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THE SIDEDNESS OF CARNITINE ACETYLTRANSFERASE AND CARNITINE OCTANOYLTRANSFERASE OF RAT LIVER ENDOPLASMIC RETICULUM *

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The location of carnitine acetyltransferase and carnitine octanoyltransferase on the inner and outer surfaces of rat liver microsomes was investigated. Latency of mannose-6-phosphate phosphatase showed that the microsomes were 90–94% sealed. All of the octanoyltransferase is associated with the cytosolic face, while the acetyltransferase is distributed between the cytosolic face (68–73%) and the lumen face (27–32%) of the endoplasmic reticulum membrane. Small amounts of trypsin inhibit the carnitine octanoyltransferase equally in either sealed or permeable microsomes but the acetyltransferase of sealed microsomes is stimulated. Large amounts of trypsin inhibit all transferase activities by about 60%, except for acetyltransferase of sealed microsomes. Other studies show that 0.1% Triton X-100 partially inhibits carnitine octanoyltransferase of microsomes but does not inhibit the acetyltransferase or any of the mitochondrial carnitine acyltransferase.

Introduction

Carnitine acyltransferases catalyze the transfer of short-chain, medium-chain, and long-chain acyl groups of CoA to the β-hydroxyl group of carnitine to form acylcarnitines which can be transported across some intracellular membranes. Carnitine acetyltransferase and carnitine octanoyltransferase occur in the microsomal fractions from both rat liver and heart [1–5]. The function of these transferases in the endoplasmic reticulum is not known. If any carnitine acyltransferase activities are located on the lumen side of the endoplasmic reticulum, they could provide a mechanism for generating acyl-CoA derivatives from acylcarnitines in the cisternae of this cellular structure. When microsomes are isolated they form sealed vesicles which retain proper sidedness with the external surface of the vesicles corresponding to the cytoplasmic surface [6].

The extent to which microsomes are sealed can be ascertained by determining the mannose-6-phosphate phosphohydrolase activity of the glucose-6-phosphate phosphatase enzyme. This has been used as a quantitative index of microsomal membrane integrity under various assay conditions [6–9]. Here, we show that carnitine acetyltransferase is located on both microsomal surfaces while carnitine octanoyltransferase is only associated with the cytosolic side of the endoplasmic reticulum.

Materials and Methods

Reagents and chemicals

Mannose 6-phosphate, phosphomannose isomerase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, trypsin, trypsin inhibitor and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma. DL-[*methyl*-

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^{3}H]Carnitine hydrochloride and [1-^{14}C]acetyl-CoA were purchased from Amersham. Acetyl-CoA, octanoyl-CoA, and palmitoyl-CoA were purchased from P-L Biochemicals. L-(−)-Carnitine was a gift from the Otsuka Pharmaceutical Co., Naruto, Tokushima, Japan.

Isolation of liver microsomes

Liver from 18-h-fasted male Spraque-Dawley rats (150–200 g) were rapidly removed and homogenized in ice-cold 0.25 M sucrose, 0.05 M Hepes, 5 mM EDTA buffer (pH 7.5) with a Potter-Elvejhem homogenizer. The homogenate was centrifuged at $500 \times g$ and the supernatant fluid was spun at $20000 \times g$. After recentrifugation at $20000 \times g$, the supernatant fluid was centrifuged at $100000 \times g$ for 60 min. The pellet was resuspended in the sucrose buffer (15–20 mg protein/ml) and assayed immediately.

Assays

Carnitine acyltransferases were assayed spectrophotometrically and by following the rate of formation of radiolabeled acylcarnitines. The spectrophotometric method measures the release of CoASH by using the thiol reagent DTNB. The reaction mixture contained 0.116 M Hepes (pH 8.0), 1.25 mM L-(−)-carnitine and one of the following: 10^{-4} M octanoyl-CoA, 10^{-4} M acetyl-CoA, or 37.5 μM palmitoyl-CoA. The reaction was initiated by the addition of enzyme and the rate was monitored at 412 nm with a Gilford 250 spectrophotometer [10]. The formation of radiolabeled acylcarnitine used the same reaction media except DTNB was omitted and either DL-[*methyl*-^{3}H]carnitine for the palmitoyl- and octanoyltransferases or [1-^{14}C]acetyl-CoA for the acetyltransferase assays was added. The reaction was stopped by boiling for 4 min and the octanoylcarnitine and palmitoylcarnitine were isolated by extraction into *n*-butanol [11]. Acetylcarnitine was separated by passing over Dowex 1-Cl$^-$ [12].

Peroxisomal catalase [13], mitochondrial glutamate dehydrogenase [14], microsomal NADPH-cytochrome *c* reductase [15], and protein [10] were assayed as referenced. Mannose-6-phosphate phosphatase was determined by modifying the procedure of Nordlie and Arion [16]. A 0.1 ml aliquot of isolated microsomes 915 mg protein/ml) was incubated in 1.4 ml of reaction medium containing 0.06 M sodium cacodylate buffer (pH 7.0), 1.30 μmol mannose 6-phosphate, 0.1 mM acyl-CoA, and 0.025% Triton X-100 when present, until termination by 0.5 ml of cold 12% perchloric acid. The protein was pelleted by centrifugation and the supernatant fluid was adjusted to pH 7.6 with 6 M KOH. Potassium perchlorate was precipitated by centrifugation and a 0.8 ml aliquot of the supernatant fluid was added to 2.2 ml reaction medium containing 0.4 units of both phosphomannose isomerase (EC 5.3.1.8) and phosphoglucoisomerase (EC 5.3.1.9) in 0.1 M Tris-HCl buffer, pH 7.6. Any mannose 6-phosphate which was not hydrolyzed by glucose-6-phosphatase during the initial incubation would be converted to glucose 6-phosphate by the isomerase and the amount of glucose 6-phosphate formed was then determined by the change in absorbance at 340 nm after addition of 0.2 ml of 25 mM NADP and excess glucose-6-phosphate dehydrogenase. Trypsin inactivation of the microsomal carnitine acyltransferases was determined by incubating the microsomes with varying amounts of trypsin (0–100 μg/ml protein) at 37°C for 15 min. Subsequently, a 3-fold excess of trypsin inhibitor was added [17] and the transferase activities and the latency were determined.

Results

Purity of microsomes

Table I shows that the microsomes contained little contamination by mitochondria and peroxisomes. NADPH-cytochrome *c* reductase was used as the microsomal marker, catalase was the peroxisomal marker and glutamate dehydrogenase was used as the intact mitochondrial marker. Both catalase and glutamate dehydrogenase were very low or negligible in the microsome preparations. Since the microsomes could contain mitochondrial fragments, the amount of fragmented mitochondrial membrane was determined by measuring the amount of cytochrome aa_3 in the microsome preparations by determining the difference spectrum using an American Instrument Co., dual-beam, dual-wavelength spectrophotometer (Aminco DWZA) and the extinction coefficients as referenced [18]. The amount of cytochrome aa_3

TABLE I

DISTRIBUTION OF MARKER ENZYMES DURING MICROSOME ISOLATION

Fractions were isolated and enzymes assayed as described in Materials and Methods.

Fraction	Catalase (μmol/min per mg protein)	Protein (total) (mg)	Glutamate dehydrogenase (nmol/min per mg protein)	NADPH-cytochrome *c* reductase (nmol/min per mg protein)
500× *g* supernatant fluid	917	1625	18.2	23.9
20000× *g* supernatant fluid	616	1251	11.9	16.4
20000× *g* pellet	–	360	80.0	36.9
Microsomes	7	112	0.0	127
100000× *g* supernatant fluid	539	1168	1.0	1.2

was small (3.1–3.7%) when compared to the amount of microsomal cytochrome b_5 (100%) (data not shown).

Permeability of microsomes

The method used to determine the sidedness of the acyltransferases required sealed or impermeable microsomes. When microsomes are treated under the assay conditions used for either the spectrophotometric DTNB assay or the radiolabel assay, the microsomal membrane remains impermeable to mannose 6-phosphate in the absence of

TABLE II

PERMEABILITY OF MICROSOMES DURING THE ASSAYS USED TO DETERMINE THE SIDEDNESS OF CARNITINE ACETYL- AND OCTANOYLTRANSFERASE

The % latency and the percent of sealed microsomes were determined for each of the assay conditions used for measuring the sidedness of carnitine acyltransferases described in Figs. 1–4 by measuring the amount of glucose 6-phosphate produced in the presence and absence of 0.025%. Triton X-100 as described in Materials and Methods. % latency is based on the amount of the original 0.2 mM acyl-CoA was used in each assay mixture. Each assay; $n = 4$, S.D. $< 8\%$.

Assay conditions	Acetyl-CoA present				Octanoyl-CoA present			
	Time (min)	Triton X-100	μmol glucose 6-phosphate	% latency	Time (min)	Triton X-100	μmol glucose 6-phosphate	% latency
Spectrophotometric and radiolabel	0	–	1.20	92	0	–	1.19	91
	5	–	1.18	90	5	–	1.16	89
	10	–	1.16	86	10	–	1.15	88
	5	+	0.32	24	5	+	0.31	24
	10	+	0.27	20	10	+	0.25	19
Freeze-thawing	0	–	1.23	94	0	–	1.20	92
	5	–	1.20	92	5	–	1.16	89
	10	–	1.17	85	10	–	1.14	88
Trypsin digestion	0	–	1.17	89	0	–	1.15	88
	5	–	1.12	86	5	–	1.14	88
	10	–	1.09	84	10	–	1.10	85
	5	+	0.51	37	5	+	0.49	38
	10	+	0.49	33	10	+	0.50	38

Triton X-100 (see Table II). Thus, only acyltransferases on the cytosolic surface should be measured using these conditions. Addition of 0.025% Triton X-100 to the assay apparently disrupts the membranes with subsequent hydrolysis of mannose 6-phosphate. Adding more Triton X-100 did not increase the amount of mannose 6-phosphate hydrolyzed; thus, 0.025% Triton X-100 should expose both the lumen and cytosolic acyltransferases. Attempts to disrupt microsomal membranes by repeated freeze-thawing produced little hydrolysis of mannose 6-phosphate. Trypsin treatment did not affect the permeability of microsomes to mannose 6-phosphate in the absence of Triton X-100, but addition of 0.025% Triton X-100 lowered the phosphatase activity in the presence of trypsin, as expected for disrupted microsomes [19,20]. These data indicate that only the carnitine acyltransferases on the cytosolic face would be measured by the DTNB and the radiochemical assays even when microsomes are treated with small amounts of trypsin in the absence of Triton X-100.

Location of carnitine acyltransferases in microsomal vesicles

The acetyltransferase is apparently distributed on both sides of the microsomes, since the activity of intact microsomes was 71% of that measured with Triton X-100-treated microsomes (see Fig. 1). In contrast, the carnitine octanoyltransferase activity of intact microsomes was the same as that of Triton X-100-treated microsomes (0.025% Triton X-100), indicating that all of this transferase activity is located on the cytosolic surface of the endoplasmic reticulum. The 0.10% Triton X-100 normally used to disrupt membranes for our carnitine acyltransferase assay is partially inhibitory to the microsomal carnitine octanoyltransferase. Thus, 0.025% Triton X-100 was used to disrupt the microsomal membrane in all subsequent studies. Triton X-100 inhibition (0.10%) of carnitine octanoyltransferase was found only with microsomes. The detergent was not inhibitory to peroxisomes or mitochondrial carnitine acyltransferases (Fig. 2) and did not inhibit the carnitine acetyl- or palmitoyltransferases.

The distribution of carnitine acyltransferases of microsomes was also investigated using a spectro-

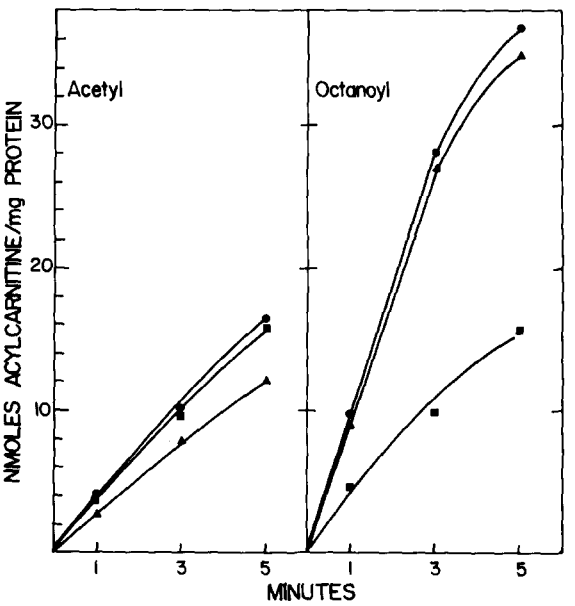

Fig. 1. Rate of formation of acetylcarnitine and octanoylcarnitine in Triton X-100-treated and intact microsomes. Acylcarnitine formation was determined by measuring the amount of radiolabeled acylcarnitines as described in Materials and Methods. (▲——▲) No Triton X-100; (●——●) 0.025% Triton X-100; (■——■) 0.10% Triton X-100.

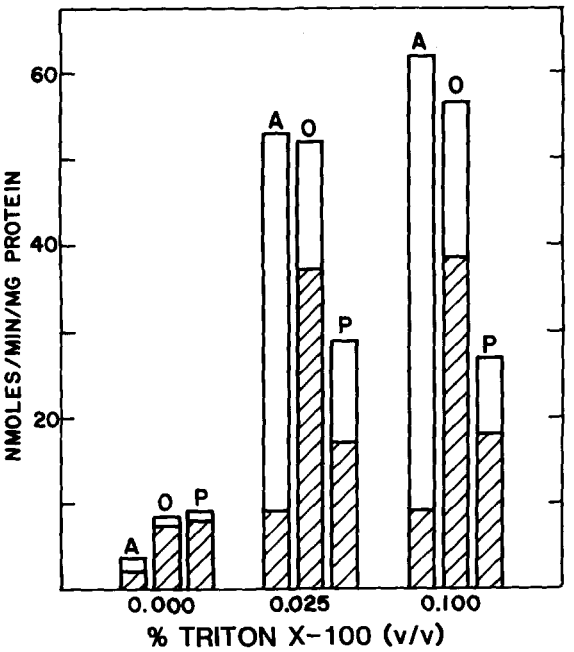

Fig. 2. Effect of Triton X-100 on mitochondrial carnitine acyltransferase. The transferase activity (striped area) and hydrolase activity (open area) were determined by the spectral method as described in the Materials and Methods. A, carnitine acetyltransferase; O, carnitine octanoyltransferase; P, carnitine palmitoyltransferase.

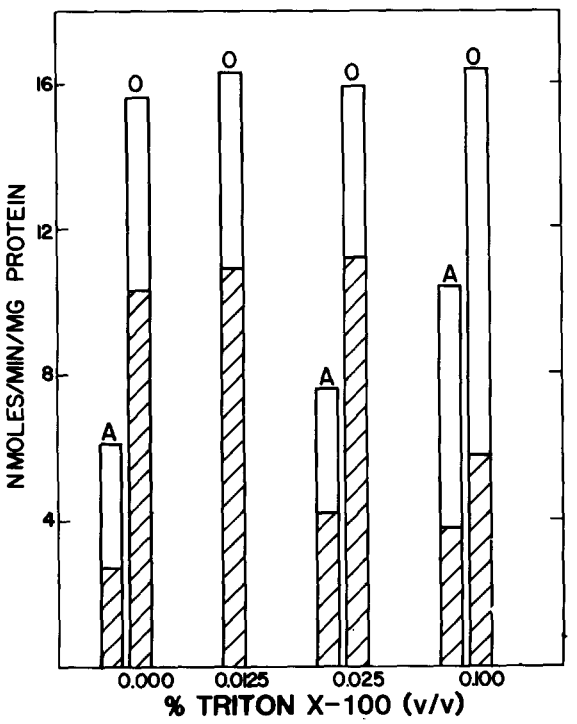

Fig. 3. Effect of Triton X-100 on microsomal carnitine acyltransferases. The transferase activities (striped area) and the hydrolase activity (open area) were determined by the spectral assay as described in Materials and Methods. A, carnitine acetyltransferase; O, carnitine octanoyltransferase.

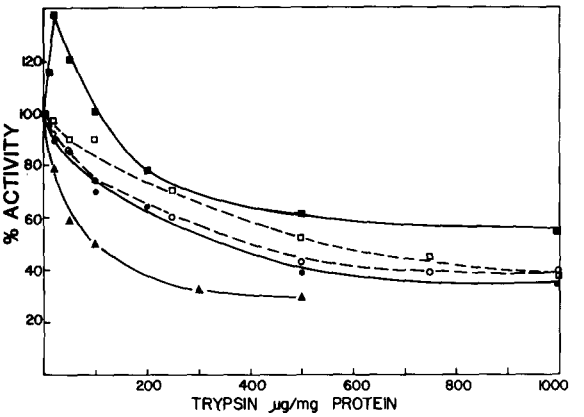

Fig. 4. Effect of trypsin on microsomal carnitine acyltransferases of disrupted and intact vesicles. Microsomes were incubated with various amounts of trypsin for 15 min at 30°C and the percentage of transferase activities remaining was determined by measuring the formation of radioactive acylcarnitines. Carnitine acetyltransferase of intact microsomes (■——■) and disrupted microsomes (□——□) after trypsin treatment. Carnitine octanoyltransferase of intact (●——●) and disrupted (○——○) microsomes. Purified carnitine palmitoyltransferase (beef heart) (▲——▲) was kindly provided by Peter Clarke of this laboratory.

photometric assay which measures release of CoASH rather than production of labeled acylcarnitines. The DTNB (Fig. 3) method gave results similar to those observed with the radiolabel method, with 68% of the acetyltransferase and 95% of the octanoyltransferase association with the cytosolic face. Since there is a high background for the DTNB method with microsomes, the results are subject to greater error than the radiolabel assay.

Trypsin treatment

Since some enzymes associated with the external face of microsomes can be inactivated by trypsin, we tested the effect of trypsin on the carnitine octanoyl- and acetyltransferases. Treatment with small amounts of trypsin (10–50 µg/mg protein in the absence of Triton X-100 stimulated the acetyltransferase (Fig. 4) but inhibited octanoyltransferase. The addition of detergent plus trypsin or the addition of large amounts of trypsin inhibited both acetyl- and octanoyltransferases. The octanoyltransferase activity in permeable microsomes decreased with increasing amounts of trypsin to the same extent as in sealed non-Triton X-100-treated microsomes. The maximum of 60% reduction in carnitine octanoyltransferase is similar to that observed when purified carnitine palmitoyltransferase (see Refs. 26 and 27 for a description of this enzyme) was treated with Triton X-100.

Discussion

We previously reported that carnitine acetyl- and octanoyltransferases are associated with rat liver microsomes [21]. The microsomal carnitine acetyltransferase activity in rat liver is low but in rat brown adipose tissue it has an about 10-fold higher specific activity than liver microsomes [22]. At this time the function(s) have not been elucidated. A major function for carnitine and carnitine acetyltransferase in mitochondria appears to

be buffering of the acetyl-CoA/CoASH ratio [23]. This role has been broadened to include a general role for carnitine in buffering the CoASH/acyl-CoA ratio via the short-chain and medium-chain carnitine acyltransferases in mitochondria [24]. Although such functions for carnitine seem likely in mitochondria, the necessity for modulating the CoASH/short-chain acyl-CoA ratio by microsomes seems unlikely. A definitive determination of the membrane face with which the carnitine acyltransferases are associated in the endoplasmic reticulum could provide clues about their function. Since the cytosolic compartment of liver cells has considerable synthetic capacity utilizing acetyl-CoA, a possible role for microsomal carnitine acetyltransferase could be to provide cytosolic acetyl-CoA from acetylcarnitine which has been generated via peroxisomal and/or mitochondrial β-oxidation. Both organelles have a large capacity for generating acetylcarnitine. However, such a role is not likely for the acetyltransferase associated with the lumen face of the endoplasmic reticulum. Since some posttransciptional modifications including acetylations occur on endoplasmic reticulum, it seems likely that lumen carnitine acetyltransferase-CoA could be used for acetylations that are part of packaging processes in the lumen, possibly for export of macromolecules.

The results presented here using two independent assays show that approx. 30% of the carnitine acetyltransferase of the rat liver endoplasmic reticulum is latent in microsomes which are impermeable to mannose 6-phosphate. It has been shown by others [6,7,25] that such latency is due to the location of the latent enzyme on the lumen side of the endoplasmic reticulum. For rat liver, this represents about 1.0 μol acetylcarnitine being converted to acetyl-CoA/mg protein on the cisternal side of the endoplasmic reticulum. However, we have not shown that microsomes are permeable to acetylcarnitine and have not demonstrated that endoplasmic reticulum contains coenzyme A in the lumen.

Both assays used here show that most, if not all, of the carnitine octanoyltransferase is associated with the cytosolic face of endoplasmic reticulum. Its function is unknown, but it could provide a mechanism for converting peroxisomally generated medium-chain acylcarnitine derivatives, which have been translocated to the cytosol, to medium-chain length acyl-CoA derivatives. These could then be elongated or modified. Rat liver peroxisomes have the capacity to convert medium-chain acyl-CoA derivatives to the respective acylcarnitines [28,29], most likely derived from peroxisomal β-oxidation [30,31].

The carnitine octanoyltransferase is partially inhibited by 0.1% but not by 0.025% Triton X-100. In our previous investigations of microsomes [2,21], we probably underestimated the amount of carnitine octanoyltransferase associated with the organelles because 0.1% detergent was used. This detergent inhibition was found only with the carnitine octanoyltransferase associated with microsomes. No inhibition was obtained with any of the mitochondrial or peroxisomal carnitine acyltransferase nor with the acetyltransferase associated with microsomes. Our microsome preparations contained small amounts of carnitine palmitoyltransferase activity, however, they also contained small amounts of the inner mitochondrial membrane components as indicated by cytochrome oxidase. If the mitochondrial membrane fragments containing cytochrome oxidase also contained palmitoyltransferase activity in a ratio similar to that of intact mitochondria, then the carnitine palmitoyltransferase activity present in the microsomal fraction could be due to mitochondrial contamination.

Although trypsin inhibited the carnitine octanoyltransferase in intact microsomes and the acetyl- and octanoyltransferase activity in Triton X-100-treated microsomes, small quantities of trypsin activated carnitine acetyltransferase activity of intact microsomes. The extent of the activation was variable but was seen in all of the several experiments done. This activation implies that microsomal enzyme has more enzymatic capacity than we normall measure.

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