The Malonyl-CoA-sensitive Form of Carnitine Palmitoyltransferase Is Not Localized Exclusively in the Outer Membrane of Rat Liver Mitochondria*

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The data used to support the idea that malonyl-coenzyme A (CoA)-sensitive carnitine palmitoyltransferase (CPT-I) is localized on the outer mitochondrial membrane are based on harsh techniques that disrupt mitochondrial physiology. We have turned to the use of the French press, which produces a shearing force that denudes mitochondria of their outer membrane without the physiologically disruptive effects characteristic of phosphate swelling. Our results indicate that the mitoplasts contain just 15–19% of the outer membrane marker enzyme activity while retaining 85% of the total CPT activity and 50% of both CPT-I, as well as long-chain acyl-CoA synthase activity, the latter two supposed outer membrane enzymes. These mitoplasts were shown by electron microscopy to have the configuration of mitochondria that merely have been divested of their outer membranes. Carnitine-dependent fatty acid oxidation was retained in the mitoplasts, showing that they were physiologically intact. Moreover, protein immunoblotting analysis showed that CPT-I, as well as the inner membrane CPT-II, was localized in the mitoplast fraction. The outer membrane fraction, which consisted of membrane “ghosts,” contained most (50–60%) of marker enzyme activity, monoamine oxidase-B and porin proteins, but only about 27–29% CPT-I activity. Because CPT-I and long-chain acyl-CoA synthetase appear to be associated with both inner and outer membranes, we postulate that these enzymes reside in contact sites, which represent a molding of both limiting membranes.

The two carnitine palmitoyltransferase (CPT) enzyme activities are essential in the mitochondrial oxidation of long-chain fatty acids. The malonyl-CoA-sensitive or outer form of CPT (variously termed CPT-I, CPT-I, CPT\textsubscript{\textsc{o}}, overt, or outer form) is the first committed step in mitochondrial long-chain fatty acid oxidation. The intramitochondrial localization of this enzyme is controversial. Murthy and Pande (1) reported that CPT-I is present in the outer membrane and the catalytic site is specifically localized on its inner surface, whereas Fraser et al. (2) recently have proposed that this site is on the outer surface. In contrast, two groups of investigators have reported that the enzyme is localized to the outer surface of the mitochondrial inner membrane (3–5).

Previous studies on the localization of CPT have relied upon techniques for separating inner and outer membranes that potentially could alter the membranes by producing breaks with subsequent annealing in a non-native configuration and which might not yield membranes of absolute purity. To overcome these potential pitfalls in localizing the malonyl-CoA-sensitive CPT, we have utilized a method of mitochondrial fractionation that does not rely on methods that use either organellar swelling to rupture the outer membrane (1, 2), or exposure to detergents (3), or enzymatic digestion of membrane components (5). Instead, we have utilized physical shearing produced by the French press to skin isolated rat liver mitochondria so that the outer membrane can be detached and separated from the residual mitoplasts without exposure to potentially harmful agents. We have carried out functional and enzyme marker studies to monitor the procedure and the separated membranes were used to determine the localization of CPT. Moreover, we used electron microscopy to monitor the purity and structural integrity of the various membrane fractions. Our data show that CPT-I and long-chain acyl-CoA synthase, supposed outer membrane enzymes, are associated with a component of the mitoplast fraction.

MATERIALS AND METHODS

Isolation and Purification of Mitochondria—Livers from 4–5 rats were pooled, yielding a gross weight of 50 g for each experiment. The livers were minced, rinsed with 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4 (MSM, isolation buffer) containing 2 mM EGTA and a 10% homogenate prepared using two strokes of a Potter-Elvehjem loose-fitting Teflon pestle. The mitochondria were isolated by differential centrifugation, washed twice in isolation buffer, and resuspended in 0.2 ml of isolation buffer/g of liver, yielding a mitochondrial protein concentration of 80–100 mg/ml (6). Further purification of mitochondria was achieved using self-forming Percoll gradient centrifugation. One ml aliquots of mitochondrial suspensions were layered on top of 20 ml of 30% Percoll in MSM (a total of eight tubes were prepared in this manner for each experiment) and centrifuged for 30 min with 35,000 rpm (50.2 Ti rotor, Beckman LS-M). The tubes each contained two distinct layers separated by a clear zone. The upper layer (which electron microscopy revealed to consist of peroxisomes) and the clear zone were removed and the lower brownish layer consisting of mitochondria were collected. Following a 2-fold dilution with MSM, the mitochondria were collected by centrifugation (10 min, 7000 × g) and washed once with...
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isolation buffer. This preparation was designated as the Percoll-purified rat liver mitochondria.

French Press Treatment of Percoll-purified Mitochondria—The final pellet from above was resuspended in 2× MSM containing 0.5 mg of defatted BSA/ml and protease inhibitors (1.0 μg/ml each aprotinin, antipain, trasylol, N-ethylmaleimide, 0.2 mM palmitoyl-CoA, and benzamidine) and 0.5 mM phenylmethyl sulfonium fluoride) and after 10 min was loaded into the French press (American Instrument Co., Silver Spring, MD) fitted with a 12-ml cell according to the directions of the manufacturer and to the protocol of Decker and Greenawalt (7). The mitochondria were subjected to a pressure of 2000 p.s.i., while being extruded at a rate of 7–8 ml/min. An adequate pressure was selected after a series of preliminary experiments wherein the pressure was varied from 1400 to 2600 p.s.i. (see “Results”). The material exiting the French press was collected, diluted with 12 ml of 2× MSM by resuspension and centrifugation as above, and the pellet, designated as the mitoplast fraction, resuspended in 3.0 ml of MSM containing protease inhibitors. The combined supernatant fluids were subjected to centrifugation for 30 min with 45,000 rpm (50.2 Ti rotor, Beckman L8-M) and the pellet, outer membrane fraction, resuspended in approximately 1.0 ml final volume of MSM. During the entire isolation procedure, the volumes of different fractions were recorded and aliquots saved for subsequent morphological, enzymological and balance studies.

Electron Microscopy—An aliquot of each and every mitochondrial fraction was added to an equal volume of phosphate-buffered, full-strength Karnovsky’s fixative (8), mixed, and immediately spun down for 30 s in a microcentrifuge. The resultant pellets were removed from the centrifuge tubes, and the fixation continued in half-strength Karnovsky’s fixative for a total of 2 h. The pellets were thoroughly rinsed in distilled water, then postfixed for 2 h in an unbuffered 1:1 mixture of 2% osmium tetroxide and 3% potassium ferricyanide (9). After rinsing with distilled water, the specimens were soaked overnight in an acidified solution of 0.25% uranyl acetate (10). After another rinse in distilled water, they were dehydrated in ascending concentrations of ethanol, passed through propylene oxide, and embedded in an Epon-Marasglas mixture (11). Thin sections were sequentially stained with acidified methanolic uranyl acetate (10) and lead tartrate (12) and examined in a Zeiss CEM 902 electron microscope.

Enzyme and Protein Assays—CPT activity was determined using three different radiochemical assays (3). 1) Assay-forward measures the forward reaction (palmitoyl-l-carnitine formation) in the presence of N-ethylmaleimide to prevent the reverse direction using 25 μg of mitochondrial fractions. The assay system contained in a final volume of 250 μl: 80 mM KCl, 50 mM MOPS, 1.0 mM EDTA, 0.1% defatted BSA, 1.0 mM N-ethylmaleimide, 0.2 mM palmitoyl-CoA, and 15 μM L-[14C]-carnitine, pH 7.0. After preincubation, the reaction was initiated with 10 μl (25 μg) of sample solubilized with 10 μl Lubrol PX. Following a 5-min incubation at 37 °C, the reaction was terminated with 0.5 ml of 1:1 mixture of 2% water-saturated n-butanol. The butanol phase was washed with 2.0 ml of butanol-saturated water, and 0.4 ml of the organic phase was used to determine the formation of radioactive palmitoyl-l-carnitine (13). The CPT activity was calculated on the basis of the specific radioactivity of L-[14C]-carnitine and free CoASH concentration. The assay system contained in a final volume of 200 μl: 80 mM KCl, 50 mM MOPS, 1.0 mM EGTA, 4.0 mM diethiothreitol (DTT), 0.1% defatted BSA, 2.0 mM CoASH, and 2.0 mM palmitoyl-l-[14C]-carnitine, pH 7.0. After preincubation, the reaction was initiated with 10 μl (25 μg) of sample. Following a 5-min incubation at 37 °C, the reaction was stopped with 0.2 ml of cold 6% perchloric acid and the precipitated protein and unreacted palmitoyl-l-[14C]-carnitine removed by centrifugation. The radiolabeled free carnitine formed during the reaction was determined by counting 0.3-ml aliquots of the acid supernatant. 3) Assay-modified forward is optimized for measuring the malonyl-CoA-sensitive CPT activity (CPT-II), and contains in a final volume of 250 μl: 80 mM KCl, 50 mM MOPS, 1.0 mM EGTA, 4.0 mM DTT, 0.4 mM NaCN, 0.4 mM deoxycholate, 0.02% defatted BSA, 50 μM palmitoyl-CoA, 2 μg/ml bovine serum albumin, 0.5 mM malonyl-CoA, 0.5 mM potassium phosphate, 0.5 mM EGTA, and 10 μl (25 μg) of sample. Following preincubation, the reaction was initiated with L-[14C]-carnitine (5.0 mM final concentration). The reaction was terminated after a 3-min incubation at 37 °C and the formation of radioactive palmitoyl carnitine determined as described above. The difference in rate obtained in the absence and presence of malonyl-CoA represents CPT-I activity (14). Since malonyl-CoA inhibition of CPT-I is subject to changes due to desensitization of the enzyme to the inhibitor (15, 16), the activity also was determined using the active-site-directed covalent inhibitor, etomoxir-CoA (17). For this, samples at 2.5 mg/ml 150 mM NaCl, 10 mM potassium phosphate, 0.1 mM EGTA, and 0.5 mM DTT were preincubated for 15 min. at room temperature with 0.5 μM etomoxir-CoA. This pressure was selected after a series of preliminary experiments wherein the pressure was varied from 1400 to 2600 p.s.i. (see “Results”). The material exiting the French press was collected, diluted with 12 ml of 2× MSM by resuspension and centrifugation as above, and the pellet, designated as the mitoplast fraction, resuspended in 3.0 ml of MSM containing protease inhibitors. The combined supernatant fluids were subjected to centrifugation for 30 min with 45,000 rpm (50.2 Ti rotor, Beckman L8-M) and the pellet, outer membrane fraction, resuspended in approximately 1.0 ml final volume of MSM. During the entire isolation procedure, the volumes of different fractions were recorded and aliquots saved for subsequent morphological, enzymological and balance studies.

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Decker and Greenawalt might be due to a difference in the size of the pressure cell used in the respective experiments. In an effort to increase the degree of detachment of the outer membrane from mitoplasts so that these two components could be cleanly separated, we carried out a systematic study varying the pressure in our French press (Fig. 1). To judge the efficacy of a particular pressure, we measured the monoamine oxidase activity of the putative mitoplasts. Proceeding from 1500 to 2200 p.s.i., there was a progressive decrease in the recovery of monoamine oxidase activity associated with the mitoplast fraction (Fig. 1) from 80% of the beginning total mitochondrial activity to 20% at 2000 p.s.i. There is no change between 2000 and 2200 p.s.i.; above 2200 p.s.i., mitoplast damage increases. In addition to studies of the recovery of monoamine oxidase, parallel studies of oxidative phosphorylation were carried out on the mitoplasts resulting from the different pressures. Glutamate oxidation was 50% of the beginning mitochondrial activity in mitoplasts prepared at 2000 p.s.i. while the RCR was 2.6 ± 0.1 and the ADP/O ratio was 2.38 ± 0.20, showing retention of the ability to couple oxidative phosphorylation in the mitoplasts. The oxidation of palmitoyl carnitine plus L-malate, as well as palmitoyl-CoA plus carnitine and L-malate was 60–70% of the starting activity. Therefore, we selected 2000 p.s.i. as the optimal pressure to use in fractionating rat liver mitochondria.

**Percoll-purified Mitochondria—**Not only were the mitochondria used in our studies examined biochemically, but morphologically as well by electron microscopy. The starting sample consisted almost entirely of intact mitochondria with a very slight contamination by other types of organelles (Fig. 2). The mitochondria were spherical, had retained both limiting membranes, and had a relatively dense inner compartment. Most showed a modest dilatation of their cristae, but some had cristae that were identical in configuration to those in their in situ counterparts. A few bodies, presumably damaged mitochondria, had lost their outer membrane, the resultant mitoplast being swollen, cristae-free, and of relatively low density.

Although the isolated rat liver mitochondria were intact and had little contamination from other subcellular organelles, we elected to subject the mitochondria to a self-forming Percoll gradient to eliminate any possible peroxisomal contamination. The mitochondrial fraction resulting from passage through Percoll strongly resembled the starting material, except that there were fewer overtly damaged organelles and no obvious other type organelles (Fig. 3).

**FIG. 1.** French press treatment of rat liver mitochondria: effect of increasing pressure on the removal of outer membrane and on the functional integrity of resultant mitoplasts. Rat liver mitochondria were treated at the indicated pressures (p.s.i.) and the mitoplast fraction isolated as described under “Materials and Methods.” Removal of the outer membrane was assessed by following the MAO activity and the functional integrity of resultant mitoplasts determined by measuring their capacity to oxidize glutamate, palmitoyl-L-carnitine plus malate, and palmitoyl-CoA plus carnitine and malate. The results are expressed as percentage of activity remaining with the mitoplasts, where 100% activity represents the values obtained with intact, untreated mitochondria. For MAO the specific activity was 14.8 ± 6.1 nmol/min/mg, and the 2 mM ADP stimulated rates of oxygen consumption for glutamate, palmitoyl carnitine, and palmitoyl CoA plus carnitine were 109.3 ± 9.2, 106.3 ± 6.2, 101.5 ± 11.5 nanouats of oxygen/min/mg, respectively (values expressed are averages and standard deviations for n = 5).

**FIG. 2.** Initial pellet of rat liver mitochondria. Although relatively rare, a few contaminants are present. Original magnification, ×7,000.
French Press-treated Mitochondria—The Percoll-purified mitochondria were washed and then resuspended in hyperosmolar (2×) MSM followed 10 min later by extrusion through the French press at 2000 p.s.i. After passage through the French press, the mitochondrial fraction appeared to comprise three morphologically distinct subclasses. The first consisted of structurally unaltered organelles identical to those in the untreated sample. The second consisted of mitoplasts that completely lacked any trace of outer membrane. Of these, some retained their characteristic matrix density, whereas others were rather pallid. In both cases, the cristae had kept their inward orientation, no mitoplasts exhibiting everted cristae in the manner of those illustrated by Schnaitman and Greenawalt (21). The final category of mitochondria consisted of organelles that in terms of density and of configuration of cristae resembled those in the starting mitochondria, but from which a portion of the outer membrane had been stripped. The residual outer membrane either lay flat against the inner membrane in its original position or in part formed small vesicles that seemed to adhere to the organelles.

The French press-treated mitochondria were fractionated using differential centrifugation to produce a low speed mitoplast (inner membrane-matrix) preparation and a supernatant. The low speed supernatant was ultracentrifuged to produce a high speed pellet (the outer membrane fraction) and a supernatant. The biochemical data associated with the mitochondrial fractionation are shown in Table I. The specific activity of the marker enzymes is presented as well as the percentage of the marker enzymes retained in the outer membrane vesicles, there is only a 5.8-fold enrichment of malonyl-CoA-sensitive CPT in the mitoplasts is 2.5 times greater than that observed for monoamine oxidase activity. The lack of parallelism between the enrichment of monoamine oxidase and CPT in these two fractions raises the question as to whether the malonyl-CoA-sensitive CPT activity is exclusively localized in the mitochondrial outer membrane.

Because the competitive inhibitor, malonyl-CoA, depends on the ratio of malonyl-CoA to palmitoyl-CoA and sundry other factors such as type and amount of protein, we used the specific irreversible inhibitor of CPT-I, etomoxiryl-CoA. CPT-I activity was expressed as the etomoxiryl-CoA-sensitive activity in the fractions. The results using etomoxiryl-CoA were identical to those obtained using malonyl-CoA (Table I).

Because the foregoing results strongly suggest that CPT-I does not reside in the same site as monoamine oxidase and RINCR, we used an additional enzyme marker for the outer membrane, namely long-chain acyl-CoA synthetase. The data for long-chain acyl-CoA synthetase resemble those for CPT-I (Tables I and II).

Mitoplast Fraction—The inner membrane-matrix fraction consisted of a mixture of mitochondria in various stages of denudation (Fig. 4), ranging from intact outer membrane (whole mitochondria), through mitoplasts with partial outer membrane, to “naked” mitoplasts (Fig. 5). In every case the cristae were inwardly directed. The matrix compartment retained its characteristic density. The retained outer membrane appeared sufficient to account for the small amount of monoamine oxidase activity (19%) present in this fraction. Strewed among the mitoplasts were some membranous vesicles of unrecognizable identity.
Table I

Specific activity and recovery of mitochondrial outer membrane, inner membrane and matrix marker enzymes, and carnitine palmitoyltransferase in rat liver mitochondrial subfractions

Values represent the average and standard deviation of three separate experiments. Specific activities are expressed as nanomoles/min/mg, except for cytochrome c oxidase (1/s/mg).

| Enzyme                           | Specific activity of French press mitochondria | Percent recovery |
|----------------------------------|-----------------------------------------------|------------------|
|                                  | Mitoplast          | Supernatant | Outer membrane | Total     |
| Monoamine oxidase                | 8.1 ± 0.8          | 19.1 ± 10.4 | 20.8 ± 2.9    | 48.0 ± 10.0 | 87.9 ± 20.0 |
| Rotenone-insensitive NADH        | 157.0 ± 20.7       | 15.1 ± 1.2  | 18.5 ± 8.4    | 53.0 ± 22.4 | 91.6 ± 30.7  |
| Long-chain acyl-CoA synthetase   | 76.5 ± 11.5        | 41.6 ± 7.6  | 13.7 ± 2.6    | 19.8 ± 5.7  | 75.2 ± 9.9   |
| Cytochrome c oxidase             | 6.1 ± 3.7          | 90.6 ± 20.3 | 0.4 ± 0.2     | 5.6 ± 2.9   | 88.7 ± 21.9  |
| Citrate synthase                 | 168.6 ± 8.2        | 93.7 ± 8.5  | 4.1 ± 3.8     | 1.5 ± 1.3   | 99.3 ± 3.8   |
| CPT assay-modified forward       |                  |            |              |            |
| Mal-CoA-sensitive                | 9.6 ± 1.6          | 50.7 ± 6.8  | 12.8 ± 4.2    | 28.9 ± 7.0  | 92.4 ± 3.3   |
| Et-CoA-sensitive                 | 10.5 ± 1.4         | 53.9 ± 7.2  | 12.2 ± 3.6    | 28.1 ± 7.0  | 94.2 ± 3.0   |
| CPT assay-reverse               | 191.2 ± 10.3       | 84.7 ± 6.7  | 2.7 ± 0.7     | 2.7 ± 0.9   | 90.2 ± 8.0   |
| CPT assay-forward                | 74.9 ± 8.6         | 86.3 ± 7.5  | 2.9 ± 0.0     | 2.3 ± 1.1   | 91.5 ± 8.7   |
| Protein (mg total)               | 806.9 ± 65.1       | 66.1 ± 0.8  | 30.7 ± 4.8    | 6.7 ± 1.2   | 103.6 ± 4.3  |

* Et-CoA, etomoxiry-CoA.

Table II

Specific activity ratios (enrichment) of mitochondrial inner and outer membrane, mitochondrial matrix (mitoplasts) marker enzymes, and carnitine palmitoyl transferase

Values represent the average and standard deviation of three separate experiments. OM, outer membrane.

| Enzyme                           | OM | Mitoplast | RLMfp |
|----------------------------------|----|-----------|-------|
| Monoamine oxidase                | 4.0 ± 28.0 | 7.2 ± 0.9 |
| Rotenone-insensitive NADH        | 37.5 ± 9.3 | 9.0 ± 2.8 |
| Long-chain acyl-CoA synthetase   | 4.8 ± 1.1  | 3.1 ± 1.0 |
| Cytochrome c oxidase             | 0.6 ± 0.2  | 0.9 ± 0.3 |
| Citrate synthase                 | 0.1 ± 0.1  | 0.2 ± 0.1 |
| CPT assay-modified forward       | 5.8 ± 0.8  | 4.2 ± 0.4 |
| Mal-CoA-sensitive                | 5.9 ± 1.0  | 4.2 ± 0.4 |
| Et-CoA-sensitive                 | 0.4 ± 0.2  | 0.6 ± 0.3 |
| CPT assay-reverse               | 0.3 ± 0.1  | 0.3 ± 0.1 |

* Et-CoA, etomoxiry-CoA.

known provenance.

A functional assessment was used as an additional approach to the determination of the presence of the enzymes in mitoplasts. Oxidative phosphorylation with glutamate as the substrate provided measurement of state 3 (ADP-stimulated), state 4 (ADP-limited), RCR (state 3/state 4), and ADP:O ratios (amount of ADP added/amount of O used). These measurements also were done in the presence of 3.2 μM cytochrome c, because maximal rates of oxidation in mitoplasts were stimulated at least 2-fold and were similar to that observed in intact mitochondria. The data in Table III show that although there was little effect of added cytochrome c to mitochondria, there was a marked stimulation of the rate of glutamate oxidation in the mitoplasts. The mean RCR was 4.0 ± 0.4, and the ADP:O ratio was 2.06 ± 0.10, showing retention of the ability to couple oxidative phosphorylation in the mitoplasts. Maximal oxidation with high ADP was used to characterize substrate utilization by the mitoplasts. Table IV shows data for oxidative rates in the absence and in the presence of cytochrome c for both mitochondria and the resultant mitoplasts. Substrates that provide reducing equivalents to specific sites in the mitochondrial electron transport chain were used. Glutamate (using glutamate dehydrogenase) and pyruvate (pyruvate dehydrogenase complex) were used to assess substrates producing NADH. Succinate (plus rotenone) enters through complex II, didehydroquinone (plus rotenone) enters through complex III, and

N,N',N'-tetramethyl-p-phenylenediamine and ascorbate combine to reduce cytochrome c for complex IV (Table IV). With mitochondria the addition of cytochrome c to the incubation had little effect except for succinate. In contrast, the maximum rate of mitoplast oxidation of all substrates was stimulated by 2–3-fold providing rates similar to those seen in mitochondria.

The oxidation of fatty acid substrates in the presence of l-malate was determined using palmitoylcarnitine as a substrate and compared with oxidation of palmitoyl-CoA plus carnitine, which requires in addition CPT-I activity, and to palmi-
tate, which requires long-chain acyl-CoA synthetase and CPT-I. As shown in Table IV, the oxidation rates of palmitoyl-carnitine, palmitoyl-CoA plus carnitine and palmitate are similar in mitoplasts and in intact mitochondria when supplemented with cytochrome c.

**Outer Membrane Vesicles**—This fraction consisted almost wholly of a series of membrane vesicles that, in keeping with the terminology of Parsons (31), can be called “ghosts” (Figs. 6 and 7). Some of these ghosts contained entrapped small membrane vesicles. A few scattered mitoplasts in both damaged or pristine condition also were present.

**Immunoblotting**—To further verify the localization of CPT enzymes, we subjected membrane fractions to SDS-polyacrylamide gel electrophoresis immunoblotting. As a yardstick, we used a polyclonal antibody against MAO (in rat liver mitochondria) and a monoclonal antibody against porin. Using identical amounts of proteins for the respective blots, both proteins are enriched in the outer membrane fraction (Fig. 8). Because the monoclonal antibody against porin was derived from a human B cell antigen, we verified that the antibody cross-reacted with purified porin from rat liver mitochondria (lane 12, Fig. 8).

Fig. 9 shows that the antibody directed against CPT-II decorates the immunoblots obtained by loading 1 milliunit of total forward reaction CPT activity per fraction not only from the isolated rat liver mitochondria and Percoll-purified mitochondria, but especially that from the mitoplasts. In contrast, there is little reaction with the immunoblot from the outer membrane fraction. For comparative purposes, lanes 2 and 7 contain 1 and 0.5 milliunit, respectively, of purified beef liver mitochondrial CPT-II activity.

To test the distribution of CPT-I by the same approach, we utilized a polyclonal antibody generated against a peptide representing CPT-I3–17 with a terminal cysteine coupled to keyhole limpet hemocyanin. This antibody recognized a single protein in rat liver mitochondria with a subunit mass of 87–88 kDa, the apparent mass of CPT-I (Fig. 9). Using this antibody to probe the membranes obtained from the mitochondrial fraction where 0.5 milliunit of CPT-I activity, measured as the malonyl-CoA-sensitive activity in the modified forward assay, was loaded onto the gel for each fraction, there was enhanced decoration of the mitoplasts as compared with the outer membrane (Fig. 9). It is especially noteworthy that, although present, there is no indication of enrichment of CPT-I in the outer membrane fraction.

### DISCUSSION

The results of our study show that CPT activity resides mainly in the mitoplast membranes and to a lesser extent in the outer membrane. This is true of total CPT, whether measured as the forward or reverse reaction. About 85% of the activity measured in either direction is present in mitoplasts, whereas less than 3% activity is present in the outer membrane fraction. Furthermore, the malonyl-CoA-sensitive activity showed a similar distribution with 52% of the activity in the mitoplasts and 29% in the outer membrane. To determine the composition of the membrane fractions, we carried out various biochemical tests. The purity and integrity of our mitoplast fraction is indicated by the fact that 91–94% of the marker enzymes for the inner membrane (cytochrome c oxidase) and matrix (citrate synthase) is retained by these structures. In addition, the capacity for coupled oxidative phosphorylation is maintained, the slightly reduced respiratory control ratios notwithstanding. Not only were these mitoplasts functionally normal, but electron microscopy showed that morphologically most of these bodies are a near simulacrum of their in situ counterparts, i.e. they have inwardly directed cristae.

In contrast to mitoplasts, the outer membrane ghosts contained less that 3% of the total CPT activity whether measured in the forward or reverse direction. These ghosts retained 29% of the malonyl-CoA-sensitive CPT activity. That these ghosts actually consisted of outer membrane is shown by the almost 10-fold increase in the specific activity and 7–9-fold enrichment

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**Table III**

2 mM ADP stimulated respiration (nA oxygen/min/mg) of Percoll-purified rat liver mitochondria (RLMpp) and mitoplasts in the absence and presence of exogenous cytochrome c.

|                | iii     | iv     | RCR   | ADP/O  | 2 mM ADP | 0.2 mM DNP |
|----------------|---------|--------|-------|--------|----------|------------|
| 20 mM glutamate |         |        |       |        |          |            |
| RLMpp          | 91.8 ± 9.7 | 9.0 ± 1.2 | 10.5 ± 2.2 | 2.56 ± 0.1 | 114.4 ± 16.8 | 114.0 ± 17.8 |
| Mitoplasts     | 41.4 ± 1.8 | 15.5 ± 0.7 | 2.7 ± 0.1 | 2.11 ± 0.1 | 39.9 ± 1.6  | 43.5 ± 4.8  |
| + 3.2 μM cytochrome c |       |        |       |        |          |            |
| RLMpp          | 104.2 ± 12.7 | 9.1 ± 1.7 | 11.9 ± 1.5 | 2.36 ± 0.1 | 131.1 ± 24.4 | 111.0      |
| Mitoplasts     | 90.6 ± 4.7  | 23.1 ± 3.3 | 4.0 ± 0.4  | 2.06 ± 0.1 | 95.8 ± 14.8 | 111.8      |
of monoamine oxidase and RINCR, outer membrane markers. Although a small amount of inner membrane-matrix enzymes was found in this fraction (<5%), it should be noted that electron microscopy revealed it to contain a small number of mitoplasts, enough to account for this contamination.

If in fact the CPT activity is confined mainly to the mitoplast fraction, then these structures should be capable of oxidizing palmitoyl-CoA plus carnitine as well as palmitoylcarnitine. In a similar fashion, if long-chain acyl-CoA synthetase also is confined mainly to the mitoplast fraction, then palmitate oxidation by mitoplasts should be retained. It is obvious that if these key enzymes reside in and are removed with the outer membrane, then the mitoplast fraction would be incapable of carrying out those oxidations. In contrast, our mitoplast preparations retain 100% of the capacity to oxidize not only the non-lipid substrates tested, but palmitoylcarnitine and, above all, palmitoyl-CoA plus carnitine, and palmitate. If the malonyl-CoA-sensitive form of CPT had been removed from the mitoplasts, the rate for the oxidation of palmitoyl-CoA plus carnitine should have fallen virtually to zero, which it did not. Long-chain acyl-CoA synthetase and palmitate oxidation behave in a like fashion.

Although as early as 1963 Fritz and Yue (32) proposed dual localization of CPT activity within mitochondria, subsequent studies could detect CPT activity only in the inner membrane (3, 5). In 1972, Hoppel and Tomec (3), using a multifaceted approach, reported that CPT activity exists in two sites associated with the inner membrane, one termed CPT-A on the outer aspect, the other termed CPT-B on the inner aspect. This interpretation was supported in a subsequent study by Fritz (5), who employed a technically different approach. That CPT was localized on the inner membrane was generally accepted until 1987, when Murthy and Pande (1) offered an alternative conclusion based on their new data. These workers suggested that the malonyl-CoA regulatory site is on the cytosolic side of the outer membrane, whereas CPT actually is on the inner surface of the outer membrane. In order to separate the membranes, these investigators used the hypotonic phosphate swelling procedure developed by Parsons et al. (31). Starting with the same swelling procedure of mitochondria, Fraser et al. (2) then studied the effect of proteolytic enzymes on the regulation of malonyl-CoA-sensitive CPT activity and concluded that both the regulatory and catalytic site of CPT-I are on the cytosolic face of the outer membrane.

When we repeated the hypotonic swelling experiments, we

**Table IV**

| Substrate       | 2 mM ADP | 0.2 mM DNP | + 3.2 mM cytochrome c |
|-----------------|----------|------------|-----------------------|
| 20 mM pyruvate  | RLMpp    | 54.3 ± 0.7 | 40.1 ± 2.9            |
|                 | Mitoplasts | 38.0 ± 6.4 | 38.0 ± 7.3            | 54.9 | 38.7 |
| 20 mM succinate | RLMpp    | 62.0 ± 34.1| 41.5                  | 128.2 ± 52.7| 145.1 ± 28.7 |
|                 | Mitoplasts | 39.2 ± 3.6 | 46.1                  | 129.0 ± 8.8 | 156.9 |
| 20 mM durohydroquinone | RLMpp | 122.2 ± 14.1 | 119.2 | 166.1 ± 73.2 | 113.3 |
|                 | Mitoplasts | 69.2 ± 15.3 | 56.6 | 201.2 ± 26.8 | 148.4 |
| 1 mM TMPD, 10 mM ascorbate | RLMpp | 266.3 ± 13.4 | 305.2 | 309.1 ± 76.9 | 360.7 ± 104.0 |
|                 | Mitoplasts | 84.8 ± 6.7 | 89.5 | 336.5 ± 50.2 | 388.0 ± 80.6 |
| 40 μM palmitoyl carnitine | RLMpp | 109.5 ± 10.7 | 133.1 ± 7.5 | 118.8 ± 16.1 | 125.0 ± 40.5 |
|                 | Mitoplasts | 46.4 ± 3.0 | 54.1 ± 5.7 | 103.6 ± 8.3 | 127.1 ± 15.9 |
| 40 μM Palmitoyl-CoA + 5 mM carnitine | RLMpp | 83.0 ± 1.8 | 98.2 ± 10.2 | 91.8 ± 3.9 | 112.9 ± 5.6 |
|                 | Mitoplasts | 44.8 ± 1.6 | 52.6 ± 2.1 | 89.4 ± 13.0 | 113.5 ± 21.9 |
| 25 μM palmitate + 5 mM carnitine | RLMpp | 69.5 | 81.7 |
|                 | Mitoplasts | 44.0 | 62.3 |
found that the enrichment of malonyl-CoA-sensitive CPT in the outer membrane vesicles was only 20% that of monoamine oxidase, the premier marker of outer membrane (33). This lack of agreement may be explained by the fact that hypotonic phosphate treatment dislodges at least part of the CPT activity from mitochondria. Such liberated CPT conceivably could end up in an outer membrane fraction by entrapment within the membranous vesicular structures that Parsons et al. (31) found to be plentiful in this particular fraction. The history of intramitochondrial localization of enzymes is rife with examples of erroneous localizations that were based on the application of inappropriate methodologies.

Not only does hypotonic swelling have its drawbacks, but most other procedures for localizing CPT have associated pitfalls. For example, digitonin not only fragments the outer membrane by complexing with its cholesterol, but it also distorts the membrane phospholipids. The major problem with all three of these techniques is that the resultant mitoplasts manifest little to no respiratory control ratios, which is the hallmark of mitochondrial integrity.

A decided asset in our study is the use of electron microscopy to monitor the structural integrity and purity of each and every fraction. Although such an approach was commonplace in the early days of modern cell fractionation, the advantages of this protocol have largely been lost sight of nowadays. If electron micrographs accompany recent papers, they tend to be at relatively high magnifications. Thus, they provide a highly restricted view of the organelles under study and may not be representative of the sample as a whole.

To guard against sampling error, all of our samples are fixed in suspension, then pelleted. This fixation technique forestalls the stratification that occurs when organelle suspensions are first pelleted, then fixed. The pellets, which we have fixed in suspension, are subdivided, then embedded without regard to orientation. Embeddings are randomly selected for sectioning, and micrographs of random fields are taken at low magnification to obtain an overall picture of the sample under investigation. Of course, some organelles are examined at higher magnification to provide details of structure not discernible at lower power.

Using these protocols, we do not have to rely solely on biochemical evidence. We have direct visualization of the phenomenon of outer membrane removal, and the electron microscopy evidence can be roughly quantitated and correlated with biochemical data. Moreover, it becomes a simple matter to determine if our samples have been contaminated with any other cellular components.

As a further confirmation of our enzymic and morphological findings, we turned to immunoblotting to localize specific proteins. Although this technique is at best semi-quantitative, it provides additional information on enzyme localization. Using antibodies against MAO-B and porin, we found that these two proteins are enriched in the outer membrane fraction consistent with the localization of MAO activity. With an antibody directed against beef liver CPT-II, the inner form of CPT, the presence of MAO-B and porin, we found that these two proteins are enriched in the outer membrane fraction consistent with the localization of MAO activity. With an antibody directed against beef liver CPT-II, the inner form of CPT, the protein localized to the mitoplast fraction again consistent with
the enzymic activity data. With an antibody directed against a peptide representing amino acids 3–17 of CPT-I, the bulk of the protein that was decorated is present in the mitoplast fraction. The immunologically determined distribution of the various proteins reflects the fact that CPT-I is associated not only in some fashion with the inner membrane, but that it has a relationship with the outer membrane as well. A candidate for a structure that encompasses both inner and outer mitochondrial membranes is the so-called contact sites, which are localized segments where the inner and outer membranes are fused. Various functions, such as protein translocation (36, 37) and adenine nucleotide transport (38, 39), have been ascribed to contact sites. Recently, Fraser and Zammit (40) reported that CPT-I and CPT-II proteins, identified by immunoblot analysis, had a non-uniform distribution with enrichment in the contact site fraction from rat liver mitochondria. The localization of CPT-I in contact sites offers an explanation for the discrepant results obtained by the use of different membrane isolation techniques. We propose that hypotonic swelling results in the contact sites being removed in conjunction with the outer membrane, whereas treatment with the French press results in the contact sites being retained by the mitoplasts.

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