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Characterization of a solubilized malonyl-CoA-sensitive carnitine palmitoyltransferase from the mitochondrial outer membrane as a protein distinct from the malonyl-CoA-insensitive carnitine palmitoyltransferase of the inner membrane

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By using octyl glucoside in the presence of glycerol, it is possible to obtain a solubilized malonyl-CoA-sensitive carnitine palmitoyltransferase (CPT_o) from the outer membranes of rat liver mitochondria. H.p.l.c. on hydroxyapatite column has now allowed a clear separation of the CPT_o from the malonyl-CoA-insensitive CPT activity of the inner membranes (CPT_i). The separated CPT_o activity showed inhibition by low micromolar concentrations of malonyl-CoA, 2-tetradecylglycidyl-CoA and etomoxir-CoA. On solubilization and fractionation, the CPT_o rapidly lost activity, unlike the relatively stable CPT_i activity. Reconstitution into asolectin liposomes enhanced the activity and the malonyl-CoA-sensitivity of the CPT_o fractions, whereas it had no such effect on the activity or malonyl-CoA insensitivity of the CPT_i fractions. A polyclonal antibody raised against the malonyl-CoA-insensitive enzyme, purified from the inner membranes, precipitated the CPT_i activity, but showed no reactivity with the CPT_o fractions. In Western blots, the above antibody did not react with any polypeptide of the CPT_o fractions. Incubation of the outer-membrane preparations with [^{3}H]etomoxir, in the presence of ATP and CoA, led to labelling of a 90 kDa polypeptide that in the above hydroxyapatite chromatography was eluted in the same region as the CPT_o. No such polypeptide labelling was seen in the CPT_i fractions. With heart and skeletal-muscle mitochondria, the correspondingly labelled polypeptide was of about 86 kDa. These results show that the CPT_o and CPT_i are distinct proteins, that a subunit of 90 kDa for liver and 86 kDa for muscle constitutes a component of their respective CPT_o systems, and that the 66 kDa subunit of the CPT_i does not constitute a part of the CPT_o system.

INTRODUCTION

The inner and outer carnitine palmitoyltransferases (CPT_i and CPT_o) of mitochondria catalyse the interconversion of fatty acyl-CoAs to fatty-acylcarnitines and play a role in the mitochondrial transport of fatty acyl groups and in the regulation of fatty acid oxidation (Bremer, 1983; Pande, 1983; Bieber, 1988; McGarry *et al.*, 1989). We have recently shown that, whereas the malonyl-CoA-insensitive CPT_i activity is known to be localized in the inner membrane, the malonyl-CoA-inhibitable CPT_o is localized in the outer membrane of mitochondria (Murthy & Pande 1987*a*,*b*). The question of whether these two CPT activities of mitochondria represent manifestations of the same or of different protein(s) is currently being debated (Miyazawa *et al.*, 1983; Brady & Brady, 1987, 1989; Murthy & Pande, 1987*a*,*b*; Woeltje *et al.*, 1987; Bieber, 1988; Zammit *et al.*, 1988).

Evidence supporting the distinct identities of the CPT_o and CPT_i activities may be summarized as follows. Whereas the subunit molecular mass of CPT_i activity from liver and other tissues is estimated as 69 kDa (Bieber, 1988; McGarry *et al.*, 1989; 80 kDa in Declercq *et al.*, 1987), tetradecylglycidyl-CoA, an irreversible inhibitor of the CPT_o activity, was found to label a polypeptide of a higher subunit molecular mass. The latter was estimated as 94 kDa (Kiorpes *et al.*, 1984) or 90 kDa (Declercq *et al.*, 1987) in experiments with liver mitochondria, and about 86 kDa in experiments with muscle mitochondria (Declercq *et al.*, 1987). The possibility that the CPT_o may be a protein of larger molecular mass than CPT_i has been indicated also by irradiation-inactivation experiments; the initial estimates for CPT_o and CPT_i were about 96.7 and 69.7 kDa respectively in the experiments with liver mitochondria (Zammit *et al.*, 1988), but subsequent experiments, using mitochondrial outer membrane as the source of CPT_o, have indicated a molecular mass of about 83 kDa for CPT_o activity (Zammit *et al.*, 1989). Although immunological evidence has not been uniform, some polyclonal antibodies that could distinguish between the two mitochondrial CPT activities have been described (Brosnan *et al.*, 1973; Declercq *et al.*, 1987; Woeltje *et al.*, 1987; Lilly *et al.*, 1989).

An examination of the properties of the CPT_o and CPT_i in mitochondrial outer and inner membrane (Murthy & Pande, 1987*b*) has also supported the possibility of their being distinct entities. A clear-cut assessment of this, after solubilization and separation of the two CPT activities, has not been possible until now, although it had become known that under appropriate conditions a malonyl-CoA-sensitive CPT activity could be solubilized with octyl glucoside (Lund, 1987; Murthy & Pande, 1987*b*). We report here that the separation of solubilized CPT_o activity from that of CPT_i has now been attained. Our results lend support to the possibility that these two CPT activities are distinct and have different subunit molecular masses. Moreover, they have revealed that housing in asolectin liposomes enhances not only the malonyl-CoA-sensitivity of the solubilized and

Abbreviations used: CPT, carnitine palmitoyltransferase; CPT_o, outer, malonyl-CoA-sensitive, CPT of outer membrane; CPT_i, inner, malonyl-CoA-insensitive, CPT of inner membrane; OMV, outer-membrane vesicles; IMV, inner-membrane vesicles; etomoxir, 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate.

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fractionated CPT_o activity, as reported previously (Murthy & Pande, 1987*b*), but also its basal CPT_o activity. We believe that these findings would aid in the further functional characterization of CPT_o, an apparently transmembrane protein (Murthy & Pande, 1987*a*). An abstract of some of these results has appeared (Murthy & Pande, 1988).

EXPERIMENTAL

[1-^{14}C]Octanoyl-CoA was obtained from P.L. Biochemicals. [1-^{14}C]Palmitoyl-CoA was synthesized from [1-^{14}C]palmitic acid (Amersham) as described by Hajra & Bishop (1986). [^{3}H]Etomoxir and unlabelled etomoxir-CoA were gifts from Dr. H. P. O. Wolf, Byk-Gulden Pharmazeutika, Konstanz, Germany. 2-Tetradecylglycidyl-CoA was synthesized as described by Kiorpes *et al.* (1984) from 2-tetradecylglycidic acid, kindly supplied by McNeil Laboratories, Spring House, PA, U.S.A. Octyl glucoside was from Calbiochem.

Mitochondrial outer- and inner-membrane vesicle (OMV and IMV) preparations were as described previously (Murthy & Pande, 1987*a*,*b*). Activity of CPT was assayed at 30 °C either with 50 μM-[1-^{14}C]palmitoyl-CoA and 10 mM-carnitine as in Pande *et al.* (1986), or with octanoyl-CoA and carnitine. In the latter case the assay system in 100 μl contained 50 mM-potassium phosphate, pH 7.4, 50 μM-[1-^{14}C]octanoyl-CoA and 10 mM-carnitine, and the reactions were stopped by the addition of 0.7 ml of a charcoal suspension [6.7% (w/v) charcoal, 91% (v/v) ethanol and 2% (v/v) H_3PO_4]. After vortex-mixing and centrifugation (10000 *g*, 5 min), the supernatant was monitored for radioactivity corresponding to [1-^{14}C]octanoylcarnitine. Because of the higher sensitivity, simplicity and rapidity of the latter assay procedure, in many experiments we have used [1-^{14}C]octanoyl-CoA as the substrate for monitoring the CPT activity and its inhibition by malonyl-CoA; our intermittent comparisons showed that the results obtained were similar to those with [1-^{14}C]palmitoyl-CoA as the acyl-CoA substrate.

Purification of CPT_i

Rat liver mitochondrial IMV were used as the starting material for this purification; the CPT activity of the IMV was not noticeably inhibited by up to 200 μM-malonyl-CoA, in agreement with that described earlier (Murthy & Pande, 1987*b*). The IMV (5 mg of protein/ml) were extracted with a mixture containing 5 mM-potassium phosphate, pH 7.4, 5% Tween-20, and protease inhibitors [leupeptin, 10 μg/ml; pepstatin, 10 μg/ml; tosyllysylchloromethane (Tos-Lys-CH_2Cl), 50 μg/ml] for 20 min at 0–4 °C. After centrifugation at 150000 *g* for 1 h, the supernatant was applied to a DEAE-cellulose column (DE 52, Whatman) and the proteins were eluted with a linear gradient of 5–100 mM-potassium phosphate as described by Miyazawa *et al.* (1983). Fractions with CPT activity were pooled, concentrated and equilibrated with 20 mM-potassium phosphate, pH 7.4, containing 2% Tween-20 and protease inhibitors as above, and then applied to a hydroxyapatite h.p.l.c. column and the proteins were eluted with a 20–500 mM-potassium phosphate gradient. Fractions with CPT activity were eluted at about 260 mM-phosphate; these were combined, equilibrated with 25 mM-imidazole/HCl, pH 7.0, containing 0.5% Tween-20 and protease inhibitors, and applied to a PBE-94 chromatofocussing column. For elution, Polybuffer 74, pH 5.0, containing 0.5% Tween-20 and protease inhibitors was used. The CPT activity emerged at about pH 6.0 as a near-homogeneous protein as monitored by SDS/PAGE and silver staining. This preparation of CPT_i, after the removal of Polybuffer, was used to raise polyclonal antibodies in female rabbits.

Solubilization and h.p.l.c. separation of CPT_o

Rat liver mitochondrial OMV were solubilized with octyl glucoside. The solubilization medium contained 100 μM-potassium phosphate, pH 7.4, 20% (v/v) glycerol, leupeptin (10 μg/ml), Tos-Lys-CH_2Cl (50 μg/ml), pepstatin (10 μg/ml) and OMV (1.2 mg/ml). Octyl glucoside was added last, to a final concentration of 40 mM. The ratio (w/w) of OMV protein to octyl glucoside was maintained at 1:10. The mixture was left at 0 °C for 45 min and then centrifuged at 150000 *g* for 60 min. CPT activity was found only in the supernatant. Approx. 1 ml of the above supernatant, containing about 1 mg of solubilized OMV protein, was applied to the hydroxyapatite h.p.l.c. column at 4 °C, equilibrated in 100 mM-potassium phosphate, pH 7.4, containing 10% glycerol, 30 mM-octyl glucoside and protease inhibitors (leupeptin, 1 μg/ml; Tos-Lys-CH_2Cl, 25 μg/ml; pepstatin, 1 μg/ml); the proteins were eluted with a 100 mM–1 M gradient of potassium phosphate, pH 7.4, containing 30 mM-octyl glucoside, 10% glycerol and the protease inhibitors. Fractions containing CPT activity emerged at around 260 mM-phosphate (peak I) and at 500 mM-phosphate (peak II). These were separately pooled and their proteins concentrated by using Centriprep 30 (Amicon).

Reconstitution of CPT activities was done with asolectin liposomes. About 50 μg of protein of the test fraction was mixed with 2 mg of asolectin liposomes containing 100 mM-potassium phosphate, pH 7.4, and 0.1 mM-EDTA in a final volume of 400 μl. This mixture was frozen on solid CO_2, then thawed at 30 °C and sonicated for 5 s at 4 °C. The resulting proteoliposomes were used the same day.

RESULTS AND DISCUSSION

It is generally accepted that mitochondria have two differentially localized CPT activities, that both exist in membrane-bound forms and that only one of them is sensitive to malonyl-CoA inhibition (Bremer, 1983). After solubilization of liver and heart mitochondrial membranes with detergents, and purification, several workers have obtained only one protein with a subunit of 66–69 kDa having CPT activity (Bieber, 1988). However, it has been observed by many that, once detached from the membranes with detergents, the resulting solubilized CPT does not show malonyl-CoA inhibition. Two interpretations have been offered from these results. The first assumes that a loss (dissociation) of a malonyl-CoA-inhibition-conferring peptide occurs on solubilization and that the catalytic activities of both mitochondrial CPT enzymes reside in the same protein (Bieber, 1988). The second view ascribes these results to the detergent-lability of the CPT_o such that, after detergent solubilization of membrane proteins, the CPT activity that survives is the one derived exclusively from CPT_i, the malonyl-CoA-insensitive enzyme of mitochondria (Woeltje *et al.*, 1987; Lund, 1987; Murthy & Pande, 1987*b*).

Unlike in the previous studies where, for purification of mitochondrial CPT, mitochondrial membranes having both malonyl-CoA-sensitive as well as insensitive CPT activities were subjected to detergent solubilization, in the present work we have tried to solubilize and fractionate the malonyl-CoA-sensitive and -insensitive CPT activities of liver mitochondria separately by proceeding with IMV as the exclusive source of the malonyl-CoA-insensitive CPT activity and with OMV as the exclusive source of the malonyl-CoA-sensitive one (Murthy & Pande, 1987*b*). This has allowed us to follow the fate of the CPT_i and CPT_o activities separately during their solubilization and subsequent fractionation. Our results have shown that from the IMV the malonyl-CoA-insensitive CPT activity could be purified

to near-homogeneity as a protein with a subunit of about 66 kDa by using protocols as described by others (Miyazawa *et al.*, 1983; Brady & Brady, 1987); this molecular-mass assignment is based on that of the BSA present in the Bio-Rad molecular-mass marker kit, with which the CPT_i subunit co-migrated in the SDS/PAGE gels and which was assigned a mass of 66 kDa; others have mentioned the value for CPT_i subunit as 68 kDa (Brady & Brady, 1987) or 69. 2 kDa (Miyazawa *et al.*, 1983). Our use of the hydroxyapatite h.p.l.c. step in the present work helped us to obtain a purer final preparation of CPT_i, as monitored by the SDS/PAGE separation and silver staining, than was otherwise possible. At this hydroxyapatite step, the CPT activity was eluted with 260 mM-potassium phosphate, and in high yields regardless of whether the detergent present was Tween-20 or octyl glucoside (results not shown).

The situation was quite different during the solubilization of the CPT_o, the malonyl-CoA-sensitive CPT activity, from OMV. We have described previously that solubilization with a number of detergents led to a loss of this activity but that, under appropriate conditions, in the presence of 20% glycerol, by using octyl glucoside it was possible to obtain a malonyl-CoA-sensitive CPT activity in the solubilized state, but with only about 50% loss of activity (Murthy & Pande, 1987*b*). Without glycerol the activity was lost nearly completely. For more effective solubilization and preservation of activity, we have since evaluated other detergents, different conditions of solubilization, and use of stabilizers described for labile membrane proteins, but none offered any improvement over the octyl glucoside/glycerol procedure mentioned above. In these experiments, using increasing ratios of detergents to protein, and by monitoring the CPT activity appearing in the 150000 *g*/60 min supernatant and that remaining with the residual pellet, we observed that a major activity loss and/or inactivation of CPT occurred instantly at the point of the enzyme detachment from the membrane, the extent of which varied with different detergents. The solubilized enzyme then continued to lose activity, but at a slower pace, in a relative sense. For example, the near-50% loss of CPT activity that occurred on solubilization with octyl glucoside in the presence of glycerol occurred immediately after octyl glucoside addition, and then in the next 24 h at 0 °C the activity declined to $\leqslant 10\%$. It is likely that, because of these losses, separation of proteins by several procedures, including affinity chromatography using CoA- or carnitine-coupled columns, did not yield fractions with increased CPT_o specific activity. However, h.p.l.c. on hydroxyapatite column allowed the protein-fractionation step to be completed in less than 90 min and, despite lack of increase in the specific activity of CPT_o, yielded a clear separation of the malonyl-CoA-sensitive from the malonyl-CoA-insensitive activity for the first time. This has allowed further characterization of the two mitochondrial CPT activities and, as described below, these results have strongly supported the possibility of CPT_i and CPT_o being distinct proteins.

Fig. 1 shows that the solubilized preparations of OMV on hydroxyapatite h.p.l.c. fractionation yielded two CPT activity peaks, the first of which was eluted at about 260 mM- and the second at about 500 mM-potassium phosphate. When IMV were similarly solubilized and fractionated, all the CPT activity was eluted as a single peak near 260 μM-potassium phosphate (results not shown). From this and other evidence (see below) we have concluded that the peak-I activity represented CPT_i, whereas peak-II CPT activity was due to CPT_o; clearly these proteins had different surface charges, inasmuch as their retention times on the hydroxyapatite column varied. Although the peak-I CPT activity accounted for $\leqslant 10\%$ of the peak-II activity, the former corresponded to the presence of less than 1% of total CPT activity in the OMV, as illustrated below. Thus about 50% of the CPT_o activity of the OMV was lost at the octyl glucoside/glycerol solubilization step and the combined CPT activity of peaks I and II accounted for only about $\frac{1}{6}$th of that applied on the hydroxyapatite column. If we assume that the malonyl-CoA-insensitive CPT_i activity did not undergo any loss in these steps, as was observed during comparable processing of the IMV, then the peak-I activity is calculated as $(50/100) \times (1/6) \times (1/10) \times 100 = 0.8\%$ of the total OMV CPT activity. This could easily have come from the presence of IMV as a minor contaminant in the OMV. Other evidence that the peak II activity represented CPT_o may be summarized as follows.

First, the peak-II activity proved very labile, unlike the peak-

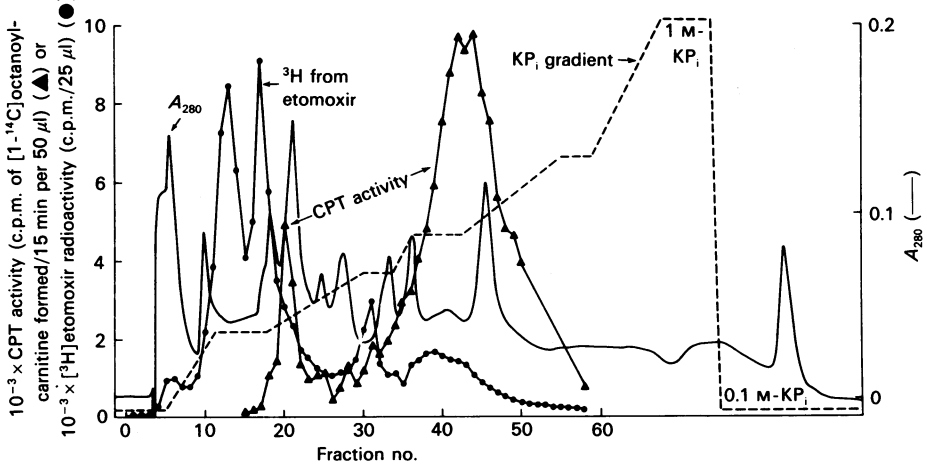

Fig. 1. Fractionation of liver mitochondrial outer-membrane proteins after [^{3}H]etomoxir labelling by h.p.l.c. on a hydroxyapatite column

Rat liver OMV (3 mg of protein) were suspended in 1 ml of 100 mM-potassium phosphate, pH 7.4, containing 1 mM-ATP, 1 mM-$MgCl_2$, 50 μM-CoA, 0.5 mM-dithiothreitol and 1 μM-[^{3}H]etomoxir and incubated for 30 min at 30 °C. Then 4 ml of ice-cold 20 mM-potassium phosphate, pH 7.4, was added and the mixture was centrifuged at 150000 *g* for 60 min. The membrane pellet was solubilized with octyl glucoside as described in the Experimental section. Approx. 1 mg of the solubilized protein was injected into the hydroxyapatite column, and the proteins were eluted as described in the Experimental section (KP_i, potassium phosphate). All the fractions were monitored for ^{3}H radioactivity (●) and for CPT activity (▲). For the latter assays, 50 μM-[1-^{14}C]octanoyl-CoA plus 10 mM-carnitine were used as substrates.

Table 1. Specific inhibition of peak-II CPT activity from hydroxyapatite h.p.l.c. of OMV proteins by 2-tetradecylglycidyl-CoA, etomoxir-CoA and malonyl-CoA

Octyl glucoside-solubilized OMV proteins were fractionated by hydroxyapatite h.p.l.c., and CPT peaks I and II were obtained. The details of reconstitution and CPT assays are described in the Experimental section. Results are averages of three or four experiments using different batches of membrane preparations: N.D., not determined.

	Inhibitor (μM)		CPT activity (nmol of octanoylcarnitine/min per mg) Before reconstitution	After reconstitution
Solubilized OMV	None		140	146
	2-Tetradecylglycidyl-CoA	(1)	34	22
	Etomoxir-CoA	(0.2)	57	28
	Malonyl-CoA	(20)	86	N.D.
	Malonyl-CoA	(100)	42	32
Solubilized IMV	None		147	111
	2-Tetradecylglycidyl-CoA	(1)	148	115
	Etomoxir-CoA	(0.2)	136	103
	Malonyl-CoA	(20)	149	N.D.
	Malonyl-CoA	(100)	149	119
Hydroxyapatite-h.p.l.c. CPT peak I	None		12	11
	2-Tetradecylglycidyl-CoA	(1)	12	12
	Etomoxir-CoA	(0.2)	12	13
	Malonyl-CoA	(20)	13	11
	Malonyl-CoA	(100)	12	11
Hydroxyapatite-h.p.l.c. CPT peak II	None		66	158
	2-Tetradecylglycidyl-CoA	(1)	9	6
	Etomoxir-CoA	(0.2)	33	57
	Malonyl-CoA	(20)	41	38
	Malonyl-CoA	(100)	22	21

I activity. Thus the peak-II activity showed non-linear kinetics with time when assayed at 30 °C with octanoyl-CoA as the acyl-CoA substrate. Vigorous or continuous mixing of the assay system led to greater loss of activity. Whereas the presence of carnitine or of octanoyl-CoA alone had little effect on these activity losses, inclusion of 2 mg of BSA/ml appreciably stabilized the activity. The subsequent assays were therefore carried out in the presence of BSA. We verified that under our assay conditions, with octanoyl-CoA as substrate, the presence of BSA had no significant effect on the activity measurement or on the malonyl-CoA inhibition of the CPT_o activity. Our attempts to prevent activity losses in the CPT activity of peak II by the inclusion of additional protease inhibitors and other known protein stabilizers have not been successful. The h.p.l.c.-fractionated CPT_o continued to lose its catalytic activity even when rapidly frozen and stored at −20 °C. This instability of CPT_o made its further fractionation impractical.

Second, the peak-II CPT activity was inhibited by malonyl-CoA, but that of peak I was not (Table 1). We have previously described that the octyl glucoside solubilization of CPT_o led to a marked decrease in its malonyl-CoA-sensitivity, and that this was increased by the reconstitution of the CPT_o into asolectin liposomes (Murthy & Pande, 1987*b*). We have made similar observations in the present experiments; thus, whereas 20 μM- and 100 μM-malonyl-CoA inhibited the membrane-bound CPT_o activity by 80 and 95% respectively, the corresponding inhibition was only 38 and 66% for the CPT activity of peak II before reconstitution, and 76 and 81% after reconstitution. In the present series, we found that reconstitution markedly increased the basal activity also (Table 1). Overall these results were about the same regardless of whether the reconstitution was by freeze–thaw and sonication or by detergent dialysis. Importantly, reconstitution had no effect on the basal CPT activity of the peak-I fractions or its insensitivity to malonyl-CoA inhibition. These results indicate not only that phospholipid housing is important for the optimal expression of the regulatory property of CPT_o, as was observed by Murthy & Pande (1987*b*), but that this may apply also to the basal activity of CPT_o. This indicates that the marked loss of basal CPT_o activity and its malonyl-CoA inhibition on detergent solubilization resulted, at least in part, from the separation of the CPT_o from its lipid environment *in situ*.

Third, Table 1 shows that at low micromolar concentrations both tetradecylglycidyl-CoA and etomoxir-CoA, known potent and selective inhibitors of CPT_o of mitochondria, inhibited the CPT activity of peak II, but not that of peak I.

Fourth, Table 2 shows that polyclonal anti-(rat liver CPT_i) antibody effectively precipitated the CPT activity present in the octyl glucoside-solubilized supernatants of IMV as well as of peak I, but under matching conditions it had no such effect on the CPT activity in the octyl glucoside supernatants derived from OMV or that of peak II activity. In a previous preliminary report, we described similar results with anti-(bovine liver CPT_i) antibody and that the octyl glucoside-solubilized CPT_o activity was not precipitated by the antibody to the peroxisomal CPT (Murthy & Pande, 1988). It is known that CPT_i is immunologically different from the peroxisomal CPT (Healy *et al.*, 1988; Ramsay, 1988). The above findings therefore indicate that CPT_o, CPT_i and the peroxisomal CPT are immunologically distinct proteins.

Kiorpes *et al.* (1984) and Declercq *et al.* (1987) observed that, after incubation of liver mitochondria with [^{3}H]2-tetradecylglycidyl-CoA, an irreversible inhibitor of the malonyl-CoA-sensitive CPT, a 94–95 kDa polypeptide became labelled, and inferred that this polypeptide represented CPT_o. With skeletal muscle the corresponding labelled polypeptide was of 86 kDa (Declercq *et al.*, 1987). Woeltje *et al.* (1987) reported that open hydroxyapatite chromatography of liver mitochondrial-mem-

Table 2. Effect of anti-CPT$_i$ antibody on the CPT activities of solubilized OMV and IMV and of the hydroxyapatite h.p.l.c. fractions

Liver mitochondrial OMV and IMV were solubilized as described in the Experimental section. CPT peaks I and II were from the hydroxyapatite h.p.l.c. of OMV proteins. CPT activity was assayed with 50 μM-[1-^{14}C]octanoyl-CoA plus 10 mM-carnitine. Solubilized membrane proteins (2–5 μg of protein) or 10 μl samples of the CPT peaks I and II were separately incubated with 15 μl of anti-CPT$_i$ antibody (250 μg of protein) in phosphate-buffered saline (0.5 M-potassium phosphate, pH 7.4, 0.15 M-NaCl) in a final volume of 50 μl at 4 °C for 4 h. Then the tubes were centrifuged at 10000 *g* for 5 min and the supernatants were assayed for CPT activity. For the details see the Experimental section. Two controls were set up, one with BSA and the other with pre-immune IgG with matched amounts of total protein, and both gave similar results. Results are averages of three or four experiments.

	Antibody used	CPT activity (nmol of octanoylcarnitine/min per mg of protein)
Solubilized outer membranes	Control IgG	150
	Anti-CPT$_i$	140
Solubilized inner membranes	Control IgG	149
	Anti-CPT$_i$	30
Hydroxyapatite-h.p.l.c. CPT peak I	Control IgG	13
	Anti-CPT$_i$	2
Hydroxyapatite-h.p.l.c. CPT peak II	Control IgG	68
	Anti-CPT$_i$	66

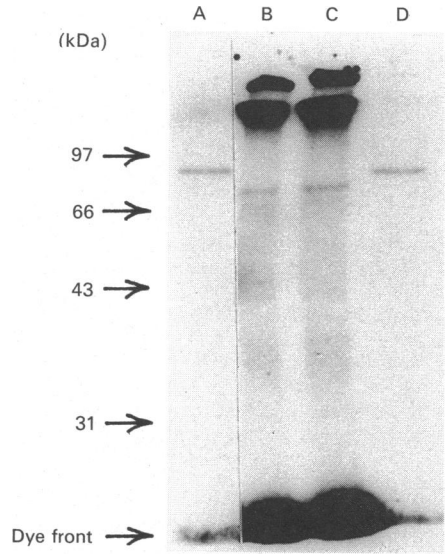

Fig. 2. Fluorography of SDS/PAGE-separated outer-membrane proteins from rat liver, heart and skeletal-muscle mitochondria after exposure to [^{3}H]etomoxir, CoA and ATP-Mg

OMV (25 μg of protein) from liver, heart and skeletal-muscle mitochondria were exposed to [^{3}H]etomoxir under conditions described for Fig. 1, but in 50 μl final volume. After labelling, the outer-membrane pellet was dissolved directly in SDS/PAGE sample buffer and processed for SDS/PAGE as described by Laemmli (1970). After electrophoresis, the gel was processed for fluorography by using Amplify according to the manufacturer's (Amersham) instructions, by using an X-ray film (Kodak X-OMAT RP) and exposure time of 24 h at −70 °C. Lanes: A and D, liver OMV; B, heart OMV; C, skeletal-muscle OMV. Positions of molecular-mass standards (kDa) are indicated at the left.

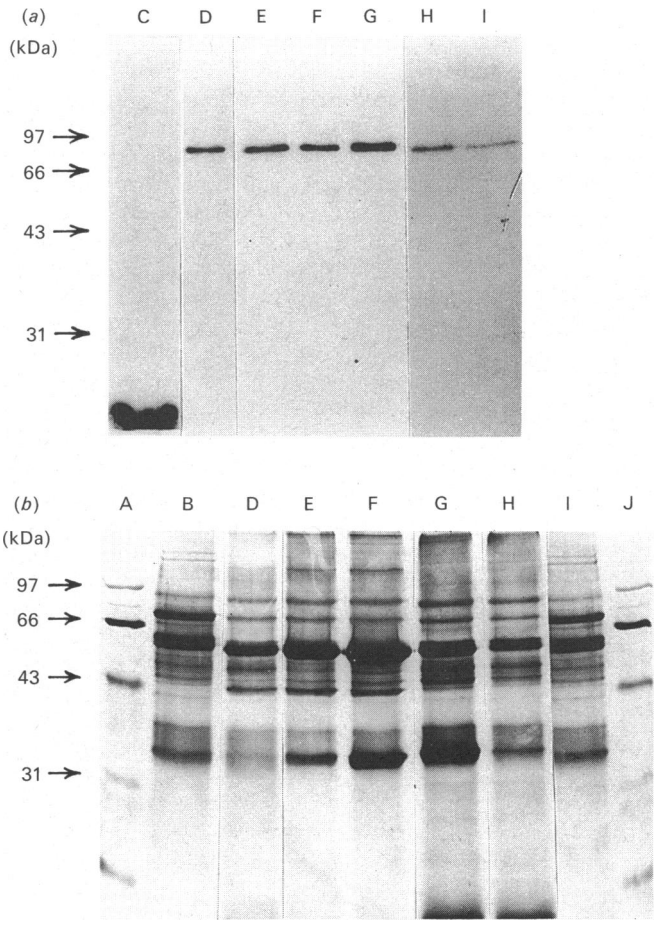

Fig. 3. Elution profile of the [^{3}H]etomoxir-labelled 90 kDa protein during hydroxyapatite h.p.l.c. of the liver OMV preparation

OMV (2–3 mg) from liver mitochondria were exposed to [^{3}H]etomoxir and processed as described for Fig. 1. Pooled fractions from CPT peak I and fractions 39, 41, 42, 44 and 46 from CPT peak II were processed for SDS/PAGE and then for fluorography (panel *a*) or silver staining (panel *b*). Lanes: A and J, molecular-mass markers (kDa); B and I, etomoxir-labelled liver OMV; C, pooled CPT peak I; D–H, CPT peak-II fractions 39, 41, 42, 44 and 46 respectively.

brane proteins after exposure to [^{3}H]tetradecylglycidyl-CoA led to the elution of a CPT activity peak (probably CPT$_I$) and another peak having the [^{3}H]tetradecylglycidyl-CoA-bound protein, and inferred this to indicate the separation of CPT$_i$ from putative CPT$_o$ (the tetradecylglycidyl-binding protein). However, a verification of whether the latter peak would have had any CPT activity in the absence of tetradecylglycidyl-CoA was not made.

A clear correspondence between the labelled polypeptide and the CPT_o activity has now been obtained, as described below.

We observed that, after incubation of OMV with [^{3}H]etomoxir, the CoA ester of which is another irreversible inhibitor of CPT_o (Wolf & Engel, 1985; Declercq *et al.*, 1987), along with ATP, $MgCl_2$ and CoA, a 90 kDa polypeptide became labelled in the case of liver OMV and a 86 kDa polypeptide in the case of skeletal-muscle and heart mitochondrial OMV (Fig. 2). No such protein labelling was observed with liver IMV (results not shown; however, see Fig. 3*a*, lane C). These results were in line with those of Kiorpes *et al.* (1984) and Declercq *et al.* (1987), and showed further that the subunit molecular mass of the labelled polypeptide was the same for heart and skeletal-muscle mitochondria. To examine the relationship of the labelled peptide to the CPT activity of the OMV, we incubated liver OMV with [^{3}H]etomoxir under conditions where only about 10 % of the CPT_o activity was inhibited. These labelled OMV were solubilized, subjected to hydroxyapatite h.p.l.c. and the fractions were monitored for CPT activity and ^{3}H radioactivity and processed for SDS/PAGE and fluorography. These analyses showed that, of the four ^{3}H radioactivity peaks in Fig. 1, only the one eluted near 500 mM-potassium phosphate, in the same region as the CPT_o, had a polypeptide (90 kDa) that bound radioactivity. The radioactivity of the other three peaks moved ahead of polypeptide bands on SDS/PAGE (with the dye front), and most likely resulted from left-over unreacted [^{3}H]etomoxir and its metabolites, such as [^{3}H]etomoxir-CoA, produced in the incubation system containing OMV, ATP, CoA etc. Furthermore, we observed that the intensity of the radioactive labelling of the 90 kDa polypeptide in the individual fractions of peak II closely matched the elution profile of the CPT_o activity (Fig. 3*a*). Prolonged exposure of the SDS/PAGE gel to the X-ray film (more than 4–5 days) during fluorography led to the appearance of other minor polypeptide bands, of which a 45 kDa polypeptide was more prominent (results not shown). SDS/PAGE of the peak-II fractions and silver staining showed that there was an enrichment of the 90 kDa polypeptide in the fractions with the highest CPT_o activity. However, many other polypeptides, including a major 60 kDa polypeptide, were also present in these fractions (Fig. 3*b*). In the earlier stage of these studies, as described in an abstract (Murthy & Pande, 1988), only one CPT activity peak was obtained by using a hydroxyapatite h.p.l.c. column (from Rainin, Woburn, MA, U.S.A.), which on SDS/PAGE showed a major 77 kDa and a minor 60 kDa polypeptide on Coomassie Blue staining. However, in the later work, as described here, refinement of conditions using another hydroxyapatite column (from Toyosoda, Tokyo, Japan) and silver staining have given substantially different results. In recent experiments, Zammit *et al.* (1989) have obtained evidence indicating that a 60 kDa polypeptide is responsible for conferring the malonyl-CoA inhibition, whereas the catalytic activity of CPT_o itself resides in a 83 kDa polypeptide.

Collectively, the present results strengthen the view that the CPT_i and CPT_o activities reside in different proteins and that the malonyl-CoA-sensitive CPT activities of liver and muscle mitochondrial outer membranes are due to different isoenzymic forms of CPT_o.

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