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STUDIES ON THE TRANSVERSE LOCALIZATION OF LYSOPHOSPHOLIPASE IN BOVINE LIVER MICROSOMES USING PROTEOLYTIC ENZYMES

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Summary

1. Sonication of bovine liver microsomes completely solubilized the membrane-bound lysophospholipase II (EC 3.1.1.5). Co-chromatography with purified ^{125}I-labelled lysophospholipase indicated that the enzyme was solubilized from microsomes in a lipid-free state.

2. In the presence of residual microsomal membranes, the solubilized lysophospholipase could only be partly degraded by trypsin (EC 3.4.21.4). Therefore, trypsin could not be used to study the transmembrane disposition of lysophospholipase in intact microsomes.

3. Chymotrypsin (EC 3.4.21.1) destroyed the solubilized lysophospholipase activity, even in the presence of residual microsomal membranes.

4. Lysophospholipase in intact microsomal vesicles was resistant to chymotrypsin digestion.

5. When microsomal vesicles were made leaky with lysophosphatidylcholine, chymotrypsin destroyed more than 95% of the lysophospholipase activity.

6. It is concluded from these experiments that at least the active center of lysophospholipase is located at the luminal side of the bovine liver microsomal membrane.

Introduction

In previous work, two enzymes with lysophosphatidylcholine deacylating activity, provisionally denoted lysophospholipase I and lysophospholipase II (EC 3.1.1.5), were purified from bovine liver [1]. Subsequent subcellular distri-

bution studies showed lysophospholipase I to be a soluble enzyme, whereas lysophospholipase II was found to be located in the microsomal fraction [2]. The initial experiments used *n*-butanol treatment to solubilize the enzyme from microsomal membranes [2]. The solubilized enzyme could be purified in aqueous buffers without the use of detergents [1] and did not require phospholipids or detergents to express full enzymatic activity. In fact, the hydrolysis of lysophosphatidylcholine was inhibited by the presence of phosphatidylcholine in a manner that could be explained most conveniently by dilution of the lysophosphatidylcholine substrate in the plane of the lipid bilayer [3]. These characteristics, together with the observation reported in this paper that the enzyme could be solubilized from the membrane in a lipid-free state by sonication, would seem to classify the enzyme as a peripheral protein [4].

In recent years the transverse localization of peripheral and integral membrane proteins has received considerable attention (refs. 5—7, for reviews). For endoplasmic reticulum membranes such determinations were made possible after it became apparent that microsomes, largely derived from these membranes, maintained proper sidedness [8,9], i.e. with the cytoplasmic surface of the endoplasmic reticulum forming the outside surface of the microsomal vesicles. Proteolytic digestion of intact microsomes left the microsomal permeability barrier intact [10,11] thus providing a sensitive tool to determine the localization of microsomal proteins in the transverse plane of the membrane. By utilizing this technique, evidence has recently been reported which indicated that the enzymes involved in phospholipid biosynthesis, with the possible exception of phosphatidate phosphohydrolase (EC 3.1.3.4) are all located on the cytoplasmic surface of the endoplasmic reticulum of rat liver [12,13]. During investigations reported here on the topography of the microsomal lysophospholipase in the transverse plane of the membrane some limitations of the proteolytic procedure became apparent. Nevertheless, it was possible to show that at least the active center of the lysophospholipase is located at the internal, luminal side of the microsomal membrane.

Materials and Methods

Materials. Chymotrypsin A_4 and trypsin (both bovine), trypsin inhibitor from soya bean and trypsin inhibitor from hen egg white were obtained from Boehringer (Mannheim, F.R.G.). AcA 44 was purchased from LKB (Uppsala, Sweden). *N*-Acetyl-L-tyrosine ethylester was obtained from Merck, Darmstadt, F.R.G. [^{125}I]Iodide (carrier free) was obtained from The Radiochemical Center (Amersham, U.K.). Fresh bovine liver was obtained from the local slaughterhouse.

Isolation of microsomes. A 10% homogenate of bovine liver in 0.25 M sucrose, 2 mM EDTA, 10 mM Tris-HCl buffer (pH 7.3), 0.02% NaN_3 (sucrose medium) was prepared by using a Waring Blendor for 1 min. After filtration through two layers of cheesecloth and successive centrifugation in a Sorvall SS 34 rotor for 10 min at 1000 × *g*, 9000 × *g* and 20 000 × *g*, the final supernatant was centrifuged for 1 h at 105 000 × *g* in a Beckman L265 ultracentrifuge. The microsomal pellet was resuspended in the sucrose medium at 20—30 mg protein/ml.

Treatment of microsomes. Incubations with protease were done in sucrose medium for 30 min at 37°C using a ratio of 5 : 1 (w/w) for microsomal protein to proteolytic enzyme. Trypsin activity was stopped by a 2-fold excess (w/w) of trypsin inhibitor from hen egg white. Chymotrypsin activity was inhibited totally by addition of a 5-fold excess of trypsin inhibitor from soya bean.

Sonication of the microsomes was done with a Branson Sonifier B12 with an output of 60 W. Unless otherwise indicated, 8 ml sucrose medium containing 2.5 mg microsomal protein/ml were sonicated at 0°C under an N_2 stream.

Enzyme assays. Lysophospholipase activity was assayed as described by de Jong et al. [1] using either 1-[1-^{14}C]palmitoyllysophosphatidylcholine or 1-[9,10-$^{3}H_2$]stearoyllysophosphatidylcholine as substrates. These synthetic compounds were diluted with egg lysophosphatidylcholine to specific activities of 100 dpm/nmol for the ^{14}C-labelled substrate and 400 dpm/nmol for the ^{3}H-labelled substrate.

Mannose-6-phosphatase activity was determined essentially as described by Arion et al. [14], except that 0.225 M sucrose, 2 mM EDTA, 38 mM Tris/malic acid (pH 6.3) was used as buffer and egg lysophosphatidylcholine was used as detergent.

Protein techniques. Protein was determined according to Lowry et al. [15]. Lysophospholipase II was purified from bovine liver as described previously [1]. Iodination of purified lysophospholipase II was carried out following the chloramine-T method as described by Hudson and Hay [16].

Results

Previous studies [2] on the subcellular localization of bovine liver lysophospholipase II had shown that the enzyme distributed in sucrose gradients in a manner following exactly the microsomal markers glucose-6-phosphatase and NADPH-cytochrome *c* reductase. Incubation of the microsomal fraction in 0.25 M sucrose containing 2 mM EDTA and 10 mM Tris-HCl buffer (pH 7.3) for 2 h at either 0°C or 37°C and subsequent ultracentrifugation for 1 h at 100 000 × *g* released only 5 and 11%, respectively, of the activity into the supernatant. Likewise, by freezing and thawing after 15 min only 4% of the enzymic activity appeared in the supernatant. However, a hypotonic wash, by resuspending the microsomal pellet in 20 mM Tris-HCl buffer (pH 8.0), released about 20% of the lysophospholipase activity. It was noticed that freezing and storing of the microsomes at −16°C also led to increased solubilization of the enzyme, so that about 20% appeared in the supernatant after storing for 5 days. By sonication of a relatively dilute microsomal suspension the lysophospholipase activity was solubilized completely after 10 min (Fig. 1). The sonication procedure gave rise to only a minor loss of total lysophospholipase activity.

The use of proteolytic enzymes to determine the transverse localization of proteins in membranes requires that the protein under investigation is sufficiently modified by the proteolytic enzymes so as to detect activity changes when the proteolytic enzyme has access to the protein. The finding that the lysophospholipase could be completely solubilized by sonication provided the possibility for a rigorous test of this prerequisite. The sonicated microsomes

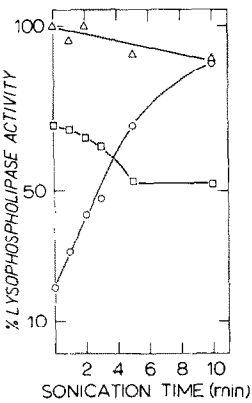

Fig. 1. Lysophospholipase inactivation by trypsin in sonicated bovine liver microsomes. Microsomes (18.75 mg protein) in 7.5 ml sucrose medium were sonicated for the indicated time periods at 0°C under N_2. At the indicated times samples of 1 ml were withdrawn. Total lysophospholipase activity was determined using 0.1 ml aliquots (△———△). 0.5 ml samples were centrifuged for 1 h at 200 000 × *g* to determine lysophospholipase activity in the high-speed supernatant (○———○). 0.2 ml sonicated samples were incubated with trypsin and after addition of trypsin inhibitor used for lysophospholipase determinations (□———□).

were incubated with trypsin for 30 min at 37°C. After addition of sufficient trypsin inhibitor to block further proteolysis, aliquots of the total incubation mixture were assayed for lysophospholipase activity. Unexpectedly, when the lysophospholipase was completely solubilized after 10 min sonication, only about half of the lysophospholipase activity was destroyed by trypsin treatment in this experiment. Control experiments showed that trypsin had exerted this inactivation already after 10 min incubation. Although incubation for longer periods did not result in further lysophospholipase inactivation the samples were routinely incubated with trypsin for 30 min to ensure that inactivation had reached a plateau value. The amount of lysophospholipase inactivated varied from about 25% to about 70% in various microsomal preparations. In the experiment depicted in Fig. 1 the ratio of trypsin to microsomal protein amounted 1 to 5. In another experiment, ratios of 1 : 20, 1 : 5 and 1 : 2 were used and these yielded 27%, 26% and 44% lysophospholipase inactivation, respectively. Since purified lysophospholipase II was readily degraded by trypsin under the conditions employed the possibility was considered that the lysophospholipase after sonication of microsomes was present in or bond to small, lipid vesicles which were not pelleted at 200 000 × *g* but protected the lysophospholipase from proteolytic attack.

To investigate this possibility a batch of sonicated microsomes was divided in four portions. One part was centrifuged for 1 h at 200 000 × *g*. In this particular experiment, 43% of the total lysophospholipase activity present in the sonicate before centrifugation appeared in the supernatant. Presumably, this less efficient solubilization was due to the higher protein concentration of the microsomal suspension. Another part of the sonicate, after addition of a small quantity of ^{125}I-labelled pure lysophospholipase II, was chromatographed on an AcA 44 column (Fig. 2). Two peaks of lysophospholipase activity were eluted from the column, one in the void volume coinciding with the micro-

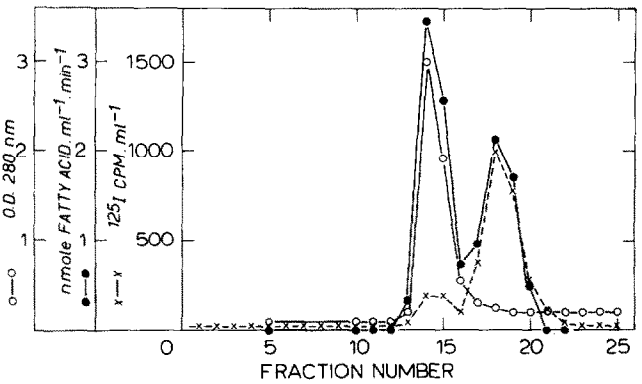

Fig. 2. AcA 44 chromatography of sonicated bovine liver microsomes. Microsomes (40 mg protein) in 8 ml sucrose medium were sonicated during 10 min at 0°C under N_2. The sample was divided in four fractions of 2 ml. To one fraction, 7 μg pure ^{125}I-labelled lysophospholipase II (11 000 cpm ^{125}I) was added. To a second fraction, 2 mg trypsin, and, to a third fraction, 2 mg chymotrypsin were added (see Figs.3 and 4). All fractions were incubated for 30 min at 37°C and then applied to an AcA 44 column (40 × 2 cm). The column was eluted with sucrose medium (flow rate, 10.5 ml/h, 3.5-ml fractions). 0.4 ml aliquots were used for lysophospholipase determination and 1-ml aliquots for radioactivity measurements.

somal membranes and a second one eluting together with the soluble ^{125}I-labelled enzyme. Since the enzymatic activity of the added ^{125}I-labelled enzyme amounted to only 5% of the total lysophospholipase activity applied to the column, this result indicated that sonication had released the lysophospholipase in monomolecular lipid-free form. Moreover, this second peak contained 43% of the lysophospholipase activity in the column eluate, in good agreement with the amount appearing in the 200 000 × *g* supernatant. A third portion of the sonicated microsomes was first incubated with trypsin and subsequently chromatographed on a similar AcA 44 column. It is clear from Fig. 3 that trypsin did not inactivate all the monomolecularly solubilized lysophospholipase in the presence of sonicated beef liver microsomal membranes. In contrast, when the sonicate was first centrifuged at 200 000 × *g* and the supernatant then treated with trypsin a complete loss of lysophospholipase activity was noticed. This indicated that trypsin could not be used to determine the

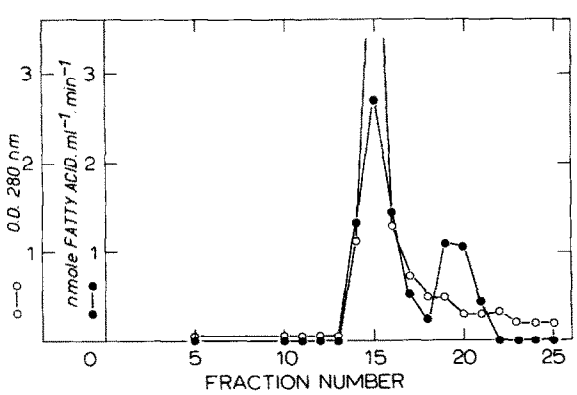

Fig. 3. AcA 44 chromatography of sonicated bovine liver microsomes after trypsin treatment. For experimental details see legend of Fig. 2.

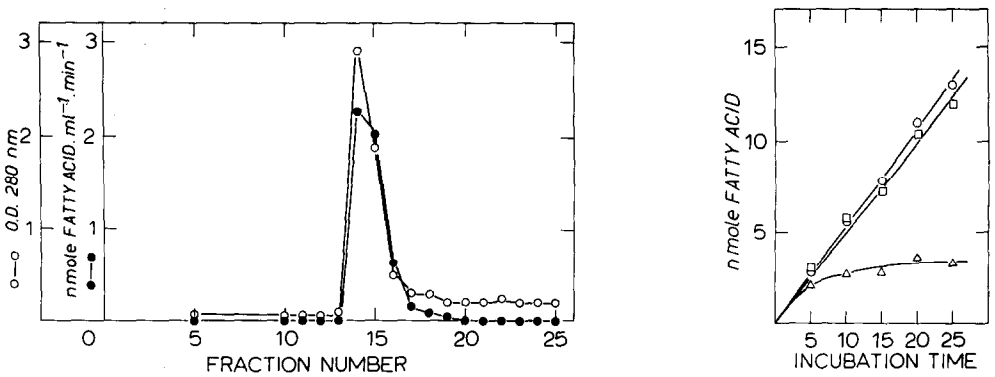

Fig. 4. AcA 44 chromatography of sonicated bovine liver microsomes after chymotrypsin treatment. For experimental details see legend of Fig. 2.

Fig. 5. Influence of chymotrypsin on lysophospholipase activity in bovine liver microsomes. Microsomes (10 mg protein) were incubated for 30 min at 37°C with or without 2 mg chymotrypsin in a total volume of 1 ml sucrose medium. In one tube, chymotrypsin activity was blocked after 30 min incubation by addition of 10 mg trypsin inhibitor from soya bean. The incubation mixtures were diluted with sucrose medium to a final volume of 2 ml and then centrifuged for 1 h at 200 000 X *g*. The pellets were resuspended in 5 ml sucrose medium and 0.125 ml aliquots were assayed for the indicated time periods to determine lysophospholipase activity. ○———○, microsomes incubated without chymotrypsin; △———△, microsomes incubated with chymotrypsin; □———□, microsomes incubated with chymotrypsin and trypsin inhibitor from soya bean added prior to centrifugation.

transverse localization of lysophospholipase in the microsomes. Apparently, the microsomes contained some inhibiting factor(s), which prevented the tryptic inactivation of even the solubilized lysophospholipase. In an attempt to find a useful alternative for trypsin the experiment described in Fig. 3 was repeated with chymotrypsin. As can be seen in Fig. 4 at least the solubilized lysophospholipase activity was completely degraded by this proteolytic enzyme. This made chymotrypsin a suitable tool to investigate the transverse distribution of microsomal lysophospholipase. Resistance of the lysophospholipase in intact microsomes to proteolysis by chymotrypsin could then at least be interpreted as evidence that the enzyme is present in a locus which is spatially inaccessible to chymotrypsin. In initial experiments intact microsomes were treated for 30 min with chymotrypsin. The mixture was then centrifuged for 60 min at 200 000 X *g* to remove the proteolytic enzyme as described by Nilsson and Dallner [11], after which the resuspended microsomes were assayed for residual lysophospholipase activity. It was noticed in these experiments that the percentage of remaining lysophospholipase activity dependend strongly on the incubation time used to determine the lysophospholipase activity. It can be seen in Fig. 5 that the lysophospholipase activity in control microsomes is linear for at least 30 min. Linearity with time was greatly diminished in chymotrypsin-treated microsomes. However, linearity in the lysophospholipase assay could be restored when proteolysis was blocked by addition of trypsin inhibitor from soya bean. This protein is known to inhibit also chymotrypsin activity [17] and this was confirmed experimentally by using *N*-acetyl-L-tyrosine ethylester as substrate [18]. The inactivation of lysophospholipase activity as depicted in the lower curve of Fig. 5 was relatively fast when lysophospholipase

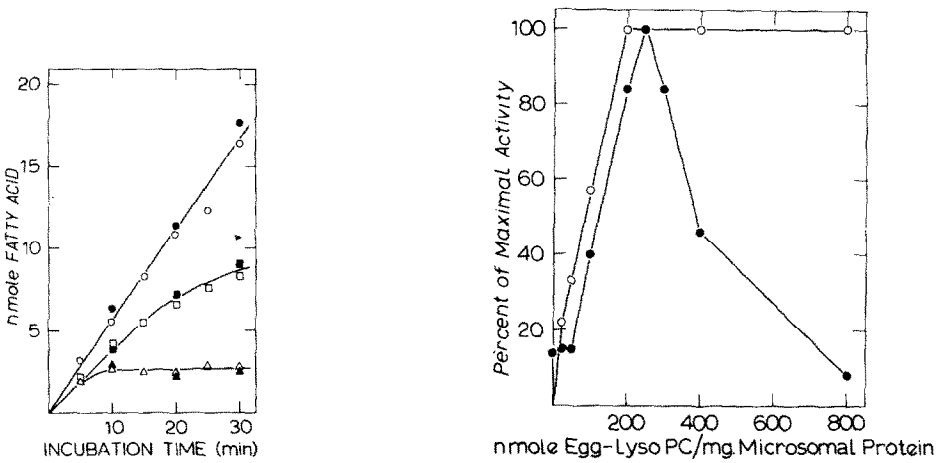

Fig. 6. Effect of preincubation with proteolytic enzymes on lysophospholipase activity in bovine liver microsomes. Microsomes (250 μg protein) were preincubated for 30 min at 37°C with or without 50 μg protease and then assayed for the indicated time periods for lysophospholipase activity by addition of 200 nmol 1-[1-^{14}C]palmitoyllysophosphatidylcholine at zero time (closed symbols). In parallel experiments, the protease, when indicated, was added together with lysophosphatidylcholine substrate at zero time for the lysophospholipase assay (open symbols). Microsomes preincubated without protease (●———●), with trypsin (■———■) or with chymotrypsin (▲———▲). Microsomes and either no protease (○———○) or trypsin (□———□) or chymotrypsin (△———△) only present during the lysophospholipase assay.

Fig. 7. Influence of egg lysophosphatidylcholine on mannose-6-phosphatase and lysophospholipase activity. Mannose 6-phosphate hydrolysis and lysophospholipase activity were determined in the presence of varying amounts of egg lysophosphatidylcholine with 250 and 500 μg microsomal protein, respectively. ●———●, mannose 6-phosphate hydrolysis; ○———○, lysophospholipase activity. Egg-lyso PC, 1-acyllysophosphatidylcholine derived from egg phosphatidylcholine.

activity was assayed with 800 nmol of lysophosphatidylcholine/mg microsomal protein. A slower inactivation of the lysophospholipase was noticed when the assays were done with 200 nmol lysophosphatidylcholine/mg microsomal protein. These results suggested that the centrifugation procedure had not completely removed chymotrypsin and that the remaining chymotrypsin gained only access to the microsomal lysophospholipase during the lysophospholipase assay. Attempts to remove the chymotrypsin completely by washing the microsomes once in sucrose medium were unsuccessful in that similar curves as depicted in Fig. 5 were obtained. The idea that chymotrypsin got only access to the microsomal lysophospholipase during the assay for lysophospholipase activity, i.e. in the presence of lysophosphatidylcholine, was born out in the experiments described in Fig. 6. Preincubation with either trypsin or chymotrypsin caused no further loss of lysophospholipase activity when compared with incubations where the protease was added only together with lysophosphatidylcholine at the zero time for the lysophospholipase assay. Under either conditions chymotrypsin inactivated lysophospholipase activity totally in 10 min in the presence of 800 nmol lysophosphatidylcholine/mg microsomal protein. It is obvious again that trypsin does not destroy all lysophospholipase activity under the same conditions. These experiments led to the preliminary conclusion that the lysophospholipase in intact microsomes was inaccessible

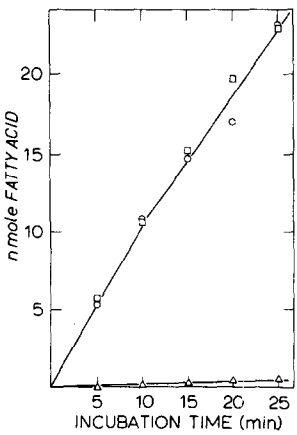

Fig. 8. Accessibility of lysophospholipase to chymotrypsin in bovine liver microsomes in presence and absence of lysophosphatidylcholine. Microsomes (500 μg protein) were preincubated for 30 min at 37°C with 100 μg chymotrypsin in the presence (△———△) or absence (○———○) of 200 nmol egg-lysophosphatidylcholine. Control microsomes (□———□) received neither chymotrypsin nor lysophosphatidylcholine. Chymotrypsin activity was blocked by addition of 500 μg trypsin inhibitor from soya bean. Microsomes to which no lysophosphatidylcholine had been added in the preincubation received 190 nmol egg lysophosphatidylcholine prior to addition of 200 nmol 1[9,10-$^3H_2$]stearoyllysophosphatidylcholine to determine lysophospholipase activity for the indicated time periods. The amount of 190 nmol egg lysophosphatidylcholine was chosen to account for some decrease in the lysophosphatidylcholine in the preincubated mixture containing both chymotrypsin and lysophosphatidylcholine. As can be seen in Fig. 6 (lower curve), the lysophospholipase is only completely inactivated after about 10 min incubation with chymotrypsin.

to both chymotrypsin and trypsin and that inactivation of enzyme only occurred after the microsomal membrane became leaky due to the presence of lysophosphatidylcholine. The experiment in Fig. 7 indeed indicated that the latency of mannose 6-phosphate hydrolysis in beef liver microsomes was completely abolished at lysophosphatidylcholine levels of 250 nmol/mg microsomal protein. A further increase in the lysophosphatidylcholine concentration gave rise to a rather steep decrease in mannose 6-phosphate hydrolysis, probably because of inactivation of the glucose-6-phosphatase activity due to changes in membrane structure. Such an inactivation has also been observed after phospholipase C treatment of microsomal membranes [19]. Maximal lysophospholipase activity in the microsomes was reached already with 200 nmol lysophosphatidylcholine/mg microsomal protein. Routinely, the lysophospholipase was assayed with 400 or 800 nmol lysophosphatidylcholine/mg microsomal protein to ensure linearity with time during a 30 min assay period. It is likely that these amounts of lysophosphatidylcholine destroy the microsomal permeability barrier not only for mannose 6-phosphate but also for chymotrypsin. In this view the lysophospholipase would become only accessible to chymotrypsin in the presence of lysophosphatidylcholine. This view was substantiated in the experiments depicted in Fig. 8. When microsomes were treated with chymotrypsin in the presence of egg lysophosphatidylcholine and then, after blocking further chymotryptic action, were assayed for lysophospholipase with [^{3}H]-lysophosphatidylcholine, virtually no lysophospholipase activity was detected. In contrast, microsomes preincubated with chymotrypsin in the absence of lysophosphatidylcholine retained the full lysophospholipase activity of control microsomes.

Discussion

Proteolytic enzymes are widely used to study the transverse disposition of proteins in membranes [5,7]. Inactivation of the protein by proteases under conditions leaving the permeability barrier of the membrane vesicles intact is generally taken as evidence for an outside surface localization of the protein. Conversely, resistance to proteolytic attack is taken as evidence that the protein is either buried in the lipid phase of the membrane or is located on the inside of the membrane vesicles. However, when lysophospholipase was solubilized from beef liver microsomes by sonication it could only partially be inactivated by trypsin, despite the fact that trypsin should have full accessibility to the lysophospholipase (Fig. 1). Complete inactivation of the lysophospholipase was only obtained after removal of residual microsomal membranes by centrifugation. At present, the reason why trypsin is not able to completely inactivate the lysophospholipase in the presence of residual beef liver microsomal membranes is not completely understood. A likely explanation could be that microsomal membranes contain a trypsin-inhibiting activity which slowly inactivates trypsin. Trypsin-inhibiting activities have indeed been reported for rat liver microsomes and cytosol [20] and for beef liver [21]. The latter inhibitor was isolated and shown to be identical with pancreatic trypsin inhibitor by Chauvet and Archer [21]. Anyway, since trypsin could not degrade solubilized lysophospholipase in the presence of residual beef liver microsomal membranes (Figs. 1 and 3) it could not be used as a tool to study the transmembrane localization of lysophospholipase. In this respect, chymotrypsin (Fig. 4) proved to be a good alternative. It is interesting to note that similar findings were made by Bell and colleagues. In studying the sensitivity of rat liver microsomal glycero-3-phosphate acyltransferase to proteolysis, these authors reported that more than 90% of the activity could be destroyed by chymotrypsin [13], whereas proteolysis with trypsin reached a plateau after about 65% of the acyltransferase had been inactivated [22]. The experiments with chymotrypsin showed that lysophospholipase in intact microsomes is virtually resistant to proteolysis (Figs. 5, 6 and 8). Complete inactivation was observed only when the microsomal permeability barrier was destroyed by addition of egg lysophosphatidylcholine, thus allowing chymotrypsin access to the inside of the microsomes or, alternatively, allowing the lysophospholipase to leak out of the microsomes. The most likely explanation for these findings is that in intact microsomes at least the active center of the lysophospholipase is located on the inner, luminal monolayer of the microsomes. However, two other possibilities for this localization have to be considered, i.e. the active center could be present in the lipid core of the membrane or the enzyme could be located inside the microsomal vesicle. The latter possibility is unlikely in view of the long sonication times required to solubilize most of the enzyme. According to Chang et al. [23], the intracisternal content of microsomes is released completely after three bursts of sonication for 20 s each and, even this mild sonication procedure, leads to release of some membranous material.

As mentioned in the introduction lysophospholipase is a water-soluble protein which does not require the presence of detergents during the course of its purification. Although these properties would seem to argue against a localiza-

tion of the enzyme in the lipid core of the membrane this possibility cannot be excluded completely. Recent evidence obtained by ^{31}P NMR techniques has shown that a considerable portion of microsomal phospholipids experience isotropic motion suggesting the presence of intramembrane non-bilayer lipid configurations [24,25]. Thus, it is conceivable that integral microsomal enzymes are not highly hydrophobic proteins. On the other hand, immunological experiments have shown that no antigenic sites of lysophospholipase can be detected on the outiside of beef liver microsomes (unpublished data). These suggest that the luminal surface of the microsomal vesicle is the most likely locus for the active center of lysophospholipase.

Under conditions where mannose-6-phosphatase activity in microsomal membranes is latent (i.e. up to 50 nmol lysophosphatidylcholine/mg microsomal protein (Fig. 7)), there is no evidence for latency of lysophospholipase. The maximal specific activity of the lysophospholipase in the microsomes used in the experiment depicted in Fig. 7 amounted to 3.2 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. It can be seen in Fig. 7 that the specific activity of the enzyme at 50 nmol lysophosphatidylcholine/mg microsomal protein amounted to one-third of the maximal value (i.e. 1.1 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}). At this rate, half of the substrate is hydrolyzed in about 30 min. If we assume that the transbilayer movement of the lysophosphatidylcholine is the rate-determining step in the deacylation, this sets an upper limit of about 30 min to the half-time for the transbilayer movement of lysophosphatidylcholine in microsomal membranes at 37°C. This is a large decrease compared to that of 100 h observed for this process by de Kruijff et al. [26] and van den Besselaar et al. [27] in phosphatidylcholine vesicles containing 5 mol% lysophosphatidylcholine. The calculated value is, however, in the same order as the half-times for transbilayer movement of other phospholipids in microsomal membranes, determined with phospholipid exchange proteins [28,29] and the half-time of transbilayer movement of 1-palmitoyllysophosphatidylcholine in phosphatidylcholine vesicles containing glycophorin [30].

The method used by Nilsson and coworkers [11,31] to stop proteolytic action by removing the proteolytic enzyme by ultracentrifugation at 0°C led to erroneous results in this study (Fig. 5). Even after a second ultracentrifugation, some of the chymotrypsin remained bound to the microsomes. We recommend, therefore, the use of inhibitors to block the action of the proteolytic enzymes. Alternatively, when no inhibitor for the protease is available, it should be established that the protease does not gain access to the interior of the membrane vesicles in further incubations with the vesicles, e.g. during assay of an enzyme in the presence of detergents.

Using proteolytic enzymes evidence has been reported recently [12,13] which indicates that the enzymes involved in lipid biosynthesis, with the possible exception of phosphatidate phosphohydrolase [13], are all located in the cytoplasmic surface of rat liver endoplasmic reticulum. However, when glycero-3-phosphate acyltransferase was localized cytochemically by detection of released thiolgroups from acyl-CoA an exclusively luminal surface localization was claimed [32]. No explanation for this discrepancy has been offered. In contrast to the cytoplasmic surface localization of diacylglycerol cholinephosphotransferase in rat liver microsomes [12,13] the enzyme in microsomes from

small intestine villus was resistant to nagarse treatment [33]. Likewise, in the latter membranes, lysophosphatidylcholine : acyl-CoA acyltransferase was not exposed to the extent that it could be inactivated by a proteolytic enzyme [33]. Whether these asymmetric dispositions of enzymes involved in phospholipid biosynthesis or degradation bears any relationship to phospholipid asymmetry in microsomal membranes remains to be seen. In this respect, it should be noted that investigation of phospholipid asymmetry in microsomal membranes itself has led to conflicting results. Nilsson and Dallner [11] and Higgins and Dawson [34] concluded that phospholipids are asymmetrically distributed over outer and inner monolayer of rat liver microsomal membranes, but a completely reversed distribution for especially phosphatidylethanolamine and phosphatidylserine was reported by both groups. In contrast, Sundler et al. [35] concluded that phospholipids were distributed symmetrically over both halves of the microsomal membrane. Although the reasons for these discrepancies are difficult to define at present, it is likely that such factors as the selectivities of the lipolytic enzymes [35,36] and the incubation temperatures used to determine the distributions are involved. In this respect, the recent observation [24, 25] that part of the microsomal phospholipids experience isotropic motion at 35°C but not at 8°C [24] may be of utmost importance.

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