The relationship of rat liver overt carnitine palmitoyltransferase to the mitochondrial malonyl-CoA binding entity and to the latent palmitoyltransferase

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1. Confirming previous work [Murthy & Pande (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 378–382], malonyl-CoA-inhibitable carnitine palmitoyltransferase (CPT₁) from rat liver was found to be localized in outer rather than in inner mitochondrial membranes. 2. Antisera were raised against a liver mitochondrial CPT of *M_r* 68 000, which was presumed to be the latent form of the enzyme (CPT₂). These antisera cross-reacted with solubilized CPT extracted from liver inner mitochondrial membranes and with polypeptides of *M_r* 68 000 and 60 000 in immunoblots of both inner and outer mitochondrial membranes. The antisera also precipitated CPT activity from detergent-treated total membrane and outer-membrane preparations. 3. The antisera did not precipitate [¹⁴C]malonyl-CoA binding material obtained either from total membranes or from outer membranes. 4. It was concluded that liver CPT₁ and CPT₂ have some epitopes in common and may have a similar subunit size. In addition, CPT₁ and the entity that binds malonyl-CoA must be separate polypeptides.

INTRODUCTION

Two forms of carnitine palmitoyltransferase are recognized in mitochondria, the overt (CPT₁) and the latent (CPT₂). Besides being localized in different mitochondrial membranes [1], the major distinguishing feature between the two forms is that CPT₁ is potently inhibited by malonyl-CoA, whereas CPT₂ has little or no sensitivity to this ligand.

Kinetic studies have provided evidence that the binding site for malonyl-CoA must be distinct from the catalytic site of CPT₁ [2,3]. Evidence for a topographical separation across the outer membrane of these two sites was shown by Murthy & Pande [1]. Their studies with Nagarse-treated mitochondria suggested that the malonyl-CoA binding site is on the outer side of the outer membrane and that the catalytic site of CPT₁ is located on the inner aspect of this membrane. In addition, the observation that liver CPT₁ activity may alter several-fold in different physiological states, whereas malonyl-CoA maximum binding at a high-affinity site is unaltered [4], raised the conjecture that the catalytic and malonyl-CoA-binding entities could reside on different polypeptides. This is supported by radiation inactivation studies [5], which suggest *M_r* values of 60 000 and 83 000 for malonyl-CoA binding and CPT₁ respectively. Indeed, Lund & Woldegiorgis [6] chromatographically separated a fraction that bound malonyl-CoA from one containing CPT activity in Triton X-100 extracts of rat liver mitochondrial membranes. However, Woeltje *et al.* [7] provided evidence that the Triton X-100 extraction procedure used in [6] would have totally inhibited CPT₁. By contrast with the studies supporting the concept of two separate entities [4–6], Declercq *et al.* [8] have suggested that liver CPT₁ is represented by a single tetradecylglycidyl-CoA-binding polypeptide of *M_r* 94 000, which also bears the binding site for malonyl-CoA.

Controversy also surrounds the question of the similarity or dissimilarity of CPT₁ and CPT₂. West *et al.* [9] suggested that they are different enzymes, on the basis of their different susceptibility to inhibition by bromopalmitoyl-CoA. However, the importance of membrane environment in the response of CPT activities to this inhibitor has since been demonstrated [10].

Several attempts at purification [10–13] have only succeeded in extracting one type of CPT, of *M_r* 68 000–69 000, from rat liver mitochondria. Furthermore, antibodies raised against purified rat liver CPT of *M_r* 69 000 recognized only one product in a rat liver mRNA translation system [13]. The possibility that CPT₁ and CPT₂ are at least immunologically similar was supported by Brady & Brady [14], who were able to immunoprecipitate, with the aid of Protein A, all CPT activity extracted from hepatic mitochondria by sonication or by Tween-20. By contrast, Woeltje *et al.* [7], having raised an antiserum against a presumed CPT₂ of *M_r* 68 000, could not show cross-reactivity on immunoblotting with either the *M_r*-94 000 tetradecylglycidyl-CoA-binding polypeptide which they assumed to be CPT₁, or with membrane residues thought to be enriched in CPT₁ by prior extraction with Tween-20. Furthermore, Zammit *et al.* [15], using radiation inactivation analysis, estimated the minimum *M_r* values of CPT₁ and CPT₂ to be approx. 97 000 and 70 000 respectively, although subsequently these workers have provided a revised estimate of *M_r* 83 000 for CPT₁ [5].

The present study has been addressed to these two controversies. It provides evidence for at least some immunological similarity between CPT₁ and CPT₂. It also provides further evidence that the malonyl-CoA-binding entity is distinct from the catalytic entity of CPT₁.

MATERIALS AND METHODS

Animals

Male rats (180–200 g) were of the Sprague–Dawley strain bred at University College London. These were maintained at 21 °C and had constant access to drinking water and to Rat & Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.), which contained (by wt.) 21 % digestible crude protein, 4 % digestible crude oil and 39 % starches and sugars. The light/dark cycle was 13 h/11 h, with light from 06:00 to 19:00 h. Rabbits were purchased from Goreside (Berkhamsted, Herts., U.K.) and were maintained on Rabma cube diet from Special Diet Services.

Abbreviations used: CPT₁ and CPT₂, the overt and latent forms respectively of carnitine palmitoyltransferase (EC 2.3.1.21); PMSF, phenylmethanesulphonyl fluoride.

Chemicals

These were as described in [4,16,17], except for Tween-20, sodium cholate and M_r standards, which were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), and n-octyl glucoside, which was from Boehringer Mannheim U.K. (Lewes, East Sussex, U.K.).

Isolation of mitochondria

Rat liver mitochondria were obtained essentially as described in [18]. Livers were quickly removed, finely chopped and washed in ice-cold 'isolation medium' [210 mM-mannitol, 70 mM-sucrose, 0.1 mM-EDTA, 10 mM-Tris/HCl buffer (pH 7.4) containing PMSF (0.1 mg/ml)]. The livers were homogenized by three to four strokes of a motor-driven Teflon pestle in a glass Potter/Elvehjem homogenizer (radial clearance 0.19 mm) at a ratio of 1 g of tissue per 7–10 ml of isolation medium. The homogenate was centrifuged for 10 min at 600 g_{av} and the resulting supernatant kept on ice. The pellet was rehomogenized in fresh isolation medium and re-centrifuged as above. This process was repeated once more. The three supernatants were combined and centrifuged at 9200 g_{av} for 15 min to give a pellet, which was in turn washed three times by resuspension in isolation medium followed by re-centrifugation at 9200 g_{av} .

Mitochondria from rat heart and interscapular brown adipose tissue were also isolated essentially as described above. Bovine heart mitochondria were obtained as in [19].

Isolation of total mitochondrial membranes

Liver mitochondria obtained as above were resuspended in 20 mM-potassium phosphate buffer (pH 7.2) containing 0.3 M-KCl and PMSF (0.1 mg/ml) at a protein concentration of 8–10 mg/ml and sonicated in ice with a probe sonicator (MSE Soniprep 150). This was performed 8 times for 15 s bursts at 10 μ m amplitude, with 15 s cooling periods. After centrifugation at 115000 g_{av} for 40 min, the supernatant was discarded and the pellet resuspended in the phosphate/KCl/PMSF buffer. The centrifugation and resuspension process was repeated twice, and the final pellet was resuspended in a minimum volume of the same buffer and stored frozen at -70°C . Table 1 shows the distribution of marker enzymes for two such preparations. The washed membranes showed enrichment of succinate dehydrogenase relative to both the original homogenate and intact mitochondria. Specific activities of the microsomal marker NADPH:cytochrome *c* reductase and the peroxisomal marker uricase were decreased in washed membranes relative to intact

mitochondria by 5- and 3-fold respectively, although for uricase the final specific activity was not appreciably different from that in the initial homogenate. The specific activity of rotenone-insensitive NADH:cytochrome *c* reductase was similar in all three fractions; however, instability of this marker was observed on washing membranes in the phosphate/KCl/PMSF medium [a decrease of $79 \pm 2\%$ in the total activity (mean $\pm$ S.E.M., $n = 6$)]. The reason for choosing to isolate and wash total membranes in the phosphate/KCl/PMSF buffer was that this procedure significantly ($P < 0.001$) increased the proportion of the membrane-bound CPT that was responsive to malonyl-CoA [100 μ M-malonyl-CoA inhibited CPT in unwashed membranes by $21 \pm 3\%$ and in phosphate/KCl/PMSF-washed membranes by $42 \pm 3\%$ (means $\pm$ S.E.M., $n = 6$)]. At the same time approx. 40% of the CPT was recovered in the wash supernatants. This activity, whose subcellular origin is unknown, was essentially unresponsive to malonyl-CoA. This phenomenon of enriching the malonyl-CoA-responsive CPT component was not seen if the total membranes were washed in phosphate/PMSF buffer without KCl.

Separation of inner and outer mitochondrial membranes

Outer membranes were isolated essentially as described in [20]. Liver mitochondria prepared as described above were resuspended in ice-cold 20 mM-potassium phosphate buffer (pH 7.2) containing fatty acid-poor albumin (0.2 mg/ml). After 20 min to allow swelling, ATP (dissolved in 20 mM- K_2HPO_4) and $MgCl_2$ were each added to give final concentrations of 1 mM. After 5 min on ice to allow shrinkage of the mitoplasts, the suspension was centrifuged at 35000 g_{av} for 20 min. The resulting pellet was resuspended in fresh 20 mM-phosphate buffer (pH 7.2) containing albumin (0.2 mg/ml), and most of the inner membrane (as mitoplasts) was then removed by centrifugation at 1900 g_{av} for 15 min. The supernatant was removed and centrifuged at 35000 g_{av} for 20 min, followed by resuspension of the pellet in 20 mM-phosphate buffer (pH 7.2) without albumin. This outer-membrane fraction was then applied to the top of a three-layered density gradient consisting of the following sucrose solutions (dissolved in 20 mM-phosphate buffer, pH 7.2): 1.2 ml of 0.74 M ($d = 1.094$), 2.4 ml of 1.1 M ($d = 1.142$), 2.4 ml of 1.5 M ($d = 1.192$). After centrifugation for 1 h at 110000 g_{av} , the band at the 0.74 M/1.1 M interface was recovered, diluted by at least 1:4 with chilled distilled water, and centrifuged at 115000 g_{av} for 1 h. The resulting pellet was resuspended in 250 mM-sucrose medium containing 5 mM-potassium phosphate buffer (pH 7.2) and stored at -70°C . The pellet below the 1.5 M-sucrose layer was combined with the mitoplast fraction obtained above, resuspended in medium consisting of 210 mM-mannitol, 70 mM-sucrose, 10 mM-Tris/HCl buffer (pH 7.4) and 0.5 mM-EDTA at a protein concentration of approx. 10 mg/ml, and sonicated in ice for 8 $\times$ 15 s at 10 μ m amplitude with 15 s cooling periods. The sonicated suspension was centrifuged at 115000 g_{av} for 40 min. The pellets were washed by resuspension in the mannitol/sucrose/Tris/EDTA medium, re-centrifuged at 115000 g_{av} and the resulting inner-membrane fractions resuspended in the same medium and stored frozen at -70°C . As seen in Table 2, 98% of the outer-membrane CPT activity was inhibitable by malonyl-CoA in a radiochemical assay and, on the basis of succinate dehydrogenase distribution, the outer-membrane preparation showed only 2% contamination by inner-membrane material. Although none of the inner-membrane CPT was inhibitable by malonyl-CoA, the distribution of rotenone-sensitive NADH:cytochrome *c* reductase suggested approx. 13% cross-contamination of inner membranes by outer-membrane material. Essentially the same conclusion is reached from Table 3, which additionally demonstrates that both inner- and outer-membrane preparations were

Table 1. Marker enzyme activities during the isolation and washing of liver total mitochondrial membranes

Total mitochondrial membranes were isolated from rat livers in phosphate/KCl/PMSF buffer as described in the text. The specific activities are expressed as nmol/min per mg of protein and are means $\pm$ the range of values for two experiments.

Enzyme	Initial homogenate	Mitochondria	Washed membranes
Succinate dehydrogenase	14 $\pm$ 1	27 $\pm$ 1	42 $\pm$ 4
Rotenone-insensitive NADH:cytochrome <i>c</i> reductase	307 $\pm$ 6	307 $\pm$ 19	296 $\pm$ 6
Uricase	10 $\pm$ 1	32 $\pm$ 1	9 $\pm$ 1
NADPH:cytochrome <i>c</i> reductase	56 $\pm$ 12	23 $\pm$ 5	10 $\pm$ 1

Table 2. Characteristics of separated outer and inner mitochondrial membranes

The values are means $\pm$ S.E.M. for four separate preparations and are expressed as nmol/min per mg of protein. Malonyl-CoA-insensitive CPT represents the activity that remains in the presence of 100 μ M-malonyl-CoA.

Mitochondrial membrane preparation	Rotenone-insensitive NADH:cytochrome <i>c</i> reductase	Succinate dehydrogenase	Radiochemical assay		Spectrophotometric assay	
			Malonyl-CoA-sensitive CPT	Malonyl-CoA-insensitive CPT	Malonyl-CoA-sensitive CPT	Malonyl-CoA-insensitive CPT
Outer	824 $\pm$ 63	0.9 $\pm$ 0.3	5.2 $\pm$ 1.0	0.1 $\pm$ 0.1	3.5 $\pm$ 0.6	0.7 $\pm$ 0.2
Inner	107 $\pm$ 7	40 $\pm$ 7	0	10.6 $\pm$ 0.4	0	9.9 $\pm$ 0.7

Table 3. Marker enzyme activities during the isolation of liver mitochondrial outer and inner membranes

The values are specific activities (nmol/min per mg of protein) and are from a single typical fractionation of rat liver homogenate (19400 mg of protein) to yield inner mitochondrial membranes (559 mg of protein) and outer membranes (56 mg of protein) as described in the text.

Enzyme	Initial homogenate	Mitochondria	Inner membrane	Outer membrane
Succinate dehydrogenase	19	35	34	0.6
Rotenone-insensitive NADH: cytochrome <i>c</i> reductase	191	239	60	848
Catalase	7890	9390	15200	3480
Uricase	3.5	15	37	11
NADPH:cytochrome <i>c</i> reductase	54	6.7	13	15

not totally freed from the microsomal marker NADPH:cytochrome *c* reductase. Furthermore, there was cross-contamination by peroxisomal markers, in particular uricase.

Measurement of [14 C]malonyl-CoA binding

This was based on the procedure of Bird & Saggerson [4] with several modifications. Incubations were performed at 4 $^{\circ}$ C in a final volume of 1.0 ml containing 20 mM-potassium phosphate buffer (pH 7.4), PMSF (0.1 mg/ml), fatty acid-poor albumin (1.3 mg/ml), 1 mM-dithiothreitol and [2- 14 C]malonyl-CoA over the range 5 nM to 20 μ M. Binding was initiated by addition of 0.1 ml of the sample (0.1–1 mg of protein). After 20 min the mixtures were transferred to ice-cold tubes containing 0.1 ml of γ -globulin (20 mg/ml) and 0.35 ml of poly(ethylene glycol) 6000 (130 mg/ml). After mixing and centrifugation at 7000 g_{av} . (5 min at 5 $^{\circ}$ C) the supernatants were discarded and any remaining medium was removed with tissue paper. The pellets were dissolved in 0.5 ml of Triton X-100 (10%, v/v) at room temperature and quantitatively transferred to vials containing 13 ml of scintillation fluid [80 g of naphthalene plus 4 g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen in 600 ml of toluene plus 400 ml of 2-methoxyethanol] for scintillation counting. Non-specific entrapment of [14 C]malonyl-CoA was calculated by including 250 μ M unlabelled malonyl-CoA in parallel incubations covering the range of test [14 C]malonyl-CoA concentrations. These values were subtracted from the measurements of total binding to obtain values for specific binding.

Assay of CPT

All assays were in the direction of palmitoylcarnitine formation and were performed at 25 $^{\circ}$ C in a final volume of 1.0 ml. Reactions were initiated by addition of 0.4 mM-L-carnitine. The purification of CPT was monitored spectrophotometrically at

412 nm and pH 7.4 by following the reaction of released CoASH with 0.1 mM-5,5'-dithiobis-(2-nitrobenzoic acid) [16]. In one instance, to assess malonyl-CoA responsiveness (see Table 2), CPT was assayed as the incorporation of [3 H]carnitine into palmitoylcarnitine [17], except that the assay was at pH 6.8. All other assays were performed spectrophotometrically at 324 nm and contained 150 mM-sucrose, 60 mM-KCl, 25 mM-Tris/HCl buffer (pH 6.8), 1 mM-EDTA, fatty acid-poor albumin (1.3 mg/ml), 0.1 mM-4,4'-dithiopyridine and 40 μ M-palmitoyl-CoA. These conditions were chosen because the thiol-group reagent 4,4'-dithiopyridine is more reactive than 5,5'-dithiobis-(2-nitrobenzoic acid) at lower pH values (e.g. 6.8), at which CPT₁ has substantially greater sensitivity to malonyl-CoA [2], thereby minimizing the effect of thiol-group reagents to interfere with the malonyl-CoA responsiveness of the enzyme [21]. This assay satisfactorily measured inner- and outer-membrane enzyme and, though not as adequately as the radiochemical assay, also provided assessment of the malonyl-CoA responsiveness of CPT (Table 2).

Assays of marker enzymes

Succinate dehydrogenase [22], rotenone-insensitive NADH:cytochrome *c* reductase [23], NADPH:cytochrome *c* reductase [24], uricase [25] and catalase [26] activities were measured respectively as described in the cited references.

Purification of CPT

Liver total mitochondrial membranes were thawed and diluted with 20 mM-potassium phosphate buffer (pH 7.2) containing PMSF (0.1 mg/ml) and 0.3 M-KCl to a protein concentration of 8–10 mg/ml. During a 40 min period the mixture was continuously stirred on ice with Tween-20 at a ratio of 3:1 (μ l of detergent to mg of protein) and sonicated at 10 min intervals for

1 min bursts in a Kerry KS 200 ultrasonic bath. Sonication and the presence of KCl in the buffer appeared to enhance solubilization of CPT. The mixture was then centrifuged at $115\,000\ g_{av.}$ for 60 min. Poly(ethylene glycol) 6000 was added to the supernatant to a concentration of 53 mg/ml. After mixing and centrifugation at $30\,000\ g_{av.}$ for 30 min, poly(ethylene glycol) 6000 was added to the resulting supernatant to a concentration of 210 mg/ml, followed by centrifugation at $30\,000\ g_{av.}$ for 30 min. The pellet was resuspended in 20 mM-potassium phosphate buffer (pH 7.2) containing PMSF (0.1 mg/ml) and Tween-20 (0.5%, v/v) and then adjusted to 40% saturation with $(NH_4)_2SO_4$. After standing in ice for 1 h, followed by centrifugation for 30 min at $30\,000\ g_{av.}$, the supernatant was decanted, passed through three layers of butter muslin and then adjusted to 50% saturation with $(NH_4)_2SO_4$. After standing in ice for 1 h, the precipitate was collected by centrifugation as above, dissolved in the phosphate/PMSF/Tween-20 buffer and then desalted and equilibrated with 20 mM-bis-Tris-propane buffer (pH 7.0) containing Tween-20 (0.5%) by applying the sample to a Sephadex G-25 column. The mixture was concentrated by Amicon ultrafiltration with a lower cut-off point of M_r 30000 and then applied to a Pharmacia f.p.l.c. Mono Q 10/10 column previously equilibrated with 20 mM-bis-Tris-propane buffer (pH 7.0) containing Tween-20 (0.5%, v/v). The enzyme was eluted with a shallow linear gradient of 0.01–0.15 M-NaCl dissolved in the same buffer. Most of the CPT activity was eluted between 0.05 M- and 0.1 M-NaCl. These fractions were pooled, desalted and concentrated by ultrafiltration and applied to a chromatofocusing column as described in [10] except that Tween-20 (0.5%, v/v) was included in the buffers instead of n-octyl glucoside. A preliminary description of this method has been given in [27].

Raising of antisera to rat liver CPT

Non-immune sera were obtained before inoculation with CPT preparations. A 1 ml portion of purified CPT (50–70 μ g of protein) from the chromatofocusing column was emulsified with

1 ml of Freund's Complete Adjuvant. Then 0.1 ml portions were injected subcutaneously into rabbits at 10 different sites across the back. Similar booster inoculations were then given at three 4-weekly intervals, with Freund's Incomplete Adjuvant. At 10 days after the last injection blood was taken and serum obtained. Cross-reactivity with inner-membrane CPT was observed in the experiment shown in Fig. 1. Although the CPT preparation obtained from the chromatofocusing column showed only a single band of M_r 68000 on a SDS/10%-polyacrylamide gel after staining [0.25% (w/v) Coomassie Blue R, 7.5% (v/v) acetic acid in 50% (v/v) methanol] and destaining (7% acetic acid in 10% methanol), the antisera exhibited cross-reactivity with more than one band on immunoblotting of mitochondrial membrane proteins (see Figs. 2 and 3). Accordingly, rabbits were also immunized as follows. CPT-containing fractions obtained from the chromatofocusing column (see above) were pooled, concentrated and subjected to electrophoresis on a SDS/10%-polyacrylamide gel. After staining and destaining the gel, the band corresponding to M_r 68000 was cut out, homogenized in 2 ml of 20 mM-potassium phosphate buffer (pH 7.2), emulsified with Freund's Incomplete Adjuvant and injected into rabbits as above. The antisera prepared in this way showed similar cross-reactivity to those described above (see the Results and discussion section).

Immunoblotting

Polypeptides were separated by SDS/PAGE [28], electroblotted on to nitrocellulose sheets, made to react with anti-CPT serum, and cross-reacting bands were made visible by using peroxidase conjugated to anti-rabbit IgG [29]. In some experiments anti-rabbit IgG conjugated with peroxidase and ^{125}I -labelled anti-rabbit IgG were used together, so that the bands observed could be cut out and quantified by γ -radiation counting. In double-staining experiments the nitrocellulose sheet was firstly stained for protein [0.2% (w/v) Amido Black, 25% (v/v) isopropanol, 10% acetic acid] and then destained in the same solution without the dye. The nitrocellulose sheets were then made to react with antisera as above and then probed with the peroxidase-conjugated second antibody.

Immunoprecipitation studies

A portion (0.15 ml) of mitochondrial total membranes or outer membranes (0.3–0.75 mg of protein) resuspended in ice-cold 250 mM-sucrose medium containing 5 mM-potassium phosphate buffer (pH 7.2) and PMSF (0.1 mg/ml) was mixed either with 0.05 ml of 40% Tween-20 (v/v, in 20 mM-phosphate buffer, pH 7.2) or with 0.05 ml of 15 mM-sodium cholate, also dissolved in phosphate buffer. After addition of 0.15 ml of anti-CPT serum or of pre-immune serum and 0.15 ml of the phosphate buffer, the samples were kept on ice for 2 h, followed by centrifugation ($7000\ g_{av.}$ for 15 min at $5^\circ C$). The supernatants were decanted and the pellets resuspended in the phosphate buffer.

Measurement of protein concentration

This was done either by the method of Lowry *et al.* [30] or by a modification of that of Bradford [31] which achieved linearity over the range 10–100 μ g of BSA. Samples (0.1 ml) were added to 3 ml of reagent [0.0125% (w/v) Coomassie Brilliant Blue G, 0.003% (w/v) SDS, 4.7% (v/v) ethanol, 8.5% (v/v) H_3PO_4]. After mixing and then standing for 5 min, the A_{595} was measured.

Statistical methods

Statistical significance was evaluated by using Student's *t* test for paired or unpaired samples as appropriate. Unless stated otherwise, values in Tables are given as means $\pm$ S.E.M.

Fig. 1. Immunoprecipitation or inactivation of CPT extracted from liver mitochondrial inner membranes

Inner membranes (0.35 mg/ml) were incubated in ice for 30 min in 0.25 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4) and 30 mM n-octyl glucoside. The mixture was then centrifuged for 1 h at $110\,000\ g_{av.}$. The resulting supernatant contained 50% and 85% of the initial protein and CPT activity respectively. Samples (0.2 ml) of the supernatant were then incubated on ice for 2 h with various quantities of anti-CPT serum from rabbit B or with preimmune serum in a final volume of 0.3 ml. After centrifugation at $7000\ g_{av.}$ for 15 min, CPT activity was assayed in the supernatant.

Fig. 2. Immunoblotting of proteins from liver mitochondrial membranes

SDS/PAGE gels were probed with anti-CPT serum raised in rabbit A. (a) The gel was first stained for protein, destained and then treated with the antiserum. (b) The gel was only treated with the antiserum. Lane 1(a), M_r standards (phosphorylase, M_r 94000; bovine albumin, M_r 67000; ovalbumin, M_r 43000; carbonic anhydrase, M_r 30000). Lane 1(b), CPT (5 μ g) semi-purified but with the final chromatofocusing stage omitted. Lanes 2–5 are identical in both gels: lane 2, total membranes (40 μ g); lane 3, inner membranes (40 μ g); lane 4, outer membranes (40 μ g); lane 5, purified CPT (2 μ g).

RESULTS AND DISCUSSION

Immunoblotting experiments

The malonyl-CoA-insensitive CPT purified from Tween-20 extracts of liver mitochondrial membranes was observed on SDS/PAGE as a single polypeptide of M_r approx. 68000 [27]. Typically these preparations had specific activities in the range of 12–16 μ mol/min per mg of protein. These characteristics are similar to those previously reported for rat liver CPT preparations [8,13,14]. Fig. 2(a) shows an immunoblot in which the cross-reacting bands were highlighted by pre-staining with Amido Black. Fig. 2(b) shows a duplicate blot in which the bands were simply detected with the antiserum alone. The antiserum cross-reacted mainly with a band of M_r 68000 with semi-purified CPT (lane b1) and only with this band with purified CPT (lane b5). Since this polypeptide has been presumed to be CPT₂ [7], it was not unexpected that the antiserum cross-reacted at the same M_r value with total membranes (lanes 2) or with inner membranes (lanes 3). Additionally, the antiserum cross-reacted with all of the 85% of the inner-membrane CPT that could be extracted by a single treatment with n-octyl glucoside (fig. 1). It was surprising, however, that the antiserum also showed cross-reactivity with a M_r -68000 band in outer membranes (lanes 4), which contained only minor contamination by the inner-membrane marker succinate dehydrogenase (Tables 2 and 3) and contained almost exclusively malonyl-CoA-sensitive CPT (Table 2). In a subsequent experiment four other preparations of inner and outer membranes were immunoblotted and then probed with the ¹²⁵I-labelled second antibody. The inner membranes showed CPT activities of 9.3 ± 0.2 nmol/min per mg of protein and 4630 ± 670 d.p.m. of cross-reactivity in the M_r -68000 band per

50 μ g of protein. Outer-membrane preparations had CPT activities of 5.2 ± 1.0 nmol/min per mg of protein (97% inhibitable by 100 μ M-malonyl-CoA) and exhibited 702 ± 142 d.p.m. of cross-reactivity in the M_r -68000 band per 50 μ g of protein. After correction for $1.7 \pm 0.6\%$ of cross-contamination of outer membranes by inner-membrane protein (on the basis of succinate dehydrogenase distribution), this value was corrected to 602 ± 143 d.p.m. per 50 μ g of outer-membrane protein; i.e. this cross-reactivity cannot simply be explained as cross-contamination by M_r -68000 immunoreactive material derived from the inner membrane. It is, however, clear from this experiment that there is a difference of approx. 4-fold between the inner and outer membranes in the relationship between CPT activity *in situ* and the cross-reactivity at this M_r -68000 band. Although the M_r -68000 polypeptide in the outer membranes cannot be CPT₂, these data alone do not mean that this band must represent CPT₁. However, the only other major band of cross-reactivity (approx. M_r 60000) was also present in inner membranes which were devoid of malonyl-CoA-sensitive CPT₁ (Table 2). It should be noted that the antiserum had no appreciable cross-reactivity with polypeptides of M_r > 68000. However, assignment of the subunit M_r of CPT₁ as 68000 would be at variance with Declercq *et al.* [8], who concluded that the M_r -94000 polypeptide from liver mitochondria that binds [³H]tetra-decylglycidyl-CoA represents CPT₁, and also at variance with Zammit *et al.* [15], who initially estimated an M_r of 96700 for liver CPT₁ by radiation inactivation analysis and subsequently provided a revised value of 83000 [5].

Woeltje *et al.* [7] obtained an antiserum against a CPT activity purified from detergent extracts of whole mitochondria obtained from rats treated with the peroxisomal proliferating agent di(2-ethylhexyl)phthalate. This activity, of M_r 68000, was presumed to be CPT₂. That antiserum did not cross-react with the SDS/PAGE blot of liver mitochondrial membranes after their extraction with Tween-20. Since it was presumed that the residual CPT left in these membranes was CPT₁, it was concluded [7] that CPT₁ and CPT₂ are distinct polypeptides. We cannot dispute this contention, but we do suggest first that they have some common epitopes (see the immunoprecipitation studies below) and second that they may have broadly similar subunit sizes. Zammit *et al.* [5] have also speculated about similarity of antigenic determinants between CPT₁ and CPT₂. Our antiserum must recognize a broader range of immunological determinants than that used in ref. [7], because it also cross-reacted with the polypeptide of M_r 60000, whereas that used in ref. [7] did not. We considered whether cross-reactivity with this second band might be due to inadequate purification of the initial CPT antigen used to raise the antiserum. This possibility was discounted after raising an antiserum solely against the M_r -68000 band obtained after SDS/PAGE of purified CPT (see the Materials and methods section). This antiserum showed cross-reactivity similar to that seen in Fig. 2 (results not shown). Another possibility considered was that the band at M_r 60000 might represent a proteolytic degradation product of CPT arising during the isolation and storage of mitochondrial membranes. This also was discounted, because the band was just as apparent in whole liver mitochondria freshly prepared in the presence of several proteolytic inhibitors in addition to the customary PMSF (Fig. 3, lane 3). We also observed that this second band was still apparent after fractionation of liver mitochondria on Percoll [32] to decrease peroxisomal contamination (Fig. 3, lane 4). It was also noteworthy that our antiserum cross-reacted with a M_r -68000 band in rat brown adipose tissue and heart mitochondrial membranes (Fig. 3). The brown-fat preparation contained less of the M_r -60000 material, and this band was essentially absent from heart membranes. Fig. 3 (lane 7) also shows cross-reactivity of the

Fig. 3. Cross-reactivity of antiserum raised against purified liver CPT

SDS/PAGE gels were probed with anti-CPT serum without prior staining for protein. Lane C, M_r standards [as for Fig. 1, plus soya-bean trypsin inhibitor (M_r 20 100)]. Lane 1, liver mitochondrial total membranes (80 μ g); lane 2, liver intact mitochondria (80 μ g); lane 3, liver intact liver mitochondria (80 μ g) prepared as described in the Materials and methods section but with pepstatin (2 μ g/ml), leupeptin (1 μ g/ml) and antipain (1 μ g/ml) additionally present in the isolation media; lane 4, liver intact mitochondria (80 μ g) prepared as described in the Materials and methods section and then further fractionated by using Percoll [17.5% (v/v) in isolation medium] as described in [32]; lane 5, total membranes (80 μ g) from brown-adipose-tissue mitochondria; lane 6, total membranes (80 μ g) from heart mitochondria; lane 7, bovine heart mitochondria (80 μ g).

antiserum with bovine heart mitochondria. The relative faintness of this band may be related to the low total CPT activity in these mitochondria (only 2–3 nmol/min per mg of protein).

Use of anti-CPT sera to discriminate between catalytic units and malonyl-CoA binding entities

Immunoprecipitation experiments were performed under two sets of conditions, namely in the presence of either cholate or Tween-20. Detergents were added to facilitate solubilization and/or mobility and redistribution of membrane components in the presence of antibodies. Several preliminary experiments were performed to define the status of these preparations. Adopting the criterion of non-sedimentability at $115\,000 \times g_{av.}$ $\times$ 60 min to represent 'solubilization', treatment of total membranes with cholate solubilized $38 \pm 7\%$ of CPT activity, whereas Tween-20 solubilized $65 \pm 5\%$ (means $\pm$ S.E.M., $n = 3$ in both cases). Neither detergent caused any loss of CPT activity. Treatment of total membranes with cholate (without centrifugation) did not appreciably impair the binding of [14 C]malonyl-CoA (5 nM–20 μ M) to either high- or low [4]-affinity binding sites (results not shown). By contrast, Tween-20 interfered in a reversible manner with malonyl-CoA binding to total membranes, decreasing that seen with 5–400 nM-[14 C]malonyl-CoA by approx. 85%. Reversibility was inferred since, after centrifugation at $115\,000 \times g$, the pellets and the supernatants added together showed approximately normal binding relative to protein (results not shown). It appears that it is membrane-bound rather than 'solubilized' entities whose binding of malonyl-CoA is most affected by Tween-20. In further preliminary experiments, the two detergents were tested against outer membranes under the conditions used for the immunoprecipitation studies. Neither detergent caused any significant change in CPT₁ activity (four experiments in each case). Again it was found that cholate had no significant effect on malonyl-CoA binding and, for Tween-20, that interference with binding was reversed after centrifugation (results not shown). These preliminary experiments provided assurance that CPT activities and malonyl-CoA binding could both reasonably be measured under the chosen conditions without serious interference by the two detergents. To provide an empirical discrimination of immunoprecipitation, we tested for changes in sedimentability at $7000 \times g$. Clearly this treatment will leave some membrane-bound

Table 4. Effect of anti-CPT sera on the sedimentability of CPT activity and of [14 C]malonyl-CoA binding in total mitochondrial membranes treated with detergents

Total mitochondrial membranes were treated as described for immunoprecipitation studies in the Materials and methods section. Preimmune sera and anti-CPT sera were obtained from two separate rabbits (A and B, as indicated). [14 C]Malonyl-CoA binding was measured with 200 nM-malonyl-CoA. The values for CPT activity (nmol/min) and malonyl-CoA binding (pmol) were obtained by summing the values measured in the supernatants and pellets after centrifugation ($7000 \times g$ for 15 min). n refers to the number of separate membrane preparations. $^A P < 0.05$, $^B P < 0.01$ and $^C P < 0.001$ for comparison of the effects of the anti-CPT sera with those of the preimmune sera.

Membranes	CPT		Malonyl-CoA binding		X/Y	n
	nmol/min	% sedimented (X)	pmol	% sedimented (Y)		
Alone	22 $\pm$ 2	—	8 $\pm$ 3	—	—	5
Tween-20 + preimmune A	29 $\pm$ 2	23 $\pm$ 4	7 $\pm$ 1	56 $\pm$ 10	0.4 $\pm$ 0.1	4
Tween-20 + antiserum A	10 $\pm$ 1 ^C	80 $\pm$ 4 ^C	9 $\pm$ 1	47 $\pm$ 10	1.8 $\pm$ 0.3 ^B	4
Cholate + preimmune A	19 $\pm$ 2	53 $\pm$ 3	10 $\pm$ 3	75 $\pm$ 4	0.7 $\pm$ 0.1	5
Cholate + antiserum A	9 $\pm$ 1 ^B	89 $\pm$ 2 ^C	10 $\pm$ 2	64 $\pm$ 5	1.4 $\pm$ 0.1 ^B	5
Tween-20 + preimmune B	29 $\pm$ 2	16 $\pm$ 3	6 $\pm$ 1	56 $\pm$ 12	0.3 $\pm$ 0.1	4
Tween-20 + antiserum B	22 $\pm$ 2 ^A	83 $\pm$ 4 ^C	8 $\pm$ 1	42 $\pm$ 6	2.6 $\pm$ 0.4 ^B	4
Cholate + preimmune B	22 $\pm$ 3	55 $\pm$ 7	10 $\pm$ 3	65 $\pm$ 6	0.9 $\pm$ 0.1	5
Cholate + antiserum B	13 $\pm$ 2 ^A	91 $\pm$ 2 ^B	10 $\pm$ 2	69 $\pm$ 3	1.3 $\pm$ 0.1 ^A	5

Table 5. Effect of anti-CPT serum on the sedimentability of liver mitochondrial outer-membrane CPT activity and of [¹⁴C]malonyl-CoA binding in the presence of detergents

Preparations of outer membranes were firstly mixed with detergents with or without antiserum, sonicated as for preparation of CPT (see the Materials and methods section), and then treated as described for immunoprecipitation studies in the Materials and methods section. [¹⁴C]Malonyl-CoA binding was performed with 200 nM of the ligand. The values for CPT activity and malonyl-CoA binding ($n = 4$ in each case) were obtained by summing the values measured in the supernatants and pellets after centrifugation (7000 g for 15 min).

Detergent treatment	Antiserum B added	CPT activity		[¹⁴ C]Malonyl-CoA binding	
		nmol/min	% sedimentable	pmol	% sedimentable
Cholate	–	7.3 ± 1.0	31 ± 5	11.8 ± 2.1	59 ± 1
	+	7.3 ± 0.4	100 ± 0	17.5 ± 1.9	69 ± 3
Tween-20	–	5.0 ± 0.3	34 ± 4	7.1 ± 1.9	78 ± 5
	+	5.0 ± 0.3	100 ± 0	11.4 ± 3.5	42 ± 3

material in the 7000 g supernatant, which may lead to the underestimation of malonyl-CoA binding in this fraction in the presence of Tween-20 (see above). However, this in no way alters the interpretation of the findings shown below.

Two antisera were tested against total membranes (Table 4). Both, in particular that from rabbit A, inhibited CPT activity from total membranes, whereas pre-immune sera did not. By contrast, neither of the antisera interfered with the binding of malonyl-CoA. Table 4 shows that both antisera significantly increased the proportion of CPT activity that could be sedimented by centrifugation without altering the sedimentability of malonyl-CoA binding. This was also reflected by a significant increase in the sedimentation ratio (X/Y). Essentially the same experiment is repeated in Table 5, except that outer membranes were used. In preliminary experiments (results not shown), it was found that sonication of the membranes in the presence of the detergents (in the presence or absence of the antisera) assisted in the discrimination by immunoprecipitation between the CPT₁ activity and malonyl-CoA binding. Presumably sonication results in some disruption of their association in the outer membrane. Table 5 shows that the antiserum (from rabbit B) enhanced the precipitation of outer-membrane CPT, but did not enhance the sedimentation of the malonyl-CoA-binding entity. It was also observed that this antiserum did not inhibit the outer-membrane CPT₁ activity. These findings reinforce our earlier conclusion (see above) that the antiserum recognizes CPT₁ as well as CPT₂.

Conclusions and general considerations

Two conclusions emerge from this study. First, in accord with [5], CPT catalytic and malonyl-CoA-binding entities can be distinguished separately by the use of an antiserum which causes selective precipitation of CPT activity. This was seen with total membranes where, of course, CPT₂ is also present, but again with outer membranes, which have little contamination by inner membranes. Second, CPT₁ and CPT₂ have some common epitopes. It is clear that the antiserum recognizes inner-membrane CPT₂ with M_r 68000, and immunoprecipitates outer-membrane CPT₁. Furthermore, the antiserum shows appreciable recognition of the M_r 68000 band in immunoblots of outer-membrane preparations that cannot be explained by contamination from inner membranes. The lack of recognition of a band of M_r 94000 [8] or of M_r 83000 [5] in the immunoblot of outer membranes suggests that the subunit size of CPT₁ may be more similar to the M_r of 68000 assigned to CPT₂. Although this conclusion would be at variance with that of Zammit *et al.* [5], those authors [5] and others [33] have stressed the need for caution in the interpretation of radiation-inactivation analysis of membrane proteins.

We have been mindful that liver peroxisomes [34] and microsomal fractions [35] are reported to contain malonyl-CoA-inhibitable CPT activities and that our membrane preparations contain marker enzyme activities derived from these fractions. Peroxisomal CPT is reported as constituting approx. 20% of peroxisomal plus overt-mitochondrial activity in fed-rat liver [34]. The brief abstract reporting microsomal CPT [35] provides no data as to the abundance of this activity. Since neither of these activities are reported as cross-reacting with polyclonal antisera against mitochondrial CPT [35–37], and since our antiserum appeared to recognize all available inner-membrane CPT (Fig. 1) or outer-membrane CPT (Table 5), we reject the conjecture that our findings could be explained by peroxisomal or microsomal contamination of membrane preparations. Furthermore, the peroxisomal CPT is reported as being easily soluble [37,38], and should therefore be decreased through washing procedures used in the isolation of mitochondrial membranes.

On the basis of our conclusions, further studies to distinguish mitochondrial CPT₁ and CPT₂ may require the use of monoclonal antibodies. This report may also encourage studies aimed at separation and characterization of the entity that confers malonyl-CoA sensitivity on CPT.

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