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ENZYME ASYMMETRY IN HEPATIC MICROSOMAL VESICLES

CRITERIA FOR LOCALIZATION OF LUMENAL ENZYMES WITH PROTEASES

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Summary

Chymotrypsin inactivation of lysophosphatidic acid acyltransferase activity in detergent-disrupted rat liver microsomes, but not in intact microsomes, falsely indicated a lumenal location for the enzyme. Inhibition by several other proteases in the absence of detergent showed that lysophosphatidic acid acyltransferase activity is located on the cytoplasmic surface of microsomes. Chymotrypsin inactivation did not occur in vesicles disrupted by nitrogen cavitation unless deoxycholate was present, suggesting that deoxycholate exposes a cryptic chymotrypsin cleavage site. Criteria for localization of lumenal microsomal enzymes should include studies using several proteases and/or employ more than one method of microsomal disruption.

Introduction

The asymmetric transmembrane distribution of proteins provides a molecular basis for the functional asymmetry of many biological membranes. Localization of the enzymes of the endoplasmic reticulum has been facilitated by investigations employing sealed microsomal vesicles which maintain proper sidedness, i.e. the vesicles' outer surfaces correspond to the cytoplasmic surface of the endoplasmic reticulum [1,2]. Since the vesicles are impermeable to charged molecules and to macromolecules [1,3], proteases have been exten-

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sively employed to localize microsomal enzymes [3]. Unless the microsomal vesicles have been disrupted or rendered permeable to the protease, only the enzymes and proteins located on the cytoplasmic surface of the microsomal vesicles are susceptible to proteolysis [1,3]. Low levels of detergents render microsomal vesicles permeable to macromolecules [4,5]. It has been generally assumed that microsomal enzymes which are inactivated by proteases only in the presence of a detergent are located on the lumenal surface of the vesicle [6]. DePierre and Dallner have suggested, however, that a detergent might remove membrane components and unmask a cryptic site for proteolytic attack [3]. Therefore, if enzyme inactivation occurred only after detergent treatment, it might be mistakenly assumed that the enzyme was located on the lumenal surface. In this paper, we report the first instance in which the protease procedure falsely indicated a lumenal location for a microsomal enzyme.

Materials and Methods

Materials. Hexokinase, mannose, mannose-6-*P*, sodium taurocholate, bovine serum albumin (essentially fatty acid free), and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma. Elastase, α-chymotrypsin, microprotease and trypsin-TPCK were from Worthington. Thermolysin and pronase were from Calbiochem. Subtilisin was from Nutritional Biochemicals Corp. Proteinase K was from Beckman. 1-Oleoylglycerol-3-*P* was from Serdary. Oleoyl-CoA, and ATP were from PL Biochemicals. $^{32}P_i$ and Aquazol were from New England Nuclear. [^{32}P]ATP was synthesized as reported previously [7]. Mannose-6-^{32}P was synthesized by a modification [8] of the method of Slein [9] and employed at a specific activity of 1.36 Ci/mol.

Isolation of liver microsomes. Livers from 200—250 g Charles River CD strain female rats were homogenized by ten rapid up and down strokes using a motor-driven, Teflon-glass homogenizer in 3 vols. of cold medium I (0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 22 000 × *g* for 15 min. The resulting supernatant was centrifuged at 100 000 × *g* for 1 h. The pellet was suspended in medium I and centrifuged at 100 000 × *g* for 1 h. The resulting pellet was suspended in medium I without EDTA. Protein was determined by the method of Lowry et al. [10] using bovine serum albumin as the standard.

Protease treatment of microsomes. The method was essentially that of Nilsson and Dallner [6]. Microsomes in 0.25 M sucrose/50 mM Tris-HCl (pH 7.5)/50 mM KCl were disrupted by incubation with 0.05% deoxycholate. After 10 min at 4°C protease treatment of disrupted and non-detergent-treated microsomes (30 mg microsomal protein) was carried out at 30°C for 25 min. Control intact and disrupted microsomes were treated similarly except that the protease was omitted. At the end of the incubation, the samples were cooled in ice/water and centrifuged immediately at 100 000 × *g* for 2 h. The recovered microsomes were suspended in medium I without EDTA, and protein was determined by the method of Lowry et al. [10]. Enzyme assays were performed immediately or within 3 days on samples stored at −15°C. No activity was lost by freezing and thawing once.

Protease treatment of nitrogen-cavitated microsomes. Microsomes (10 mg/ml

in medium I without EDTA) were disrupted under nitrogen by passage through a French Pressure Cell at 20 000 lb/inch^2 and immediately treated for 25 min at 30°C with chymotrypsin (100 μg/mg microsomal protein). Control disrupted and non-disrupted microsomes were treated similarly except that chymotrypsin was omitted. Following the incubation the microsomes were collected by centrifugation and resuspended as described above.

Enzyme assays. Mannose-6-*P* phosphatase (assayed at 2 mM mannose-6-*P*) was assayed as previously described [11]. Lysophosphatidic acid acyltransferase was determined spectrophotometrically [12] using 50 μM oleoylglycerol-*P* and 50 μM oleoyl-CoA. All assays were proportional to the time and to the amount of protein employed using either intact or disrupted microsomes.

Results and Discussion

Treatment of microsomal vesicles with chymotrypsin did not affect mannose-6-*P* phosphatase latency and resulted in only a 6% loss of mannose-6-*P* phosphatase activity (Table I). However, when the integrity of the microsomal membrane was disrupted with deoxycholate, 88% of the mannose-6-*P* phosphatase activity was inactivated by chymotrypsin. Since mannose-6-*P* phosphatase is a lumenal enzyme activity [11,13—15], these data demonstrate that the non-deoxycholate-treated microsomes remained largely intact during the chymotrypsin digestion [8]. Under these conditions lysophosphatidic acid acyltransferase activity was reduced 19% in the intact microsomes, while about 90% of the activity was inactivated by chymotrypsin in the presence of deoxycholate. Although these data suggest a lumenal location for lysophosphatidic acid acyltransferase, experiments using pronase suggested that lysophosphatidic acid acyltransferase was located on the cytoplasmic surface of microsomal vesicles (Ref. 8 and Table I). After incubation with pronase, 87% of the lysophosphatidic acid acyltransferase activity was lost under conditions in which greater than 94% of the mannose-6-*P* phosphatase activity was retained, indicating that pronase inactivated the acyltransferase in intact microsomes. Upon addition of deoxycholate, 74% of the mannose-6-*P* phosphatase activity was inactivated by pronase. This demonstrated that mannose-6-*P* phosphatase was susceptible to pronase digestion after microsomal disruption.

To resolve the conflicting interpretations of the effects of chymotrypsin and pronase proteolysis, similar experiments were performed with subtilisin, proteinase K, and microprotease (Table I). In each case 40—90% of the lysophosphatidic acid acyltransferase activity was inactivated in microsomes whose impermeability to the protease employed was demonstrated by the high recoveries of mannose-6-*P* phosphatase activity (Table I). When intact microsomes were incubated with trypsin, collagenase, clastase, or thermolysin (100 μg/mg microsomal protein), lysophosphatidic acid acyltransferase was inactivated 44%, 25%, 20%, or 50%, respectively. (Since the microsomes employed in the latter experiments were not reisolated by centrifugation, solubilized but active lysophosphatidic acid acyltransferase would not be distinguished from the microsomal-bound activity.) These data clearly indicate that the lysophosphatidic acid acyltransferase of intact vesicles is susceptible to protease inactivation and strongly support the conclusion that the enzyme is

TABLE I

EFFECT OF PROTEASES ON MANNOSE-6-*P* PHOSPHATASE AND LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE ACTIVITIES

Mannose-6-*P* phosphatase latency was over 90% in each initial microsomal preparation. After incubation and collection of microsomes, latencies ranged between 80 and 87% and between 77 and 85% for the intact controls and the protease-treated microsomes, respectively. The chymotrypsin data express the average of two independent experiments. Protein recoveries from intact control and chymotrypsin-treated microsomes were 21.1 and 18.5 mg, respectively; protein recoveries from disrupted control and chymotrypsin-treated microsomes were 18.9 and 10.8 mg, respectively. For pronase, protein recoveries from intact control and pronase-treated microsomes were 23.5 and 19.5 mg, respectively; protein recoveries from disrupted control and pronase-treated microsomes were 15.2 and 12.4 mg, respectively. These data have been previously reported [8]. For subtilisin, protein recoveries from intact control and subtilisin-treated microsomes were 20.6 and 16.4 mg, respectively; protein recoveries from disrupted control and subtilisin-treated microsomes were 14.7 and 9.2 mg, respectively. For proteinase K, protein recoveries from intact control and proteinase K-treated microsomes were 20.3 and 16.4 mg, respectively; protein recoveries from disrupted control and proteinase K-treated microsomes were 14.1 and 7. mg, respectively. For microprotease, protein recoveries from intact control and microprotease-treated microsomes were 20.3 and 16.4 mg, respectively; protein recoveries from disrupted control and microprotease-treated microsomes were 14.1 and 14.2 mg, respectively. To facilitate comparison, the percent of control activity is underlined.

Protease (μg/mg microsomal protein)	Enzyme	Control + protease % of control					
		Intact microsomes			Disrupted microsomes		
		Control	+Protease	% of control	Control	+Protease	% of control
Chymotrypsin (100)	Mannose-6-*P* phosphatase	1522	1434	94.2	1181	218	11.5
	Lysophosphatidic acid acyltransferase	1173	973	82.9	993	80	8.1
Pronase (50)	Mannose-6-*P* phosphatase	2260	2140	94.7	1882	492	26.1
	Lysophosphatidic acid acyltransferase	1152	150	13.0	122	18.5	15.2
Subtilisin (125)	Mannose-6-*P* phosphatase	2090	1831	87.6	1700	248	14.6
	Lysophosphatidic acid acyltransferase	1322	100	7.6	764	85	11.1
Proteinase K (32)	Mannose-6-*P* phosphatase	2269	2142	94.4	1197	34	2.8
	Lysophosphatidic acid acyltransferase	1240	100	8.1	517	0	0
Microprotease (64)	Mannose-6-*P* phosphatase	2269	1961	86.4	1197	546	45.6
	Lysophosphatidic acid acyltransferase	1240	503	40.6	517	131	25.3

located on the cytoplasmic surface of the endoplasmic reticulum. We eliminated the possibility that lysophosphatidic acid acyltransferase has a non-cryptic cytoplasmic chymotrypsin cleavage site(s) which does not result in enzyme inactivation in the absence of detergent, by exposing chymotrypsin-treated microsomes to 1% deoxycholate after the microsomes were reisolated by centrifugation. No inactivation occurred. The chymotrypsin proteolysis data indicate that the lysophosphatidic acid acyltransferase does not contain readily accessible chymotrypsin cleavage sites exposed on the cytoplasmic surface of microsomal vesicles.

Two alternatives are consistent with the data: the enzyme might span the membrane and contain a chymotrypsin site on the lumenal surface, or deoxycholate might unmask a chymotrypsin site on the cytoplasmic surface. To distinguish between these possibilities, microsomes disrupted by nitrogen cavitation rather than detergent were exposed to chymotrypsin for 25 min at 30°C and then recollected by centrifugation (see Materials and Methods). Disruption was evidenced by the chymotrypsin-dependent inactivation of greater than 60% of the mannose-6-*P* phosphatase activity. Lysophosphatidic acid acyltransferase activity, however, was not inactivated by the chymotrypsin treatment. When deoxycholate plus chymotrypsin was added to the nitrogen-cavitated microsomes, 85% of the lysophosphatidic acid acyltransferase activity was inactivated. Deoxycholate treatment, rather than access of chymotrypsin to the lumenal surface, appears to be the important determinant in the sensitivity of the lysophosphatidic acid acyltransferase activity. The detergent may be unmasking a site of chymotrypsin cleavage which is buried within the membrane.

These data provide the first example in which protease inactivation falsely indicated a lumenal location for a microsomal enzyme. The criteria for localization of lumenal enzymes of microsomal vesicles should therefore include studies using several proteases and/or employ more than one method of microsomal disruption.

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