached to the outer surface of erythrocyte and other plasma membranes [3, 4]. On the other hand, only a few studies have been performed to date concerning the distribution of protein-bound sugar residues in intracellular membranes. Experiments with lectins and hydrolytic enzymes indicate that some glycoproteins may be localized on the outer surface of chromaffin granules from the adrenal medulla, and of microsomes and Golgi vesicles from liver [5—7]. In contrast, on the basis of experiments using ferritin conjugates of lectins, it was proposed that saccharide residues of rough and smooth microsomal membranes prepared from myeloma cells are located at the inner, luminal surface [8]. In this study we describe the effect of proteolytic enzymes and neuraminidase on the

protein-bound sugar residues of microsomal vesicles and Golgi membranes.

Adult male albino rats weighing 180—200 g were used. The animals were starved for 20 h before killing. Rough and smooth microsomes and plasma membranes were prepared as described earlier [9, 10]. For isolation of Golgi fraction 1.2 ml 50% ethanol per 100 g body weight was administered to the rats by stomach tube 90 min before decapitation [11]. The total Golgi fraction floating on the top of the 1.10 M sucrose layer was used. The subfractions were suspended in 0.25 M sucrose/50 mM Tris-HCl, pH 7.5, at a concentration of 10 mg protein/ml (rough and smooth microsomes) or 5 mg/ml (Golgi fraction). In the case of treatment with proteases the subfractions were treated with 100 μg trypsin (Boehringer, Mannheim, G.F.R.) and 100 μg pronase, an unspecific bacterial protease from *Streptomyces griseus* (Sigma Chemical Co., St. Louis, Mo.), per mg protein. Incubation was carried out at 30°C for 10 min. The particles were then pelleted by centrifugation (105 000 × *g* for 60 min). When the distribution of sialic acid was to be determined, the pellets were again suspended after proteolytic treatment in the sucrose/Tris-HCl medium, 20 μg neuraminidase (Type VI, Sigma) per mg protein was added, and this suspension was incubated at 30°C for 10 min. The particulate fraction was collected by ultracentrifugation and subjected to the Tris-water-Tris washing procedure in order to remove adsorbed and luminal, secretory proteins [9]. This method has proved to be very efficient in removing all adsorbed and secretory proteins from rough and smooth microsomes, but is probably less effective for the removal of secretory proteins from Golgi vesicles. The pellets were finally suspended in water at a concentration of 5 mg protein/ml. The controls were incubated without enzymes and washed in the same manner as the treated samples.

For the determination of neutral sugars the fractions were delipidated and hydrolyzed (1 M HCl, 100°C, 8 h) and the sugars purified by ion-exchange chromatography as described earlier [6]. Alditol acetate derivatives were prepared and the neutral sugars were estimated by gas-liquid chromatography [12, 13]. Amino sugars were released by hydrolysis (2 M HCl, 100°C, 16 h) and purified by ion-exchange chromatography [6] and the amount of hexosamine was determined [14]. Since microsomes and Golgi vesicles do not contain galactosamine [6], all of the hexosamine present was taken to be glucosamine. Sialic acid was liberated by hydrolysis (0.1 M H_2SO_4, 80°C, 60 min), purified as described earlier [15], and quantitated by the Warren procedure [16]. Protein, phospholipid and enzyme activities were measured as described before [2, 15]. The values in the tables represent the mean of 5—7 experiments.

Rough and smooth microsomes and Golgi membranes are modified in a characteristic manner by the combined treatment with trypsin and pronase (Table I). 40% of rough microsomal and about 30% of smooth microsomal and Golgi membrane proteins are liberated without removal of membrane phospholipids. In addition, measurement of membrane permeability for macromolecules using radioactive dextran demonstrated, in agreement with previous investigations [2, 17], that the protease treatment caused no increase in membrane permeability and 90% of the vesicles are impermeable to dextran with a molecular weight of 15 000 (not shown in Table I). NADPH-cytochrome *c* reductase, an enzyme localized at the cytoplasmic surface of rough and

smooth microsomes [18], was removed completely by the proteolysis. In contrast, IDPase, an enzyme thought to be associated with the inner surface of microsomal vesicles [19], was not affected by the proteolytic treatment. These experiments demonstrate that it is possible to solubilize the outer surface of the intact vesicles without destroying the membrane structure, e.g. without increasing their permeability to macromolecules such as hydrolytic enzymes.

Cytoplasmic membranes contain the neutral sugars mannose and galactose in a protein-bound form (Table II). The mannose content on a phospholipid basis is higher in microsomes than in the Golgi fraction. Proteolysis removes as much as 60% of the protein-bound mannose from both microsomal subfractions, while the amount liberated from the Golgi fraction is about 40%. The amount of protein-bound galactose/mg phospholipid is least in rough microsomes, intermediate in smooth microsomes, and highest in Golgi vesicles; but in all three cases this sugar is affected by hydrolysis to about the same extent. Approximately half of the membrane protein-bound galactose is solubilized by treatment with the two proteases.

A relative large amount of protein-bound glucosamine is associated with

TABLE I

EFFECT OF COMBINED TRYPSIN AND PRONASE TREATMENT ON CYTOPLASMIC MEMBRANES

Isolated subfractions in 0.25 M sucrose/50 mM Tris-HCl, pH 7.5, were incubated without proteolytic enzymes (control) or with 100 μg of both trypsin and pronase (unspecific protease from *Streptomyces griseus*) per mg protein. After incubation for 10 min at 30°C the particles were sedimented by centrifugation (105 000 × *g*, 60 min) and subjected to the Tris-water-Tris washing procedure [9]. The pellets were suspended in water (5 mg protein/ml) and analyzed.

Fractions	Protein (mg/g liver)	Phospholipid (mg/g liver)	NADPH-cytochrome *c* reductase (μmol NADPH oxidized/min per mg phospholipid)	IDPase (μmol P_i released/ min per mg phospholipid)
Rough microsomes				
Control	4.4	2.28	0.11	1.85
Treated	2.5	2.08	0.011	1.79
Smooth microsomes				
Control	2.5	1.81	0.12	1.96
Treated	1.7	1.70	0.011	1.82
Golgi fraction				
Control	0.25	0.28		
Treated	0.18	0.25		

TABLE II

NEUTRAL SUGAR CONTENT OF INTACT CYTOPLASMIC MEMBRANES AFTER PROTEOLYSIS

The isolated fractions were either incubated without addition of proteases (control) or incubated at 30°C for 10 min in the presence of trypsin and pronase, both 100 μg/mg protein. The mannose and galactose contents of the Tris-water-Tris washed fractions were determined by gas-liquid chromatography.

Fractions	Mannose		Galactose	
	μg/g liver	%	μg/g liver	%
Rough microsomes				
Control	27.0	100	9.1	100
Treated	11.3	41.9	4.4	48.4
Smooth microsomes				
Control	18.9	100	10.9	100
Treated	7.6	40.2	5.9	54.1
Golgi vesicles				
Control	1.76	100	2.32	100
Treated	1.08	61.4	1.10	47.4

cytoplasmic membranes. On a phospholipid basis there is about twice as much protein-bound glucosamine in the Golgi fraction as in the two microsomal subfractions (Table III). Proteolytic treatment liberated about 55% of this sugar from rough and Golgi vesicles and the corresponding value for smooth microsomes was about 40%. Protein-bound sialic acid was also found in all three washed subfractions. In rough microsomes the amount was about half as much per mg phospholipid as in the two other subfractions. In previous investigations it was found that the protein-bound sialic acid of smooth microsomes is affected only after both protease and neuraminidase treatment [20]. For this reason the fractions were treated first with trypsin and pronase and thereafter with neuraminidase. After this treatment the vesicles were still intact, as indicated by the finding that they were still not permeable to high molecular weight dextran and that IDPase was neither released nor inactivated. The effect of this sequential hydrolytic treatment is very pronounced; almost all protein-bound sialic acid (about 80%) is removed from rough microsomes and Golgi vesicles while the amount removed from smooth microsomes is about 50%.

Intact microsomal vesicles do not bind lectins [21, 22] which raise the possibility that all glycoproteins are localized at the inner, luminal side. Therefore, the presence of sugars like sialic acid in microsomes, particularly in rough microsomes, could arise from contaminating plasma membrane fragments. AMPase activity, which is present in rough microsomes [23, 24] but mostly enriched in plasma membranes, exhibits a 20 times higher specific activity in the latter fraction (Table IV). On the other hand, sialic acid content in the plasma membrane fraction is only 4 times higher than in rough microsomes. Consequently, the lack of lectin-binding to the surface of

TABLE III

GLUCOSAMINE AND SIALIC ACID CONTENT OF INTACT CYTOPLASMIC MEMBRANES AFTER PROTEOLYSIS

Treatment in the case of glucosamine determination involved incubation of the fraction with trypsin and pronase. Sialic acid was measured on fractions which were incubated with neuraminidase after pretreatment with trypsin and pronase.

Fractions	Glucosamine		Sialic acid	
	μg/g liver	%	μg/g liver	%
Rough microsomes				
Control	22.2	100	10.5	100
Treated	9.7	43.6	2.4	22.9
Smooth microsomes				
Control	21.0	100	14.8	100
Treated	12.6	60.0	7.5	50.7
Golgi vesicles				
Control	6.12	100	2.2	100
Treated	2.76	45.0	0.33	15.0

TABLE IV

DISTRIBUTION OF SIALIC ACID AND AMPase ACTIVITY IN PLASMA MEMBRANES AND ROUGH MICROSOMES

Rough microsomal and plasma membrane fractions were subjected to the Tris-water-Tris washing procedure before measurements.

Fractions	Sialic acid (μg/mg protein)	AMPase (μmol P_i released/min per mg protein)
Rough microsomes	2.44	0.04
Plasma membranes	10.80	0.83

microsomal vesicles is not caused by the absence of sugar residues but by the lack of sterically available lectin acceptors.

The experiments described demonstrate clearly that a sizeable portion of the proteins of the rough and smooth microsomal and Golgi vesicles are localized at the cytoplasmic surface. The nature of these proteins is not yet known; they may include enzyme proteins such as cytochromes b_5 and *P*-450 [25–27]. Some of them may have non-enzymic functions such as participating in binding or recognition processes [28, 29]. About 50% of the neutral and amino sugar residues are removed by proteolysis, but it is possible that a larger portion or almost all of these sugars are associated with proteins at the outer surface. It is most reasonable to suppose that the hydrophilic oligosaccharide chains, especially those containing negatively-charged sialic acid, are situated mainly at the outer, cytoplasmic surface of the membrane. Proteolytic digestion of the membrane is by no means an ideal approach for studying the transverse topology; surface proteins may be protected by neighbouring membrane components or their exposed portions may be resistant to hydrolysis by the enzymes used [30]. Such considerations are emphasized by the finding that after sequential treatments with proteases and neuraminidase almost all sialic acid is removed from the intact vesicles of rough microsomes and the Golgi apparatus. Using the deoxycholate-salt procedure it is possible to introduce hydrolytic enzymes into the vesicle lumen and thus to study the composition of the inner surface [31]. However, this method may not be effective in determining to what extent sugar residues are associated with the inner surface. It is very difficult to remove all of the sugar-containing secretory proteins from the luminal compartment, particularly from the Golgi vesicles [11]; thus, these proteins may contain a relatively large portion of the remaining protein-bound sugar residues after proteolytic treatment. In spite of the fact that the proteolysis is not an ideal approach and may lead to a subtle reorganization of the membrane the large number of studies performed using this approach all indicate that only proteins from the cytoplasmic surface are solubilized [1]. Consequently, the conclusion that glycoproteins are associated to a large extent with the outer surface of microsomal and Golgi vesicles appears to be justified.

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