The soluble carnitine palmitoyltransferase from bovine liver

A comparison with the enzymes from peroxisomes and from the mitochondrial inner membrane

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The properties of two carnitine acyltransferases (CPT) purified from bovine liver are compared to confirm that they are different proteins. The soluble CPT and the inner CPT from mitochondria differ in subunit M_r , native M_r , pI and reactivity with thiol reagents. All eight free thiol groups in soluble CPT react with 5,5'-dithiobis-(2-nitrobenzoate) in the absence of any unfolding reagent, and activity is gradually lost. The inner CPT activity is completely stable in the presence of 5,5'-dithiobis-(2-nitrobenzoate), and only one thiol group per molecule of subunit is modified in the native enzyme. Antisera to each enzyme inhibit that enzyme, but do not cross-react. CPT activity in subcellular fractions can now be identified by titration with these antibodies. The soluble CPT from bovine liver is probably peroxisomal in origin, but, although antigenically similar, it differs from the peroxisomal carnitine octanoyltransferase found in rat and mouse liver in its specificity for the longer-chain acyl-CoA substrates.

INTRODUCTION

Carnitine acyltransferases catalyse the transfer of acyl groups between CoA and carnitine to facilitate the transfer of activated fatty acids into mitochondria. Carnitine palmitoyltransferase (CPT; EC 2.3.1.21) is thought to be exclusively mitochondrial (see [1] for references), but the specificity of carnitine octanoyltransferase (COT) from peroxisomes [2,3] overlaps with that of CPT, making it difficult to determine the intracellular location of each enzyme. Little is known about the microsomal enzyme [4]. Within the mitochondrion, there are two functional pools of CPT with very different kinetic and regulatory properties (for a review, see [1]). Although it has been suggested that these two pools (the overt, using cytoplasmic CoA and carnitine, and the latent, located facing the mitochondrial matrix) are expressed by one protein whose properties have been modified by the membrane [3,5,6], they are now thought to be two separate proteins [7–9].

Recently a soluble CPT, first identified in bovine liver homogenates [10], has been purified to homogeneity from bovine liver mitochondria [11]. It differs from the tightly membrane-bound enzyme isolated from bovine heart and rat liver mitochondria in its native and subunit M_r values, its pI and its sensitivity to inhibitors [3,5,6]. However, liver mitochondria, unless prepared by gradient centrifugation, are significantly contaminated with peroxisomes and microsomes [12,13]. The contamination by peroxisomes was estimated to be less than 5%, but 15–20% of the total CPT activity was solubilized by homogenizing the mitochondria in water, suggesting that the soluble enzyme was more than could be accounted for by the peroxisomal contamination and that it could be the enzyme responsible for the overt CPT activity in intact mitochondria.

Three findings make the latter identification unlikely. Firstly, Healy *et al.* [13] found only one soluble enzyme in rat liver homogenates. That enzyme was more active with C_{10} than with C_{16} substrates, and it cross-reacted with antibody to mouse peroxisomal COT. Secondly, Murthy & Pande [7] demonstrated CPT activity in mitochondrial outer membranes which was very difficult to solubilize and which retained the malonyl-CoA sensitivity characteristic of overt CPT. Thirdly, it has been demonstrated that detergents destroy the activity of the overt CPT and that it cannot be extracted in active form [8,9].

Antibodies to the purified enzymes now permit an examination of the location of the soluble CPT in bovine liver and further characterization of its properties in comparison to those of the inner (latent) CPT and of the soluble, peroxisomal COT.

MATERIALS AND METHODS

Assays

The assay system (25 °C; 1 ml) for carnitine acyltransferase activity was as described previously [11] and contained 25 mM-potassium phosphate, pH 7.5, 125 μ M-4,4'-dithio(bis)pyridine, acyl-CoA (25 μ M-palmitoyl-CoA, 100 μ M-octanoyl-CoA or 200 μ M-acetyl-CoA), enzyme and 10 mM-L-carnitine. Protein was assayed as described previously [11].

Marker enzymes were assayed by standard methods: catalase, adenylate kinase, malate dehydrogenase and cytochrome oxidase [14]; monoamine oxidase [15].

Enzymes

Mitochondria were prepared by differential centrifugation as described previously [11], and peroxisomes

Abbreviations used: COT, carnitine octanoyltransferase; CAT, carnitine acetyltransferase; CPT, carnitine palmitoyltransferase; soluble CPT is the peroxisomal form obtained in water extracts of bovine liver; outer (overt) CPT is that expressed in intact mitochondria; inner (latent) CPT uses matrix substrates and is solubilized by Triton X-100.

by a rapid Nycodenz-density-gradient centrifugation [16].

Enzymes were purified from bovine liver by the published procedures for soluble CPT [11] and for mitochondrial inner CPT [5]. The latter is solubilized with 2% Triton X-100 from pelleted material after hypo-osmotic treatment of previously frozen mitochondria and has the same properties as preparations previously characterized as inner CPT [3,6]. The conditions used are now thought to inactivate the outer (overt) CPT of the mitochondria [9].

Immunology

Antibodies to the purified enzymes (soluble CPT and inner CPT) were raised in rabbits and precipitated from the immune serum with $(\text{NH}_4)_2\text{SO}_4$ [17]. Antibodies were stored at -20°C in 0.9% NaCl/0.03% NaN_3 . Rabbit antiserum to mouse peroxisomal COT was kindly given by Dr. L. L. Bieber, Michigan State University. Immunotitration incubations contained, in 100 μl total volume, 20 mM-potassium phosphate, pH 7.5, 0–30 μl of antibody preparation plus 30–60 μl of bovine serum albumin (30 mg/ml) to keep the amount of added protein constant, and approx. 0.03 unit of CPT. The samples were placed in a rotary mixer for 2 h at 4°C or for 15 min at 25°C . For the purified enzymes, the immunoprecipitate was removed by centrifugation (40 000 g -min). For particulate samples centrifugation would remove membrane-bound enzyme even if it had not reacted with antibody, so the incubation mixture was assayed directly.

Western blots

Purified enzymes and particulate samples were subjected to SDS/polyacrylamide-gel electrophoresis in 7.5%-acrylamide gels. The gels were blotted, made to react with antiserum to soluble CPT (1:15 000 dilution), inner CPT (1:10 000 dilution) or COT (1:4 000 dilution), and detected by the horseradish peroxidase system [18].

Mitochondrial fractionation

Digitonin fractionation was performed as described previously [19,20]. The mitochondria were incubated for 5 min in the presence of digitonin. Mitoplasts and outer membrane were prepared by a swell-shrink cycle followed by sonication and separated by sucrose-density-gradient centrifugation [21].

RESULTS

Comparison of the properties of the acyltransferases in liver

Long-chain acyltransferase activity has been found in four locations in liver cells, namely the interior (latent) and exterior (overt) of mitochondria, peroxisomes and microsomes. From bovine liver, only the latent, tightly membrane-bound, inner CPT and the soluble CPT, whose location is in question, have been purified. The properties of these two enzymes and those published for rat or mouse liver peroxisomal COT are compared as a basis for identifying their intracellular location.

The soluble CPT purified from bovine liver mitochondria is monomeric (M_r 63 500) and has a pI of 6 [11]. The inner CPT is very different, with properties similar to those of the enzyme from bovine heart [5], namely, a subunit M_r of 67 000, a very high native M_r , and a pI of

Table 1. Free thiol groups in the CPTs from bovine liver

Free thiol groups were measured by their reaction with 5,5'-dithiobis-(2-nitrobenzoate) in 0.1 M-Tris/HCl, pH 8.1, containing 1 mM-EDTA. To measure the total free thiol content, the enzyme (which was previously dialysed to remove dithioerythritol and glycerol) was incubated for 1 h at 30°C in the same buffer containing 2% SDS. Values are averages of two determinations. Subunit M_r values were: soluble CPT, 63 500; inner CPT, 67 000.

	Free thiol groups (mol/mol of subunit)	
	Soluble CPT	Inner CPT
Native	7.1	1.0
SDS-treated	7.3	5.8

8. In addition, the sensitivity of the two enzymes to thiol reagents differs (Table 1). The free thiols in the monomeric soluble enzyme all react with 5,5'-dithiobis-(2-nitrobenzoate) even in the native form. The titre obtained (7.3 mol/mol) suggests that there may be no disulphide bonds in this enzyme, because the amino acid analysis demonstrated 8 mol of cysteine per mol of subunit (M_r 63 500). There is no reactive titratable 'essential thiol', but activity is lost slowly ($t_{1/2} = 9$ min) when the enzyme is incubated with the thiol reagent 4,4'-dithio(bis)pyridine (Fig. 1). Substrates protect the enzyme from loss of activity, palmitoyl-CoA better than carnitine (Fig. 1). In contrast, the inner CPT is completely stable for at least 1 h under the same conditions (results

Fig. 1. Loss of CPT activity in the presence of a thiol reagent

Purified soluble CPT (0.31 $\mu\text{g}/\text{ml}$) was incubated at 30°C in 20 mM-potassium phosphate, pH 7.5, with 25 μM -palmitoyl-CoA (○), or with 125 μM -4,4'-dithio(bis)pyridine, alone (●), or plus 2.7 mM-L-carnitine (□) or plus 25 μM -palmitoyl-CoA (■). The missing assay ingredients were added to the cuvette at a given time and the residual activity was measured.

Fig. 2. Acyl-CoA specificity of the purified CPT enzymes from bovine liver

The apparent V_{max} values were measured in the 4,4'-dithio(bis)pyridine assay by varying the acyl-CoA concentration in the presence of 2 mM-L-carnitine for soluble CPT (○) or of 10 mM-L-carnitine for inner CPT (●).

not shown). Only 1 mol of thiol/mol of subunit is titratable in the native enzyme, and 6 mol/mol of subunit after SDS treatment. Cysteine was not determined in the amino acid sequence of the inner CPT from heart [5].

The acyl-CoA specificities of the two enzymes are shown in Fig. 2. The maximum velocity for the soluble CPT is at C_{16} and that for the inner CPT at C_{10} – C_{12} . Clearly, the enzymes differ under these assay conditions.

The maximum velocity for rat liver peroxisomal COT is at C_6 [3], so the soluble CPT is also different from the peroxisomal COT, but bovine liver peroxisomal COT could have very different kinetic properties from the rat liver enzyme (see below). In substrate specificity, the bovine enzymes resemble more closely the peroxisomal and mitochondrial CPT from chick-embryo liver, which both have similar acyl-chain-length profiles with maximum activity at C_{12} [22].

In order to determine how similar the bovine liver CPT proteins were, the effect of antibodies to each one on CPT and COT activity was tested. Rabbit anti-(soluble CPT) and anti-(inner CPT) antisera inhibit the corresponding purified enzymes completely, but did not cross-react (Fig. 3). Anti-(peroxisomal COT) antiserum inhibited the soluble enzyme completely (Fig. 4), but not the inner enzyme (results not shown). It is curious to note the differences between the inhibition in the presence of palmitoyl-CoA and that with octanoyl-CoA. The cross-reaction of COT and soluble CPT is seen clearly in the Ouchterlony double-diffusion pattern in Fig. 5. Thus the antibody data suggest that the soluble CPT could indeed be the same as the peroxisomal COT.

Intracellular location of the soluble CPT

The antisera were used to quantify the enzymes responsible for CPT activity in mitochondria prepared by differential centrifugation. Thawed mitochondria were diluted, sonicated and centrifuged at 100000 g for 1 h, a procedure which has been suggested to separate the outer CPT (along with CPT from contaminating peroxisomes) and leave the inner CPT in the pellet [23]. The supernatant contained activity of 0.012 μ mol/min per mg of protein for the original mitochondria (39%) and the pellet contained 0.019 μ mol/min per mg (61% of the total mitochondrial activity). The CPT activity of the pellet was completely inhibited by anti-(inner CPT)

Fig. 3. Inhibition of the purified CPTs by polyclonal antibodies

In (a) soluble CPT, and in (b) inner CPT, were incubated with control serum (●) or with antiserum to inner CPT (○) or to soluble CPT (Δ) for 1 h at 4 °C, centrifuged (Eppendorf) for 5 min, and the supernatant was assayed for CPT activity as described in the Materials and methods section.

Fig. 4. Inhibition of bovine liver soluble CPT by anti-(peroxisomal COT) antiserum

Purified soluble CPT (0.65 μ g) in 20 mM-potassium phosphate, pH 7.5 (to give a final volume of 50 μ l), was incubated for 15 min at 25 °C with samples of anti-(mouse peroxisomal COT) antiserum plus bovine serum albumin (30 mg/ml) so that the same amount of protein was added to each sample. After centrifugation at 9500 g for 5 min, 5 μ l samples were assayed for activity with 50 μ M-palmitoyl-CoA (●) or with 100 μ M-octanoyl-CoA (□). The 100% values were 0.4 μ mol/min per ml of incubation mixture with palmitoyl-CoA and 0.25 μ mol/min per ml with octanoyl-CoA.

antiserum only. The supernatant was inhibited by 70% by anti-(inner CPT) antiserum and by 40% by anti-(soluble CPT) antiserum. Irreversible inhibition by 2-bromopalmitoyl-CoA plus carnitine was observed only in the supernatant. The 34% inhibition suggests that only the soluble CPT was affected. Thus, of the total mitochondrial CPT activity, 16% was soluble CPT. Of the remainder, the distribution was 31% in the supernatant and 69% in the pellet, similar to the expected distribution for overt and latent CPT.

In order to identify the source of the soluble CPT in the mitochondria prepared by differential centrifugation, the preparation was incubated with 0–0.2 mg of digitonin/mg of protein for 5 min on ice. After dilution, the remaining intact particles were separated from the liberated components by centrifugation [20]. Fig. 6(a) shows the appearance of marker enzymes in the supernatant in the classic pattern: catalase from contaminating peroxisomes, followed by adenylate kinase from the mitochondrial intermembrane space, monoamine oxidase from the outer membrane, malate dehydrogenase from the matrix, and lastly cytochrome oxidase, from the inner membrane. Fig. 6(b) shows the release of CAT, COT and CPT. CAT is released slightly more slowly than malate dehydrogenase, but consistent with a matrix location. COT and CPT follow very similar patterns, and show at least bimodal distribution. The activity released at lowest concentrations of digitonin seems to follow the pattern of adenylate kinase, consistent with the conclusions in previous work [19,20]. However,

Fig. 5. Ouchterlony double-diffusion patterns of CPTs from bovine liver

Agar gel (1%, w/v) contained 0.1 M-potassium phosphate, pH 7.0, 0.85% NaCl, and 0.1% NaN₃. The centre well contained a mixture of 3.5 μ g of soluble CPT and 25 μ g of inner CPT. Well 1, control serum; wells 2 and 5, anti-(inner CPT) antiserum; wells 3 and 6, anti-(soluble CPT) antiserum; well 4, anti-(mouse peroxisomal COT) antiserum.

when the CPT activity released is inhibited by antibodies, to distinguish between the contributions from the soluble and inner enzymes, a different pattern for the early part of the curve emerges. The activity released most easily is inhibited only by antibody to the soluble enzyme, as shown in Fig. 6(c). Expressed as a percentage of the maximum activity inhibited by anti-(soluble CPT) antiserum, the release pattern follows that of catalase. Thus the soluble CPT may originate from the intact peroxisomes contaminating the mitochondrial preparation.

The second part of the release curve begins at the same concentration of digitonin as is required to release monoamine oxidase, but its slope is less, suggesting that its release trails that of the outer-membrane marker. Such a pattern is consistent with two locations for this second part of the CPT activity, namely tightly bound to the outer membrane and fairly loosely attached to the matrix side of the inner membrane, as suggested by Pande's group [7].

If the soluble enzyme were solely responsible for the CPT activity on the outer side of the mitochondrial inner membrane, it should be possible to prepare mitoplasts which oxidize palmitoylcarnitine but not palmitoyl-CoA. In one preparation of mitoplasts, prepared by a swell-shrink cycle [21], palmitoyl-CoA oxidation was indeed deficient, but this method could also remove any CPT associated with the outer membrane, as postulated by Murthy & Pande [7]. In the digitonin experiment, there was no significant difference between the loss of coupled oxidation of palmitoyl-CoA and of palmitoylcarnitine (Fig. 6d). The significance of this experiment is, however, limited by the likelihood that damaged mitochondria are produced by the higher digitonin concentrations, so that inner CPT from these mitochondria can produce external palmitoylcarnitine from added palmitoyl-CoA.

Western-blot analysis of samples from these experiments (Fig. 7) shows that the bovine liver mitochondria prepared by differential centrifugation (lane 2) contained both the soluble and inner bands (M_r 63 500 and 67 000 respectively). Mitoplasts prepared by the swell-shrink method [21] (lane 3) and bovine heart mitochondria (lane 1) (generally considered to be free of peroxisomes)

Fig. 6. Digitonin fractionation of bovine liver mitochondria

Fresh bovine liver mitochondria were fractionated as described in the Materials and methods section. Respiration by the treated pellets and the activity of catalase in the supernatants were measured immediately, followed by the other assays in succession. Inhibition of the total CPT activity in the supernatants by antibodies was determined the next day. Results are given as percentages of the total recovered activity (supernatant plus pellet) at each concentration of digitonin. (a) Release of marker enzymes: ●, catalase; ○, adenylate kinase; ▲, monoamine oxidase; △, malate dehydrogenase; ■, cytochrome oxidase. (b) Acyltransferase activity released was measured with (○) palmitoyl-CoA (50 μM), (●) octanoyl-CoA (100 μM) or (▲) acetyl-CoA (200 μM). (c) Amount of the released CPT activity (expressed as percentage of the total recovered activity) inhibited by antiserum to soluble CPT (■) or to inner CPT (□). (d) Rate of respiration in digitonin-treated mitochondrial pellets as a percentage of the rate in intact mitochondria in the presence of 50 μM-palmitoyl-CoA plus 10 mM-L-carnitine (●), or 25 μM-palmitoyl-carnitine (○), or 40 μM-octanoate (▲).

contained only inner CPT. This pattern confirms the data above suggesting that the soluble enzyme is not mitochondrial.

Finally, the crude mitochondrial fraction from bovine liver was layered over Nycodenz (density 1.15 g/ml) and centrifuged at 131000 g_{av} for 1 h to separate the peroxisomes and mitochondria [16]. The properties of these fractions are shown in Table 2. The kinetic constant which differs most is the apparent K_m for L-carnitine. The peroxisomes exhibit the low K_m (0.17 mM) characteristic of the soluble enzyme, and the mitochondria exhibit a high K_m (2.8 mM) characteristic of the inner CPT. This segregation is confirmed by the antibody-sensitivity of the CPT activity in each fraction. The peroxisomal fraction (Fig. 7, lane 6, which is rather underloaded) reacts with antiserum to soluble CPT, but not with antiserum to inner CPT, whereas the purified mitochondria (Fig. 7, lane 5) react only with antiserum to inner CPT. Quantitatively, the peroxisomal activity is inhibited (92%) by antibody to the soluble enzyme, but partly (26%) also by that to inner CPT, which may be due to mitochondrial contamination (see marker enzyme amounts, Table 2). The mitochondrial enzyme is com-

pletely inhibited by antibody to inner CPT and not at all by that to soluble CPT. Thus the source of the soluble enzyme must be the peroxisomes.

DISCUSSION

Localization of the CPT activities from bovine liver in subcellular organelles has been achieved with the aid of antibodies which both inhibit the activity and react with the enzyme subunits after SDS/polyacrylamide-gel electrophoresis. In agreement with previous reports [3,5,6], the mitochondrial activity, which survives solubilization with Triton X-100, seems to be a homogeneous pool which is completely inhibited by antibody to the purified inner enzyme (Table 2). The acyl-CoA specificity of the purified inner enzyme (Fig. 2) is clearly modulated in the mitochondria. The ratio of V for C_{16}/C_8 is 0.5 in the purified enzyme, but is 2.1 in mitochondria (Table 2). The purified enzyme is not malonyl-CoA-sensitive, consistent with the properties of the latent enzyme in mitochondria [23,24].

The bovine liver peroxisomal activity has properties similar to those of the purified soluble CPT. Con-

Fig. 7. SDS/polyacrylamide-gel electrophoresis and Western blots of CPTs and liver cell organelles

Purification of samples and conditions for the Western blots are described in the Materials and methods section. The samples were: M_r standards [lane 0, low- M_r standard mixture, from Bio-Rad, which includes phosphorylase b (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soya-bean trypsin inhibitor (21 500) and lysozyme (14 400)], bovine heart mitochondria (lane 1), bovine liver mitochondria prepared by differential centrifugation (lane 2), mitoplasts prepared by the swell-shrink method (lane 3), 0.5 μ g of soluble CPT plus 1 μ g of inner CPT (lane 4), bovine liver mitochondria after centrifugation through Nycodenz (lane 5), bovine liver peroxisomes after centrifugation through Nycodenz (lane 6). In (a), the proteins were stained with Coomassie Blue R-250. The Western blots were probed with antisera to inner CPT (b) and to soluble CPT (c).

Table 2. Properties of the acyltransferases in bovine liver peroxisomes and mitochondria

Fractions enriched in peroxisomes or mitochondria were prepared by centrifugation through Nycodenz [16]. The assays are described in the Materials and methods section. In (c) the K_m for L-carnitine was measured in the presence of 100 μ M-octanoyl-CoA, and the apparent V_{max} ($V_{app.}$) in the presence of 10 mM-L-carnitine. In (d) the assays contained 25 μ M-palmitoyl-CoA and 10 mM-L-carnitine. Western-blot analysis of the mitochondria (lane 5) and peroxisomes (lane 6) are shown in Fig. 7.

	Peroxisomes	Mitochondria
Specific activities (μ mol/min per mg)		
(a) Marker enzymes		
Catalase	3.50	0.18
Glutamate dehydrogenase	0.57	0.77
Cytochrome oxidase	0.26	0.67
(b) Acyltransferases		
CPT	0.065	0.060
COT	0.065	0.010
CAT	0.061	0.064
(c) Characteristics		
K_m for L-carnitine (mM)	0.17	2.8
K_m for octanoyl-CoA (μ M)	2.9	4.5
K_m for palmitoyl-CoA (μ M)	1.4	2.2
$V_{app.}$ with octanoyl-CoA (μ mol/min per mg)	0.071	0.100
$V_{app.}$ with palmitoyl-CoA (μ mol/min per mg)	0.095	0.208
(d) Antibody-sensitivity of CPT activity		
Inhibition by anti-(soluble CPT) antiserum (%)	92	0
Inhibition by anti-(inner CPT) antiserum (%)	26	100

tamination with peroxisomes could explain at least part of the amount of soluble enzyme found in bovine liver mitochondrial preparations, although the possibility remains that the soluble form could result from partial degradation of the mitochondrial outer enzyme [7]. Curiously, the acyl-CoA specificity of this bovine peroxisomal enzyme is very different from that of the COT observed in rat [3] or mouse [2] liver peroxisomes. It has higher activity with the longer-chain substrates, the ratio of V for C_{16}/C_8 being 1.13, in comparison with that of 0.1 in mouse [2] or 0.3 in rat [3]. Activities measured with long-chain acyl-CoA substrates are very variable, depending on the assay conditions. However, in mouse [2] the ratios of C_{16}/C_8 activities for the peroxisomal and mitochondrial enzymes are quite different. Here, in crude bovine preparations, they are significantly closer (1.13 and 2.1). The net effect could be to remove acyl-CoA substrates from the peroxisomes at a longer acyl-chain length than in rat and channel them into the more energy-efficient β -oxidation pathway of mitochondria. The role of the peroxisomes in the overall oxidation of fatty acids by the liver cell has not been well defined, except that very-long-chain fatty acids ($> C_{18}$) are much better oxidized in peroxisomes than in mitochondria. The well-established role of the mitochondrial acyltransferases in the entry of fatty acids into the mitochondria is in no way changed by the results reported here.

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