Solubilization and separation of two distinct carnitine acyltransferases from hepatic microsomes: characterization of the malonyl-CoA-sensitive enzyme

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INTRODUCTION

Carnitine acyltransferases capable of catalysing the interconversion of medium- or long-chain fatty acyl-CoA esters with the corresponding acylcarnitine esters have been described in several mammalian tissues and in a number of organelles within tissues [1–3]. In liver, carnitine acyltransferase activities are found in mitochondria, in peroxisomes and in the microsomal fraction [1–7]. Because of its likely importance in regulating entry into mitochondria of fatty acyl units for subsequent β-oxidation, the mitochondrial carnitine acyltransferase system has so far been studied in the greatest detail and has been shown to consist of a malonyl-CoA-inhibitable acyltransferase activity (CPT₂) on the inner face of the outer membrane, a carnitine acylcarnitine translocase in the inner membrane and a malonyl-CoA-insensitive acyltransferase activity (CPT₁) on the inner face of the inner membrane. cDNA clones corresponding to CPT₁ and CPT₂ from rat liver have been obtained and sequenced [8,9], indicating distinct polypeptides of 88000 and 70000 Da respectively.

It is now also suggested that liver peroxisomes contain two distinct carnitine acyltransferase proteins [10]. One is readily solubilizable, is unaffected by physiological concentrations of malonyl-CoA and has been purified as a polypeptide of approx. 63000 Da [11,12]. The other peroxisomal activity is malonyl-CoA-inhibitable and is more tightly associated with the membrane of the organelle [10].

The presence of carnitine acyltransferase activity in rat liver microsomes was first reported in 1976 [13] and in 1990 Lilly et al. [7] reported that all of the acyltransferase activity observed in ‘intact’ microsomes could be inhibited by malonyl-CoA. However, an acyltransferase of approx. 54000 Da was subsequently purified to apparent homogeneity and was found to be insensitive to malonyl-CoA [14,15]. At first sight, and in view of previous proposals [1,16–19] that are now realized to be incorrect, these findings suggested that a malonyl-CoA-sensitivity-conferring factor or regulatory subunit might have been lost during the purification of this enzyme. We therefore set out to try to isolate this putative factor/subunit using rat liver microsomes as the starting material. However, in the course of our attempts to solubilize microsomal carnitine acyltransferase activity with retention of malonyl-CoA-sensitivity it became very apparent that these microsomal fractions contained two discrete carnitine acyltransferase activities, one that was sensitive to and one that was insensitive to malonyl-CoA. In the present paper we describe the solubilization, partial purification and partial characterization of the malonyl-CoA-inhibitable carnitine acyltransferase of hepatic microsomes. We provide evidence that it is distinct in properties both from the microsomal malonyl-CoA-insensitive acyltransferase previously isolated [14,15] and from CPT₂ of mitochondrial outer membranes. While this study was in progress and after a preliminary presentation of some of our findings (N. M. Broadway and E. D. Saggerson, unpublished work), Murthy and Pande [21] reported the finding of two carnitine acyltransferases in microsomal fractions and also demonstrated that the readily solubilized, malonyl-CoA-insensitive microsomal acyltransferase may be a previously reported stress-related protein [22]. Our study complements and extends the findings of these workers in providing other lines of evidence for the existence of two microsomal carnitine acyltransferases.

MATERIALS AND METHODS

Chemicals

Fatty acyl-CoA esters, soybean l-α-lecithin (approx. 40% phosphatidylcholine, product no. P3644), proteinase inhibitors,
sodium cholate (98%) and octyl glucoside (> 98%) were obtained from Sigma. Sodium deoxycholate (> 99%) was from Fluka. L-carnitine was from Lonza. Superdex 200 and Resource Q columns and Sephadex G-50 were from Pharmacia. Sodium etomoxir (sodium 2-[6-(4-chlorophenoxy)hexyl]oxirane 2-carboxylate) was a gift from Dr. H. P. O. Wolf, Byk Gulden Pharmazeutika, Konstanz, Germany. All other reagents were of the highest grade commercially available.

Isolation of liver microsomes

Fed male Sprague-Dawley rats (200–250 g) were killed by cervical dislocation, the livers removed immediately, washed in ice-cold isolation medium (220 mM mannitol, 80 mM sucrose, 5 mM Tris/HCl, pH 7.4, 1 mM EDTA, 5 μg/ml bestatin, 5 μg/ml pepstatin, 5 μg/ml leupeptin and 0.1 mM PMSF) and then homogenized in 4 vol. of the same medium with 4 strokes of a motor-driven Potter-type homogenizer. The homogenate was centrifuged at 50,000 g$_{av}$ for 10 min; the supernatant was then centrifuged at 100,000 g$_{av}$ for 60 min. The resulting crude microsomal pellet was then either (i) resuspended in isolation medium and re-centrifuged at 100,000 g$_{av}$ for 60 min and the final pellet resuspended in KH$_2$PO$_4$/K$_2$HPO$_4$ buffer (10 mM, pH 7.2) containing 20 μM (v/v) glycerol plus PMSF (0.1 mM) and bestatin/leupeptin (50 μg/ml each) or (ii) resuspended in potassium phosphate/glycerol buffer (as above), frozen overnight at −70°C, thawed at room temperature, centrifuged at 100,000 g$_{av}$ for 60 min and then resuspended in the minimum volume of potassium phosphate/glycerol medium. In some cases rough microsomes were prepared by sucrose-density-gradient centrifugation as described in [23] except that the final microsomal pellet was resuspended in potassium phosphate/glycerol buffer as above.

Isolation of liver mitochondrial outer membranes

This was as described previously [24] except that the final outer membrane pellet was resuspended in potassium phosphate/glycerol medium as for microsomes.

Assay of carnitine acyltransferase activity

Carnitine acyltransferase was assayed spectrophotometrically at 25°C in the direction of acylcarnitine formation. Unless stated otherwise, assays (1 ml) contained 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$ buffer, pH 7.5, 20 μM 4,4'-dithiodipyridine, 20 μM decanoyl-CoA and 20–100 μg of sample protein, and were initiated by addition of 2 mM L-carnitine. Rates were corrected for carnitine-independent CoASH release. In some experiments fatty-acid-poor BSA (1.3 mg/ml) was included in the assay medium, in which case the concentrations of decanoyl-CoA and 4,4'-dithiodipyridine were increased to 100 μM and 125 μM respectively. Activity was calculated using $\varepsilon_{242} = 19.4$ mM$^{-1}$ cm$^{-1}$.

Solubilization of microsomal carnitine acyltransferase activity

Microsomal membranes (approx. 5 mg of protein/ml) in KH$_2$PO$_4$/K$_2$HPO$_4$ buffer (10 mM, pH 7.2), 20% (v/v) glycerol, PMSF (0.1 mM) and pepstatin/bestatin/leupeptin (50 μg/ml of each) were solubilized with 7 mM sodium deoxycholate. After 2 h on ice with frequent vortex mixing, non-solubilized material was removed by centrifugation at 200,000 g$_{av}$ for 90 min.

Separation of microsomal carnitine acyltransferases

For gel filtration, Superdex 200 (Pharmacia; 16/60 column) was equilibrated with KH$_2$PO$_4$/K$_2$HPO$_4$ buffer (25 mM, pH 7.2)/20% (v/v) glycerol/5 mM sodium deoxycholate/0.1 mM PMSF/20 μg/ml each of pepstatin/bestatin/leupeptin. Generally, 4–5 ml of deoxycholate-solubilized microsomes (after filtration through a 0.22 μm-pore-size filter) were loaded on to the column and eluted at 0.5 ml/min, with 2 ml fractions being collected. The column was maintained at approx. 4°C. For anion-exchange chromatography, Resource Q (Pharmacia; 1 ml column) was equilibrated with KH$_2$PO$_4$/K$_2$HPO$_4$ buffer (10 mM, pH 7.2)/20% (v/v) glycerol/1.5 mM sodium cholate/proteinase inhibitors as for the Superdex 200 column. Generally 3–5 ml of sample was loaded, the column was washed with 10 column vol. of equilibration buffer, and then bound proteins were eluted with a linear gradient (over 20 column vol.) of 0–1 M KCl in the equilibration buffer. The flow rate was 5 ml/min and 1 ml fractions were collected.

Reconstitution into liposomes

Liposomes were formed by sonicating soybean L-α-lecithin (100 mg/ml in distilled water) with a probe sonicator until a clear lipid suspension was obtained. The carnitine acyltransferase sample to be reconstituted was mixed with 0.2 vol. of preformed liposomes, frozen at −70°C, thawed at room temperature and then sonicated twice for 5 s each time at 4 μm amplitude with the microtip of an MSE probe sonicator.

Inhibition of microsomal carnitine acyltransferase by etomoxir-CoA

Microsomal membranes (depleted of readily solubilized, malonyl-CoA-insensitive carnitine acyltransferase by freeze-thawing/centrifugation) were incubated in potassium phosphate buffer (10 mM, pH 7.2) containing 20% (v/v) glycerol, 50 μg/ml each of bestatin/pepstatin/leupeptin, 0.1 M PMSF, 5 mM ATP, 10 mM MgCl$_2$, 1.9 mM CoASH and 100 μM sodium etomoxir for 20 min at room temperature, after which the incubation was diluted 4-fold with potassium phosphate/glycerol buffer, centrifuged at 200,000 g$_{av}$ for 30 min and finally resuspended in potassium phosphate/glycerol/proteinase inhibitor medium. Parallel controls were run from which either etomoxir or CoASH was omitted.

Marker enzyme assays

NADPH: cytochrome c reductase and succinate dehydrogenase were assayed as described previously [24]. Monoamine oxidase was assayed as described previously [4]. Protein was determined using a bicinchoninic acid assay kit (Sigma) or by the method of Lowry et al. [25].

Statistical methods

Statistical significance was determined by Student’s $t$-test for unpaired samples.

RESULTS AND DISCUSSION

Studies with microsomal membranes

We used microsomes isolated either by conventional differential centrifugation or by sucrose-density-gradient centrifugation (see the Materials and methods section). The specific activity of carnitine acyltransferase and the degree of inhibition by malonyl-
CoA were virtually identical regardless of the method of microsomal isolation. This is despite the fact that differential centrifugation yields a preparation containing both rough and smooth microsomes whereas sucrose-density-gradient centrifugation yields rough microsomes only. A similar observation has been reported [7]. On the basis of marker enzyme assays (results not shown), gradient-purified microsomes were contaminated by <0.8 % with mitochondrial inner membranes (succinate dehydrogenase) and by <0.6 % with mitochondrial outer membranes (monooamine oxidase). After freeze-thawing of ‘intact’ microsomes in potassium phosphate/glycerol buffer, the specific activity of total microsomal acyltransferase was 6–8 nmol/min per mg of protein, and this was inhibited by 55 ± 4 % (mean ± S.D.; n = 6) by 50 µM malonyl-CoA (i.e. representing 3.3–4.4 nmol/min per mg of protein of malonyl-CoA-inhibitable activity). We found, in agreement with [21], that if total microsomes were freeze–thawed or sonicated and then ultracentrifuged at 200000 g, for 30 min an essentially malonyl-CoA-insensitive carnitine acyltransferase activity was released into the high-speed supernatant, whereas the microsomal membrane residues displayed increased inhibitory ability by malonyl-CoA. Such microsomal membranes displayed a specific activity of 6–8 nmol/min per mg of protein and were inhibited by 72 ± 3 % (mean ± S.D.; n = 6) by 50 µM malonyl-CoA.

Under the same assay conditions, CPT1 in mitochondrial outer membrane vesicles displayed a specific activity of approx. 70 nmol/min per mg of protein and was inhibited by 70–80 % by 50 µM malonyl-CoA (i.e. representing 49–56 nmol/min per mg of protein of malonyl-CoA-inhibitable activity). On the basis of the specific activities of microsomal and mitochondrial outer membrane acyltransferases and the cross-contamination reported above, CPT1 derived from mitochondrial outer membranes accounted for at most 8.6 % of the acyltransferase activity measured in microsomal membranes.

In agreement with [21] we found the malonyl-CoA-sensitive acyltransferase of microsomal membranes to be irreversibly inhibited by etomoxiryl-CoA (generated in situ). Incubation under our conditions routinely resulted in >90 % inhibition compared with controls from which either etomoxir or CoASH was omitted. In some cases, after inhibition by etomoxir-CoA, we solubilized microsomal membranes with sodium deoxycholate (see below) and passed the solubilized extract through a Sephadex G-50 column (10 mm × 300 mm) to remove traces of etomoxir-CoA. Malonyl-CoA-inhibitable acyltransferase activity was still inhibited by >90 % compared with the enzyme in control microsomes that were solubilized and chromatographed in an identical manner.

In rat liver the mitochondria and the endoplasmic reticulum constitute 20.2 % and 21.5 % of total cellular protein respectively [26]. The mitochondrial outer membrane constitutes approx. 4 % of total mitochondrial protein [27] and thus accounts for approx. 0.8 % of total cellular protein. On the basis of an approx. 14-fold greater specific activity (with decanoyl-CoA as substrate) of malonyl-CoA-inhibitable CPT1 as compared with malonyl-CoA-inhibitable carnitine acyltransferase of ‘intact’ microsomes, the relative contributions of mitochondrial CPT1 and microsomal carnitine acyltransferase to total cellular malonyl-CoA-inhibitable carnitine decanoyltransferase activity are 1:1.9 respectively. Regardless of assay conditions, the microsomal malonyl-CoA-inhibitable carnitine acyltransferase activity we measured with palmityoyl-CoA was at most 25 % of that seen with decanoyl-CoA as substrate (see below). Other workers [28] have reported liver CPT1 to display with palmityoyl-CoA approx. 50 % of the activity seen with decanoyl-CoA. On this basis the relative contributions of mitochondria and microsomes to total hepatic malonyl-CoA-inhibitable carnitine palmitoyltransferase activity are 1:0.95 respectively. Clearly the contribution of the microsomal malonyl-CoA-inhibitable enzyme to total liver malonyl-CoA-inhibitable acyltransferase activity is not trivial.

**Solubilization of microsomal malonyl-CoA-inhibitable carnitine acyltransferase**

Sodium deoxycholate proved the most effective of a number of detergents tested. Total microsomal carnitine acyltransferase activity was solubilized in 70–80 % yield by 7 mM sodium deoxycholate with 20 % (v/v) glycerol also present. These conditions solubilized 80–90 % of total microsomal protein and there were no obvious differences between microsomes prepared by differential centrifugation or by sucrose-density-gradient centrifugation. Importantly, in contrast to the octyl glucoside solubilization protocol employed in [14], the use of deoxycholate allowed solubilization of microsomal carnitine acyltransferase in a stable, malonyl-CoA-inhibitable state. The solubilized microsomal acyltransferase activity was stable for at least 4 months (the longest period tested) with no significant change in malonyl-CoA sensitivity when stored at −70 °C. We attempted to solubilize CPT1 from mitochondrial outer membranes using exactly the same protocol but found that 85–90 % of the CPT1 activity was lost, with the small residual activity being recovered in the 200000 g, supernatant. This inability to solubilize mitochondrial outer membrane CPT1 under conditions that readily solubilize microsomal carnitine acyltransferase is taken as evidence that the malonyl-CoA-sensitive carnitine acyltransferase we have solubilized from microsomes is not CPT1.

**Fractionation of solubilized microsomal carnitine acyltransferase activities**

Gel filtration of deoxycholate-solubilized ‘intact’ microsomes on Superdex 200 in the presence of deoxycholate gave two peaks of acyltransferase activity in the included volume (Figure 1). Peak I
corresponded to a molecular mass of approx. 300 kDa and was typically inhibited by approx. 25–35% by 50 μM malonyl-CoA. Pooled fractions from this peak typically had a specific activity of approx. 12 nmol/min per mg of protein. At present it is not known whether a size of approx. 300 kDa represents the native acyltransferase or the transferase in tight association with other microsomal proteins. Obviously the presence of detergent will alter the apparent molecular size observed on gel filtration, but any such change will be small in this case because deoxycholate has an aggregate number of 4–10 and a molecular mass of only 415 Da. Peak II corresponded to a molecular mass of approx. 60 kDa (which is not dissimilar to the 54 kDa subunit size assigned to the malonyl-CoA-insensitive enzyme in [14]), was unaffected by 50 μM malonyl-CoA and pooled fractions typically had a specific activity of approx. 19 nmol/min per mg of protein. Activity recoveries of approx. 45–70% were obtained from the Superdex column. Both Peak I and Peak II carnitine acyltransferases were completely stable for at least 4 months at −70 °C. When microsomes were freeze–thawed and then ultracentrifuged before solubilization, Peak II activity was essentially abolished (decreased by > 90%) whereas Peak I was unaffected (results not shown).

When deoxycholate-solubilized total microsomes were subjected to anion-exchange chromatography on Resource Q at pH 7.2, two distinct peaks of activity bound to the column (Figure 2). A small, variable amount of activity eluted in the wash-through. This wash-through material was turbid and presumably represented acyltransferase activity associated with detergent micelles. Peak A activity was unaffected by 50 μM malonyl-CoA whereas Peak B activity was typically inhibited by 30% under the same conditions. Activity recoveries of 50% were typically obtained from the Resource Q column.

It was particularly noteworthy that when Peak I acyltransferase from the Superdex 200 column was re-chromatographed on the Resource Q column it eluted in the same position as Peak B, retaining its sensitivity to malonyl-CoA, with no activity ever eluting in the position of Peak A (results not shown); i.e. there was never any indication that the Peak I activity could be resolved into malonyl-CoA-sensitive and -insensitive activities. This suggested that the Peak I carnitine acyltransferase was not merely Peak II carnitine acyltransferase that had a higher apparent molecular mass and sensitivity to malonyl-CoA by virtue of its association with a putative regulatory subunit. Similarly, when Peak II from the Superdex column was run on Resource Q it eluted in the same position as Peak A and remained insensitive to malonyl-CoA (results not shown).

Differences between the microsomal malonyl-CoA-sensitive and -insensitive carnitine acyltransferase activities

Peak I and Peak II carnitine acyltransferases clearly have distinct fatty-acyl-chain-length specificities (Figure 3), which suggests that they are different catalytic entities. Regardless of assay conditions (i.e. acyl-CoA concentration, presence or absence of albumin) the maximum activity of Peak I with palmitoyl-CoA was never more than 25% of the maximum activity observed with decanoyl-CoA (results not shown). We observed the same with microsomal membranes depleted of readily solubilized malonyl-CoA-insensitive carnitine acyltransferase (results not shown), indicating that poor activity with palmitoyl-CoA is not an artifact of solubilization.

We examined the effect of the detergent octyl glucoside on acyltransferase activities. Overnight incubation at 4 °C of Peak II enzyme (0.3 mg of protein/ml in the Superdex 200 equilibration buffer) together with 1% (w/v) octyl glucoside had no effect on activity. In contrast, incubation of Peak I enzyme under the same conditions resulted in a complete loss of activity. Similarly, when 1% (w/v) octyl glucoside was added to deoxycholate-solubilized extracts of freeze–thawed microsomal residues (i.e. microsomes depleted of malonyl-CoA-insensitive acyltransferase) over a period of hours, carnitine acyltransferase was inactivated. This is further evidence that the malonyl-CoA-sensitive and -insensitive acyltransferase activities reside on distinct proteins. The observation that octyl glucoside inactivated the malonyl-CoA-sensitive microsomal acyltransferase is particularly noteworthy.

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**Figure 2** Resource Q anion-exchange chromatography of deoxycholate-solubilized rat liver microsomes

The Resource Q column (1 ml) was equilibrated at room temperature with 10 mM potassium phosphate buffer (pH 7.2) containing glycerol [20% (v/v)], 1.5 mM sodium cholate and protease inhibitors (see the Materials and methods section). Solubilized microsomal extract (4 ml, 12 mg of protein) was loaded and eluted with a linear KCl gradient. ●, Carnitine acyltransferase activity; ★★, absorbance at 280 nm; ---, KCl gradient.

**Figure 3** Fatty acyl-CoA chain-length specificity of Peak I and Peak II microsomal acyltransferase fractions from the Superdex 200 column

Activities were assayed in 50 mM potassium phosphate buffer (pH 7.5) with 20 μM fatty acyl-CoAs in the absence of albumin. For Peak I 100% = 7.2 nmol/min per mg of protein and each assay contained 22.5 μg of protein. For Peak II 100% = 8.0 nmol/min per mg of protein and each assay contained 32.0 μg of protein. Values are from a representative experiment. ●, Peak I; ○, Peak II.
Microsomal carnitine acyltransferase

Bieber's laboratory used octyl glucoside both for initial solubilization and throughout the purification of malonyl-CoA-insensitive microsomal acyltransferase [14], and it is therefore likely that they inactivated the malonyl-CoA-sensitive enzyme that we describe here. Indeed when we chromatographed Peak I acyltransferase on Resource Q under our standard conditions (see the Materials and methods section), except that 1% (w/v) octyl glucoside was used instead of 1.5 mM cholate, no activity was recovered from the column. We therefore propose that the malonyl-CoA-insensitive microsomal acyltransferase purified in [14] corresponds to our Peak II/Peak A acyltransferase and that the malonyl-CoA-sensitive acyltransferase of Peak I/Peak B is a totally different protein.

Effects of membrane/phospholipid environment on inhibition by malonyl-CoA

In preliminary experiments (results not shown) it was found that reconstitution of Peak I (Superdex 200) or Peak B (Resource Q) acyltransferase activity into soybean l-α-lecithin liposomes markedly increased the extent of inhibition by 50 μM malonyl-CoA. We therefore performed the studies shown below using: (i) the malonyl-CoA-sensitive enzyme that remained in situ in microsomal membranes after freeze-thawing/ultracentrifuging to remove the malonyl-CoA-insensitive (Peak II) activity; (ii) deoxycholate-solubilized Peak I acyltransferase; or (iii) Peak I acyltransferase that had been incorporated into l-α-lecithin liposomes. In the absence of malonyl-CoA the profile for the dependence of acyltransferase activity upon decanoyl-CoA concentration was essentially the same in all three preparations (Figure 4). In contrast, the kinetic profiles observed in the presence of malonyl-CoA were substantially altered by the membrane/phospholipid environment. The detergent-solubilized Peak I activity, though showing some inhibitability by malonyl-CoA, was appreciably less affected by the inhibitor than the enzyme in situ in membranes or in phospholipid vesicles. This was seen in terms both of an effect of inhibition by a variable concentration of malonyl-CoA at a fixed concentration of decanoyl-CoA (Figure 5) or of the effect of a fixed malonyl-CoA concentration at a varied concentration of decanoyl-CoA (Figure 4). The key finding from these experiments was that the incorporation of the previously solubilized enzyme into phospholipid vesicles restored it to a state that was at least as inhibitable by malonyl-CoA as the enzyme in situ in freeze-thawed microsomal membranes (Table 1). Reconstitution of Peak II enzyme into liposomes had no effect on activity, which remained insensitive to malonyl-CoA (results not shown).

Comparison of the kinetics of microsomal malonyl-CoA-sensitive carnitine acyltransferase and of mitochondrial outer membrane CPT1

Values are shown in Table 1. Experiments 1–3 summarize findings from Figures 4 and 5. Without albumin the microsomal enzyme obeyed Michaelis–Menten kinetics when decanoyl-CoA was the substrate. Although malonyl-CoA had a slight effect in increasing the $K_m$ for decanoyl-CoA, the main effect of the inhibitor was to decrease the $V_{max}$, and this was most pronounced with the enzyme in situ in microsomal membranes or with the Peak I activity after reconstitution into liposomes. With CPT1 in situ in mitochondrial outer membranes we observed significant inhibition by substrate at low micromolar concentrations of decanoyl-CoA when assays were performed in the absence of albumin (results not shown). On inclusion of albumin (experiment 5) CPT1 no longer showed substrate inhibition although there was some deviation from ideal hyperbolic kinetics. Hence a value for the $K_m$ for decanoyl-CoA rather than a $K_i$ value was estimated. In order to make a direct comparison between the mitochondrial and the microsomal enzymes we re-assayed the microsomal malonyl-CoA-sensitive enzyme in the presence of albumin (experiment 4). Not surprisingly, albumin increased the

Figure 4 Fatty acyl-CoA substrate-dependence of malonyl-CoA-inhibitable microsomal carnitine acyltransferase preparations

Activity was assayed in 50 mM potassium phosphate buffer (pH 7.5) in the absence of albumin. Assays of Peak I activity (before and after reconstitution) additionally contained 0.25 mM sodium deoxycholate (due to carry-over). After reconstitution, assays also contained 0.8 mg of l-α-lecithin. 100% represents the activity measured at 20 μM decanoyl-CoA (8.0, 11.6 and 9.4 nmol/min per mg of protein for microsomal membrane residues, Peak I and reconstituted Peak I respectively). Values are from one experiment that is representative of three separate experiments. A, Microsomal membrane residues depleted of malonyl-CoA-insensitive acyltransferase; O, Peak I from the Superdex 200 column; □, Peak I after reconstitution in l-α-lecithin liposomes. Open symbols, malonyl-CoA absent; closed symbols, with 5 μM malonyl-CoA.

Figure 5 Effect of malonyl-CoA concentration on microsomal carnitine acyltransferase preparations

Assay conditions were as for Figure 4 but with [decanoyl-CoA] fixed at 20 μM. 100% = 8.0, 7.8 and 5.9 nmol/min per mg of protein for microsomal membrane residues, Peak I and reconstituted Peak I respectively. Values are from one experiment that is representative of three separate experiments. A, Microsomal membrane residues depleted of malonyl-CoA-insensitive acyltransferase; O, Peak I from the Superdex 200 column; □, Peak I after reconstitution in l-α-lecithin liposomes.
apparent $K_m$ for decanoyl-CoA of the microsomal enzyme from 1.1 $\mu$M to 6.8 $\mu$M. However, albumin had other unexpected effects in that malonyl-CoA now had only a small (though still significant) effect on $V_{\text{max}}$ but a 7-fold effect on $K_m$ (experiment 4). We can offer no explanation for this effect of albumin. The key observation is that under identical assay conditions the $K_m$ for decanoyl-CoA of mitochondrial CPT$_1$ was nearly 5-fold ($P < 0.001$) greater than the $K_m$ of the malonyl-CoA-sensitive microsomal enzyme. We discount the possibility that these findings reflect different environments in microsomal and mitochondrial outer membranes because we have shown that the membrane/phospholipid environment has little effect on the $K_m$ for decanoyl-CoA of the microsomal enzyme (experiments 1–3). This is further evidence that mitochondrial CPT$_1$ and the malonyl-CoA-sensitive acyltransferase of microsomes are different enzymes.

**General discussion**

This study provides evidence for two distinct carnitine acyltransferases associated with microsomal fractions from liver. It complements the recent findings of Murthy and Pande [21] who reached the same conclusion from submicrosomal fractionation studies, from use of the inhibitor tetradecylglycycyl-CoA and from use of antibodies directed against the malonyl-CoA-insensitive enzyme. The potential contribution of microsomes to hepatic malonyl-CoA-sensitive fatty acylcarnitine formation is not trivial; indeed it appears to be comparable with that of the mitochondria. Whether this has significant bearing on our understanding of the regulation of hepatic fatty acid oxidation remains to be established.

We initially considered the possibility that the malonyl-CoA-sensitive enzyme was the same as CPT$_1$, from mitochondrial inner membranes. However, three lines of evidence did not support this conjecture, namely the differences between the two enzymes in facility of solubilization by detergents, in their stability after solubilization and in some kinetic properties. Further evidence was provided by Murthy and Pande [21], who showed that $[^{3}H]$tetemoxiryl-CoA labelled a polypeptide of 47000 Da in liver microsomes without labelling the 88000 Da polypeptide assigned to CPT$_1$. There is, however, one similarity that may be noteworthy. Previous studies [29,30] have suggested that the effect of malonyl-CoA in inhibiting mitochondrial CPT$_1$ is considerably altered by membrane composition/environment. Our findings (Figures 4 and 5, Table 1) indicate that the same is true of the microsomal enzyme. The great difficulty that is generally encountered in solubilizing and fractionating the mitochondrial outer membrane CPT$_1$ in a stable, active form is a major obstacle to fully defining the phospholipid/cholesterol requirements of that enzyme. In this regard, therefore, the solubilized/reconstituted microsomal enzyme may prove to be a useful model system in future studies.

In more general terms, the similarity of the mitochondrial and microsomal acyltransferase systems, namely a membrane-bound, malonyl-CoA-sensitive enzyme (presumed to have a cytoplasmic orientation) and a malonyl-CoA-insensitive activity (associated with the microsomal lumen [22]) is striking. It is tempting to suggest that the two microsomal enzymes may act in concert in a manner analogous to that of the mitochondrial CPT$_1$ system to enable transport of fatty acyl units between the cytosol and the lumen of the endoplasmic reticulum. If this is indeed the case then it is necessary, by analogy with the mitochondrial system, that the microsomal membranes should also contain a carnitine:acylcarnitine translocase. Such a transport system could be necessary if fatty acyl-CoA was required in the lumen because the endoplasmic reticulum membrane is essentially impermeable to fatty acyl-CoA [31]. The physiological role of such a system in the microsomal fraction, however, remains to be elucidated. A general involvement of fatty acyl-CoA in promoting transport of proteins through the Golgi apparatus has been demonstrated [32–34], and important secreted proteins such as apoprotein B$_{100}$ or major histocompatibility antigens are known to be fatty acylated somewhere in the secretory pathway [35–38]. The microsomal carnitine acyltransferases may possibly play a role in provision of fatty acyl units for these key processes. Several studies [39–41] have led to the conclusion that triacylglycerol stored in a hepatic cytoplasmic pool is largely unable to be incorporated into secreted triacylglycerol without prior hydrolysis. One possible corollary to this finding is the need for a spatial separation of the enzymic machinery used for synthesis of

**Table 1** Comparison of the kinetic parameters of microsomal malonyl-CoA-sensitive carnitine acyltransferase and mitochondrial CPT$_1$.

$K_m$ and $K_p$ were calculated from Hanes plots. $V_{\text{max}}$ values were normalized so that 100 refers to activity measured in the absence of malonyl-CoA with 20 $\mu$M or 100 $\mu$M decanoyl-CoA in the absence or presence of albumin (1.3 mg/ml) respectively. $IC_{50}$ indicates the concentration of malonyl-CoA needed to achieve 50% of maximum inhibition ($V_{\text{max}}$) with 20 $\mu$M decanoyl-CoA as substrate. All values are means $\pm$ S.E.M of three independent measurements except for the measurement of $IC_{50}$ in Experiment 2 ($n = 6$) and in Experiment 5 ($n = 7$). $IC_{50}$ indicate $P < 0.05$, $< 0.01$ and $< 0.001$ respectively for effects of 5 $\mu$M malonyl-CoA. $IC_{50}$ indicate $P < 0.01$ for comparison with Experiment 2. $IC_{50}$ indicate $P < 0.001$ for comparison with Experiment 4. n.d., not determined.

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<th>Experiment</th>
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<th>Malonyl-CoA</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$ for decanoyl-CoA ($\mu$M)</th>
<th>$IC_{50}$ for malonyl-CoA ($\mu$M)</th>
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<td>1.1 $\pm$ 0.2</td>
<td>&gt; 10</td>
<td>31 $\pm$ 4†</td>
</tr>
<tr>
<td>3</td>
<td>Peak I (reconstituted)</td>
<td>—</td>
<td>—</td>
<td>110 $\pm$ 2</td>
<td>1.0 $\pm$ 0.4</td>
<td>13 $\pm$ 1</td>
<td>77 $\pm$ 3</td>
</tr>
<tr>
<td>4</td>
<td>Microsomes*</td>
<td>+</td>
<td>—</td>
<td>107 $\pm$ 3</td>
<td>6.8 $\pm$ 0.3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>CPT$_1$ (mitochondrial outer membranes)</td>
<td>+</td>
<td>—</td>
<td>124 $\pm$ 2</td>
<td>32.5 $\pm$ 2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* Membrane residues recovered by centrifugation after freezing and thawing to release the malonyl-CoA-insensitive acyltransferase (see the Materials and methods section).
† Percentage inhibition achieved with 50 $\mu$M malonyl-CoA.
‡ Measurement of $K_{50}$ rather than $K_m$ (see text for further details).
the cytoplasmic pool from that used for synthesis of very-low-density lipoprotein (VLDL) triacylglycerol. If all or part of the assembly of VLDL triacylglycerol took place in the secretory compartment, fatty acyl-CoA would have to be provided there.

Finally, why should the microsomal form of carnitine acyltransferase that is firmly associated with the membrane be inhibitable by malonyl-CoA? It is tempting to speculate that this may be one of the mechanisms contributing to the well-documented effect of insulin in acutely decreasing VLDL triacylglycerol secretion [40, 42–46]. Further studies are needed to substantiate or disprove this speculation. It may also be of interest to investigate the activities and distributions of the microsomal carnitine acyltransferases in other triacylglycerol-secreting tissues such as the intestine or the mammary gland.

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REFERENCES

20. Reference deleted