Submitochondrial and subcellular distributions of the carnitine-acylcarnitine carrier

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Abstract The submitochondrial and subcellular distributions of the carnitine-acylcarnitine translocase (CAC) have been studied. CAC is enriched to a much lesser extent than the carnitine palmitoyltransferases within the contact sites of mitochondria. A high-abundance protein of identical molecular size as the mitochondrial CAC that is immunoreactive with an anti-peptide antibody raised against a linear epitope of mitochondrial CAC is present in peroxisomes but not in microsomes. This suggests that CAC is targeted to at least two different locations within the liver cell and that acylcarnitine transport into peroxisomes is CAC mediated.

Key words: Carnitine; Carnitine-acylcarnitine translocase; Carnitine palmitoyltransferase; Mitochondria; Contact site; Peroxisome; Microsome

1. Introduction

It is well established that mitochondria have overt (outer membrane) and latent (inner membrane) carnitine palmitoyltransferases (CPT). In conjunction with the carnitine-acylcarnitine translocase of the inner membrane (CAC, [1,2]) they effect the transfer of acyl moieties from the cytosol into the mitochondrial matrix. The sensitivity of the overt, cytosol-facing CPT (CPTo or CPT I) to malonyl-CoA inhibition makes it an important regulatory locus for acylcarnitine formation, and thus for the β-oxidation of fatty acids. We have recently shown that both the overt and latent (CPTi or CPT II) carnitine palmitoyltransferases are enriched within the contact sites of mitochondria [3]. This submitochondrial distribution of the two enzymes at sites known to be important for lipid traffic between the cytosolic and intramitochondrial compartments [4], suggests that acylcarnitine formed by CPTo is preferentially channelled to CPTi. We were interested, therefore, in establishing whether CAC is also enriched within the contact sites, or whether its other functions (e.g. transport of acetylcarnitine) necessitate its wider distribution within the inner membrane.

It has become apparent relatively recently that peroxisomes and microsomes also have overt and latent carnitine palmitoyltransferase activities [5–8]. These are assumed to be involved in the transport of acyl moieties across these membranes. As the overt and latent transferases of organelles require the presence of a mechanism for the transport of acylcarnitines across the membrane in order to fulfil their function, we carried out experiments to find out whether mitochondrial CAC is also expressed in microsomes or peroxisomes.

2. Materials and methods

2.1. Preparation of submitochondrial membrane fractions

Percoll-purified mitochondria (see below) were resuspended in 2.14 ml of 10 mM phosphate buffer (pH 7.4) and incubated on ice for 20 min, followed by the addition of 0.86 ml of 60% sucrose in 10 mM phosphate buffer. The suspension was incubated for a further 20 min on ice and then sonicated for three periods of 30 s, over a period of 3 min with constant cooling on ice, as described previously [3]. After a low speed centrifugation ($300\times g$) to remove debris, the resulting supernatant was layered on top of 10 ml of a continuous sucrose gradient (0.82–1.71 M sucrose) and centrifuged for 16 h at 37 000 rpm in a swing-out rotor (Sorvall TH641) as described previously [3]. Fractions collected by vertical displacement.

2.2. Preparation of subcellular fractions

Livers of 24 h-starved rats (200–220 g) were homogenised in medium containing 300 mM sucrose, 5 mM Tris-HCl and 1 mM EGTA (pH 7.4) and a cocktail of protease inhibitors (Boehringer Complete). After centrifugation at $500\times g$, a crude mitochondrial pellet was sedimented from the supernatant by centrifugation at $5800\times g$ for 20 min. This pellet was used to prepare purified mitochondria by Percoll density gradient centrifugation [9] and purified peroxisomes by Nycodenz density gradient centrifugation [10]. Microsomes were prepared from 12 000 supernatants by centrifugation at $100\,000\times g$ for 60 min.

2.3. Western blotting and enzyme assays

The protein components of the membrane fractions were separated on 8% acrylamide Laemmli gels and blotted onto nitrocellulose, followed by probing with appropriate antibodies. Detection of CPTs I and II was performed using sheep anti-peptide (residues 428–441 of rat liver CPT I) and anti-CPT II antibodies, respectively, as described previously [3]. Porin was detected using mouse anti-human porin 3HL (Calbiochem). CAC was detected by an anti-peptide antibody raised in sheep against residues 96–110 of rat liver CAC. Secondary antibodies conjugated to alkaline phosphatase and the 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium system were used throughout for detection. Quantification of the bands obtained was performed using phosphorimage analysis (Molecular Dynamics).

Rotenone-insensitive cytochrome c-NADPH oxidoreductase (outer membrane marker), adenylate kinase (inter membrane space marker) and cytochrome c oxidase (inner membrane marker) were assayed as described previously [3]. Total and membrane protein (after centrifugation at $160\,000\times g$) were measured by the method of Lowry et al. [11].

3. Results

3.1. Submitochondrial distribution of CAC

Separation of outer membrane, contact site and inner membrane fractions from rat liver mitochondria on continuous sucrose gradients is shown in Fig. 1a, together with the distribution of membrane protein (Fig. 1b). Characteristic distributions of NADPH-cytochrome c oxidoreductase, porin and

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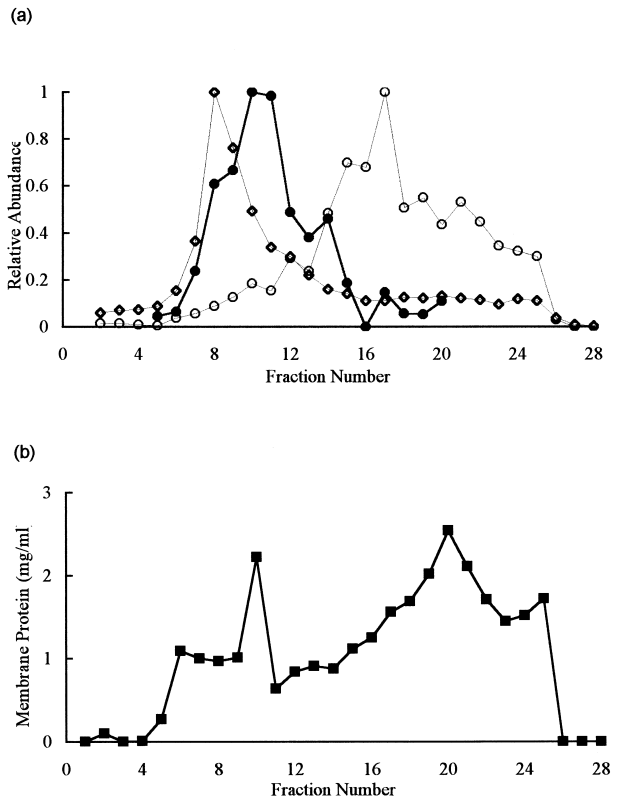

Fig. 1. The distribution of three marker proteins and total membrane protein in submitochondrial fractions separated by sucrose density gradient centrifugation. a: Mitochondria prepared and purified on Percoll gradients (see Section 2) were subfractionated on continuous sucrose density gradients. The relative activity or abundance of rotenone-insensitive NADPH-cytochrome c reductase (◇), porin (●) and cytochrome c oxidase (○) in the different fractions is shown. For each parameter, the value for the fraction showing the highest activity/abundance is set at unity and the values for other fractions are expressed relative to it. b: Distribution of membrane protein in the different fractions. The values shown are from an experiment representative of three separate preparations that gave qualitatively similar results. OM, outer membrane; CS, contact sites; IM, inner membrane.

cytochrome oxidase were observed (cf. [3]). In Fig. 2a the distribution of immunodetectable CAC is shown. It can be seen that CAC is primarily distributed within the inner membrane fraction, but that a smaller but discrete peak also occurs within the contact site fraction of intermediate density. The distributions of CPTs I and II observed for the same gradient are shown in Fig. 2b. The greater enrichment of both CPTs within contact sites compared to CAC is evident from Fig. 3 in which the abundance of the individual components of the mitochondrial acylcarnitine transferase-translocase system are expressed relative to the distributions of the outer and inner membrane marker enzymes. (Values higher than 1.0 indicate enrichment relative to the marker.) Fig. 3a shows that CPT I and CAC are enriched respectively within the contact sites and inner membrane fractions. When the distributions of CPT II and CAC are expressed relative to cytochrome oxidase (Fig. 3b) it is evident that whereas CPT II is highly enriched within the contact sites (cf. [3]) CAC is distributed much more uniformly relative to the inner membrane marker (values close to unity throughout).

3.2. Subcellular distribution of CAC

A specific antibody raised against a linear epitope of mitochondrial CAC as expected detected the protein in mitochondrial extracts (Fig. 4, lane 1) having the molecular size (approx. 33 kDa) anticipated from the deduced amino acid sequence of the carrier [12]. A protein corresponding to an identical molecular size was detected in peroxisomes (Fig. 4, lane 3), but not in microsomes (lane 2) even when twice the amount of protein was loaded onto the gel (lane 4). Quantification of the relative intensities of reaction of peroxisomal and mitochondrial CAC indicates that the peroxisomal protein is present at a 1.5-fold higher specific abundance (per mg protein) than that in mitochondria. This is 10-fold higher than that which can be accounted for by the low level of cross-contamination of peroxisomes by mitochondria (Table 1). Consequently, we conclude that CAC is highly expressed in peroxisomes and mitochondria but not in microsomes.

4. Discussion

The previously described enrichment of CPT I and CPT II within mitochondrial contact sites suggested that a degree of channelling of long-chain acylcarnitines occurs between the two enzymes. Other observations tend to support this contention. For example, there is evidence that carnitine generated

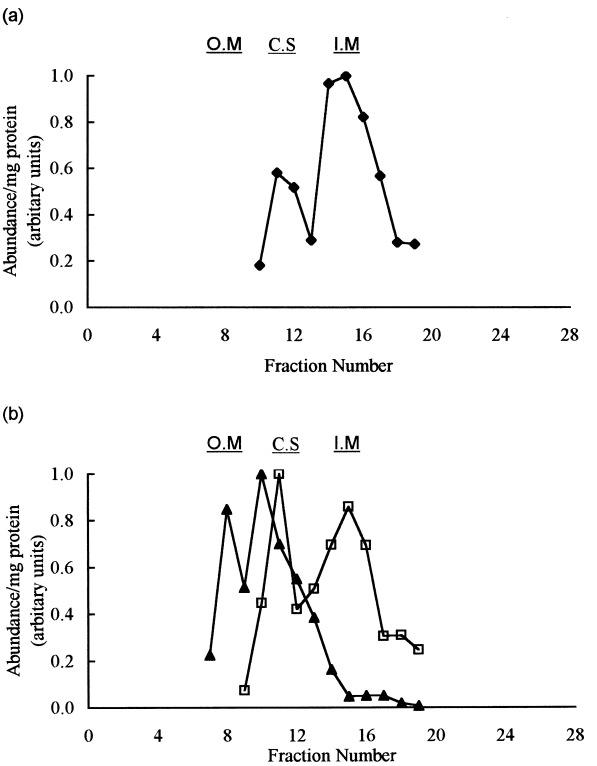

Fig. 2. Distribution of carnitine acylcarnitine carrier, CPT I and CPT II in submitochondrial fractions. Gradient distribution of the specific abundance (per mg membrane protein) of immunodetectable (a) CAC and (b) CPT I (▲) and CPT II (□). The value for the fraction with the highest specific abundance (per mg membrane protein) is set at unity. Values shown are for the same preparation used for Fig. 1 and are representative of those obtained for three preparations. OM, outer membrane; CS, contact sites; IM, inner membrane.

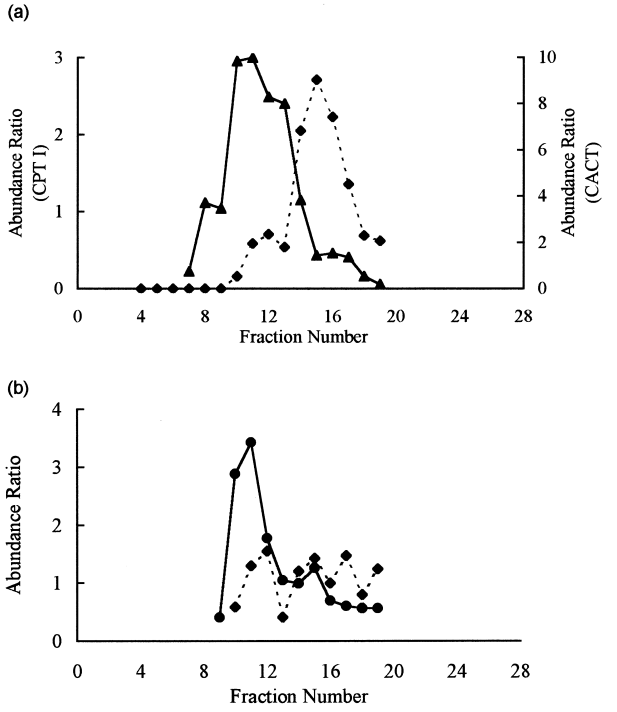

Fig. 3. Submitochondrial distributions of CAC, CPT I and CPT II relative to outer and inner membrane markers. In (a) the relative abundance of CAC (♦) and CPT I (▲) in each fraction is plotted as a fraction of that of the outer membrane marker rotenone-insensitive NADPH cytochrome c oxidoreductase. CPT I is highly enriched within the contact sites (values >1.0) whereas CAC is much more highly enriched within the inner membrane fraction. In (b) the relative abundance of CAC (♦) and of CPT II (●) in each fraction is plotted as a fraction of that of cytochrome c oxidase (inner membrane marker). CPT II is highly enriched within the contact site fraction (values >3.0), whereas CAC has a similar profile to that of cytochrome oxidase (values ~ 1.0 throughout).

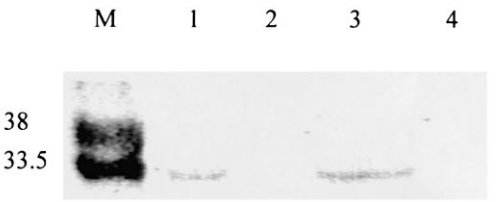

Fig. 4. Expression of CAC in peroxisomal membranes and mitochondria but not in microsomes. Mitochondria, microsomes and peroxisomes were prepared as described in Section 2 (see also Table 1 for purity). Following SDS-PAGE electrophoresis of membrane fractions and transfer onto nitrocellulose, the blots were probed with anti-CAC peptide antibody (raised against residues 96–110). Lanes 1, 2 and 3: 100 μg protein of mitochondrial, microsomal and peroxisomal fractions, respectively. Lane 4: 200 μg microsomal protein. M, molecular weight markers of the sizes indicated.

from long-chain acylcarnitines by CPT II does not equilibrate with a larger pool of carnitine within the matrix, and is preferentially used by CAC in exchange for further acylcarnitine uptake by mitochondria [13,14]. Also, a mixture of CPT I and CPT II has been isolated in a complex with β-oxidation enzymes and electron transport chain components [15], suggesting that they may exist in a complex [16] in which channelling of acylcarnitines to β-oxidation occurs. This raised the question as to whether CAC is concentrated at contact sites to a similar extent as CPT I and CPT II [3]. The present data show that this is not the case. Although CAC is present in contact site membranes, it is also uniformly distributed within the inner membrane of mitochondria. Its submitochondrial distribution is very similar to that of cytochrome oxidase, and much less concentrated at the contact sites than CPT II. The reason for this more uniform distribution of CAC may be that it fulfils functions other than those linked to the transport of acylcarnitine formed by CPT I. For example, a considerable proportion of acylcarnitine esters formed in peroxisomes after partial β-oxidation of very long-chain fatty acids are transported into the mitochondria matrix for complete oxidation [17]. Similarly, short-chain acylcarnitines (acetylcarnitine, propionylcarnitine) formed in peroxisomes during β-oxidation there are known to be further metabolised by mitochondria of rat liver [18] and yeast [19]. In the reverse direction, it is well established that mitochondria have a high capacity to form and release acetylcarnitine and other short-chain carnitine esters formed through the activity of the matrix located carnitine acetyltransferase (CAT). For example, under ketogenic conditions, the liver is known to release acetylcarnitine of mitochondrial origin into the circulation [20]. Therefore, CAC has to transport acylcarnitines that are neither necessarily generated by the CPT I nor used by CPT II for the generation of intramitochondrial long-chain acyl-CoA. We suggest that this wider role of CAC is reflected in its more generalised distribution within the mitochondria inner membrane.

Acyl moieties need to be moved across intracellular membranes for a wide range of cellular functions. However, esterification to CoA within the cytosol [21] renders the resulting acyl-CoA esters impermeant through biological membranes. The relatively recent description of pairs of carnitine palmitoyltransferases not only in mitochondria, but also in microsomes [5,6] and peroxisomes [7], has raised the prospect that carriers that fulfil the same function as the mitochondrial CAC also exist in other organelles and that CAC itself may be targeted to organelles other than mitochondria. In the present study we found that a peroxisomal protein with an identical molecular size to that of mitochondrial CAC (32.5 kDa [12]) was immunodetectable when Western blots were probed with a specific anti-peptide antibody raised against a linear epitope of mitochondrial CAC. Its high level of expres-

Table 1
Marker enzyme activities in the preparations of Percoll-purified mitochondria, microsomes and Nycodenz-purified peroxisomes used for Western blotting experiments

	Mitochondria	Microsomes	Peroxisomes
Cytochrome c oxidase	1280 ± 120	5.0 ± 2.0	189.0 ± 56.9
NADPH-cytochrome c oxidoreductase	7.6 ± 2.2	72.3 ± 5.4	5.9 ± 3.8
Uricase	14.6 ± 3.6	8.3 ± 1.9	257.3 ± 35.9

See Section 2 for details of subcellular fractionation and membrane purification. Enzyme activities (nmol/min per mg protein) were measured at 30°C. Values are for four different preparations.

sion in peroxisomes was 10-fold higher than could be accounted for by cross-contamination between the two subcellular fractions. By contrast, no CAC could be detected in the microsomal fraction. These observations suggest that CAC is not limited to the mitochondrial inner membrane and that it may be involved in the transfer of acylcarnitine esters across the peroxisomal membrane. There is increasing evidence that transfer of acyl moieties across the peroxisomal membrane is carnitine-dependent in the intact cell [22,23]. It is noteworthy that, in common with other members of the mitochondrial anion carrier family [24,25] CAC is not proteolytically processed upon import into the mitochondrial inner membrane [12]. The motif(s) responsible for targeting CAC (and other carriers) to the mitochondrial inner membrane have not been elucidated and no obvious sequences have emerged yet that direct the targeting of proteins to the peroxisomal membrane [26].

CAC is absent from microsomal membranes (Fig. 4). Recent studies by Gooding et al. [27] have concluded that transfer of acylcarnitines across the endoplasmic reticular membrane is not carrier mediated but occurs by a pore mechanism. Suggestions for a pore or channel of 5–8 Å diameter that allows the entry of metabolites across the endoplasmic reticular membranes have been made previously [28]. The existence of such a pore may explain the absence of CAC from this membrane system. However, acylcarnitine transfer across the endoplasmic reticular membrane does occur [27]. In tissues (like the liver) that secrete triacylglycerol-rich lipoproteins its functions may include the provision of acyl moieties for the intra-lumenal endoplasmic reticular synthesis of triacylglycerol [29,30].

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