RESEARCH COMMUNICATION

Enrichment of carnitine palmitoyltransferases I and II in the contact sites of rat liver mitochondria

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The submitochondrial distribution of the overt and latent carnitine palmitoyltransferases (CPT I and II respectively) of rat liver mitochondria were studied. Separation of outer and inner membranes, as well as of a fraction of intermediate density consisting of contact sites between the two membranes, was achieved, as judged by the distribution of marker enzymes. Both CPT I and CPT II were found to be enriched within the contact-site fraction of mitochondria. These data show that the two carnitine acyltransferases are distributed non-uniformly within their respective membranes, and that subpopulations of the two enzymes occur in close proximity within the mitochondrial membrane structure, while retaining their different accessibilities to cytosolic and matrix pools of metabolites. As the number of contact sites is known to vary with changes in the energy status of mitochondria, the possibility that such changes may acutely affect the proportion of CPT I within the distinctive lipid environment of the contact sites, and thus its overall kinetic characteristics, is discussed.

INTRODUCTION

Transfer of long-chain acyl moieties into the mitochondrial matrix is achieved through the successive and opposing reactions catalysed by two carnitine palmitoyltransferases (CPTs): (i) an overt outer membrane carnitine palmitoyltransferase (CPT I), which transfers the acyl group from CoA to carnitine, and (ii) a latent inner membrane CPT II which catalyses the reverse reaction of that catalysed by CPT I. The two reactions are linked by an inner membrane carnitine–acylcarnitine carrier. CPT II is synthesized with an N-terminal mitochondrial inner membrane/matrix signal peptide that is cleaved when the protein is processed to its mature form and inserted into the inner membrane [1]. In contrast, CPT I, in common with all other outer membrane proteins that have been studied, lacks a cleavable signal peptide, and the mature protein is the same size as the nascent protein, incorporating its N-terminus [2]. Membrane topography of CPT I has indicated that both the active and regulatory (malonyl-CoA-binding) sites of CPT I are exposed on the outer face of the outer membrane [3]. Consequently, CPT I can interact with the cytosolic pool of long-chain acyl-CoA (complexed to acyl-CoA binding protein [4]). CPT II is not accessible to cytosolic acyl-CoA esters in intact mitochondria, since its active site is located on the inner aspect of the inner membrane [5]. However, work by Kerner and Bieber [6] has indicated that both malonyl-CoA-sensitive (CPT I) and -insensitive (CPT II) carnitine acyltransferase acitivities can be isolated in a complex with β-oxidation enzymes which are known to reside in the inner membrane of mitochondria. These data raised the prospect that both CPTs I and II could be abundant within the contact sites that exist between the respective peripheral regions of the outer and inner membranes of mitochondria [7]. More recently, data published in abstract form [8] has indicated that mitoplasts prepared by French-press treatment of liver mitochondria retain considerable CPT I activity, again suggesting that any outer membrane still associated with the inner membrane may be enriched in CPT I.

Therefore we considered it necessary to investigate systematically the submitochondrial distribution of CPT I and CPT II. Our data indicate that both proteins are highly enriched within the contact sites of isolated rat liver mitochondria.

MATERIALS AND METHODS

Subfractionation of mitochondria

Rat liver mitochondria were prepared from fed female animals (200–220 g body weight) by differential centrifugation in a medium containing 300 mM sucrose/5 mM Tris/HCl/1 mM EGTA, pH 7.4, and purified on self-forming 30 % Percoll gradients prepared in the same medium, as described previously [9]. They were washed twice in the sucrose medium. Submitochondrial fractionation was performed essentially as described previously [10]. Briefly, the final mitochondrial pellet was re-suspended in hypotonic medium (10 mM potassium phosphate, pH 7.4) to give a total volume of 2.14 ml (40–60 mg of protein/ml). The suspension was incubated on ice for 20 min, followed by addition of 0.86 ml of 60 % (w/v) sucrose. After a further 20 min period of incubation on ice, the suspension was divided into two, and each aliquot was sonicated (three periods of 30 s delivered over 3 min) using the microtip of a Contes ultrasonic disintegrator set at 70 % of maximal amplitude, while cooling at 0 °C. The resulting suspension was centrifuged at 3000 ***g*** for 10 min to remove debris, and the supernatant was layered on top of a continuous sucrose gradient (density 1.113–1.227 g/l) prepared in 10 mM potassium phosphate buffer (pH 7.4) in 13 ml centrifuge tubes. Separation of the different fractions was achieved by centrifugation at 105000 ***g*** for 20 h at 4 °C, followed by positive displacement with 2.26 M sucrose and collection of 0.5 ml fractions from the top of the tube. All procedures were carried out at 4 °C. The fractions were divided into several aliquots, and frozen at −20 °C until used for enzyme activity measurements, protein determination [11] (after sedi-

Abbreviations used: CPT I and II, carnitine palmitoyltransferases I (overt) and II (latent) of mitochondria; rNCR, rotenone-insensitive NADPH-cytochrome *c* reductase; cyt-ox, cytochrome *c* oxidase.

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mentation at 100000 *g* of 20-fold dilutions in water), or SDS/PAGE.

Enzyme activity measurements and Western blotting

The marker enzymes used to identify the fractions were adenylate kinase (intermembrane space), rotenone-insensitive NADPH-cytochrome *c* reductase (rNCR; outer membrane) and cytochrome *c* oxidase (cyt-ox; inner membrane). The activities of these enzymes, and that of long-chain acyl-CoA synthetase, were all measured using the techniques described in [3]. The distribution of porin was determined by Western blotting using monospecific antibodies. Separation of the component proteins of the submitochondrial fractions was performed routinely using SDS/PAGE (8% gels, except when porin was to be detected, in which case 12% gels were used), followed by transfer on to nitrocellulose and immunodetection with the appropriate antibodies. Detection was performed using the 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium system (Promega) followed by quantitative densitometry (Molecular Dynamics).

Materials

Antibodies against a peptide corresponding to residues 428–441 of CPT I (peptide C of ref. [3]) and against purified CPT II [12] were raised in sheep. Anti-(human porin) was purchased from Calbiochem–Novabiochem U.K. Ltd. (Nottingham, U.K.), and goat anti-(mouse IgG) and anti-(rabbit IgG) fractions were from Sigma (Poole, Dorset, U.K.), as were all other biochemicals. Radioactive [1-^{14}C]palmitate and [^{3}H]carnitine were from Amersham (Amersham, Bucks., U.K.).

RESULTS

As expected, the adenylate kinase activity of the disrupted mitochondria, which serves as an intermembrane space marker, was released into the soluble phase and recovered at the top of the gradient (result not shown). Figure 1(a) shows the profile obtained for membrane protein. Figure 1(b) shows profiles for the respective marker enzymes for the outer membrane (rNCR) and the inner membrane (cyt-ox). The latter is mostly distributed in the cristae membranes, but is also present in considerable quantities within the contact sites [13,14]. The inner and outer membrane peaks were both clearly distinguishable (Figure 1b). Immunoblots for porin (Figure 1b), which is known to be present generally within the outer membrane but with a significantly enhanced expression within contact sites [15], show that the distribution of the channel-forming protein differed from that of the outer membrane marker in that it showed a very distinct and pronounced second peak in the intermediate-density region between the outer and inner membranes [15].

Figure 2 shows the distributions of CPT I and CPT II immunoreactive proteins relative to the profiles of the inner and outer membrane marker enzymes. It can be seen that the distribution of both proteins showed pronounced double peaks, with marked enrichment within the contact-site fraction. The enrichment is even more evident when the data are expressed per

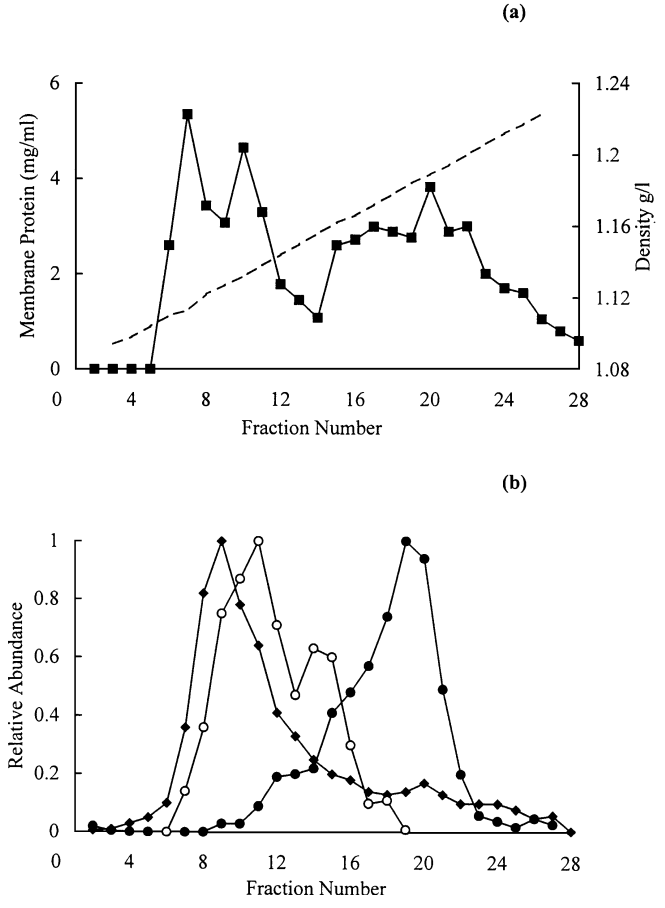

Figure 1 Distribution of (a) membrane protein, and (b) outer and inner membrane marker enzymes and porin in submitochondrial fractions obtained from rat liver mitochondria

Mitochondrial outer and inner membranes and contact sites were prepared and separated on continuous sucrose-density gradients, as described previously [7] (see the Materials and methods section). (**a**) The amount of membrane protein (sedimented at 100000 ***g***) in each fraction is given (■). The density-gradient profile (broken line, right hand *y*-axis) is also shown. (**b**) The activities or immunoreactive contents of rNCR (◆), cyt-ox (●) and porin (○) are shown. Values are representative of those obtained for four different preparations which gave qualitatively similar results. In (**b**) the activity or content of each enzyme in the fraction in which it was most abundant is set at unity, and the other values are expressed as relative values.

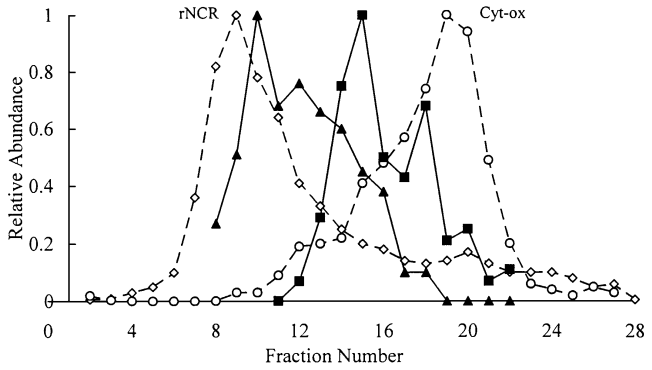

Figure 2 The distribution of CPT I and CPT II proteins in rat liver submitochondrial fractions

Immunoquantification of CPT I (▲) and CPT II (■) proteins was performed after SDS/PAGE separation of the proteins of each fraction, and Western blotting with monospecific antibodies. The profiles for the distributions of the outer and inner membrane markers (broken lines) are given for comparison. The amount of immunodetectable protein in the fraction in which it was most abundant is set at unity.

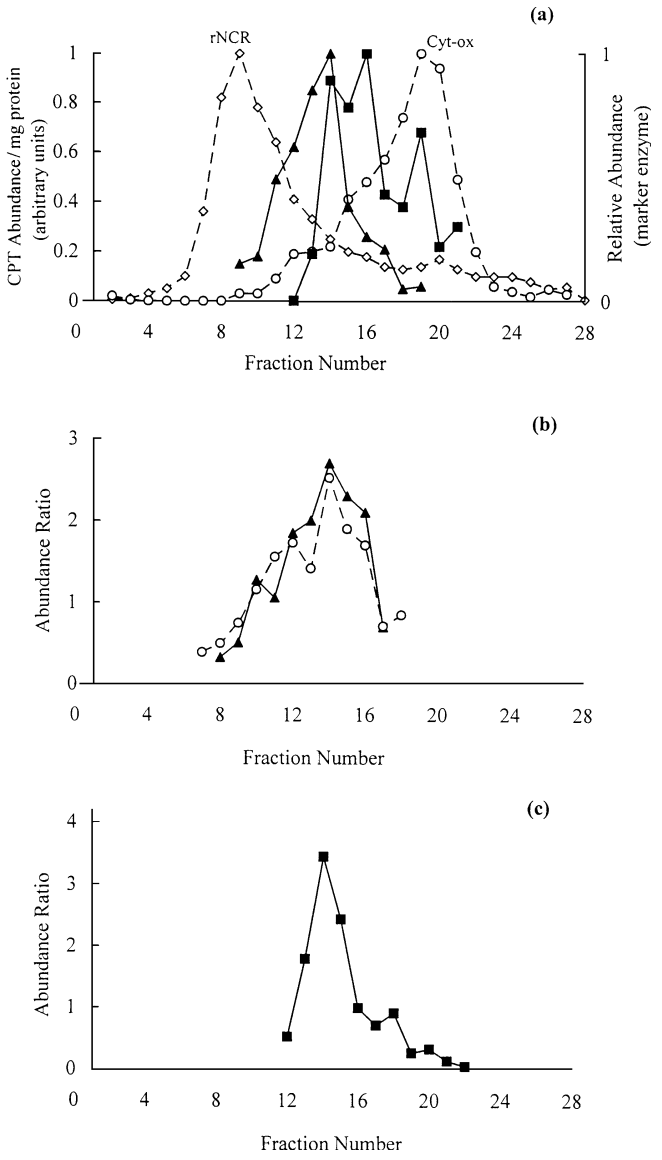

Figure 3 Relative enrichment of CPT I and CPT II with respect to (a) membrane protein content and (b,c) marker enzymes

(**a**) The relative amounts of CPT I (▲) and CPT II (■) in each fraction (see legend to Figure 2) are divided by the amount of membrane protein in that fraction. (**b**) The relative amounts of CPT I (▲) and porin (○) in each fraction are divided by the relative activity of the outer membrane marker rNCR. (**c**) The amount of CPT II is divided by the relative activity of cyt-ox in each fraction. The values are calculated from the data presented in Figures 1 and 2.

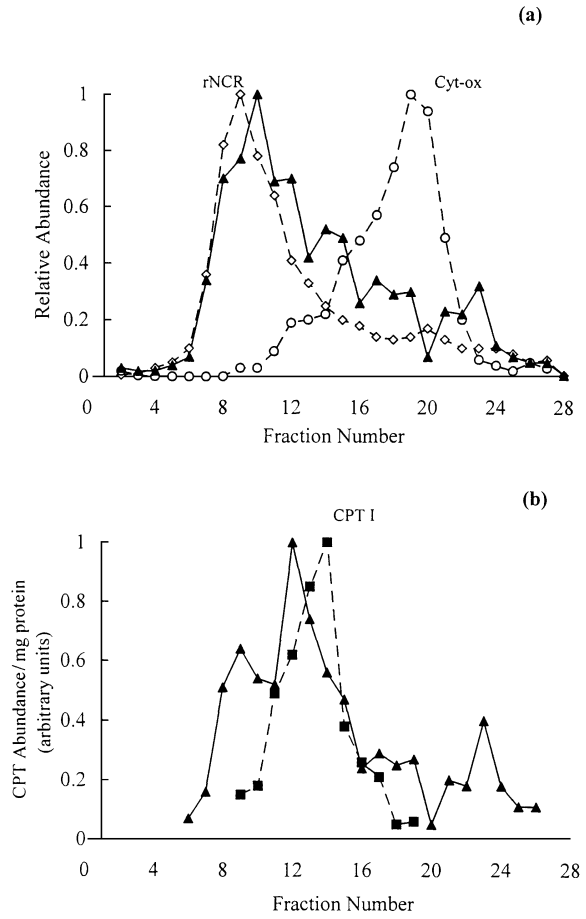

Figure 4 The distribution profile of long-chain acyl-CoA synthetase activity in submitochondrial fractions

The data were obtained from the same gradient shown in Figure 1. (**a**) The activity of acyl-CoA synthetase activity (▲) measured with [1-^{14}C]palmitate as substrate is shown (most active fraction set at unity). The profiles of the outer membrane (rNCR) and inner membrane (Cyt-ox) markers are also shown (broken lines). (**b**) The distribution of acyl-CoA synthetase (▲) relative to that of the membrane protein content of each fraction is shown. The corresponding profile for CPT I is given (■; broken line) for comparison.

unit membrane-protein content of the different membrane fractions (Figure 3a), or in relation to that of the respective marker enzymes for the outer and inner membranes (Figures 3b and c). In either case, the peaks of both CPT-I- and CPT-II-specific expression coincided with the intermediate-density fraction represented by the contact sites. In order to ascertain whether CPT I and CPT II immunoreactive material was genuinely associated with membranous material, the contact site fractions collected from a gradient similar to that shown in Figure 1 were pooled and subjected to density-gradient centrifugation for a second time. There was quantitative and monophasic recovery of both CPT I and II, as well as of porin and total protein from the membranes of intermediate density obtained after the second centrifugation (results not shown).

While this study was in progress, data appeared in abstract form [8] showing that mitoplasts prepared from rat liver mitochondria by French-press treatment retained a high proportion of their CPT I and long-chain acyl-CoA synthetase activities. These authors suggested that this residual activity could be due to the presence of both enzymes within contact sites, although contact sites were not prepared in that study. Therefore we monitored the distribution of long-chain fatty acyl-CoA synthetase activity in the submitochondrial fractions obtained. It can be seen from Figure 4 that, although the distribution of long-chain acyl-CoA synthetase is more similar to that of the outer membrane marker (rNCR) than is that of CPT I, there is considerable enrichment of the synthetase within the contact-site fraction. This is more clearly apparent in Figure 4(b), where the specific activity of the two enzymes is expressed on a membrane-protein content basis. Two characteristics of the distribution of synthetase activity are noteworthy. First, its distribution appears

to extend more widely into the outer membrane fraction than that of CPT I (see also Figure 4a). This indicates that the enrichment within the outer membrane component of the contact sites is not as pronounced as that of CPT I. Secondly, acyl-CoA synthetase activity extended into the inner membrane region of the density gradient, giving a second specific activity peak within the dense inner membrane fraction. This apparently paradoxical observation can be explained by the fact that a second acyl-CoA synthetase occurs within the inner membrane/matrix fraction of mitochondria [16,17]. This second synthetase, although having a higher preference for medium-chain acyl-CoA esters, also has considerable activity with long-chain acyl-CoA [18]. Therefore our present observations would explain why mitoplasts appear to retain a high activity of acyl-CoA synthetase [8], i.e. partly because of the enrichment of the outer membrane long-chain acyl-coA synthetase within contact sites, and partly because of the presence of the inner membrane/matrix acyl-CoA synthetase also detected in assays using palmitoyl-CoA as substrate. Methods for assay of medium-chain acyl-CoA synthetase were not sufficiently sensitive to be used with the amounts of material recovered from individual density gradients.

DISCUSSION

Contact sites represent defined areas of outer and inner boundary membranes that are in such stable and close contact that they cannot be separated by mechanical forces (such as those generated by the sonication protocols used to prepare submitochondrial fractions [13]). In isolated mitochondria, they can be morphologically distinguished as areas in which the two membranes come to within 4 nm of each other, and they represent between 5 and 10 % of the outer membrane surface area [19]. It is therefore even more remarkable that they appear to contain about 40 % of both CPT I and CPT II proteins. *In vivo*, contact sites could be more abundant, especially as it is known that their number can be increased in coupled mitochondria exposed to ADP [20]. The adenine nucleotide translocator is known to be more concentrated at the contact sites [20], and these appear to be involved in the facilitation of transfer of ATP to hexokinase [21,22] and creatine kinase [23], which are both concentrated at contact sites in mitochondria in which they occur. In addition, contact sites are known to be involved in several other mitochondrial functions, including interaction with cytoskeletal proteins [24], protein import [19,25,26], and phospholipid transfer [27,28] between mitochondria and a specialized population of endoplasmic reticular membranes [29].

The concentration of CPT I and CPT II at contact sites raises the possibility that acylcarnitine transfer into the mitochondrial matrix is thereby facilitated. This may be part of a generalized involvement of contact sites in both metabolite and energy transfer between either the intermembrane space and/or cytosol and the inner membrane/matrix compartment [7]. Such a location, however, does not appear to affect the functionally different accessibility of the two enzymes to the acyl-CoA pools in the cytosolic and mitochondrial matrix compartments respectively. Thus in intact isolated mitochondria only malonyl-CoA-sensitive CPT I activity is overtly expressed. Conversely, access of exogenously added acyl-CoA to the active site of CPT II can be gained only after detergent disruption of the inner membrane [5,30].

The extreme sensitivity of the kinetics of CPT I to membrane fluidity [31] suggests that the lipid composition of the microdomain in which CPT I resides may be important in determining its function. Because the lipid composition of contact sites is distinct from that of the rest of the outer membrane (e.g. [22]), the location of the enzyme at these sites may result in a pool of CPT I that is affected differently by their distinctive properties [27,28]. Moreover, alteration of the number of contact sites by changes in the energization state of the mitochondria [20], such as may be brought about *in vivo* by the activation of the electron-transport chain in response to specific hormonal action on the liver (see [32] for review), may alter the relative proportions of CPT I localized in the two microdomains, with consequent effects on the overall kinetic characteristics of the enzyme [31,33–35].

Our previous experimental evidence [36–38] suggested that some acute changes in CPT I activity may be mediated by interactions between the mitochondrial outer membrane and other cell membrane systems. Although CPT I may not be directly involved in such interactions, its kinetic characteristics may be influenced by changes in the lipid environment brought about by intermembrane association [35]. For example, mitochondria are associated, through contact sites, with a specialized population of endoplasmic reticular membranes (see above). It may be relevant therefore that purification of mitochondria from adhering endoplasmic reticular membranes on Percoll gradients results in a significant desensitization of CPT I to malonyl-CoA inhibition [39].

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