Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane

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The topology of carnitine palmitoyltransferase I (CPT I) in the outer membrane of rat liver mitochondria was studied using several approaches. 1. The accessibility of the active site and malonyl-CoA-binding site of the enzyme from the cytosolic aspect of the membrane was investigated using preparations of octanoyl-CoA and malonyl-CoA immobilized on to agarose beads to render them impermeant through the outer membrane. Both immobilized ligands were fully able to interact effectively with CPT I. 2. The effects of proteinase K and trypsin on the activity and malonyl-CoA sensitivity of CPT I were studied using preparations of mitochondria that were either intact or had their outer membranes ruptured by hypo-osmotic swelling (OMRM). Proteinase K had a marked but similar effect on CPT I activity irrespective of whether only the cytosolic or both sides of the membrane were exposed to it. However, it affected sensitivity more rapidly in OMRM. By contrast, trypsin only reduced CPT I activity when incubated with OMRM. The sensitivity of the residual CPT I activity was unaffected by trypsin. 3. The

proteolytic fragments generated by these treatments were studied by Western blotting using three anti-peptide antibodies raised against linear epitopes of CPT I. These showed that a proteinase K-sensitive site close to the N-terminus was accessible from the cytosolic side of the membrane. No trypsin-sensitive sites were accessible in intact mitochondria. In OMRM, both proteinase K and trypsin acted from the inter-membrane space side of the membrane. 4. The ability of intact mitochondria and OMRM to bind to each of the three anti-peptide antibodies was used to study the accessibility of the respective epitopes on the cytosolic and inter-membrane space sides of the membrane. 5. The results of all these approaches indicate that CPT I adopts a bitopic topology within the mitochondrial outer membrane; it has two transmembrane domains, and both the N- and C-termini are exposed on the cytosolic side of the membrane, whereas the linker region between the transmembrane domains protrudes into the intermembrane space.

INTRODUCTION

Mitochondrial overt carnitine palmitoyltransferase (CPT I) catalyses the conversion of long-chain acyl-CoA into acyl-carnitine, thus committing the acyl moiety to intramitochondrial β -oxidation. Carrier-mediated translocation of acylcarnitines across the inner membrane is followed by resynthesis of acyl-CoA within the mitochondrial matrix, catalysed by latent (inner-membrane) CPT II. CPT I and CPT II are separate gene products. CPT I catalyses the rate-limiting step of fatty acid utilization by mitochondria under most conditions, and thus fulfils a highly important role in the maintenance of cell function in eukaryotes (see [1]). Its most important regulatory characteristic is inhibition by malonyl-CoA [2]. This interaction is now recognized to be important not only in the direct control of the rate of fatty acid oxidation in all tissues studied but also in providing the basis for 'fuel sensing' which transduces to the cell information about the relative availability of fatty acids and glucose. This role may be important in such diverse functions as the response to ischaemia in cardiac muscle [3] and the control of insulin secretion by the pancreatic β -cell [4].

CPT I has been localized to the mitochondrial outer membrane through the use of cell and mitochondrial fractionation procedures [5,6]. The cDNAs of CPT I from rat and human liver [7,8] and from rat and human heart, skeletal muscle and brown adipose tissue [9–11] have been cloned and sequenced. The

deduced amino acid sequences and Western blot analyses [12] show that two isoforms of the enzyme exist in the rat: a 'liver' form that is also present in kidney and to a much lower extent in adult heart, and a 'muscle' form which is the predominant form in heart, skeletal muscle, adipose tissue and brain. Although the nucleotide sequences of the two isoforms are only 62.6% identical, they both predict two relatively highly conserved hydrophobic domains (residues 48–75 and 103–122) within the N-terminal region (The rat muscle form of CPT I has only one fewer amino acid residue than the 773 residues of the liver isoform.) These domains are the only ones within the protein to be sufficiently long to span the mitochondrial outer membrane and have, therefore, been considered [13] to be putative transmembrane domains (TMDs). We have previously suggested that the high concentration of positively charged residues on the N-terminal flank of the first (more N-terminal) of the two putative TMDs is likely to result in the N-terminus of the protein being located on the cytosolic side of the membrane [13], by analogy with at least one other mitochondrial outer membrane protein [14,15], and in compliance with the more generally applicable positive-inside (cytosolic) rule [16]. However, the possibility that both hydrophobic domains are actual TMDs has been disputed [7,17].

The topology of CPT I within the mitochondrial outer membrane has not been rigorously examined experimentally. Studies that were restricted to partial proteolysis experiments,

Abbreviations used: CPT I, the overt carnitine palmitoyltransferase of the mitochondrial outer membrane; CPT II, latent carnitine palmitoyltransferase of the inner membrane; TMD, transmembrane domain; OMRM, outer-membrane-ruptured mitochondria; IMS, mitochondrial intermembrane space; AEBF, 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride.

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and which preceded the cloning and sequencing of rat liver CPT I cDNA [5,18–20], suggested that the active site and the malonyl-CoA (regulatory) site are located on opposite sides of the mitochondrial outer membrane, with the latter facing the cytosol. In view of the notoriously difficult and unreliable interpretation of partial proteolysis data (see [21]), it was decided to perform a systematic study using a much wider range of topographical techniques, including the use of anti-peptide antibodies raised against linear epitopes deduced from the recently reported cDNA sequence. Our data indicate that both hydrophobic domains of CPT I of rat liver mitochondria are actual TMDs, and that both the active site and the malonyl-CoA-binding site are exposed on the cytosolic face of the mitochondrial outer membrane. This topology was confirmed by results obtained from functional studies in which the sidedness of the active and regulatory sites was determined using their respective immobilized ligands.

MATERIALS AND METHODS

Chemicals

Sepharose 4B, activated CH-Sepharose, EAH-Sepharose and palmitoyl-CoA were from Pharmacia (St. Albans, Herts., U.K.). Octanoyl-CoA was from Lipid Products (S. Nutfield, Surrey, U.K.). 4-(2-Aminoethyl)benzenesulphonylfluoridehydrochloride (AEBSF) was from ICN-Pharmaceuticals (Thame, Oxon., U.K.). Proteinase K was from Boehringer-Mannheim (Lewes, E. Sussex, U.K.). Malonyl-CoA, trypsin and soya bean trypsin inhibitor were from Sigma (Poole, Dorset, U.K.). *N*-Ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide hydrochloride and succinic anhydride were from Aldrich (Gillingham, Kent, U.K.). Immobilized papain was from Warriner and Pierce (Chester, Cheshire, U.K.).

Malonyl-CoA and octanoyl-CoA were immobilized on to Sepharose 4B through the amino group of the adenine moiety of CoA. Two types of spacer arm were used: 6-atom and 14-atom. The starting materials were activated CH-Sepharose 4B and EAH-Sepharose 4B respectively. In the latter instance the 10-atom spacer arm was extended by reaction with succinic anhydride to form the succinylaminoethyl derivative (SAE-Sepharose), to which the CoA esters were coupled by using *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide hydrochloride. Gels were washed exhaustively with several cycles of low- and high-pH buffers (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0, and 0.1 M Tris/HCl, 0.5 M NaCl, pH 8.0, respectively), and finally dialysed against distilled water containing 1% (w/v) BSA, followed by washing in distilled water only.

Preparation of mitochondria and treatment with proteases

Mitochondria were prepared from livers of fed female Wistar rats. All mitochondrial preparations were purified by Percoll-gradient centrifugation [6] to minimize contamination with microsomes and peroxisomes, which also contain malonyl-CoA-sensitive carnitine acyltransferases. Mitochondria were exposed to proteases either when intact (in isotonic medium containing 250 mM sucrose, 5 mM potassium phosphate, 0.1 mM EGTA, pH 7.4) or after their outer membrane had been ruptured (OMRM) by incubation in hypo-osmotic medium (containing 25 mM instead of 250 mM sucrose) for 10 min at 30 °C. The protein concentration was 5.0 mg/ml. Trypsin (10 µg/ml) or proteinase K (5 µg/ml) was added and incubation with shaking continued for the indicated periods. Protease action was terminated by the addition of trypsin inhibitor (100 µg/ml) or AEBSF (1 mM) respectively. The tubes were placed on ice for 2 min before aliquots were taken for assay of CPT activity in the

Figure 1 Schematic representation of the primary structure of rat liver CPT I

The diagram shows the relative positions of the two putative TMDs, and of epitopes N, L and C used for the generation of anti-peptide antibodies.

presence or absence of 20 µM malonyl-CoA. Aliquots were also frozen in liquid nitrogen for later measurement of the activity of marker enzymes.

When Sepharose-malonyl-CoA or -octanoyl-CoA was used, control incubations contained an equivalent amount of Sepharose 4B gel in order to standardize all incubations for any physical damage sustained by the mitochondria during mixing with the solid matrices.

Assay of CPT and marker enzyme activities

The standard assay medium for CPT I contained 150 mM KCl, 1 mM EGTA, 5 mM Tris/HCl (pH 7.4), 5 mM MgATP, 1% defatted BSA, 1.6 mM dithiothreitol, 4 µg of rotenone/ml, 2 µg of antimycin A/ml and 0.52 mM L-[³H]carnitine (specific radioactivity 770 d.p.m./nmol). In assays containing soluble palmitoyl-CoA or octanoyl-CoA, these were added at 10 µM and 60 µM respectively. In experiments using Sepharose-malonyl-CoA, the reaction mixture consisted of 80 mM sucrose, 70 mM KCl, 50 mM imidazole, 1 mM EGTA, 5 mM MgATP, 10 µM palmitoyl-CoA, 1% BSA, 5 mM glutathione, 2 µg of antimycin A/ml, 4 µg of rotenone/ml and 0.52 mM L-[³H]carnitine (770 d.p.m./nmol). The final pH was 6.5. The reactions were performed at 25 °C in a total volume of 0.5 ml (of which 0.25 ml was represented by the packed gel volume). The rationale underlying these conditions was to optimize the sensitivity of CPT I to malonyl-CoA through the use of a lower pH [22], decreased membrane fluidity (lower temperature; see [23]) and lower competition by the acyl-CoA substrate. Quantification of CPT I activity was performed as described previously [23], by extraction of [³H]acylcarnitine into butanol.

Adenylate kinase was used as a marker for the inter-membrane space and was assayed as described in [24]. Rotenone-insensitive NADPH-cytochrome *c* reductase was used as a marker for the outer face of the outer membrane and was assayed as described in [25]. Long-chain acyl-CoA ligase was used as an outer-membrane marker with transverse plane membrane topology (see below) and was assayed as described in [26].

Anti-peptide antibodies: preparation and binding to mitochondria

Peptides corresponding to residues 14–29 (epitope N), 86–100 (epitope L) and 428–441 (epitope C) of rat liver CPT I, as deduced from its cDNA sequence [7], were synthesized with the additional inclusion of a C-terminal cysteine residue. The relationship of these epitopes to the two putative TMDs in the primary sequence of rat liver CPT I is shown in Figure 1. The peptides were cross-linked to *n*-maleimidobenzoyl-*N*-hydroxy-succinimide-activated keyhole-limpet haemocyanin or BSA and used to immunize sheep. IgG fractions were purified from serum by ammonium sulphate precipitation and batch-wise anion-exchange (DE 52) chromatography. They were used for Western blotting, and in back-titration experiments after binding either to

intact mitochondria or to preparations in which the outer membrane was permeabilized by incubation (2 min at 0 °C) with digitonin {0.24 g/mg of protein; sufficient to release approx. 95 % of adenylate kinase from the intermembrane space (IMS), as described previously [4]}. Unbound antibodies remaining in the supernatant after incubation with these preparations were quantified by ELISA using microtitre plates coated with the respective peptide.

RESULTS AND DISCUSSION

Functional localization of substrate- and malonyl-CoA-binding sites of CPT I using immobilized ligands

A major complication in the study of the topology of mitochondrial outer-membrane proteins is that this membrane is permeable to molecules with a molecular size smaller than approx. 6 kDa. Consequently, selectively permeant ligands and/or protein modification reagents cannot be used to study the functional sidedness of catalytic or regulatory sites. We sought to overcome this through the use of Sepharose-immobilized octanoyl-CoA and malonyl-CoA. We studied the ability of these immobilized ligands to interact with CPT I in both intact mitochondria and OMRM. The data in Table 1 show that both Sepharose–octanoyl-CoA and Sepharose–malonyl-CoA were able to interact entirely effectively with CPT I in intact mitochondria. (Preliminary experiments showed that octanoyl-CoA linked to Sepharose through either a 6- or 14-atom spacer was equally effective in acting as substrate for CPT I; however, a 14-atom spacer was required for significant inhibition to be achieved by immobilized malonyl-CoA, suggesting the the malonyl-CoA site may be less accessible within the tertiary structure of the protein.) Rupture of the outer membrane did not increase either the activity of CPT I expressed with Sepharose–octanoyl-CoA or inhibition by Sepharose–malonyl-CoA (Table 1). These data

indicate that both the active site and the malonyl-CoA-binding site of the enzyme are exposed on the outer (cytosolic) face of the membrane. This conclusion is at variance with that drawn previously by other authors [5,18,19] from experiments in which the effects of proteases on the activity and malonyl-CoA sensitivity of CPT I were studied. Consequently, correlations between protease action and CPT I function were sought.

Effects of proteinase K and trypsin treatment of mitochondria with intact or ruptured outer membranes

Effects on activity and malonyl-CoA sensitivity of CPT I

The validity of the use of mitochondrial swelling to distinguish the sidedness of action of proteases on either soluble or monotopic enzymes was ascertained through the measurement of the proteolytic effects on marker enzymes: rotenone-insensitive NADPH–cytochrome *c* reductase and adenylate kinase for the outer face of the outer membrane and the IMS respectively (see Figures 2 and 3). Moreover, the effects of trypsin on the activity of acyl-CoA ligase (Figure 3d) illustrate the behaviour of a bitopic outer-membrane enzyme which has domains crucial to its activity exposed on either side of the membrane (see legend to Figure 3), and also illustrate the limitations of proteolysis data when used in isolation to elucidate the topology of membrane proteins (see the Introduction section).

In intact mitochondria, only proteinase K had any effect on either the activity or the malonyl-CoA sensitivity of CPT I (Figures 2a and 2b); trypsin was completely ineffectual on both activity (Figure 3a) and sensitivity (results not shown). By contrast, proteinase K treatment of intact mitochondria induced the simultaneous loss of activity and sensitivity, indicating that domains crucial for the optimal expression of these parameters (although not necessarily the respective binding sites themselves) occur on the outer face of the membrane and that proteinase K-sensitive site(s) within them are exposed to protease action. After rupture of the outer membrane, the loss of activity induced by proteinase K was not increased significantly, but the effect of the protease on malonyl-CoA sensitivity was accelerated (Figure 2b). This indicated that (an) additional site(s) sensitive to proteinase K was exposed on the IMS side of the membrane and that cleavage at this site resulted primarily in an accelerated loss of malonyl-CoA sensitivity.

Similarly, rupture of the outer membrane must have exposed tryptic site(s) on the IMS side, because trypsin treatment of OMRM resulted in the marked loss of activity of CPT I (compared with intact mitochondria; Figure 3a). However, in this instance, loss of activity was not accompanied by loss of sensitivity to malonyl-CoA, as the residual CPT I activity always displayed the same sensitivity as the intact enzyme. This observation suggested that the initial action of trypsin from the IMS side of the membrane induced subsequent extensive proteolysis to give soluble products that were no longer catalytically active. Consequently, only the activity of intact, fully malonyl-CoA-sensitive CPT I would have been detected by the enzyme assay.

In order to test these inferences, the sizes and identity of the fragments generated by the above proteolytic treatments were studied.

Western blotting of fragments of CPT I generated by protease treatment

In these experiments, intact mitochondria or OMRM were sedimented after protease treatment so as to exclude any soluble products of protease action on CPT I and retain the membrane-bound ones (no proteolytic products were detected in super-

Table 1 Immobilized octanoyl-CoA and malonyl-CoA act as substrate and inhibitor respectively for CPT I in intact mitochondria

CH-Sepharose (6-atom spacer)-linked octanoyl-CoA and succinylaminoethyl-Sepharose (14-atom spacer)-linked malonyl-CoA were incubated with mitochondria that were either intact or which had the outer membranes ruptured (OMRM) by hypo-osmotic swelling. Aliquots of mitochondrial suspension were incubated with equal volumes of the packed gels linked to the respective ligands (see the Materials and methods section). The formation of [³H]acetylcarnitine product was measured in acid-stopped supernatants after sedimentation of the gels by light centrifugation. Controls that contained an equal volume of the supernatants of the packed immobilized preparations (gel supernatants) were run concurrently to obtain a measure of the maximal contribution of non-covalently bound ligand still present in the gels. This contribution was always significantly lower ($P \leq 0.01$; paired *t* test) than the total effects observed. The proportion of total mitochondrial adenylate kinase activity that remained associated with the mitochondrial pellet was used as an index of outer-membrane damage in OMRM. In six separate preparations, $90.5 \pm 2.5\%$ of the marker enzyme was still present in intact mitochondria after incubation with the gels, whereas only $23.2 \pm 3.1\%$ was associated with OMRM.

Ligand	CPT I activity (nmol/min per mg of protein)	
	Intact mitochondria	OMRM
Sepharose–octanoyl-CoA gel	1.43 ± 0.30	1.63 ± 0.09
Octanoyl-CoA (60 μ M)	2.91 ± 0.14	2.86 ± 0.14
Gel supernatant	0.45 ± 0.13	0.77 ± 0.14
Palmitoyl-CoA (10 μ M)	1.29 ± 0.15	1.32 ± 0.41
Palmitoyl-CoA + Sepharose–malonyl-CoA gel	0.47 ± 0.07	0.34 ± 0.05
Palmitoyl-CoA + 100 μ M malonyl-CoA	0.47 ± 0.19	0.32 ± 0.06
Palmitoyl-CoA + gel supernatant	1.01 ± 0.13	1.04 ± 0.35

Figure 2 Time courses for the effects of proteinase K on (a) the maximal activity of CPT I, and (b) activity in the presence of 20 μ M malonyl-CoA, in intact mitochondria and OMRM

Mitochondria (5 mg of protein/ml) were incubated with proteinase K (5 μ g/ml) at 30 °C for the indicated periods under either isotonic (■) or hypotonic (●) conditions (see the Materials and methods section). Controls to which no protease were added were run concurrently (▲ and ◆ respectively). Identical results were obtained when the osmotic conditions of previously swollen mitochondria were normalized before assay of CPT I activity or marker enzyme activities. The effects of the protease on marker enzymes are also shown for adenylate kinase (c) and rotenone-insensitive NADPH-cytochrome *c* reductase (d), markers for the IMS and cytosolic aspect of the outer membrane respectively. The pattern of destruction of these activities is entirely consistent with that expected from the locations of these marker enzymes and the loss of outer membrane integrity upon swelling of the mitochondria. In (a), values are given as a percentage of the activity at zero time, whereas in (b) values for the activity in the presence of malonyl-CoA are expressed as a percentage of the control activity (obtained in the absence of malonyl-CoA) at each time point. All values are means $\pm$ S.E.M. of at least four separate determinations.

natants, presumably due to further extensive proteolysis). In pilot experiments, the inability to extract membrane-bound CPT I fragments with alkali Na_2CO_3 was used to confirm that they were integral to the membrane. The proteins of the sedimented mitochondria were separated by SDS/PAGE and analysed by Western blotting using the anti-N, anti-L and anti-C anti-peptide antibodies described in the Materials and methods section (see Figure 1). In all experiments, the degree of intactness of the outer membrane of the mitochondria was quantified by the accessibility of the proteases to adenylate kinase (see Figures 2 and 3). Routinely, less than 10% of the IMS marker was inactivated by proteases in intact mitochondria, compared with > 80% in OMRM.

Treatment of intact mitochondria with proteinase K resulted in a very small (≤ 1 kDa) but reproducible decrease in the size of

CPT I. This decrease in size was detected with all the three antibodies, but was most clearly discernable with anti-C (Figure 4c), which reacted most strongly with CPT I on Western blots. This action of the protease on CPT I appeared to produce a limit polypeptide, as densitometric quantification of bands from four separations showed that the smaller band represented $102.3 \pm 15.1\%$ of the intensity of the intact CPT I band. Concomitantly, detection of the truncated polypeptide produced by anti-N antibody was markedly enhanced compared with the detection of intact CPT I (Figure 4a). These observations indicated that the action of proteinase K exclusively from the cytosolic side of the membrane resulted in the loss of the (rather hydrophobic) extreme N-terminus of CPT I (possible proteinase K-sensitive sites occur at alanine residues 4, 7 and 9), and that its removal facilitated the interaction of anti-N antibody with its

Figure 3 Time courses for the effects of trypsin on CPT I (a) and marker enzyme (b–d) activities in intact mitochondria and OMRM

Mitochondria (5 mg of protein/ml) were incubated with trypsin (10 μ g/ml) under the conditions described in the legend to Figure 2 (same symbols used). The sensitivity of CPT I remaining after trypsin action in OMRM was not different from that of intact CPT I (results not shown). The effects of trypsin on (b) adenylate kinase, (c) rotenone-insensitive NADPH–cytochrome *c* reductase and (d) long-chain acyl-CoA synthase are shown as markers for the IMS, the cytosolic aspect of the outer membrane and the transverse plane of the outer membrane respectively. The effects on acyl-CoA ligase are as expected from the observations in [35], i.e. the enzyme is integral to the outer membrane and contains domains crucial to its activity on both sides of the membrane (cf. CPT I; see the text). Values are given as percentages of the activities at zero time and are means $\pm$ S.E.M. of at least four separate determinations.

antigenic epitope (residues 14–29) when the proteolytic product was blotted (and presumably partially refolded) on to nitrocellulose.

Exposure of OMRM to proteinase K resulted in the additional formation of a smaller product (arrowed in Figure 4c) that was detected only by anti-C antibody and not by either anti-N or anti-L antibodies (Figures 4a and 4b). This product is suggested to have arisen through the loss of a larger N-terminal segment of CPT I, including at least part of the epitope recognized by anti-L antibody. On SDS/PAGE it migrated with an apparent size of 85 kDa, whereas it can be calculated that truncation up to and including epitope L would involve the loss of approx. 10 kDa. Consequently, it is suggested that this fragment migrates slightly anomalously on SDS gels. This is plausible, as anomalous migration of CPT I isoforms on SDS gels (presumed to be due to their hydrophobic nature [7]) is well documented [10,17].

Exposure of intact mitochondria to trypsin did not result in any change in the size or intensity of the CPT I band detected by any of the antibodies (Figures 5a–5c), in agreement with the inability of this protease to affect either the activity or malonyl-CoA sensitivity of the enzyme in intact mitochondria (Figure 3a). However, when OMRM were used, detection of intact CPT I by anti-N and anti-L antibodies was almost totally lost (cf. loss in activity in Figure 3a), without any accompanying appearance of smaller-sized membrane-bound fragments (Figures 5a and 5b). By contrast, at least one fragment was detected by anti-C antibody (Figure 5c), indicating that the initial action of trypsin occurred at a site between the L and C epitopes in such a way as to leave the product integral to the membrane. Nevertheless, there was a major overall loss of anti-C immunoreactive material. It is suggested that rupture of the outer membrane in OMRM allows trypsin to cleave CPT I at a site within epitope L (cf.

Figure 4 Detection of products of CPT I proteolysis by proteinase K using anti-peptide antibodies

Mitochondria were incubated under either iso- or hypo-osmotic conditions for 10 min and then for a further 15 min in the presence of proteinase K ($5 \mu\text{g/ml}$) before termination of protease action (see the Materials and methods section). After sedimentation, samples of the mitochondrial pellets were electrophoresed on SDS gels. CPT I and the proteolytic fragments generated were detected by Western blotting using (a) anti-N, (b) anti-L and (c) anti-C antibodies (see Figure 1). The products of proteinase K action on CPT I are shown in lanes 2 for intact mitochondria, and in lanes 4 for OMRM. The respective controls, run concurrently in the absence of protease, are shown in lanes 1 and 3. Results are representative of four experiments. The positions to which the 85.3 kDa marker migrated are shown. The arrow indicates a smaller product that was detected only by the anti-C antibody.

Figure 5 Detection of products of CPT I proteolysis by trypsin using anti-peptide antibodies

Experimental details were as given in the legend to Figure 4, except that trypsin ($10 \mu\text{g/ml}$) was used. Intact (lanes 1 and 2) or hypo-osmotically swollen (lanes 3 and 4) mitochondria were incubated with protease for 45 min at 30°C . After sedimentation of the mitochondria, their constituent proteins were separated by SDS/PAGE and Western blot analysis performed using (a) anti-N, (b) anti-L or (c) anti-C antibodies. Results are representative of three experiments. The positions to which the 85.3 kDa marker migrated are indicated.

proteinase K; possible trypsin-sensitive sites occur C-terminal to Lys-86 or -102 and Arg-89 or -96), and that this leads to (i) loss of detection by anti-N and anti-L antibodies, and (ii) marked alteration of the tertiary structure of the remaining anti-C-detectable fragment (anchored to the membrane through the more C-terminal of the two putative TMDs; see Figure 1), such that further tryptic sites are exposed, with consequent net loss of

anti-C-detectable material from the membrane fraction (Figure 5c).

The results obtained with the two proteases place epitope N (and therefore the N-terminus of CPT I) on the cytosolic side of the membrane, and epitope L on the IMS side of the membrane. In the deduced amino acid sequence of rat liver CPT I these two epitopes are separated by the more N-terminal of the two putative TMDs (Figure 1). Therefore these observations establish that this hydrophobic domain is an actual TMD (designated TMD 1). The fact that the fragment resulting from proteinase K action from the IMS side of the membrane remained integral to the membrane and was still detectable by anti-C antibody on Western blots indicates that the hydrophobic domain that occurs between epitopes L and C is also an actual TMD (TMD 2). It is inferred, therefore, that epitope L is on the opposite (IMS) side of the membrane from both epitopes N and C, and that the latter are both exposed on the cytosolic side of the membrane. To validate these inferences, the ability of the three anti-peptide antibodies to bind to rat liver mitochondria was studied.

Binding of antibodies to mitochondria with intact or permeabilized outer membranes

Binding of anti-C and anti-L antibodies to intact or digitonin-treated mitochondria (see the Materials and methods section) was assessed by back-titration of the antibodies remaining in the supernatant after incubation with, and sedimentation of, the mitochondria. In these experiments, digitonin treatment of mitochondria (sufficient to release $>95\%$ of their adenylate kinase content; see the Materials and methods section) was preferred to hypotonic swelling as a way to disrupt the outer membrane, as low-ionic-strength conditions resulted in unacceptably high non-specific background binding of antibodies. As shown in Figure 6(a), anti-C but not anti-L antibodies were bound and removed from solution by intact mitochondria, as would be expected if epitope C but not epitope L were exposed on the outer surface. Moreover, whereas digitonin treatment had no enhancing effect on the removal of anti-C (indeed, it had a slightly inhibitory effect), it markedly enhanced the clearance of anti-L antibody from solution by the mitochondria (Figure 6b). These observations confirmed that epitope C is exposed on the cytosolic face of the mitochondrial outer membrane, whereas epitope L protrudes into the IMS.

Although anti-N antibody could be used to detect CPT I on Western blots (Figures 4 and 5), it failed to bind to either intact or digitonin-treated mitochondria (results not shown). This could have resulted from the inaccessibility of the N-terminal domain of CPT I in the native protein (see below).

Correlation of deduced topology with kinetic behaviour of CPT I

The results of all the approaches used in this study to elucidate the topology of CPT I in rat liver mitochondria suggest the following features: (i) CPT I has two TMDs, (ii) the N- and C-termini protrude on the same (cytosolic) side of the mitochondrial outer membrane, and (iii) the short loop region (27 residues) connecting the two TMDs protrudes into the IMS. The deduced topology is shown diagrammatically in Figure 7.

Analysis of the cDNA nucleotide sequence for rat liver mitochondrial CPT I shows that the C-terminal domain is the only region of the protein that shows identity with those of CPT II and peroxisomal carnitine octanoyltransferase [7]. Both of these carnitine acyltransferases catalyse the same reaction as CPT I (although with different specificities), but are not integral membrane proteins and lack any domains corresponding to the N-terminal 130 amino acid residues (including TMDs 1 and 2) of

Figure 6 Quantification of the binding of anti-L and anti-C antibodies by rat liver mitochondria

Increasing amounts of (a) intact or (b) digitonin-treated mitochondria (see the text) were incubated with anti-L (■) or anti-C (□) antibodies for 16 h at 0 °C with continuous gentle mixing, in a medium containing 120 mM KCl, 5 mM Tris, 1 mM EGTA and 0.36% BSA (pH 7.4). After sedimentation of the mitochondria, back-titration of the unreacted antibodies remaining in the supernatant was performed by ELISA using microtitre plates coated with either L or C peptide (200 ng). Antibody binding to the plates was quantified using donkey anti-sheep IgG conjugated to horseradish peroxidase (1:6000, in medium containing 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 0.05% Tween-20). Detection of bound conjugate was performed with 3,3',5,5'-tetramethylbenzidine. Values are means $\pm$ S.E.M. for four mitochondrial preparations.

Figure 7 Deduced topology of CPT I within the mitochondrial outer membrane (om)

The main features of the topology of CPT I are shown: (i) the protein has two TMDs; (ii) TMD 1 has an $\text{N}_{\text{cyto}}-\text{C}_{\text{ims}}$ orientation (where cyto is cytoplasm) and TMD 2 has a reverse one to this; (iii) both the N- and C-termini of the protein are exposed on the cytosolic aspect of the membrane; and (iv) the loop region between the two TMDs protrudes into the IMS. The positions of the N, L and C epitopes, against which the respective anti-peptide antibodies were raised, are indicated.

the CPT I sequence. Therefore we propose that the active site of CPT I resides within the large domain C-terminal to TMD 2 (see Figures 1 and 7) and that, as this is localized by our studies to the cytosolic face of the membrane, the active site is also cytosolic. This is supported by our observation that, functionally, the active site of the enzyme is fully accessible to immobilized octanoyl-CoA in mitochondria with intact outer membranes (Table 1). It is evident, therefore, that the marked loss of activity observed upon cleavage of the extreme N-terminus by proteinase K cannot be interpreted as indicating that this event represents the loss of the active site itself. Although there was quantitative conversion of CPT I into a marginally smaller (by ≤ 1 kDa) product, this product was still appreciably ($\sim 50\%$) active. It is suggested that the ability of the extreme N-terminus to affect CPT I activity so markedly may be due to its interaction with the C-terminal domain, so as to maintain the conformation of the latter in an state that is optimal for the expression of catalytic activity. Such an inter-domain interaction is plausible in view of the location of both the N- and C-terminal domains on the cytosolic aspect of the membrane and of the close proximity of

the two TMDs linking them (separated by only a short 27-residue loop region).

The loss of malonyl-CoA sensitivity that accompanies the cleavage of the extreme N-terminus by proteinase K indicates that such an interaction may also be involved in maintaining the optimal tertiary structure required for the malonyl-CoA sensitivity of CPT I. However, in this instance, further loss of N-terminal structure (when proteinase K also acts from the IMS side of the membrane) results in a much more accelerated loss of sensitivity, suggesting that other interactions, e.g. (i) between the truncated N-terminus and the C-terminal domain, (ii) between TMD 1 and the membrane, and (iii) an intra-membrane interaction between TMD 1 and TMD 2 [27], may be involved in determining the sensitivity of CPT I to malonyl-CoA. These inferences concur with the previous demonstration [16] that the expression in yeast of a truncated CPT I cDNA lacking the coding region for the N-terminal 82 amino acids results in a protein that, although still catalytically competent, is largely malonyl-CoA-insensitive (The higher expressed activity of the truncated protein [28] could have resulted from a higher expression efficiency of this construct.) A strong interaction between the N- and C-terminal domains in the protein may also explain the inaccessibility, in the native protein, of residues 14–29 to anti-N antibody, which was totally ineffectual in binding to CPT I in isolated mitochondria (see above), whereas it could detect the intact protein on Western blots. We also found that highly pure preparations of an aminopeptidase and of two carboxypeptidases (W and Y) were also unable to decrease the size of CPT I, again indicating the highly folded nature of the protein and its general resistance to proteases when these are used at low, non-membrane-disrupting concentrations.

The membrane topology deduced from the present studies may explain the distinctive property of CPT I of displaying altered sensitivity to malonyl-CoA and affinity for palmitoyl-CoA when assayed in mitochondria isolated from rats maintained under different (patho)physiological conditions [29–31]. It has been established that this phenomenon is related to changes in the molecular order ('fluidity') of the membrane lipids [23]. Increased fluidity of mitochondrial outer membrane lipids results in an enzyme that is much less sensitive to malonyl-CoA (V. A. Zammit, C. G. Corstorphine, M. P. Kolodziej and F. Fraser unpublished work). Moreover, these physiologically induced

changes can be mimicked in isolated mitochondria and purified outer membranes by artificially altering the fluidity of membrane lipids *in vitro* [23,32] or by incorporation of additional phospholipids [33]. Changes in the disposition of the TMDs within the lipid core of the membrane [34] may be expected to affect the interaction between the N- and C-terminal domains of CPT I, with consequent effects on the kinetic characteristics of the enzyme.

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