L-Carnitine acyltransferase in intact peroxisomes is inhibited by malonyl-CoA

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Inhibition of the overt mitochondrial carnitine palmitoyltransferase by malonyl-CoA is important in the regulation of fatty acid oxidation. In the past, the contribution of peroxisomal carnitine acyltransferase activity to the generation of medium- and long-chain acylcarnitines in the cytoplasm has been ignored. On the basis of marker enzyme levels, we now estimate that peroxisomal palmitoyltransferase activity constitutes about 20% of the peroxisomal plus overt-mitochondrial pool in fed rat liver. When assayed *in situ*, both the palmitoyltransferase and decanoyltransferase activities of gradient-purified peroxisomes are sensitive to malonyl-CoA, with up to 90% inhibition reached at less than 10 μ M-malonyl-CoA. Very similar results were obtained with intact gradient-purified mitochondria from the same livers. In addition, the acyl-CoA substrate chain-length specificity was identical in both the peroxisomes and the mitochondria, with a decanoyltransferase/palmitoyltransferase ratio of 2. Thus the overt carnitine acyltransferase activities in peroxisomes and mitochondria have the same properties. Further, the malonyl-CoA sensitivity of the peroxisomal activity is lost on solubilization, as has been observed for the overt mitochondrial enzyme. It is suggested that malonyl-CoA inhibition of the peroxisomal enzyme as well as of the mitochondrial enzyme is important for the regulation of mitochondrial fatty acid oxidation.

INTRODUCTION

The L-carnitine palmitoyltransferases catalyse the reversible transfer of long-chain acyl groups from CoA to L-carnitine (EC 2.3.1.21). All the carnitine acyltransferase enzymes have a relatively wide specificity for the length of acyl chain. In this paper, the term 'carnitine acyltransferase' is used when discussing the enzyme in general. The more specific terms 'carnitine palmitoyltransferase' and 'carnitine decanoyltransferase' are used to describe the activities measured with palmitoyl-CoA and decanoyl-CoA respectively.

Together with the L-carnitine:acyl-L-carnitine exchange system, the palmitoyltransferases promote the transfer of long-chain fatty acids across the mitochondrial inner membrane (for a review, see [1]). There are two distinct pools of long-chain acyltransferase activity. One is located outside the mitochondrial inner membrane and is therefore accessible only to cytoplasmic carnitine and palmitoyl-CoA (carnitine palmitoyltransferase I). Recently, evidence has been presented that carnitine palmitoyltransferase I activity is located in the mitochondrial outer membrane [2]. The second pool (carnitine palmitoyltransferase II) is located inside the mitochondrion and can be assayed only when the inner membrane is disrupted [3–5].

McGarry *et al.* [6,7] demonstrated that carnitine palmitoyltransferase I activity in rat liver mitochondria and homogenates is sensitive to malonyl-CoA, providing a mechanism for the control of hepatic fatty acid oxidation and ketogenesis. The potential importance of carnitine palmitoyltransferase I as a regulatory metabolic enzyme has led to intensive studies of its kinetic properties

[8–13] and of the changes in activity of the enzyme in response to different physiological and pathological states (see [13]).

Studies of the subcellular distribution of carnitine palmitoyltransferase indicate that most of the activity is mitochondrial [14–17]. However, carnitine acyltransferase activity has also been reported in microsomes (microsomal fractions) [14] and in peroxisomes [18–20]. (The peroxisomal enzyme is referred to in this paper as peroxisomal carnitine acyltransferase because the range of acyl-chain-length substrates observed for it in this laboratory does not conform to the term 'carnitine octanoyltransferase' used in the literature, although the terms probably refer to the same enzyme.) Given that peroxisomal oxidation can account for up to 50% of fatty acid oxidation in mouse liver [21] and that the peroxisomal enzyme accounted for 10–15% of the whole bovine liver palmitoyltransferase activity [22], it seemed likely that this enzyme could contribute significantly to generation of acylcarnitine in the cytoplasm and thus mimic the role of carnitine palmitoyltransferase I in the mitochondria.

We have investigated the properties, quantities and regulation of the acyltransferase activities of intact peroxisomes (peroxisomal carnitine palmitoyltransferase and carnitine decanoyltransferase). The study demonstrated that the properties of these overt activities *in situ* are very similar in the two organelles, that peroxisomal acyltransferase activity is a significant part of the total, and that, like mitochondrial carnitine palmitoyltransferase I, the peroxisomal enzyme is regulated by malonyl-CoA.

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MATERIALS AND METHODS

Materials

L-[methyl-³H]Carnitine hydrochloride was obtained from Amersham Corp., Arlington Heights, IL, U.S.A. Unlabelled L-carnitine and all acyl-CoA derivatives were from Sigma Chemical Co., St. Louis, MO, U.S.A., Percoll was from Pharmacia LKB, Piscataway, NJ, U.S.A., and Nycodenz from Accurate Chemical & Scientific Corp., Westbury, NY, U.S.A. All other reagents were the best grade available.

Extraction of L-[methyl-³H]carnitine hydrochloride

As received, the radioactive L-carnitine contained small quantities of radioactive contaminants which generated a high background in the carnitine acyltransferase assay, but could be removed by extraction into butan-1-ol [23]. The L-carnitine concentration was measured spectrophotometrically [24] and the radioactivity by scintillation counting. The final specific radioactivity was 8.7 Ci/mol.

Measurement of marker enzyme activities

Marker enzymes, assayed by standard procedures, were: mitochondria, glutamate dehydrogenase [25] and monoamine oxidase [26]; peroxisomes, catalase and urate oxidase [25]; microsomes, NADPH-cytochrome *c* reductase [27]; and lysosomes, acid phosphatase [25]. Samples were diluted with 50 mM-KH₂PO₄/KOH, pH 7.5, containing 0.1% Triton X-100 before assay.

Protein concentration was measured by the Petersen [28] adaptation of the Lowry procedure.

Assay for carnitine palmitoyltransferase and carnitine decanoyltransferase activity in intact organelles

Carnitine palmitoyltransferase and carnitine decanoyltransferase were assayed by the method of Saggerson *et al.* [9], with minor modifications. The final concentrations of the assay constituents (in 100 μ l final volume)

were 220 mM-sucrose, 40 mM-KCl, 10 mM-Tris/HCl, 1 mM-EGTA, 1.3 mg of fatty-acid-free bovine serum albumin/ml, 40 μ M-palmitoyl-CoA or -decanoyl-CoA, 8 μ g of sample protein and 400 μ M-L-[³H]carnitine (8.7 μ Ci/ μ mol). The final pH of the medium was 7.4 at 30 °C. To conduct the assay, two solutions were set up: one (solution A) contained the sample (mitochondria or peroxisomes) in sucrose/KCl/Tris/EGTA medium and was kept on ice. The second solution (B) contained defatted albumin, palmitoyl-CoA or decanoyl-CoA and L-[³H]carnitine, also in sucrose/KCl/Tris/EGTA medium. Solution B was pre-warmed to 30 °C immediately before use. Malonyl-CoA was added to solution A before 20 μ l of the suspension was prewarmed (1 min) to 30 °C in a 1.5 ml mini-centrifuge tube. The reaction was started by addition of 80 μ l of solution B and terminated after an appropriate time (usually 8 min) by addition of 150 μ l of 1 M-HCl, which was kept on ice. The decanoyl- or palmitoyl-L-[³H]carnitine formed was extracted into water-saturated butan-1-ol for scintillation counting [9]. Recoveries of the long-chain acyl-L-carnitine derivatives were 100%, except for octanoyl-L-carnitine, which was 75% (results not shown). The assay was linear with time and protein concentration for both decanoyl-CoA and palmitoyl-CoA, and also for both mitochondria and peroxisomes.

Isolation of mitochondria and peroxisomes

The method used for the preparation of mitochondria and peroxisomes from fed male Sprague-Dawley rats (250 g) is illustrated in Scheme 1. Peroxisomes were centrifuged through Nycodenz iso-osmotic solution as described by Ghosh & Hajra [29]. Peroxisomal contamination was removed from mitochondria by fractionation on a Percoll gradient, by a method adapted from that described by Neat *et al.* [30]. The organelles were finally suspended in the isolation medium, which contained 0.25 M-sucrose, 10 mM-Tris/HCl, 1 mM-Na₄EDTA, pH 7.4 at 4 °C.

Scheme 1. Flow diagram for the preparation of clean peroxisomes and mitochondria

The buffer throughout is 0.25 M-sucrose/1 mM-Na₄EDTA/10 mM-Tris/HCl, pH 7.4 at 4 °C.

Table 1. Marker enzymes in gradient-purified preparations of peroxisomes and mitochondria from rat liver

Specific activities ($\mu\text{mol}/\text{min}$ per mg) are given as means $\pm$ S.D. ($n = 4$); values in brackets are those relative to the homogenate. Origin of marker enzymes: *mitochondria; †peroxisomes; ‡lysosomes; §microsomes.

Sample	Glutamate dehydrogenase*	Monoamine oxidase*	Catalase†	Uricase†	Acid phosphatase‡	NADPH-cytochrome <i>c</i> reductase§
Homogenate	1.18 $\pm$ 0.02	0.0026 $\pm$ 0.0007	820 $\pm$ 160	0.0091 $\pm$ 0.0020	0.0686 $\pm$ 0.0021	0.059 $\pm$ 0.007
Peroxisomes	0.27 $\pm$ 0.04 [0.23]	0 (< 0.001) [0]	9760 $\pm$ 1410 [11.9]	0.294 $\pm$ 0.104 [32.3]	0.0735 $\pm$ 0.0132 [1.07]	0.0271 $\pm$ 0.0043 [0.46]
Mitochondria	3.18 $\pm$ 0.49 [2.70]	0.0167 $\pm$ 0.0032 [6.43]	47.4 $\pm$ 9.0 [0.06]	0.00107 $\pm$ 0.00034 [0.12]	0.0157 $\pm$ 0.0020 [0.23]	0.040 $\pm$ 0.015 [0.68]

Fig. 1. Inhibition of carnitine decanoyltransferase and carnitine palmitoyltransferase by malonyl-CoA

The activities were measured as described in the Materials and methods section. The maximum activities for carnitine decanoyltransferase (CDT; *a*) were 9.32 nmol/min per mg in peroxisomes (○) and 8.42 nmol/min per mg in mitochondria (●). For carnitine palmitoyltransferase (CPT; *b*), the activities were 3.85 nmol/min per mg in peroxisomes (○) and 2.94 nmol/min per mg in mitochondria (●). Note the logarithmic scale for the malonyl-CoA concentration.

RESULTS

Separation of rat liver mitochondria and peroxisomes

The specific activities of marker enzymes in the homogenate and density-gradient-purified mitochondrial and peroxisomal preparations are shown in Table 1. Substantial enrichment of catalase (11.9-fold) and urate oxidase (32.3-fold) was obtained in the peroxisomes, relative to the homogenate. It is important to note that the rats were not treated with any peroxisome-proliferating drugs, so that the comparison between the L-carnitine acyltransferases in mitochondria and peroxisomes was made under normal metabolic conditions. The peroxisomal preparation was virtually free of mitochondria, on the basis of monoamine oxidase activity. On the basis of glutamate dehydrogenase activity, the mitochondrial contamination was less than 8%. Lysosomal and microsomal contamination was acceptably low.

As would be expected from their abundance in liver, the enrichment of mitochondrial enzyme activities relative to the homogenate was modest (2.7-fold for glutamate dehydrogenase and 6.4-fold for monoamine oxidase). However, the isolation process was successful in removing most peroxisomal contamination from the preparation (to less than 0.6% of the protein), and the lysosomal and microsomal markers were also substantially decreased relative to the homogenate (Table 1).

Malonyl-CoA sensitivity of carnitine palmitoyltransferase and carnitine decanoyltransferase in rat liver mitochondria and peroxisomes

The effect of malonyl-CoA on the carnitine palmitoyltransferase and decanoyltransferase activities of intact mitochondria and peroxisomes is shown in Fig. 1. The inhibition of the mitochondrial and peroxisomal activities by malonyl-CoA is very similar, if not identical. The concentration of malonyl-CoA required to give 50%

Fig. 2. Substrate specificity of the acyltransferases in peroxisomes and mitochondria

The activities were measured as described in the text with 40 μ M-acyl-CoA and 0.4 mM-L-carnitine present. The derivatives were octanoyl-CoA (C_8), decanoyl-CoA (C_{10}), lauroyl-CoA (C_{12}), myristoyl-CoA (C_{14}), palmitoyl-CoA (C_{16}), and stearoyl-CoA (C_{18}), used with peroxisomes (○) or mitochondria (●).

inhibition of carnitine palmitoyltransferase ($I_{0.5}$) is 2.2 μ M in peroxisomes and 3.6 μ M in mitochondria. These values are not significantly different and compare well with the reported $I_{0.5}$ of 5.4 μ M for malonyl-CoA in mitochondria from fed rats assayed under similar conditions [8].

Substrate specificity of the overt carnitine acyltransferases in peroxisomes and mitochondria

The specific activities of carnitine palmitoyltransferase in mitochondria and in peroxisomes are 3.9 ± 0.7 and 4.9 ± 0.4 nmol/min per mg respectively. These values are means $\pm$ S.D. for ten triplicate assays on four different liver preparations from fed rats. Carnitine decanoyltransferase specific activities were more than 2-fold higher in both of the intact organelle preparations (9.7 ± 1.3 and 10.5 ± 1.3 nmol/min per mg for mitochondria and peroxisomes respectively).

Examination of the acyl-chain-length specificities of the overt activities in the intact organelles at a fixed acyl-CoA concentration (40 μ M) indicated that mitochondrial and peroxisomal carnitine acyltransferase activities vary in the same way with the acyl-CoA substrate under the conditions used (Fig. 2). Both organelles exhibit highest activity with decanoyl-CoA (C_{10}) and with myristoyl-CoA (C_{14}). These results suggest very similar kinetic, as well as regulatory, properties for the enzyme in the two organelles.

Fractionation of rat liver peroxisomes

The properties of the acyltransferase activity in intact peroxisomes as described above differ considerably from those of the purified enzyme, which has higher activity with medium-chain acyl-CoA substrates and is insensitive to malonyl-CoA [15,16,20]. The changes which occur when the enzyme is removed from its native environment were investigated by disrupting the peroxisomes to release the carnitine acyltransferase. After dilution to 1 mg/ml

Table 2. Carnitine decanoyltransferase (CDT) and palmitoyltransferase (CPT) activities in rat liver homogenate

The amounts are calculated on the basis of the recovery of either soluble or insoluble marker enzymes in the purified preparations (see Table 1). The relative contributions are expressed as a percentage of the sum of the activities attributed to the mitochondria and peroxisomes.

	Calculated carnitine acyltransferase activities in the homogenate (nmol/min per mg)			
	From mitochondria		From peroxisomes	
	CDT	CPT	CDT	CPT
Soluble markers	3.59 (80 %)	1.43 (78 %)	0.89 (20 %)	0.41 (22 %)
Insoluble markers	1.51 (82 %)	0.56 (80 %)	0.33 (18 %)	0.15 (20 %)

with isolation medium, the peroxisomes were sonicated for 2×20 s at medium power with the micro-probe of an Ultrasonics sonicator (model W-185). The soluble and insoluble components were separated by centrifugation at 100 000 g_{av} for 1.5 h. Urate oxidase, which remains in the dense core fraction, completely sediments, whereas all the catalase activity is found in the supernatant. In contrast, the carnitine acyltransferase activities are distributed in both fractions. About 40% of the carnitine palmitoyltransferase activity and 70% of the carnitine decanoyltransferase is found in the supernatant. The activities in the supernatant are much less sensitive to malonyl-CoA, only 35% of the palmitoyl- and 20% of the decanoyl-transferase activities being inhibited by 10 μ M-malonyl-CoA. Residual malonyl-CoA sensitivity in the supernatant could be due to membrane fragments, although the centrifugation conditions are generally thought to remove all vesicular material. In contrast, both activities in the pellet fraction are inhibited by more than 90%. Further, the ratio of decanoyltransferase to palmitoyltransferase activity in the soluble fraction was increased to 4, instead of the 2-fold difference observed in the intact organelle and in the pellet. These observations suggest either a change in properties on solubilization or that two forms of the enzyme with different substrate specificities are differentially released, the malonyl-CoA-sensitive one remaining with the insoluble material.

Mitochondrial and peroxisomal contributions to carnitine acyltransferase activities in whole liver homogenate

Table 2 shows the calculated specific activities of the acyltransferases expected in the homogenate, based on the recoveries of marker enzymes in the purified organelles. On the basis of these values, the peroxisomes contribute about 20% of both the palmitoyl- and decanoyl-transferase activities, even after correction for the estimated maximum contamination of the peroxisomal fraction by mitochondria (8% of the protein).

DISCUSSION

The work presented in this paper has analysed the malonyl-CoA sensitivity and kinetic properties of carnitine palmitoyltransferase and carnitine decanoyltransferase in peroxisomes from rat liver. These appear to be similar to those for the overt carnitine palmitoyltransferase and carnitine decanoyltransferase in mitochondria (Fig. 1). In addition, the substrate-specificity profile at fixed acyl-CoA concentration is the same in both organelles (Fig. 2). These results have important implications for the study of carnitine acyltransferases in liver and their contribution to the control of mitochondrial β -oxidation.

It has been widely assumed that the carnitine palmitoyltransferase that is sensitive to malonyl-CoA is exclusively mitochondrial. This is clearly not the case. The relative contributions of mitochondrial and peroxisomal carnitine palmitoyltransferases in whole liver to the total homogenate have been estimated, based on the activities of marker enzymes in the homogenate and in each preparation (Table 2). The peroxisomal fraction accounts for approx. 20% of the combined carnitine palmitoyltransferase activity from mitochondria and peroxisomes in the livers of fed rats. A similar contribution can be estimated from the data for carnitine octanoyltransferase in an earlier study [31] if the recovery of mitochondrial markers was similar to that in the present study. However, that study found that 95% of the palmitoyltransferase activity was mitochondrial [31], in contrast with the results presented here. We propose that the peroxisomal carnitine acyltransferase activity is capable of generating significant amounts of long- as well as medium-chain acylcarnitines for oxidation by the mitochondria. The regulation of that activity by malonyl-CoA is consistent with such a role.

Carnitine acyltransferase levels change with the nutritional state of the animal and drug treatments [8–10]. A study of enzyme and mRNA levels for peroxisomal and total mitochondrial carnitine acyltransferases after administration to rats of a high-fat diet or of drugs which cause proliferation of peroxisomes indicated that the levels rose in parallel in the two organelles by 2–5-fold, depending on the treatment, suggesting concomitant regulation of mitochondrial and peroxisomal enzyme levels [32] as well as the concomitant kinetic regulation reported here.

The level and regulation of carnitine palmitoyltransferases in other subcellular locations have not been investigated. Early work [31] found that 39% of the carnitine octanoyltransferase activity of liver was non-mitochondrial and was divided between the peroxisomes and microsomes (approx. 16% and 22% respectively, by our estimates from those data). Palmitoyltransferase activity was not found in these frozen samples [31]. However, Dr. L. L. Bieber has recently found significant levels of carnitine palmitoyltransferase activity in fresh rat liver microsomes, and that activity is malonyl-CoA-sensitive (L. L. Bieber, personal communication). It is possible that extramitochondrial carnitine acyltransferase activity plays an important role in the generation of cytoplasmic long-chain acyl-L-carnitine, and hence in the regulation of mitochondrial fatty acid β -oxidation; certainly the work presented here requires that this hypothesis is given serious consideration.

Peroxisomes may be a useful organelle in which to

study the mechanism of malonyl-CoA inhibition of acyltransferases, principally because they are free of carnitine palmitoyltransferase II activity, which is present in excess in mitochondria. The role of the acyltransferases in peroxisomes remains unconfirmed. The cytoplasmic supply of CoA being limited [33], long-chain acylcarnitine derivatives may be the form presented to the peroxisome for oxidation, and some limiting effects of the acyltransferase activity on peroxisomal fatty acid oxidation have been proposed [34]. The other likely role is to shuttle chain-shortened products out of the peroxisomes for oxidation by mitochondria [35]. In this case, the role of the peroxisomal carnitine acyltransferase is equivalent to that of the outer-mitochondrial enzyme (carnitine palmitoyltransferase I), and malonyl-CoA regulation becomes important to prevent an uncontrolled by-pass of that critical step in the regulation of fatty acid oxidation.

In conclusion, the discovery of malonyl-CoA-sensitive carnitine acyltransferase activity in peroxisomes is important not only because it demonstrates that regulation of carnitine acyltransferase by malonyl-CoA is not just confined to mitochondria, but also because it offers a convenient preparation for the investigation of the mechanism of malonyl-CoA inhibition of carnitine acyltransferase.

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