Evidence that cholic acid CoA ligase is located asymmetrically on the cytoplasmic surface of hepatic microsomal vesicles

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Abstract The cholic acid CoA ligase activity of rat liver was quantitatively inactivated by proteolysis with pronase, chymotrypsin, subtilisin, or proteinase K in intact microsomal vesicles. Under the conditions employed, less than 14% of the lumenal mannose-6-phosphate phosphatase activity was lost, and the mannose-6-phosphate phosphatase activity remained highly latent. After microsomal integrity was disrupted with sodium deoxycholate, protease treatment resulted in a loss of greater than 74% of the mannose-6-phosphate phosphatase activity. Cholic acid CoA ligase activity was unaffected by preincubation of microsomes with sodium taurocholate under conditions that led to the complete expression of latent mannose-6-phosphate phosphatase activity. The data suggest that cholic acid CoA ligase activity is located on the cytoplasmic surface of hepatic microsomal vesicles.

Supplementary key words microsomes · glucose-6-phosphate phosphatase · protease · membrane asymmetry · bile salts · bile acids

The location of biosynthetic activities within the transverse plane of the endoplasmic reticulum is of particular interest in the case of enzymes whose products are secreted by the cell. Bile acids, whether synthesized de novo by the liver or absorbed from the enterohepatic circulation, must be conjugated prior to their excretion into the bile (1). The cholic acid CoA ligase (AMP) (EC 6.2.1.7) activates cholic acid to its coenzyme A thioester from which it is transferred to glycine or taurine (2, 3). Recently, the cholic acid CoA ligase activity was characterized using an improved assay method. It was shown to be present only in liver and to be associated with a microsomal enzyme distinct from the long chain fatty acid CoA ligase (AMP) (EC 6.2.1.3) (4).

Microsomes, derived from the endoplasmic reticulum, are sealed vesicles that maintain proper sidedness (5, 6). The external surface of the vesicles corresponds to the cytoplasmic surface of the endoplasmic reticulum. Since proteases neither cross the microsomal membrane nor destroy the permeability of microsomal vesicles, only the proteins or enzymes located on the cytoplasmic surface of microsomal vesicles are susceptible to proteolysis unless membrane integrity is disrupted (5, 6). Several enzymes and proteins have been localized in the transverse plane of microsomal vesicles by using proteases (5). All of the enzymes and proteins investigated, with the possible exception of cytochrome P-450, were asymmetrically localized to either the cytoplasmic or the lumenal surface (5). Glucose-6-phosphate phosphatase, associated with all hepatic microsomal vesicles (7, 8), has been demonstrated to be located exclusively on the lumenal surface by proteolysis experiments and by product localization (5, 7, 8). The enzyme also catalyzes the hydrolysis of mannose-6-phosphate, but this alternative substrate is unable to cross the permeability barrier of intact microsomes (9). Mannose-6-P phosphatase activity is thus highly latent.[1] The latency of mannose-6-P phosphatase activity appears to provide a reliable index of microsomal integrity (9).

In this communication, we report experiments on the localization of the cholic acid CoA ligase using the criteria of protease sensitivity and latency. Mannose-6-P phosphatase was employed as a lumenal control activity.

MATERIALS AND METHODS

Materials

Hexokinase, mannose-6-phosphate, mannose, cholic acid (sodium salt), sodium taurocholate, bovine serum albumin (essentially fatty acid-free), and dithiothreitol

Abbreviations: mannose-6-P, mannose-6-phosphate.

[1] Latency is defined by the equation:

$$\% \text{ latent activity} = \left[1 - \left(\frac{\text{activity observed in intact vesicles}}{\text{activity observed in disrupted vesicles}}\right)\right] \times 100$$

Thus a latency of 90% corresponds to a 10-fold increase in activity observed after microsomal disruption.

were obtained from Sigma. Triton WR-1339 was purchased from Ruger Chemical Co., Irvington, NJ. [^{3}H]-Cholic acid, $^{32}P_i$, and Aquasol were the products of New England Nuclear. α-Chymotrypsin was a Worthington product. Sodium deoxycholate was from Schwarz-Mann. Pronase was obtained from Calbiochem. Proteinase K was purchased from Beckman. Subtilisin was obtained from Nutritional Biochemicals Corp. [γ-^{32}P]ATP was synthesized as described previously (10). Mannose-6-[^{32}P]phosphate was synthesized by a modification of the method of Slein (11). The reaction mixture (2.5 ml) contained 50 mM Tris-Cl, pH 8.1, 8 mM $MgCl_2$, 20 mM mannose, 16.8 mM [γ-^{32}P]ATP (24 μCi/μmol), and about 0.5 mg of hexokinase. After 1 hr at 23°C, the mannose-6-[^{32}P]phosphate was purified from the reaction mixture by chromatography on Whatman No. 3 paper in solvent I (methanol–formic acid–water, 120:19.5:10.5 (v/v/v). The mannose-6-[^{32}P]phosphate was located by radioautography and eluted from the paper with water. Typically, 20–40% of the labeled ATP was converted to labeled mannose-6-P. Greater than 94% of the labeled material chromatographed in a manner similar to that of authentic mannose-6-P.

Isolation of liver microsomes

Livers from 200–250 g Charles River CD strain female rats were homogenized by 10 rapid up and down strokes using a motor-driven, Teflon–glass homogenizer in three volumes of cold medium I (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-Cl, 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 hr. The pellet was suspended in medium I and centrifuged at 100,000 *g* for 1 hr. The final pellet was resuspended in medium I without EDTA. Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as the standard.

Protease treatment of microsomes

The integrity of microsomal vesicles was disrupted by incubation in medium II (0.25 M sucrose, 50 mM Tris-Cl, pH 7.5, 50 mM KCl, and 0.05% sodium deoxycholate) (13) for 10 min at 4°C. Another sample (intact microsomes) was treated in the same manner in medium II lacking sodium deoxycholate. The intact and the disrupted microsomes (30 mg of protein) were then incubated for 25 min at 30°C with either pronase (50 μg/mg microsomal protein), chymotrypsin (100 μg/mg), subtilisin (125 μg/mg), or proteinase K (32 μg/mg), as described by Nilsson and Dallner (13). 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 hr. The recovered microsomal pellets were suspended in medium I without EDTA, and protein was determined according to the method of Lowry et al. (12). Enzyme assays were performed immediately or within 3 days on samples stored at −15°C. No activity was lost by freezing and thawing one time. Less than 10% of the cholic acid CoA ligase activity associated with intact microsomes was lost upon incubation at 30°C for 25 min.

Enzyme assays

Mannose-6-P phosphatase activity was assayed at 23°C using 2 mM [^{32}P]mannose-6-P (1.36 μCi/μmol) essentially as previously described (14). Cholic acid CoA ligase activity was estimated using the radiochemical assay previously developed in our laboratory (4). All assays were proportional to the time and the amount of protein employed.

RESULTS

Treatment of intact microsomes with chymotrypsin was without effect on mannose-6-P phosphatase activity (**Table 1,** experiment 1). When the chymotrypsin treatment was performed in the presence of 0.05% deoxycholate to disrupt the integrity of the microsomal membrane, more than 84% of the mannose-6-P phosphatase activity was lost. Chymotrypsin inactivation of mannose-6-P phosphatase activity was not dependent on the presence of deoxycholate, since the activity was susceptible to proteolysis in nitrogen cavitated microsomal preparations.[2] The latency of mannose-6-P phosphatase in the microsomal preparation was initially 90%. After incubation at 30°C for 25 min and reisolation, mannose-6-P phosphatase latency had dropped to 87% for the control microsomes and 85% for the protease-treated microsomes. These data indicate that the non-detergent-treated microsomal preparation remained intact during the chymotrypsin digestion. Under these conditions of chymotrypsin treatment of intact microsomal vesicles, the cholic acid CoA ligase activity was totally inactivated.

Similar experiments were performed using pronase, subtilisin, and proteinase K (Table 1, experiments 2–4). In each case at least 87% of the mannose-6-P phosphatase activity was recovered in the intact, protease-

[2] Nitrogen cavitation (15) reduced the latency of mannose-6-P phosphatase from an initial value of 90% to 50%. Chymotrypsin treatment resulted in a 55% loss of mannose-6-P phosphatase activity in nitrogen cavitated microsomes.

TABLE 1. Effect of proteases on cholic acid CoA ligase and mannose-6-P phosphatase activities[a]

Enzyme Activity	Total Activity (nmol/min)[b]					
	Intact Microsomes			Disrupted Microsomes[c]		
	Control	(+) Protease	% of Control	Control	(+) Protease	% of Control
Experiment 1—Chymotrypsin[e]						
Mannose-6-P phosphatase[d]	1453	1437	98.9	2098	324	15.4
Cholic acid CoA ligase	29.7	0	0	3.6	0	0
Experiment 2—Pronase[f]						
Mannose-6-P phosphatase	2260	2140	94.7	1882	492	26.1
Cholic acid CoA ligase	45.6	0	0	3.0	0	0
Experiment 3—Subtilisin[g]						
Mannose-6-P phosphatase	2090	1831	87.6	1701	248	14.6
Cholic acid CoA ligase	46.6	0	0	6.6	0	0
Experiment 4—Proteinase K[h]						
Mannose-6-P phosphatase	2400	2142	89.2	1197	34	2.9
Cholic acid CoA ligase	37.1	0	0	1.6	0	0
Experiment 5—Chymotrypsin[i]						
Mannose-6-P phosphatase	2719	2720	100.0	1162	302	26.0
Cholic acid CoA ligase	23.5	0	0	19.6	0	0

[a] Microsomes were incubated at 30°C for 25 min under the appropriate conditions and reisolated prior to assay, as described under Materials and Methods.

[b] Enzyme activities are expressed as total activities to permit direct comparison between samples whose protein recoveries were different. Experiments were performed on different days with different preparations whose initial enzyme activities varied.

[c] Microsomes were disrupted with 0.05% sodium deoxycholate in experiments 1–4, and with 0.5% sodium taurocholate in experiment 5.

[d] Assayed after treatment with 0.5% sodium taurocholate. Final assay mixtures contained less than 0.075% taurocholate.

[e] Protein recoveries from intact control and chymotrypsin-treated microsomes were 22.0 mg and 19.0 mg, respectively. Protein recoveries from disrupted controls and chymotrypsin-treated microsomes were 20.0 mg and 11.6 mg, respectively.

[f] Protein recoveries from intact control and pronase-treated microsomes were 23.5 mg and 19.5 mg, respectively. Protein recoveries from disrupted control and pronase-treated microsomes were 15.2 mg and 12.4 mg, respectively.

[g] Protein recoveries from intact control and subtilisin-treated microsomes were 20.6 mg and 16.4 mg, respectively. Protein recoveries from disrupted control and subtilisin-treated microsomes were 14.7 mg and 9.2 mg, respectively.

[h] Protein recoveries from intact control and proteinase K-treated microsomes were 20.3 mg and 16.4 mg, respectively. Protein recoveries from disrupted control and proteinase K-treated microsomes were 14.1 mg and 7.1 mg, respectively.

[i] Protein recoveries for intact control and chymotrypsin-treated microsomes were 23.5 mg and 19.7 mg, respectively. Protein recoveries from disrupted control and chymotrypsin-treated microsomes were 12.1 mg and 8.0 mg, respectively.

treated microsomal vesicles, while protease treatment of disrupted vesicles resulted in the loss of at least 84% of this activity. Similar results were seen when 0.5% sodium taurocholate was employed for microsomal disruption (Table 1, experiment 5). The cholic acid CoA ligase activity was quantitatively inactivated in intact vesicles by each protease tested.

The components of the cholic acid CoA ligase reac-

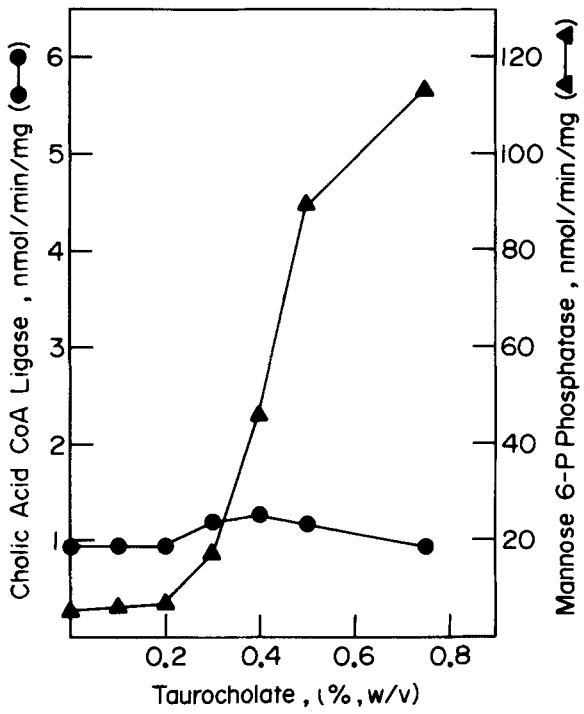

Fig. 1. Effect of taurocholate on mannose-6-P phosphatase and cholic acid CoA ligase activities. Microsomes were incubated with the indicated concentration of sodium taurocholate at a protein concentration of 3 mg/ml for 10 min at 4°C. Aliquots were assayed for mannose-6-P phosphatase and cholic acid CoA ligase as described under Materials and Methods. The aliquots tested comprised 10% of the total volume of each assay system. (△ —— △), Mannose-6-P phosphatase specific activity; (○ —— ○), cholic acid CoA ligase specific activity.

tion mixture did not disrupt the microsomal permeability barrier as judged by mannose-6-P phosphatase latency (data not shown). Therefore, the possibility was considered that protease treatment of intact microsomes caused inactivation of a protein component necessary for the transmembrane movement of substrates (cholic acid, CoASH, and ATP) for the cholic acid CoA ligase without directly affecting cholic acid CoA ligase present on the lumenal surface. If such were the case, disruption of the microsomal permeability barrier would permit the substrates access to the enzyme on the lumenal surface and thereby restore activity. However, when protease-treated intact microsomes were preincubated with sodium taurocholate or Triton X-100 at levels where mannose-6-P phosphatase activity was maximal, cholic acid CoA ligase activity was not restored (data not shown). Furthermore, cholic acid CoA ligase activity in intact microsomes was not latent, as judged by preincubation with levels of sodium taurocholate that resulted in expression of latent mannose-6-P phosphatase activity (**Fig. 1**). Similar results were obtained with Triton X-100.

DISCUSSION

The treatment of intact microsomal vesicles with chymotrypsin, pronase, subtilisin, or proteinase K (Table 1) resulted in the total inactivation of the cholic acid CoA ligase activity when less than 14% of the lumenal mannose-6-P phosphatase activity was lost. Mannose-6-P phosphatase activity was susceptible to proteolysis after disruption of microsomal vesicles with sodium deoxycholate or sodium taurocholate, (Table 1), or by nitrogen cavitation. The fact that protease exposure was without effect on mannose-6-P phosphatase latency argues that the microsomes remained intact. The data indicate that a portion of the cholic acid CoA ligase is exposed on the cytoplasmic surface of microsomal vesicles.[3] The protease data do not exclude the possibility that the cholic acid CoA ligase spans the membrane and that proteolysis of the cytoplasmic domain results in loss of activity at the lumenal surface. Evidence either for or against a spanning orientation of the cholic acid CoA ligase awaits the development of methods allowing independent access of proteases to the microsomal lumenal surface and/or the raising of antibodies to the purified enzyme.

Cholic acid CoA ligase activity was poorly recovered in control microsomes disrupted with sodium deoxycholate, (Table 1, experiments 1–4). It is likely that this effect is attributable to residual deoxycholate which remained associated with microsomes during reisolation. Vessey and Zakim (16) reported, and we have confirmed (data not shown), that deoxycholate is a powerful inhibitor of cholic acid CoA ligase activity in vitro. We have estimated that if as little as 5% of the deoxycholate present during the incubation period sedimented with the microsomal pellet during reisolation, over 80% inactivation of cholic acid CoA ligase activity would result. Since taurocholate is a much poorer inhibitor of cholic acid CoA ligase activity (16), we performed one proteolysis experiment in which microsomes were disrupted with 0.5% taurocholate, (Table 1, experiment 5). As expected, the recovery of cholic acid CoA ligase in disrupted control microsomes was considerably greater.

Since cholic acid CoA ligase did not appear to be latent (Fig. 1), it seems reasonable that the active site of the cholic acid CoA ligase as well as its protease-sensitive site are located asymmetrically on the cytoplasmic surface of the endoplasmic reticulum. The consequences of such an asymmetric location would be

[3] When protease-treated microsomes were added to intact control microsomes, cholic acid CoA ligase activity was virtually unchanged. This indicated that the intact protease-treated microsomes did not contain a cholic acid CoA ligase inhibitor.

3-fold. First, such an orientation would allow access to the required substrates, ATP and CoA, present in the cellular cytoplasm. Second, choloyl-CoA synthesized on the cytoplasmic surface of the endoplasmic reticulum would be readily available, together with cytoplasmic glycine or taurine, as a substrate for the choloyl-CoA:glycine (taurine) acyltransferase that is present in the cytoplasm or loosely associated with the microsomal membrane (3). Third, the asymmetric formation and utilization of choloyl-CoA on the cytoplasmic surface of the endoplasmic reticulum would place the resulting bile salts in the appropriate cellular compartment for active secretion (17) from the cytoplasm into the canalicular space that joins the bile duct.

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