Involvement of microsomal vesicles in part of the sensitivity of carnitine palmitoyltransferase I to malonyl-CoA inhibition in mitochondrial fractions of rat liver

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Liver mitochondrial fractions as normally isolated contain only 10–20% of total mitochondria and may not be representative of the whole mitochondrial population. This study was designed to evaluate the dependence of the sensitivity of carnitine palmitoyltransferase I (CPT I) to malonyl-CoA inhibition in mitochondrial fractions that are not normally studied. Four fractions prepared from rat liver were found to be contaminated to different extents by microsome vesicles, on the basis of marker-enzyme activities and micrographic data. Purification of mitochondrial fractions on a Percoll gradient decreased to some extent the microsomal contamination, which was due in part to the existence of close bonds between microsomes and the outer membranes of mitochondria. A greater degree of contamination of mitochondrial fractions by microsomes was correlated with a greater sensitivity of CPT I to malonyl-CoA inhibition. Attempts were made to enhance the sensitivity of CPT I to malonyl-CoA with the use of microsomes. Measurements performed by adding mitochondria and microsomes in the same CPT I assay failed to demonstrate any significant enhancement of malonyl-CoA inhibition. However, addition of ATP to a mixture of mitochondria and microsomes was shown to trigger the binding of both particles, as assessed by enzymic and micrographic data, and to increase the sensitivity of CPT I to malonyl-CoA inhibition. These results demonstrated that the binding of microsomes to mitochondria, unlike the simple mixing of both particles, was capable of altering the sensitivity of CPT I to malonyl-CoA. The data also suggest that this process could be of physiological importance, owing to the frequency of contiguous zones between mitochondria and endoplasmic reticulum observed in sections of intact liver cells.

INTRODUCTION

Mitochondrial overt carnitine palmitoyltransferase (CPT I) in liver [1,2] and other tissues [3] is inhibited by malonyl-CoA. In the liver, this mechanism enables the reciprocal regulation of fatty acid synthesis and oxidation [2]. In liver mitochondria, CPT I has been located in the outer membrane [4], whereas the latent enzyme (CPT II), which is essentially insensitive to malonyl-CoA [1,5], is found in the inner membrane [4,6]. CPT I and CPT II have been shown to be distinct proteins [7]. The most interesting property of the CPT I enzyme is the sensitivity to malonyl-CoA inhibition, which differs in various physiological situations [8–11], after pharmacological treatments [12–14] or after alteration of mitochondrial membrane components [4,15,16]. Studies performed using detergents and membrane fluidizers suggested the involvement of phospholipids in the enzymic characteristics of CPT I [9,17]. Consequently, CPT I protein whose activity depends on its interaction with membrane phospholipids may be also susceptible to other properties of the membrane. Until now, few studies have been devoted to the properties of mitochondria when in close proximity to other organelles in intact cells or in isolated fractions. Indeed, many studies related to CPT I activity were performed on mitochondrial fractions without reference to the degree of contamination by peroxisomes and microsomes. These contaminating organelles have been shown to exhibit acylcarnitine transferase activities [18,19], which are themselves sensitive to malonyl-CoA inhibition [19,20]. In addition, in micrographic sections of intact hepatocytes, the endoplasmic reticulum is often observed to be close to, and sometimes surrounding, mitochondria [21,22]. Consequently, non-mitochondrial organelles present in mitochondrial fractions might alter the apparent CPT I activity through the level of contamination and the nature of their relations with the mitochondrial outer membranes. These observations prompted us to evaluate CPT I activity and its sensitivity to malonyl-CoA in mitochondrial fractions contaminated, naturally or artificially, by microsomes. The results show that microsomal membranes, when bound to mitochondria, enhance the sensitivity of CPT-I to malonyl-CoA inhibition.

EXPERIMENTAL

Materials

L-[*methyl*-^{3}H]Carnitine was obtained from Amersham International (Amersham, Bucks., U.K.). Unlabelled L-carnitine was given by Dr. C. Cavazza of Sigma-Tau (Pomezia, Italy). Fatty-acid-free BSA (fraction V) used in homogenization mixtures was from Paesel-Lorei (Frankfurt, Germany). Percoll was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Papain was from Boehringer (Meylan, France). Coenzymes, CoA derivatives, other biochemicals and standard lipids were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and from NuChek Prep. (Elysian, MN, U.S.A.). Chemicals obtained from Prolabo (Paris, France) and Merck (Darmstadt, Germany) were of analytical grade.

Abbreviations used: CPT, carnitine palmitoyltransferase (CPT I, overt form; CPT II, latent form).

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Male Wistar rats were bred in the laboratory and kept at 23 °C in a light-controlled room (light period fixed between 08:00 and 20:00 h). They had free access to tap water and were fed on standard laboratory chow (AO3; UAR, 91360 Epinay-sur-Orge, France) containing 58.7% carbohydrate, 17% protein and 3% fat. When about 9 weeks old, they were starved for 20 h or kept feeding, then stunned and killed by exsanguination at 08:00 h.

Preparation of subcellular fractions

Mitochondrial fractions (M1–M4)

The liver was quickly removed, cooled to 4 °C in 0.25 M sucrose, cut into small pieces, rinsed several times, blotted with paper and weighed. The tissue was then homogenized in 10 vol. of 0.25 M sucrose medium containing 10 mM Tris/HCl buffer, pH 7.4, 1 mM EGTA and 1% albumin [3] by only two strokes of a Teflon pestle rotating at 120 rev./min in a cooled Potter–Elvehjem homogenizer. The homogenate was centrifuged at 2000 *g* for 2 min at 3 °C, and the supernatant was immediately centrifuged at 13000 *g* for 2 min. The pellet was resuspended in the sucrose medium without albumin and centrifuged at 13000 *g* for 2 min. The pellet was washed once again under the same conditions, resuspended in buffered 0.3 M sucrose and stored as the M1 mitochondrial fraction. The pellet of the centrifuged homogenate was then homogenized as the initial liver tissue, but by using one more stroke of the Teflon pestle, and treated similarly for the subsequent steps to obtain the M2 mitochondrial fraction. This procedure was repeated on the pellets of the centrifuged second and third homogenates, and gave rise to the M3 and M4 mitochondrial fractions respectively. Albumin was added to protect the membrane integrity by binding non-esterified fatty acids, which are likely to be released at every step of the procedure and are known to induce mitochondrial swelling [23].

Purified mitochondrial fraction (M-Percoll)

The procedure was adapted from that described by Zammit et al. [24]. The mitochondrial fraction to be purified was suspended in a mixture of 0.25 M sucrose medium containing 10 mM Tris/HCl buffer, pH 7.4, and 1 mM EGTA, and of Percoll (31% by vol. of the final mixture, pH 7.4). The diluted mitochondrial suspension was loaded in volumes of 20 ml on the top of 20 ml of a Percoll mixture prepared as above, but containing 0.3 M sucrose, in 40 ml centrifuge tubes. In some experiments, 0.25 M and 0.3 M sucrose was replaced by 0.125 and 0.15 M KCl, respectively, to enhance ionic strength. Where indicated, papain was added to the 20 ml of the upper layer. After centrifugation at 30000 *g* for 20 min, mitochondria, which sedimented as a fluffy layer, were withdrawn, diluted in buffered 0.25 M sucrose, washed free of Percoll by centrifugation at 3500 *g* for 10 min, then stored in buffered 0.3 M sucrose as the M-Percoll mitochondrial fraction.

Mitochondrial outer-membrane fraction (OM)

Mitochondrial outer membranes were prepared by the modified procedure of Parsons et al. [25] by using fractions M1–M3 purified on Percoll. Pooled mitochondrial fractions were centrifuged to remove sucrose. The pellets were gradually resuspended first with a glass rod, then by two mild hand-driven strokes of a Dounce B homogenizer in 320 ml of medium containing 20 mM KH_2PO_4, pH 7.4, and 0.02% fatty-acid-free albumin. After 20 min on ice, an amount of ATP corresponding to one-fifth of the mitochondrial protein content (w/w), estimated by u.v. assay [26], was added. After 5 min at 0 °C, outer membranes were separated from the inner-membrane/matrix fraction of mitochondria by centrifugation at 30000 *g* for 8 min. The pellet was suspended in the phosphate medium as above, and the heavier fraction of outer-membrane-free and non-disrupted mitochondria were removed by centrifugation at 3500 *g* for 8 min. The supernatant was centrifuged at 30000 *g* for 8 min and gave the crude preparation of outer membranes. Purification was performed on a discontinuous sucrose gradient at 80000 *g* for 3 h. Outer membranes were collected at the interface of the layers of density 1.1175 and 1.1390 g/ml, diluted in 3 vol. of 20 mM KH_2PO_4, pH 7.4, concentrated by centrifugation at 150000 *g* for 30 min, and stored in buffered 0.25 M sucrose as the mitochondrial outer-membrane fraction.

Microsomal fraction

The supernatant of the first M1 mitochondrial fraction was centrifuged at 18000 *g* for 20 min at 3 °C to yield a pellet containing the remaining mitochondria, peroxisomes and part of the microsomes, as shown by marker enzyme-activities. The supernatant centrifuged at 104000 *g* for 40 min at 3 °C gave a pellet which was suspended in buffered 0.25 M sucrose, distributed into 12.5 ml fractions on the top of 12.5 ml of buffered 1.2 M sucrose and sedimented as above to eliminate cytosolic proteins and albumin originating from the homogenization step. The microsomal pellet was easily separated from the lower translucent layer of glycogen by gentle shaking (see the section of pelleted crude microsomal fraction in Figure 1c), then stored in buffered 0.25 M sucrose as the microsomal fraction.

Peroxisomal fraction

The procedure for isolating a purified peroxisomal fraction from a 'light' mitochondrial fraction was that of Völkl and Fahimi [27].

Measurement of CPT I activity

Measurements were performed as described by Bremer [28] with slight modifications in a medium containing 80 mM mannitol, 75 mM KCl, 25 mM Hepes, pH 7.4, 0.2 mM EGTA, 0.5 mM dithiothreitol, 2 mM KCN, 1% fatty-acid-free albumin and 40 μM palmitoyl-CoA. Inhibition of CPT I by malonyl-CoA was carried out by incubating mitochondria in the presence of both palmitoyl-CoA and malonyl-CoA 2 min before adding L-[^{3}H]carnitine. After 4 min, acyl[^{3}H]carnitines were extracted with butan-1-ol [29] and the radioactivity was measured in Picofluor 15 (Packard Instrument Co.) by using a Packard 300 C scintillation counter. Controls were performed in the absence of palmitoyl-CoA.

Membrane markers

The following enzyme markers were used: monoamine oxidase [30] for outer membranes, cytochrome *c* oxidase [31] for inner membranes and glutamate dehydrogenase [32] for the matrix compartment of mitochondria, catalase [33] and uricase [34] for peroxisomes, and aryl-ester hydrolase [31] and glucose-6-phosphatase [35] for microsomes. Because microsomes are far richer in cholesterol (about 42 ± 2 μg/mg) than are mitochondria (0.8 ± 0.2 μg/mg, lowest values found), cholesterol can be considered as another possible microsomal marker in mitochondrial fractions. Its content was determined by g.l.c. [36]. Under the conditions used, papain was shown not to decrease either the aryl-ester hydrolase activity of microsomal fractions or the

monoamine oxidase activity of mitochondrial fractions (results not shown).

Protein

Rapid protein determinations were performed by spectrophotometry [26] just before incubations, and were later confirmed by the procedure using bicinchoninic acid as described by Smith et al. [37].

Microscopy

Mitochondrial and microsomal pellets were fixed in 4.16% glutaraldehyde for 24 h, washed in 0.33 M sucrose for 3 × 30 min and post-fixed in 2% osmium tetroxide for 1 h. All operations were carried out at 4 °C in solutions buffered to pH 7.25 with 0.1 M phosphate. Samples were dehydrated at room temperature through successive changes of 30%, 50%, 70% ethanol (10 min each) and 100% ethanol (2 × 10 min). Maximal dehydration was performed through two changes of propylene oxide. Impregnation was made in Epon/propylene oxide [1:1 and 7:3 (v/v), 45 min each]. Pellets were then transferred into pure Epon for 1 h (two changes) and left overnight. Samples were finally transferred into fresh pure Epon for 1 h and embedded. Sections were obtained with a diamond knife on a Reichert Ultracut E instrument. They were recovered with carboned collodion membrane on copper grids and treated according to the Reynolds Ur–Pb technique [38]. Observations were made with a Hitachi H600 electron microscope.

RESULTS AND DISCUSSION

Characteristics of the mitochondrial fractions

The procedure of mitochondrial isolation allowed extraction from liver of four fractions. When the mitochondrial protein content of 1 g of liver was assessed by dividing the cytochrome *c* oxidase activity found in the corresponding homogenate by that of 1 mg of protein of purified mitochondrial fraction (M-Percoll), the total amount of protein recovered in the four fractions (Table 1) represented about 50% of the total calculated mitochondrial protein content. Other measurements of monoamine oxidase and cytochrome *c* oxidase activities have shown that the non-recovered mitochondria were lost partly with lighter particles during the washing steps (8–12%), whereas the greater proportion (about 40%) was still retained in the last nuclear pellet after four homogenization/centrifugation steps as described in the Experimental section. About half of mitochondrial protein of the pooled M1–M3 fractions equilibrated at the bottom of the Percoll gradient (Table 1), suggesting that a large part of the mitochondrial population had a lower density. These data indicate that the procedures used for isolating mitochondria are selective and result in a loss of more than 50% of total liver mitochondria. These different mitochondrial fractions, which show different physical features, might also display slightly different biochemical properties. In Tables 1 and 2 similarities and differences between fractions M1–M4 are noteworthy. The specific activities of monoamine oxidase and cytochrome *c* oxidase slightly decreased in going from fractions M1 to M4. Under the conditions used, the purity of the fractions containing whole mitochondria did not exceed 75% (Table 1), implying the presence of contaminating elements. The specific activity of glutamate dehydrogenase, which is a soluble enzyme of the mitochondrial matrix, was comparable in all fractions. This result suggests that the inner membranes were not permeabilized by the successive steps of the isolation procedure. Under these

Table 1 Characteristics of the mitochondrial fractions isolated from rat liver

Fractions M1–M4 were prepared as indicated in the Experimental section. The M-Percoll fractions originated from the pooled fractions M1–M3 purified on a Percoll gradient. The outer-membrane fractions (OM) were obtained from M-Percoll fractions. The true mitochondrial protein content of fractions was given as a percentage of total protein in the mitochondrial fractions. This value, which indicates the degree of purity, was calculated by dividing the specific activity of cytochrome *c* oxidase in each mitochondrial fraction by that found in a fraction particularly pure obtained after two Percoll purifications followed by careful washings (3.8 μmol/min per mg of protein), and multiplied by 100. Results are means ± S.E.M. ($n = 6$).

Fraction	Protein per total fraction (mg/g wet wt. of tissue)	Monoamine oxidase specific activity (nmol/min per mg of protein)	Cytochrome *c* oxidase specific activity (μmol/min per mg of protein)	Glutamate dehydrogenase specific activity (μmol/min per mg of protein)	Purity of fractions (% of total protein)
M1	14.5 ± 1.0	11.3 ± 1.1	2.80 ± 0.25	2.90 ± 0.15	73.6 ± 6.5
M2	12.0 ± 1.2	11.3 ± 1.0	2.75 ± 0.25	2.80 ± 0.20	72.3 ± 6.5
M3	4.0 ± 1.2	10.9 ± 1.1	2.50 ± 0.20	2.90 ± 0.10	65.8 ± 5.2
M4	2.0 ± 1.2	9.8 ± 1.0	2.25 ± 0.30	2.90 ± 0.15	59.2 ± 7.9
M-Percoll	14.8 ± 1.5	13.1 ± 0.9	2.75 ± 0.20	3.0 ± 0.1	72.4 ± 5.2
OM	0.5 ± 0.1	240 ± 20	0.20 ± 0.20	0	—

Table 2 Enzymic activities and amounts of protein in peroxisomes and microsomes contaminating the isolated mitochondrial fractions

Fractions M1–M4 were prepared as indicated in the Experimental section. The M-Percoll fractions originated from the pooled fractions M1–M3 purified on a Percoll gradient. The outer-membrane fractions (OM) were obtained from the M-Percoll fractions. The specific activities (nmol/min per mg of protein) of uricase and catalase for peroxisomes, and of glucose-6-phosphatase (G-6-Pase) and aryl-ester hydrolase for microsomes, are means ± S.E.M. ($n = 6$). The contamination by peroxisomes and microsomes were given as a percentage of total protein in the mitochondrial fractions. Values were calculated by dividing the specific activity of uricase or aryl-ester hydrolase found in the mitochondrial fractions by that of uricase in a purified peroxisomal fraction (0.52 μmol/min per mg of protein) or of aryl-ester hydrolase in a purified microsomal fraction (4.6 μmol/min per mg of protein), and multiplied by 100.

Fraction	Uricase	Catalase	G-6-Pase	Aryl-ester hydrolase	Percentage of contamination	
					Peroxisomes	Microsomes
M1	54.0 ± 4.0	500 ± 50	1.75 ± 0.25	470 ± 70	10.4 ± 0.8	10.2 ± 1.5
M2	37.5 ± 2.5	150 ± 45	2.70 ± 0.25	800 ± 100	7.2 ± 0.5	17.4 ± 2.2
M3	27.0 ± 2.0	90 ± 40	3.45 ± 0.20	1090 ± 80	5.2 ± 0.4	23.7 ± 1.7
M4	17.0 ± 3.5	50 ± 25	3.80 ± 0.30	1180 ± 100	3.3 ± 0.7	25.6 ± 2.2
M-Percoll	5.0 ± 1.5	20 ± 9	0.65 ± 0.15	110 ± 20	0.9 ± 0.3	2.4 ± 0.4
OM	0.5 ± 0.3	0	2.35 ± 0.40	4.20 ± 130	< 0.1	9.1 ± 2.8

conditions, palmitoyl-CoA used in CPT I assays cannot reach CPT II sites on the inner face of the inner membranes, which would otherwise have interfered with measurement of the CPT I reaction. In Table 2, fractions M1 and M2 appeared to display the greatest specific activities of uricase and catalase. Peroxisomes which contain these activities have been reported to contain a carnitine acyltransferase activity sensitive to malonyl-CoA inhibition [20]. The lower specific activities of both marker enzymes in M3, M4 and M-Percoll fractions decreased the possible interference of peroxisomal carnitine acyltransferase in our CPT I assays (3.3 and 0.9% contamination in M4 and M-Percoll fractions respectively, on a protein basis).

Contamination of mitochondrial fractions by microsomes

In contrast, the specific activities of glucose-6-phosphatase and aryl-ester hydrolase, which increased from fraction M1 to fraction M4 (Table 2), attest to a progressively greater contamination of fractions by microsomes (25.6% in fraction M4). These biochemical data were supported by observations of sections of pelleted M4 fractions showing numerous microsomal vesicles bound to the outer membranes of mitochondria (see Figure 1, panel b versus panel a). Later, aryl-ester hydrolase activity was preferred as a marker of microsomes, because of its very regular distribution in the different isolated forms of microsomes, unlike glucose-6-phosphatase or NADPH–cytochrome *c* oxidoreductase [39,40]. By use of this marker, microsomes were shown to be still contaminating OM fractions (Table 2). In order to determine whether the contamination in these latter fractions was supported by unbound and/or loosely bound microsomes, the procedure of OM isolation was repeated, but with an altered mitochondrial swelling step. The mixture usually containing 20 mM phosphate, pH 7.4, and 0.02% albumin was added with 100 mM sucrose. In this instance, swelling of mitochondria and shedding of outer membranes did not occur. It was then later impossible to recover any equivalent or partial microsomal activity in the layer of the sucrose gradient in which OM were usually equilibrated, suggesting that microsomal vesicles co-migrated with OM under some firm associations. Resistance of bonds between mitochondria and microsomes was checked by applying different treatments (Table 3) to the mitochondrial M1 fraction likely to contain both unbound and bound microsomes. As glutamate dehydrogenase activity was not altered under any of these conditions, the treatments used appeared to preserve integrity of mitochondrial inner membranes. Raising of the ionic strength by using KCl in place of sucrose did not decrease microsomal contamination, as assessed by the maintenance of aryl-ester hydrolase specific activity and the relatively high cholesterol content when expressed per mg of protein. However, the Percoll gradient in sucrose or KCl medium greatly decreased both aryl-ester hydrolase activity and cholesterol content. This decrease probably corresponded to the removal of unbound and weakly bound microsomes, since another level of purification was only obtained by a treatment combining ionic strength, Percoll gradient and proteolysis by papain (Table 3). Under this latter condition, the removed and the remaining aryl-ester hydrolase activity should correspond to strongly bound microsomes and may account for a large part of the activity recovered in OM fractions (Table 2). Indeed M4 fractions appeared to be very rich in strongly bound particles, as already suggested by the electron micrographs (Figure 1b). But, unlike M1 and M2 fractions, M4 fractions did not give rise to any visible mitochondrial pellet after purification on Percoll (results not shown), the strength of whole associations being sufficient to oblige bound particles to migrate into the top layer as free microsomes. The micrographic evidence and the ability to decrease progressively the microsomal activity by appropriate treatments suggested that microsomal vesicles were mainly bound to mitochondria by contiguity. The actual extent of the associations between mitochondria and microsomes could even be underestimated, because a large part of liver mitochondria (about 80%; I. Niot, F. Pacot, P. Bouchard, J. Gresti, A. Bernard, J. Bezard and P. Clouet, unpublished work) and of microsomes (50%, as stated by Lewis and Tata [21]) were lost in nuclear pellets after centrifugation of liver homogenates. Specific procedures allow the isolation of complexes of mitochondria closely associated with rough endoplasmic reticulum [21,22], called 'mito-RER complexes' [22]. Drastic treatments [41,42] used to dissociate the complexes also suggested that tight links or even membrane continuities exist between membranes of both organelles [42,43]. It is noteworthy that microsomes exhibit a medium-chain carnitine acyltransferase activity, which is very sensitive to malonyl-CoA inhibition [19].

CPT I activity and sensitivity to inhibition by malonyl-CoA

CPT I activity was studied in mitochondrial fractions containing mainly either unbound microsomes (M1 fraction) or bound microsomes (M4 fraction) by comparison with purified M-Percoll fraction. The legend of Figure 2 indicates that the specific

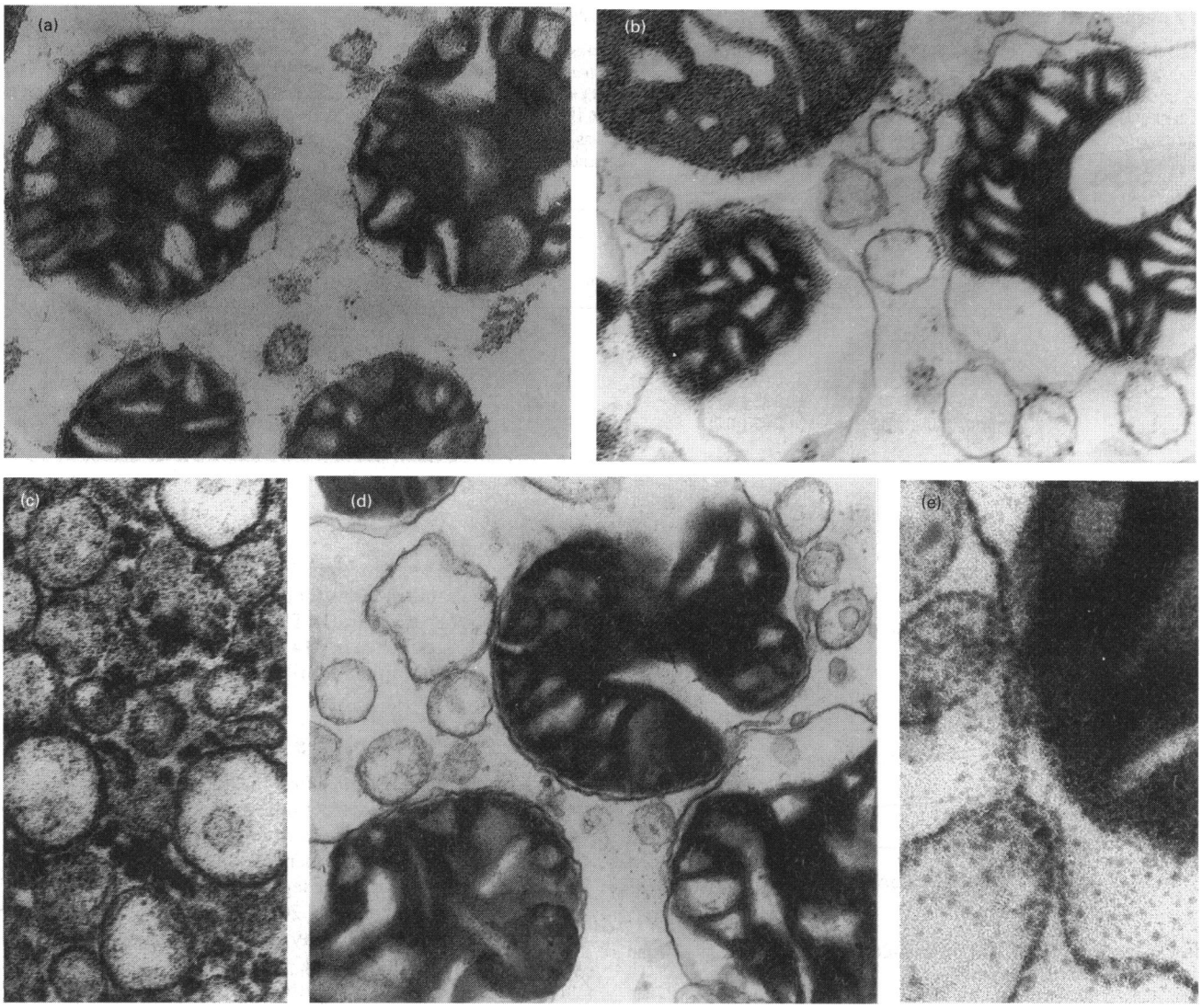

Figure 1 Sections of the mitochondrial and microsomal pellets corresponding to the main fractions used in the study

Glutaraldehyde/osmium tetroxide-fixed and Epon-embedded samples of mitochondrial and microsomal pellets were sectioned and treated by Ur/Pb technique as indicated in the Experimental section. The sections of pelleted fractions correspond to the M-Percoll mitochondrial fraction (**a**: magnification × 52500) with no vesicles bound to mitochondria, to the M4 mitochondrial fraction (**b**: magnification × 52500) with numerous microsomes bound to outer membranes of mitochondria, to the crude microsomal fraction (**c**: magnification × 90000) with microsomal vesicles of different sizes and multiple glycogen rosettes justifying its further purification as indicated in the Experimental section, to the mitochondrial fraction containing microsomal vesicles which have been artificially bound on to mitochondria (**d**: magnification × 52500) as described in Table 4, and to a detail of the preceding fraction (**e**: magnification × 168000) showing that membranes of both particles were bound contiguously.

activity of CPT I was greater in fraction M1 than in M4 and intermediary in M-Percoll fractions. Moreover, CPT I activity appeared to be slightly more sensitive to malonyl-CoA inhibition in M1 fractions than in M-Percoll fractions, and the most sensitive in M4 fractions (Figure 2). It was surprising to find nearly the same sensitivity to malonyl-CoA in purified M-Percoll fractions as in M1 fractions, in spite of their different contamination by peroxisomes and microsomes (Table 2), as both contain carnitine acyltransferase activities which are very sensitive to malonyl-CoA inhibition. The lack of specificity of carnitine acyltransferases of contaminating particles for palmitoyl-CoA as a substrate [19] may explain in part the very similar sensitivity to malonyl-CoA inhibition observed in M1 fractions. In contrast, M4 fractions poorer in peroxisomes, but richer in microsomes, displayed the greatest sensitivity. In an attempt to find whether this particular sensitivity was due to free microsomes, CPT I assays were carried out with either M-Percoll fractions alone, or M-Percoll and microsomal fractions in proportion to the degree of microsomal contamination found in M4 fractions. The specific activity of aryl-ester hydrolase, which was 4.6 ± 0.3 and 1.18 ± 0.1 μmol/min per mg of protein in microsomal and M4 fractions respectively, results in an estimate of about 25% for the degree of contamination. Under these experimental conditions, and using fractions isolated from the same 20 h-fasted rat (results representative of three different experiments), the specific activity of CPT I was 1.68 and 1.48 nmol/min per mg of protein with the mitochondrial fraction alone and the mixture of mitochondria and microsomes respectively. Moreover, the inhibition by malonyl-CoA was found not to differ to any great extent between both assays. The slight

Table 3 Influence of the washing-step procedure on aryl-ester hydrolase activity recovered in fractions obtained from the same mitochondrial preparation

A bulk M1 fraction was prepared from three livers with 0.25 M sucrose medium containing 10 mM Tris/HCl buffer (pH 7.4), 1 mM EGTA and 1% albumin, and distributed in 1 ml fractions (30 mg of protein). A final step of purification was performed by washing the fractions in a total volume of 40 ml with mixtures all containing 1 mM EGTA and 10 mM Tris/HCl (pH 7.4), and either 0.25 M sucrose (*a*, control assay) or 0.125 M KCl (*b*), or 0.25 M sucrose containing 31% Percoll (by vol.) layered on the same volume of a similar mixture containing 0.3 M sucrose (*c*), or 0.125 M KCl containing 31% Percoll (by vol.) layered on the same volume of a similar mixture containing 0.15 M KCl (*d*). In the last assay (*e*), papain (2 mg) was included in the upper layer prepared as for (*d*), and then left for 10 min at 4 °C in the presence of organelles. The mitochondrial fractions were then isolated from mixtures without Percoll (*a, b*) or containing Percoll (*c, d, e*) under the conditions used for the M1 fraction and the purified mitochondrial fraction, respectively, as described in the Experimental section. The final pellets were suspended in buffered 0.3 M sucrose. Results are means ± S.E.M. of three different experiments.

	Aryl-ester hydrolase activity (μmol/min per mg of protein)	Glutamate dehydrogenase activity (μmol/min per mg of protein)	Cholesterol content (μg/mg of protein)
(*a*) 0.25 M sucrose	0.90 ± 0.05	3.35 ± 0.13	8.0 ± 0.2
(*b*) 0.125 M KCl	1.02 ± 0.04	3.20 ± 0.15	8.1 ± 0.2
(*c*) Percoll/sucrose	0.40 ± 0.03	4.00 ± 0.12	2.00 ± 0.15
(*d*) Percoll/KCl	0.36 ± 0.03	4.05 ± 0.09	2.05 ± 0.18
(*e*) Percoll/KCl + papain	0.09 ± 0.02	4.38 ± 0.10	0.90 ± 0.18

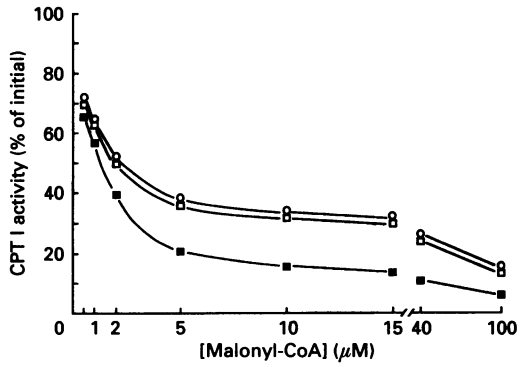

Figure 2 Sensitivity of CPT I to malonyl-CoA inhibition in the M1, M4 and M-Percoll mitochondrial fractions

The purified M-Percoll fraction (○) was prepared from the M1 fraction (□) isolated from liver of fed rats. The M4 fraction (■) originated from the third supernatant obtained after successive resuspensions and centrifugations of nuclear pellets as detailed in the Experimental section. Results are given as percentages of the values obtained in the absence of added malonyl-CoA (in the presence of 40 μM palmitoyl-CoA, initial CPT I specific activities were found to be 2.98, 2.75 and 2.82 nmol/min per mg of protein in the assayed M1, M4 and M-Percoll fractions respectively). Differences between curves are representative of three independent experiments.

difference observed between both curves (results not shown) was almost of the same order as that obtained between M-Percoll and M1 fractions (as in Figure 2). The question then arose of whether the enhanced sensitivity in M4 fractions was due to the binding of microsomes to mitochondrial outer membranes.

Artificial binding of microsomes to mitochondria

Attempts to reproduce *in vitro* the binding of microsomes to mitochondria as in M4 fractions were made by assuming that mitochondria of M4 fractions which would have been cleared of microsomes would have properties similar to those of M-Percoll fractions. As described in the legend of Table 4, a Percoll-purified fraction was prepared from 20 h-starved rats to obtain mitochondria whose CPT I activity was then relatively insensitive to malonyl-CoA inhibition [44]. Microsomal fractions were prepared the day before from either fed or starved rats (they gave qualitatively similar results) and frozen until utilization. Mixing of mitochondria and microsomes did not result in a significant binding of particles, because microsomes were largely eliminated by two washing steps, as assessed by aryl-ester hydrolase specific activity (1.6 and 0.37 nmol/min per mg of protein in the initial mixture and the final suspension, respectively) (Table 4). In

Table 4 Enzyme activities and cholesterol content of the mitochondrial fractions containing microsomal vesicles artificially bound to mitochondria

A 10 mg amount of protein of the Percoll-purified mitochondrial fraction from 20 h-starved rats was suspended in 2 ml of medium containing 210 mM mannitol, 70 mM sucrose and 10 mM Tris/HCl buffer (pH 7.4), and mixed with 5 mg of protein from the microsomal fraction (0.5 ml). ATP (5 mg in 0.5 ml of buffered 0.3 M sucrose) was then gradually added. After 5 min at 0 °C, the mixture was diluted to 40 ml with buffered 0.25 M sucrose and centrifuged at 13000 *g* for 2 min. The pellet resuspended in 40 ml of the same medium was sedimented at 3500 *g* for 8 min and finally stored in buffered 0.3 M sucrose. The washing procedure when applied only to microsomal proteins did not give rise to any visible pellet. Results are means ± S.E.M. of three different experiments.

	Percoll-purified mitochondrial fraction (10 mg protein)			
Microsomal protein (5 mg)...	0	0	+	+
ATP (5 mg)...	0	+	0	+
Enzyme activity (nmol/min per mg of protein)				
Carnitine palmitoyltransferase I	2.10 ± 0.08	2.34 ± 0.10	2.01 ± 0.05	2.21 ± 0.07
Monoamine oxidase	13.4 ± 0.9	12.1 ± 0.6	11.7 ± 0.4	10.5 ± 0.3
Aryl-ester hydrolase	172 ± 12	267 ± 20	370 ± 33	1015 ± 50
Cholesterol content (μg/mg of protein)	1.2 ± 0.2	2.3 ± 0.5	4.0 ± 0.6	14.0 ± 1.1

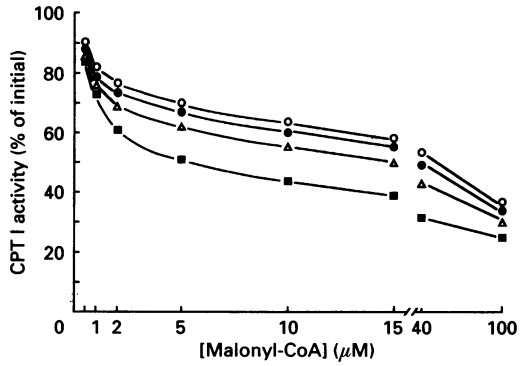

Figure 3 Sensitivity of CPT I to malonyl-CoA inhibition in the mitochondrial fractions containing microsomes artificially bound to mitochondria

The fractions were prepared as described in the legend of Table 4. They originated from the same Percoll-purified mitochondrial fraction isolated from 20 h-starved rats, which was either only submitted to the washing procedure [● (= M-Percoll)] or supplemented with ATP (○), microsomal protein (△), or with microsomal protein and ATP (■). The fractions were used after removing unbound microsomes and ATP by centrifugation. Results are given as percentages of the values obtained in the absence of malonyl-CoA. Differences between curves are representative of those obtained for three independent experiments.

contrast, under the conditions described, the addition of ATP triggered binding between particles (see Figure 1d), which resisted washing (see the Experimental section), since the final pellet exhibited a high aryl-ester hydrolase specific activity and a high cholesterol content (Table 4). When submitted to centrifugation on a Percoll gradient, all the protein of the rehomogenized pellet was found in the less-dense top layer, as observed for the M4 fraction. As shown in Figure 1(e), the microsomes and mitochondria in this fraction were bound by contiguities between their membranes. So far, the artificial binding of both organelles in the presence of ATP is not understood, but it provides a useful model for further studies.

Sensitization of CTP I to malonyl-CoA inhibition in mitochondria associated with microsomes

Figure 3 reports of the effect of the binding of microsomes to mitochondria on the sensitivity of CPT I to malonyl-CoA inhibition. It was observed that mitochondria first mixed with microsomes, and then re-isolated, gave rise to slightly higher sensitivity of the enzyme to malonyl-CoA, concomitantly with some binding of microsomes, as indicated by a slightly higher aryl-ester hydrolase specific activity of the re-isolated fraction (Table 4). Therefore surfaces of microsomal membranes have the physical ability to bind to mitochondrial surfaces even in the absence of added ATP and are able to alter the sensitivity of CPT I to the inhibitory effect of malonyl-CoA. But after binding in the presence of ATP, the sensitivity of the re-isolated mitochondrial fraction was markedly enhanced and was qualitatively what was observed with M4 fractions alone (Figure 2). ATP is not involved by itself in this process, since it did not change the level of sensitivity when added alone to mitochondria (Figure 3).

Whether associations between microsomes and mitochondria are artefacts that arise during tissue homogenization is questionable. However, such associations have also been widely described in liver cells of normal and treated rats [45] and have led to the development of particular procedures for their isolation [22]. Nevertheless, the nature of factors that induce their formation and the physiological significance of this phenomenon remain to be determined. The studies of McGarry et al. [46] suggest that the same CPT-I protein supports both the catalytic site and the regulatory part binding malonyl-CoA in the outer membrane. The efficiency of the malonyl-CoA binding site is believed to be modulated by the particular lipidic environment of the membrane [16,47]. Kolodziej and Zammit [16] have shown that the treatment of mitochondrial outer membranes with fluidizing agents enhanced CPT I activity and decreased the sensitivity of the enzyme to malonyl-CoA inhibition. Conversely, since microsomes are far richer in cholesterol than are mitochondria (see the Experimental section) and since cholesterol is known to decrease membrane fluidity, the binding of microsomes to mitochondria might confer on these latter particles a more ordered lipid environment, which would favour the binding of malonyl-CoA. All these points deserve further investigation, because associations of mitochondria and microsomes, which are present in the mitochondrial preparations as normally prepared, may represent about half of the liver mitochondrial population. In addition, owing to the frequency of associations between mitochondria and endoplasmic reticulum in liver cells, it should be of interest to study the consequences of such associations on the properties of either organelle.

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