Demonstration of N- and C-terminal domain intramolecular interactions in rat liver carnitine palmitoyltransferase 1 that determine its degree of malonyl-CoA sensitivity

Audrey FAYE*, Karen BORTHWICK†, Catherine ESNOUS*, Nigel T. PRICE†, Stéphanie GOBIN*, Vicky N. JACKSON†, Victor A. ZAMMIT†, Jean GIRARD* and Carina PRIP-BUUS*¹

*Département d'Endocrinologie, Institut Cochin, INSERM U567, CNRS Unité Mixte de Recherche 8104, Université René Descartes, 24 rue du Faubourg Saint-Jacques, 75014 Paris, France, and †Department of Cell Biochemistry, Hannah Research Institute, Ayr, Scotland KA6 5HL, U.K.

We have previously proposed that changes in malonyl-CoA sensitivity of rat L-CPT1 (liver carnitine palmitoyltransferase 1) might occur through modulation of interactions between its cytosolic N- and C-terminal domains. By using a cross-linking strategy based on the trypsin-resistant folded state of L-CPT1, we have now shown the existence of such N–C (N- and C-terminal domain) intramolecular interactions both in wild-type L-CPT1 expressed in *Saccharomyces cerevisiae* and in the native L-CPT1 in fed rat liver mitochondria. These N–C intramolecular interactions were found to be either totally (48-h starvation) or partially abolished (streptozotocin-induced diabetes) in mitochondria isolated from animals in which the enzyme displays decreased malonyl-CoA sensitivity. Moreover, increasing the outer membrane fluidity of fed rat liver mitochondria with benzyl alcohol *in vitro*, which induced malonyl-CoA desensitization, attenuated the N–C interactions. This indicates that the changes in malonyl-CoA sensitivity of L-CPT1 observed in mitochondria from starved and diabetic rats, previously shown to be associated with altered mem-

brane composition *in vivo*, are partly due to the disruption of N–C interactions. Finally, we show that mutations in the regulatory regions of the N-terminal domain affect the ability of the N terminus to interact physically with the C-terminal domain, irrespective of whether they increased [S24A (Ser²⁴ → Ala)/Q30A] or abrogated (E3A) malonyl-CoA sensitivity. Moreover, we have identified the region immediately N-terminal to transmembrane domain 1 (residues 40–47) as being involved in the chemical N–C cross-linking. These observations provide the first demonstration by a physico-chemical method that L-CPT1 adopts different conformational states that differ in their degree of proximity between the cytosolic N-terminal and the C-terminal domains, and that this determines its degree of malonyl-CoA sensitivity depending on the physiological state.

Key words: carnitine palmitoyltransferase, carnitine acyltransferase, malonyl-CoA, intramolecular interaction, cross-linking, mitochondria.

INTRODUCTION

In mammals, mitochondrial β -oxidation of long-chain fatty acids plays a major role in the regulation of energy homeostasis, especially during fasting, absolute or relative insulin deficiency, or prolonged exercise. CPT1 (carnitine palmitoyltransferase 1) (EC 2.3.1.21), which forms part of the carnitine-shuttle system, is the key regulatory enzyme of long-chain fatty acid oxidation [1,2]. This enzyme catalyses the first step in the transfer of long-chain acyl moieties from the cytosolic compartment into the mitochondrial matrix, where they undergo β -oxidation. The CPT1 activity is tightly regulated by its physiological inhibitor malonyl-CoA, the first intermediate in fatty acid biosynthesis [3]. This provides a mechanism for physiological regulation of β -oxidation in all mammalian tissues and for cellular fuel sensing, based on the availability of fatty acids and glucose [1,4,5].

In mammalian tissues, three CPT1 isoforms with various tissue distributions exist: a liver (L-CPT1 or CPT1-A), a muscle (M-CPT1 or CPT1-B) and an isoform abundant in brain (CPT1-C), which are encoded by different genes [1,2,6]. In contrast to M-CPT1, one of the distinctive properties of the liver isoform is that its sensitivity to malonyl-CoA inhibition varies markedly depending on physiological state in adult rat. For example, it is increased

by refeeding carbohydrate to fasted rats, by obesity or after insulin administration to diabetic rats, whereas it is decreased by starvation and diabetes [7–10]. These changes in malonyl-CoA sensitivity have been shown to be under the control of pancreatic hormones and to occur long term [10,11]. In a recent publication [12] it was found that residues Ser⁷⁴¹ and Ser⁷⁴⁷ of rat L-CPT1 are phosphorylated in fed rat liver OMM (mitochondrial outer membrane) isolated in the presence of protein phosphatase inhibitors. Dephosphorylation of these residues, which could only be achieved by exposure to a mitochondrial matrix phosphatase, altered the sensitivity of the enzyme to malonyl-CoA sensitivity [12]. However, cytosolic phosphatases seemed to be unable to achieve dephosphorylation, and protein kinases that are normally involved in acute modulation of metabolism (e.g. protein kinase A, AMP-activated protein kinase, protein kinase C, calcium/calmodulin protein kinase II) were ineffective in mediating phosphorylation of these residues. Only protein kinase CK2 was able to phosphorylate these residues after matrix phosphatase treatment [12]. Therefore, the physiological relevance of these findings with respect to the well-established stable changes in malonyl-CoA sensitivity observed previously in mitochondria isolated from livers *in vivo* [7–10] and cultured cells [11] – all observed in the absence of phosphatase inhibitors – is not clear, beyond the

Abbreviations used: BS³, bis(sulphosuccinimidyl) suberate; (L/M)-CPT1, (liver/muscle) carnitine palmitoyltransferase 1; E3A etc., Glu³ → Ala etc.; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; f82, 82-kDa fragment; GMBS, *N*-(γ -maleimidobutyloxy)-succinimide ester; LC-SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxy-(6-amidocaproate); MBS, 3-maleimidobenzoyl-*N*-hydroxysuccinimide ester; N–C interaction, N- and C-terminal domain interaction; OMM, mitochondrial outer membrane; sulpho-GMBS, *N*-(γ -maleimidobutyloxy)-sulphosuccinimide ester; sulpho-KMUS, *N*-(κ -maleimidoundecanoyloxy)-sulphosuccinimide ester; sulpho-MBS, 3-maleimidobenzoyl-*N*-hydroxysulphosuccinimide ester; TM, transmembrane.

¹ To whom correspondence should be addressed (email prip-buus@cochin.inserm.fr).

possible setting of the inherent properties of the protein through constitutive phosphorylation by CK2.

Changes in malonyl-CoA sensitivity of rat L-CPT1 have been shown to be associated with changes in the OMM composition environment *in vivo*. In liver mitochondria isolated from rats in different physiological states these changes are also associated with changes in membrane fluidity [13] and can be mimicked *in vitro* by temperature- or chemically-induced modulation of OMM lipid bilayer fluidity [14,15]. All these observations strengthened the hypothesis that L-CPT1 may adopt at least two conformational states that are more or less sensitive to malonyl-CoA inhibition. However, the molecular basis for the changes in malonyl-CoA sensitivity remains largely unknown, despite clues that have begun to emerge from functional mutagenesis studies. Rat L-CPT1 is an integral membrane protein anchored in the OMM that harbours two hydrophobic transmembrane segments (TM1 and TM2) [1]. Its N-terminal 47-residue domain and large C-terminal catalytic domain (residues 123–773) are both exposed to the cytosolic face of the OMM, whereas the loop connecting TM1 and TM2 protrudes into the intermembrane space [16]. The N-terminal domain, including TM1 and TM2 (1–147 residues), was shown to be responsible for mitochondrial import into the OMM and for maintenance of a folded active and malonyl-CoA-sensitive conformation [17,18]. Limited proteolysis of the extreme N terminus with proteinase K [16] and functional mutagenesis studies [19,20] have shown that the N-terminal 18 amino acids, and particularly Glu³, are required for malonyl-CoA sensitivity. In fact, the N-terminus contains predicted α -helices that are either positive (residues 1–18) or negative (residues 19–30) determinants of malonyl-CoA sensitivity [21,22], but is unable to bind malonyl-CoA with a high affinity independently of the C-terminal domain [23]. Moreover, functional study of chimeric L- and M-CPT1 proteins indicated that the nature of the cytosolic N–C interactions might be involved in the degree of malonyl-CoA sensitivity of the L-isoform [24]. The existence of such N–C (N- and C-terminal domain) interactions was supported by the identification of a highly trypsin-resistant 60-kDa folded core within the catalytic C-terminal that is masked in the native protein by its cytosolic N-terminal residues [18]. However, direct demonstration of the existence of N–C interactions, as well as the modulation of these interactions in parallel with changes in malonyl-CoA sensitivity, has not yet been made. Although the three-dimensional structure models for the catalytic domain of L-CPT1 have been published, based on the crystal structure of the carnitine acetyltransferase [25,26], these cannot be used to model the N–C interactions because the first 165 residues of L-CPT1 do not have a counterpart in carnitine acetyltransferase [2].

In the present study, we have used an alternative approach, namely, a cross-linking strategy that has been successfully used to study intramolecular interactions within proteins [27]. We examined whether the cytosolic N-terminus interacts with the catalytic C-terminal domain of rat L-CPT1, and whether these intramolecular interactions are altered in situations associated with changes in malonyl-CoA sensitivity of the enzyme. By using a cross-linking strategy, based on the trypsin-resistant folded state of L-CPT1, we demonstrated the existence of N–C interactions both in L-CPT1 expressed in *Saccharomyces cerevisiae* and in the native protein in mitochondria isolated from fed rat liver. Moreover, these N–C interactions were either totally abrogated or markedly altered by the E3A (Glu³ → Ala) and S24A/Q30A substitutions, by starvation and by streptozotocin-induced diabetes, as well as by increasing membrane fluidity of mitochondria. Altogether, these results demonstrate that rat L-CPT1 adopts different conformational states that are more, or less, sensitive to malonyl-CoA inhibition, and that involve different

degrees of proximity between specific residues within the N- and C-domains under conditions characterized by changes in malonyl-CoA sensitivity.

EXPERIMENTAL

Construction of expression plasmids, yeast culture and isolation of yeast mitochondria

To generate the E3A mutant, PCR was performed using a previously described *Pichia pastoris* expression construct [21] as template with the primers 5'-CTAAGAATTCGATGGCTGCTGCTCACC-3' and 5'-TTCCTTCATCAGTGGCCTTACAGA-3'. The primers 5'-TTAAGAATTCGATGGCAGAGGCTCACC-3' and 5'-CTCGGCCCGCAGGTAGATA-3' were used to amplify a region from S24A/Q30A construct, as described previously [22]. The purified PCR products were digested with *EcoRI* and *Acc65I*, and used to replace the corresponding fragment of rat L-CPT1 in pYEDP1/8-10 [28]. cDNA of wild-type, E3A and S24A/Q30A mutants were placed under the control of the inducible *GAL10* promoter, and the constructs were used to transform *S. cerevisiae* (haploid strain W303: *MATa*, *his3*, *leu2*, *trp1*, *ura3*, *ade2-1*, *can1-100*). The yeast expression construct for the human L-CPT1 has been described previously [29]. Yeast strains were cultivated on selective minimal lactate medium (0.17 % yeast nitrogen base without amino acids, 0.5 % ammonium sulphate, 0.1 % casamino acids, 2 % lactate, 40 mg/ml adenine sulphate, 40 mg/ml L-tryptophan) at 30 °C with shaking. After induction of protein expression by 2 % galactose for 15 h, yeast mitochondria were isolated as described previously [28], resuspended in the isolation buffer (0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4) and stored at –80 °C until used.

Isolation of rat liver mitochondria

Male Wistar rats (200–300 g) (Centre d'Élevage et de Reproduction Janvier, Le Genest St Isle, France) had continuous free access to water and were either fed *ad libitum* on a standard laboratory chow diet (62 % carbohydrate, 12 % fat and 26 % protein in terms of energy) or starved for 48 h. Diabetes was induced by a single intraperitoneal injection of streptozotocin (52 mg/kg of body mass). Only animals that 2 days after streptozotocin treatment had a blood glucose concentration higher than 20 mM were used at 5 days. All animals were kept on a light–dark cycle (light from 15:00 to 03:00 h) and were killed at 08:00 h. Rat liver mitochondria were isolated in an isolation buffer (0.3 M sucrose, 5 mM Tris/HCl, 1 mM EGTA, pH 7.4) using differential centrifugation, further purified on self-forming Percoll gradients and resuspended in the isolation buffer as described previously [17].

Chemical cross-linking

Mitochondria were washed twice either in HSY buffer (10 mM Hepes, 0.6 M sorbitol, pH 8; yeast mitochondria) or in HSR buffer (10 mM Hepes, 0.3 M sucrose, pH 8; rat liver mitochondria), and finally resuspended in the respective buffer at a protein concentration of 2 mg/ml. Where indicated, mitochondria were pre-incubated for 4 min at 30 °C in the presence of 0, 40, 100 or 200 mM of benzyl alcohol, a membrane-fluidizing agent. The polar cross-linkers used were dissolved in water, and were BS³ [bis(sulphosuccinimidyl) suberate], EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide], sulpho-GMBS [*N*-(γ -maleimidobutyloxy)-sulphosuccinimide ester], sulpho-MBS (3-maleimido-benzoyl-*N*-hydroxysulphosuccinimide ester) and sulpho-KMUS [*N*-(κ -maleimidoundecanoyloxy)-sulphosuccinimide ester]. The

apolar cross-linkers used were dissolved in DMSO, and were GMBS [*N*-(γ -maleimidobutyloxy)-succinimide ester], MBS [3-maleimidobenzoyl-*N*-hydroxysuccinimide ester] and LC-SMCC [succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxy-(6-amidocaproate)]. After addition of cross-linkers to a final concentration of either 25, 50, 100 or 250 μ M to mitochondria (100 μ g), samples were incubated for 30 min at 4°C. Excess of cross-linkers was then quenched by the addition of 7.5 μ l of TSY buffer (750 mM Tris/HCl, 0.6 M sorbitol, pH 8) (yeast mitochondria) or of TSR buffer (750 mM Tris/HCl, 0.3 M sucrose, pH 8) (rat liver mitochondria). After incubation for 15 min at 4°C, mitochondria were recovered by centrifugation, washed and finally resuspended in the respective isolation buffer at a protein concentration of 0.5 mg/ml before performing the trypsin treatment.

Treatment of mitochondria with trypsin

Mitochondria (50 μ g) were incubated for 30 min on ice either in the iso-osmotic isolation buffer or in a hypo-osmotic buffer (20 mM Hepes, pH 7.4) with various concentrations of trypsin (0–10 μ g/ml). Soya-bean trypsin inhibitor was then added in a 20-fold excess, and mitochondria were further incubated on ice for 10 min. After centrifugation for 7 min at 12 000 *g*, mitochondria were washed in the isolation buffer containing 1 mM EDTA and 1 mg/ml soya-bean trypsin inhibitor, and then lysed directly in Laemmli buffer containing 1 mM PMSF. Samples were analysed by SDS/PAGE and immunoblotting.

Western-blot analysis

Aliquots of proteins were subjected to SDS/PAGE [30] (8 % gel). The detection of L-CPT1 after blotting on to nitrocellulose was performed as described previously [28] using the ECL[®] detection system (Pierce), according to the manufacturer's instructions, and an antibody against the rat L-CPT1 [28]. The immunoblots were quantified using a Chemigenius apparatus (Syngene).

CPT assay

CPT activity was assayed using either yeast mitochondria or freshly isolated rat liver mitochondria (0.1 mg of protein/ml) at 30°C as palmitoyl-L-[methyl-³H]carnitine formed from L-[methyl-³H]carnitine (200 μ M; 10 Ci/mol) and palmitoyl-CoA (80 μ M) in the presence of 1 % (w/v) BSA as described previously [28]. Malonyl-CoA concentration ranged from 0.01 to 600 μ M for estimation of the IC₅₀ value (concentration of malonyl-CoA required to achieve 50 % inhibition at 80 μ M palmitoyl-CoA). When indicated, benzyl alcohol was included at a final concentration of 20 mM in the assay medium during the 4-min period of pre-incubation before initiation of the reaction by addition of L-carnitine, as described previously [14].

Miscellaneous and chemicals

Protein concentration was determined by the method of Lowry [31] with BSA as a standard. All cross-linkers were purchased from Pierce. Yeast culture media products were from Difco, and Zymolase 20T was from ICN Biomedicals, France. All restriction enzymes and T4 DNA ligase were from Stratagene. PCR reagents and T4 DNA polymerase were obtained from Invitrogen (San Diego, CA, U.S.A.). Other chemicals were purchased from Sigma.

Statistical analysis

The results are expressed as the means $\pm$ S.E.M. Statistical analysis was performed using the ANOVA test with the StatView program (Abacus).

RESULTS

Detection of N–C intramolecular interactions within rat L-CPT1 expressed in yeast

The first aim of the present study was to determine whether the N-terminal domain of rat L-CPT1 interacts with its catalytic C-terminal domain. For this purpose, we developed a cross-linking strategy based on the resistance of L-CPT1 towards trypsin proteolysis in its native conformation (Figure 1A). Previous studies have shown that, in intact mitochondria, L-CPT1 exhibits a native functional conformation characterized by a highly folded state that is resistant to trypsin proteolysis [16,18,23,28]. When the OMM was disrupted by swelling, trypsin was then able to cleave the loop connecting TM1 and TM2, hence generating an 82-kDa fragment (f82) (Figure 1A, a–c). We hypothesized that if a cross-linking agent was able to establish a covalent bond between the N- and C-terminal domains of L-CPT1 (Figure 1A, d), then the protein would still migrate at 88 kDa, even when cleaved by trypsin upon swelling of the mitochondria (Figure 1A, e). This should result in an increase in the L-CPT1/f82 ratio in comparison with non-cross-linked mitochondria (Figure 1A, compare c and f). Therefore, the ability of a cross-linker to reverse the L-CPT1/f82 ratio would allow us to conclude that the N-terminal domain comes into close proximity to the catalytic C-terminal domain such that the distance can be chemically bridged, suggesting the existence of N–C interactions. In order to select such cross-linkers, an initial series of experiments was performed using heterologous expression of rat L-CPT1 in *S. cerevisiae*, a system devoid of endogenous CPT [28,32]. As shown in Figures 1(B) and 1(C), in the absence of cross-linker, a concentration of trypsin of 5 μ g/ml was required to generate a ratio of approx. 1:1 between intact L-CPT1 protein and the f82 fragment in swollen yeast mitochondria expressing L-CPT1. In the presence of the polar amine/amine BS³ (which has a spacer arm of 11.3 Å between its functional groups) or amine/carboxyl EDC (no spacer arm) cross-linkers, this L-CPT1/f82 ratio remained unchanged in comparison with control mitochondria (Figures 1B and 1C). This indicated that these cross-linkers were unable to establish an N–C intramolecular bond. By contrast, the polar amine/sulphydryl sulpho-KMUS (15.7 Å spacer arm) cross-linker strongly increased the L-CPT1/f82 ratio up to more than 4:1 (Figures 1B and 1C). This clearly validated the cross-linking strategy. In order to determine the importance of the spacer-arm length of the cross-linker in the establishment of such N–C intramolecular cross-linking, we used two shorter polar analogues of sulpho-KMUS, namely, sulpho-GMBS (6.8 Å) and sulpho-MBS (9 Å). By contrast to sulpho-KMUS, both these analogues were unable to effect cross-linking of the N- and C-domains, and thus to change the L-CPT1/f82 ratio (Figure 2A), indicating that a minimal length of 15.7 Å was required for the generation of an N–C intramolecular link within L-CPT1. Moreover, as sulpho-KMUS is a polar cross-linker which is unable to cross the OMM, it was concluded that the N–C covalent bond must have been generated on the external aspect of the mitochondria.

In order to detect other intramolecular interactions, such as TM1–TM2 or intra-loop interactions, membrane-permeant analogues of sulpho-KMUS, such as the apolar GMBS (spacer arm 6.8 Å), MBS (9 Å) and LC-SMCC (16.1 Å), were tested. Similarly to what was observed for the polar sulpho-analogues, only the longest cross-linker was able to generate a cross-linked product (Figure 2B). However, LC-SMCC was less efficient than sulpho-KMUS, since it increased the L-CPT1/f82 ratio only to 7:3 at the concentration of 250 μ M, this ratio being already reached at a much lower sulpho-KMUS concentration (100 μ M) (compare Figures 2A and 2B). Thus both apolar amine/sulphydryl cross-linkers with shorter spacer arms (Figure 2B), as well as the apolar

Figure 1 Detection of intramolecular interactions between the N- and C-terminal domains of L-CPT1 using a cross-linking strategy

(A) The cross-linking strategy is based on the ability of trypsin to gain access to the trypsin-sensitive site within the inter-TM loop of L-CPT1 in the intermembrane space once the OMM is disrupted by hypo-osmotic swelling, and to generate a 82-kDa fragment (f82) (b) that can be detected by Western-blot using an antibody against rat L-CPT1 (c). Cross-linking performed on intact mitochondria (a) generates a covalent link between the N-terminus and the C-terminal domain (d); the cross-linked L-CPT1 migrates on a SDS/PAGE at 88 kDa, even when cleaved by trypsin upon swelling (e). Establishment of such a cross-link bond should lead to an increased L-CPT1/f82 ratio (f) in comparison with non-cross-linked mitochondria (c). Black rectangles, TM segments of L-CPT1; *, trypsin cleavage site. (B) Isolated yeast mitochondria expressing L-CPT1 were incubated for 30 min at 4°C with equal volumes of either water or polar cross-linkers (BS³, EDC or sulpho-KMUS) at a final concentration of 250 μ M. After quenching the excess of cross-linker, mitochondria were washed, resuspended in the isolation buffer and incubated for 30 min at 4°C in the iso-osmotic (Swelling, -) or hypo-osmotic (Swelling, +) buffer in the absence or presence of trypsin (5 μ g/ml). After addition of soya-bean trypsin inhibitor in a 20-fold excess over trypsin, samples were sedimented, washed, subjected to SDS/PAGE (8% gel), and analysed by Western blotting using anti-(rat L-CPT1) antibody. A representative Western blot is shown. (C) The L-CPT1 (open bars) and f82 (closed bars) bands from Western blots from four to five different experiments with separate mitochondrial preparations were quantified using a Chemigenius apparatus (Syngene), and the results expressed as percentage mean $\pm$ S.E.M. of total L-CPT1 protein (L-CPT1 and f82). * P < 0.05 when compared with water.

Figure 2 Effect of the length and the polarity of the cross-linkers on the efficiency to establish a N-C covalent bond

Yeast mitochondria expressing L-CPT1 were treated as described in the legend for Figure 1. Cross-linking experiments were performed in the presence of either water, sulpho-GMBS, sulpho-MBS or sulpho-KMUS (A), or DMSO, GMBS, MBS or LC-SMCC (B), at the concentrations indicated. All samples were then submitted to swelling in the presence of trypsin and analysed by SDS/PAGE and immunoblotting using anti-(rat L-CPT1) antibody. Western blots from 4–5 different experiments with separate mitochondrial preparations were quantified. L-CPT1 (open bars) and f82 (closed bars) were expressed as the percentage mean $\pm$ S.E.M. of total L-CPT1 protein (L-CPT1 and f82). * P < 0.05 when compared with water (A) or DMSO (B).

amine/amine DSG (disuccinimidyl glutarate) (9 Å) and DFDNB (1,5-difluoro-2,4-dinitrobenzene) (3 Å) cross-linkers (results not shown), did not result in the detection of intramolecular bonds within rat L-CPT1 on either side of the tryptic site within the inter-TM loop. Moreover, as only the apolar LC-SMCC was able to mimic sulpho-KMUS in generating a covalent cross-link, the detected N-C intramolecular interactions are likely to have occurred on the cytosolic face of the mitochondria, i.e. between the cytosolic N-terminus and the catalytic C-terminal domain of L-CPT1. This establishes, for the first time, the existence of N-C intramolecular interactions within the rat L-CPT1 using a physico-chemical technique.

Native rat liver mitochondrial L-CPT1 exhibits N-C intramolecular interactions that are altered by starvation and diabetes of the donor animals

In order to determine whether physio-pathological situations that are associated with changes in malonyl-CoA sensitivity are able to alter such N-C intramolecular interactions in native rat L-CPT1 in its normal membrane-environment, liver mitochondria were isolated from control fed, 48-h starved or streptozotocin-induced diabetic rats. As previously reported [10,33], both starvation and

Figure 3 L-CPT1 from starved rat liver mitochondria is more resistant to trypsin proteolysis upon swelling

(A) Liver mitochondria (50 µg of protein) isolated from fed, 48-h starved and streptozotocin-diabetic rats were analysed by SDS/PAGE and immunoblotting using anti-rat L-CPT1 antibody. (B) Liver mitochondria from fed, 48-h starved and streptozotocin-induced diabetic rats were incubated for 30 min at 4°C in the swelling buffer in the absence or presence of varied concentrations of trypsin. After addition of soya-bean trypsin inhibitor in a 20-fold excess, samples were sedimented, washed, subjected to SDS/PAGE (8% gel), and analysed by Western blotting using anti-rat L-CPT1 antibody. Representative Western blots are shown. (C) The f82 bands from Western blots from 4–5 independent trypsin experiments, as in (B), were quantified and expressed as the percentage means $\pm$ S.E.M. of total L-CPT1 (L-CPT1 and f82). $\circ$, fed rat; $\bullet$, 48-h starved rat; Δ , diabetic rat. * P < 0.05 when compared to the fed state.

diabetes increased L-CPT1 protein level (Figure 3A) and activity (Table 1) and decreased its sensitivity to malonyl-CoA inhibition, the IC_{50} value for malonyl-CoA being 10- and 4-fold higher respectively than that of L-CPT1 in mitochondria isolated from fed rat (Table 1).

Preliminary experiments were first performed to determine the concentration of trypsin required to obtain an L-CPT1/f82 ratio of approx. 1:1. In agreement with a lower L-CPT1 protein expression in fed rat liver than in yeast expressing L-CPT1 [28], a 10-fold lower trypsin concentration was needed to generate an L-CPT1/f82 ratio of 1:1 in fed rat liver mitochondria (Figures 3B and 3C) so that similar trypsin/mitochondrial protein ratios were required. However, L-CPT1 from starved rat was less susceptible to trypsin action than L-CPT1 from fed and diabetic rats, at all the trypsin concentrations tested (Figures 3B and 3C). This difference in susceptibility towards trypsin action was independent of the

Table 1 Enzyme activity and malonyl-CoA sensitivity of the wild-type, E3A and S24A/Q30A mutants L-CPT1 expressed in *S. cerevisiae* and of native L-CPT1 from rat liver

Mitochondria were isolated either from the yeast strains expressing wild-type, E3A and S24A/Q30A mutants L-CPT1 or from fed, 48-h starved and streptozotocin-induced diabetic rat liver. Mitochondria were assayed for CPT1 activity in the presence of 80 µM palmitoyl-CoA and 200 µM carnitine. Malonyl-CoA sensitivity was measured, as described in the Experimental section, in the absence or in the presence of 20 mM of benzyl alcohol. The IC_{50} value is the concentration of malonyl-CoA needed to inhibit 50% of the CPT1 activity. The results are expressed as the means $\pm$ S.E.M. for 3–7 separate preparations of mitochondria. Values that are statistically significantly different from the wild-type and from the fed state are indicated respectively; *, $\dagger P$ < 0.05. ND, not determined.

	L-CPT1 activity (nmol/min per mg of protein)	IC_{50} malonyl-CoA (µM)
Yeast strain		
Rat Wild-type	5.01 $\pm$ 0.32	6.14 $\pm$ 1.38
E3A	5.57 $\pm$ 0.49	ND ($\geq$ 600)
S24A/Q30A	3.87 $\pm$ 0.30	0.89 $\pm$ 0.30*
Rat liver		
Fed	2.06 $\pm$ 0.27	5.80 $\pm$ 0.92
48-h starved	3.53 $\pm$ 0.18†	59.28 $\pm$ 17.11†
Fed + benzyl alcohol	3.62 $\pm$ 0.13†	21.90 $\pm$ 4.49†
Streptozotocin-induced diabetic	3.12 $\pm$ 0.08†	25.41 $\pm$ 2.75†

level of L-CPT1 expression; whereas both starvation and diabetes induced a 2- to 3-fold increase in L-CPT1 protein level (Figure 3A), only L-CPT1 in mitochondria from starved rat showed a requirement for an increased trypsin concentration. These observations suggest that a change in the conformation and/or the organization of L-CPT1 within the OMM may occur during fasting, giving rise to restricted accessibility to the trypsin cleavage site within the inter-TM segment loop. These preliminary observations necessitated the use of different concentrations of trypsin, depending on the physio-pathological state of the animals used as a source for mitochondria. The trypsin concentrations used were 0.5 µg/ml for the fed and diabetic states and 2 µg/ml for mitochondria from starved rats.

Cross-linking experiments using mitochondria isolated from fed rats showed that the L-CPT1/f82 ratio was increased up to 3:1 and 19:1 in the presence of 50 µM and 250 µM of sulpho-KMUS respectively (Figure 4A). The ability of the cross-linker to increase this ratio was more pronounced for the native protein in fed rat liver mitochondria than for the protein expressed in *S. cerevisiae* (compare Figure 4A with Figure 2A). This difference may result from the over-expression of the protein in *S. cerevisiae*. By contrast, in mitochondria isolated from 48-h starved rats, the L-CPT1/f82 ratio remained constant at 1:1 at the various concentrations of cross-linker used (Figure 4B). Similarly, in mitochondria isolated from diabetic rats, concentrations of sulpho-KMUS higher than 50 µM were required to increase the L-CPT1/f82 ratio (Figure 4C), indicating the occurrence of altered N–C intramolecular interactions that make the cross-linking less favourable. These results indicated that, as observed for L-CPT1 expressed in *S. cerevisiae*, the cytosolic N-terminus interacts with the C-terminal domain of the native fed rat L-CPT1 and that these N–C interactions are altered to a greater or lesser extent by starvation and diabetes, two situations associated with a variable decrease in malonyl-CoA sensitivity.

Effect of membrane fluidization *in vitro* on the N–C intramolecular interactions

An increase in the fluidity of the OMM by a chemical agent, such as benzyl alcohol, has been shown to decrease the sensitivity

Figure 4 Starvation and diabetes induce a modification of the N-C intramolecular interactions in L-CPT1

Cross-linking experiments were performed in the presence of water or sulpho-KMUS at the indicated concentrations using liver mitochondria isolated from fed (A), 48-h starved (B) or streptozotocin-induced diabetic (C) rats. All samples were submitted to swelling in the presence of trypsin at 0.5 μg/ml (fed and diabetic) or 2 μg/ml (starved) and then analysed as in Figure 1. Representative Western blots are shown at the top of each panel. Western blots from 3–6 different experiments with separate mitochondrial preparations were quantified. L-CPT1 (open bars) and f82 (closed bars) were expressed as the percentage means ± S.E.M. of total L-CPT1 (L-CPT1 and f82). * $P < 0.05$ when compared with non-cross-linked mitochondria, † $P < 0.05$ when compared with f82 from fed rat (grey bars).

Figure 5 Decrease in malonyl-CoA sensitivity of L-CPT1 induced by membrane fluidization of fed rat liver mitochondria with benzyl alcohol is accompanied by attenuated N-C intramolecular interactions

(A) CPT activity was assayed at different concentrations of malonyl-CoA in fed rat liver mitochondria in the absence (○) or presence of 20 mM of benzyl alcohol (□), and in 48-h starved rat liver mitochondria (●). Results are expressed relative to activity in the absence of malonyl-CoA (100%) and are the means ± S.E.M. for 3–8 separate experiments. (B) Fed rat liver mitochondria were pre-treated with either 0, 40, 100 or 200 mM of benzyl alcohol for 4 min at 30 °C, and cross-linking experiments were then performed in the presence of water or sulpho-KMUS at the indicated concentrations. After swelling in the presence of trypsin, samples were analysed by SDS/PAGE and immunoblotting using anti-L-CPT1 antibody. Western blots are representative of 3–4 independent experiments with separate mitochondrial preparations.

of L-CPT1 to malonyl-CoA inhibition [14,15]. Therefore, we reasoned that this would be a good model with which to test the central hypothesis that changes in malonyl-CoA sensitivity of the enzyme are due to altered N-C spatial interactions. Firstly, we confirmed that benzyl alcohol was having the same effects as reported previously [14] in our present incubation conditions. In agreement with the results in [14], incubation of fed rat liver mitochondria with benzyl alcohol increased both L-CPT1 activity and its IC_{50} value for malonyl-CoA (Table 1 and Figure 5A) to values similar to those observed in the starved and diabetic states (Table 1). Interestingly, benzyl alcohol did not change the susceptibility of L-CPT1 towards trypsin proteolysis (results not shown); therefore, the same trypsin concentration as that used for mitochondria isolated from fed rats was used for these cross-linking experiments. In the absence of benzyl alcohol, the N-C intramolecular cross-linking could already be detected with

Figure 6 Detection of N–C intramolecular interactions by sulpho-KMUS also occurs in the human L-CPT1 which lacks the cytosolic N-terminal Cys residue

(A) Comparison of the native cysteine residues in the rat and human L-CPT1. The black boxes denote the TM segments of L-CPT1. (B) Yeast mitochondria expressing human L-CPT1 were treated as described in Figure 1. Cross-linking experiments were performed in the presence of water or sulpho-KMUS at the indicated concentrations. All samples were then subjected to swelling in the presence of trypsin and analysed by SDS/PAGE and immunoblotting using anti-(rat L-CPT1) antibody. Western blots from 3–4 different experiments with separate mitochondrial preparations were quantified. The results, L-CPT1 (open bars) and f82 (closed bars), were expressed as the percentage means $\pm$ S.E.M. of total L-CPT1 protein (L-CPT1 and f82). * $P < 0.05$ when compared with water.

25 μ M of sulpho-KMUS (Figure 5B). In the presence of increasing concentrations of benzyl alcohol, higher concentrations of cross-linker were required to observe the N–C intramolecular cross-linking (Figure 5B). These results indicated that the increase in membrane fluidity induced by benzyl alcohol, in addition to inducing a desensitization of L-CPT1 to malonyl-CoA inhibition, also altered the interactions between the cytosolic N terminus and the catalytic C-terminal domain of L-CPT1 in such a way as to decrease the facility of sulpho-KMUS-mediated cross-linking. Moreover, this concurred with the inverse relationship observed between the efficiency of sulpho-KMUS to generate a N–C covalent cross-link and malonyl-CoA sensitivity: the higher the IC_{50} value for malonyl-CoA (starvation > diabetes = benzyl alcohol > fed) (Table 1), the less efficient the cross-linker was in establishing a link between the regulatory N-terminus and the catalytic C-terminal domain respectively (compare Figure 4 with Figure 5B).

Direction of bi-functional cross-linking by sulpho-KMUS

The amine/sulphydryl sulpho-KMUS cross-linker establishes a covalent bond with the side chain of lysine through one of its functional groups, and a cysteine residue with the other. The primary amino acid sequence of the rat L-CPT1 indicates the existence of one (Cys³²) in the cytosolic N-terminus and twelve cysteine residues within the C-terminal domain [1] (Figure 6A). In order to investigate whether N–C cross-linking involved Cys³² and a lysine residue on the C-terminal domain, we made use of the fact that human L-CPT1 lacks a cysteine at position 32 [2] (Figure 6A). Therefore, we expressed human L-CPT1 in *S. cerevisiae* and performed cross-linking experiments using sulpho-KMUS. As

can be seen in Figure 6(B), sulpho-KMUS was able to cross-link the human protein equally effectively as for the rat protein, indicating that the direction of cross-linking was N-terminal lysine to C-terminal cysteine, rather than the other way round. Moreover, because in human L-CPT1 residue 29 is an arginine rather than lysine, we are also able to eliminate this position as the site for cross-linking by sulpho-KMUS. Therefore, this observation restricts the possibilities to one of the four lysine residues that occur very close to the predicted TM1–cytosol boundary, between residues 40 and 47 in the primary sequence.

Interference by N-terminal point mutations with sulpho-KMUS-mediated N–C cross-linking

The cytosolic N-terminus has been reported to contain both positive (residues 1–18) and negative (residues 19–30) determinants of malonyl-CoA sensitivity [21,22]. The point mutation E3A results in a marked decrease in malonyl-CoA sensitivity and in the loss of the high-affinity binding for malonyl-CoA [20,21], whereas the S24A/Q30A double mutation increases malonyl-CoA sensitivity [22]. Therefore, to ascertain whether these changes in malonyl-CoA sensitivity are associated with altered N–C interactions, we next examined whether the efficiency of cross-linking was modified in the E3A and S24A/Q30A mutants. As the previous data [20–22] from the E3A and S24A/Q30A mutants had been obtained on mutant proteins expressed in *P. pastoris*, first we needed to confirm that the same mutants, when expressed in *S. cerevisiae*, exhibited the expected opposite changes in malonyl-CoA sensitivity. As shown in Table 1 and Figure 7(A), this was found to be the case: the E3A mutant had an increased IC_{50} value for malonyl-CoA in comparison with the wild-type protein, whereas the S24A/Q30A mutant had a malonyl-CoA sensitivity at least one order of magnitude higher than that of the wild-type (Table 1 and Figure 7A). Importantly, for both these mutants, there was a total loss in the ability of sulpho-KMUS to affect the L-CPT1/f82 ratio, irrespective of the concentration of cross-linker used (Figures 7B and 7C). The shorter analogues, sulpho-GMBS and sulpho-MBS, were equally ineffective (results not shown). These observations indicated that the N–C intramolecular interactions that bring a lysine residue within the Lys⁴⁰–Lys⁴⁷ sequence on the N-terminal domain in close proximity to a cysteine residue in the C-terminal domain in the wild-type protein were prevented in both mutants, irrespective of whether they favoured (S24A/Q30A) or antagonized (E3A) increased malonyl-CoA sensitivity.

DISCUSSION

Existence of physical N–C intramolecular interactions within rat L-CPT1

Previous functional studies of the cytosolic N-terminus of the rat L-CPT1 suggested that this region contains both positive and negative determinants for malonyl-CoA sensitivity [21,22] which modulate the degree of malonyl-CoA sensitivity, potentially through changes in N–C intramolecular interactions [21,24]. In the present study, we used a chemical cross-linking strategy to test this hypothesis and to gain further insight into structure–function relationships of rat L-CPT1. Among several polar/apolar and homo-/heterofunctional cross-linkers with a spacer-arm ranging from 0 to 16 Å, sulpho-KMUS was the only one able to establish a N–C intramolecular link between the N- and C-terminal domains of L-CPT1 expressed in *S. cerevisiae*. Moreover, it was able to do this in intact mitochondria isolated either from yeast or rat liver (see below). As the OMM is expected to be impermeable to

Figure 7 E3A and the S24A/Q30A mutations alter both N-C intramolecular interactions and malonyl-CoA sensitivity

(A) Isolated mitochondria from yeast strain expressing the wild-type L-CPT1 (○), the E3A (◇) or the S24A/Q30A (□) mutants were assayed for CPT activity with increasing concentrations of malonyl-CoA and fixed concentrations of palmitoyl-CoA (80 μM) and carnitine (200 μM). The results are expressed relative to activity in the absence of malonyl-CoA (100%) and are the means ± S.E.M. for 4–7 separate experiments. (B, C) Yeast mitochondria expressing the E3A (B) and the S24A/Q30A (C) mutants were treated as described in Figure 1. Cross-linking experiments were performed in the presence of water or sulpho-KMUS at the indicated concentrations. All samples were then submitted to swelling in the presence of trypsin and analysed by SDS/PAGE and immunoblotting using anti-(rat L-CPT1) antibody. Western blots from 4 different experiments with separate mitochondrial preparations were quantified. L-CPT1 (open bars) and f82 (closed bars) were expressed as the percentage means ± S.E.M. of total L-CPT1 protein (L-CPT1 and f82).

such a polar cross-linker, the N-C covalent bond must have been generated on the cytosolic face of the mitochondria, i.e. between the cytosolic N-terminal and the C-terminal domains of L-CPT1. This observation demonstrated for the first time the existence of physical N-C interactions within the L-CPT1 molecule. However, the existence of other intramolecular interactions, such as TM1-TM2 interactions, cannot presently be ruled out. Indeed, the

correct TM1-TM2 interactions between transmembrane-segment pairs that occur in native isoforms have been shown to be important in determining the affinity for carnitine for those isoforms [24]. Therefore, it is possible that a wider screening of apolar cross-linkers might detect other (TM1-TM2, intra-loop) intra-molecular interactions across the tryptic cleavage site.

Modulations of the N-C intramolecular interactions by physiological state of mitochondrial donor animals

The present study shows that the N-C intramolecular interactions exist not only in fed rat liver mitochondria, but also in the yeast-expressed L-CPT1, indicating that the tertiary structure of the protein when expressed in yeast mitochondria is very similar, if not identical, to that in its native environment. However, these interactions were either totally abolished (in mitochondria from 48-h starved rats) or became less favourable (streptozotocin-induced diabetic rats) for the enzyme in mitochondria isolated from the liver of animals in physiological conditions characterized by malonyl-CoA sensitivity that was decreased to a greater or lesser degree respectively. These observations indicate that the changes (decreases) in malonyl-CoA sensitivity of rat L-CPT1 that occur *in vivo* are associated with modification of N-C intramolecular interactions. It is well established that malonyl-CoA sensitivity of L-CPT1 is dependent on the molecular order of the membrane lipids both *in vivo* [13] and *in vitro* in isolated mitochondria [14,34] or after reconstitution of the purified protein into liposomes, i.e. independently of interaction with other proteins [15] or possibility of phosphorylation [12]. Starvation and diabetes are both associated with an increase in fluidity of the membrane core lipids with the occurrence of an inverse relationship between fluidity and malonyl-CoA sensitivity [13]. In the present study, we show that benzyl alcohol, a membrane-fluidizing agent, concomitantly induced malonyl-CoA desensitization, as previously reported [14,15], and attenuation of the N-C intramolecular interactions that occur within L-CPT1; the higher the concentration of benzyl alcohol, the lower was the ability of sulpho-KMUS to cross-link the N- and C-terminal domains. Therefore, decreases in malonyl-CoA sensitivity of L-CPT1 observed in starvation and diabetes are at least partly due to the changes in N-C intramolecular interactions that render cross-linking by sulpho-KMUS more difficult. Such changes may, in turn, be mediated by an increased membrane fluidity which is presumed to facilitate the freedom of the relative movement of TM1 and TM2, and thus of the N- and C-domains with respect to each other [24]. The present observations are the first to provide positive experimental proof of this hypothesis.

Molecular organization of L-CPT1 within the OMM

More marked changes in either malonyl-CoA sensitivity or susceptibility to cross-linking by sulpho-KMUS (which occurred in parallel) were observed for L-CPT1 in the starved state (highest decrease in malonyl-CoA sensitivity accompanied by total loss of cross-linking by sulpho-KMUS). In mitochondria isolated from diabetic animals the IC₅₀ value for malonyl-CoA was 2-fold lower in comparison with that in mitochondria from fasted animals, and the facility with which sulpho-KMUS could cross-link the N- and C-terminal domains was only partially attenuated. Furthermore, the observation that higher trypsin concentrations were required to cleave the inter-TM loop of L-CPT1 in mitochondria from starved rats, than in either fed or diabetic rat liver mitochondria (Figure 3), suggested that accessibility of trypsin to its cleavage site within this loop after hypotonic swelling may be restricted in mitochondria from starved rats. One possibility is that differences

in the micro-environment of L-CPT1 exist in mitochondria isolated from animals in different physiological states. For example, differences in the outer membrane lipid composition that have previously been observed for mitochondria isolated from starved and diabetic rats [13] could affect the precise secondary structure adopted by the inter-TM loop of the L-CPT1 molecule, and hence its accessibility to cleavage by trypsin. Similarly, a modification of the organization of L-CPT1 within the OMM, and particularly its association with other proteins, may be involved. This is reminiscent of the previous observation that higher concentrations of digitonin were required to release CPT activity from liver mitochondria of starved, but not diabetic and fed, rats [35]. Moreover, L-CPT1 is distributed both within the bulk OMM and contact sites that occur between the outer and inner membranes of rat liver mitochondria [36–38] and a variation in the relative populations of enzyme in these two microenvironments, in which it appears to adopt a different conformational state as judged by its altered kinetics [39], might affect accessibility to trypsin.

Evidence for involvement of the region of the N-terminal domain immediately adjacent to the membrane in N–C cross-linking

The present study has provided an experimentally robust strategy for studying N–C interactions in L-CPT1. Although it is outside the scope of the present study to identify the precise tertiary structural features of this interaction, we have obtained important information about the cross-linking reaction, which narrows down the possibilities that need to be addressed in future studies. In particular, we have shown that either point mutation of Glu³ or the double mutation of Ser²⁴ and Gln³⁰ both disrupt the ability of the N- and C-terminal domains to approximate in the precise orientation so as to enable sulpho-KMUS to effect the cross-linking between the two residues involved. Although it is not surprising that such disruption of the secondary and tertiary structures of the N-terminal segment, induced by such mutations, should have affected the distance and/or orientation between the critical residues involved in sulpho-KMUS cross-linking, the mechanistic reasons for the effects of the respective mutations cannot be the same. Thus, in the case of the E3A mutant, the loss in sulpho-KMUS-mediated cross-linking may be ascribed to the fact that this mutant loses most of its malonyl-CoA sensitivity and, presumably, the ability of the N-terminal domain to interact normally (through the negative charge on Glu³) with one or more residues within the C-terminal domain so that this can express its high-affinity malonyl-CoA-binding properties. However, this cannot be the explanation for the loss of cross-linking in S24A/Q30A double mutant, as this is known to be much more sensitive to malonyl-CoA inhibition. The fact that this double mutant was the only one tested that has a malonyl-CoA sensitivity which is higher than that of native L-CPT1 indicates that hypersensitivity to malonyl-CoA induced by the S24A/Q30A mutation disrupts sulpho-KMUS-mediated cross-linking as much as that accompanying the extreme loss of malonyl-CoA sensitivity (E3A mutant). These observations suggest that the relative positioning of the cross-linked residues in the native enzyme is highly critical (as also indicated by the fact that only the cross-linker with a 15.7 Å spacer-arm was effective), and is totally disrupted under conditions that alter the conformation of the N-terminal domain, so as to change the critical distance/relative orientation between the residues involved. Therefore, the loss of the ability of sulpho-KMUS to cross-link the N- and C-terminal domains cannot be interpreted that all interaction between the two parts of the CPT1 molecule is also lost (indeed, in the S24A/Q30A mutant it may be increased), but that the relative spatial position of the two critical residues is altered sufficiently so as to result in a distance/

orientation between them that prevents cross-linking by sulpho-KMUS.

In conclusion, the present study demonstrates for the first time, through a physico-chemical approach, the existence of the close approximation between a lysine residue within the N-terminal domain (and particularly in the region very close to the membrane) and a cysteine residue in the C-terminal domain that enable a bi-functional molecule with a spacer arm of 16 Å (sulpho-KMUS) to effect a covalent bridge between these two domains. Moreover, we have shown that the ease with which this N–C intramolecular interaction occurs within rat L-CPT1 varies in parallel with its degree of malonyl-CoA sensitivity, in mitochondria isolated from livers of rats under different physiological states, being totally lost when extremes of hypo- or hyper-sensitivity are achieved either physiologically (in mitochondria from 48-h starved rats) or through point mutations of the N-terminal domain (as in E3A and S24A/Q30A). Finally, we have obtained strong evidence that the cluster of lysine residues within the 7-residue sequence immediately N-terminal to TM1 must be the site of the candidate lysine residue which is involved in the N–C cross-linking observed. Identification of this precise lysine residue, as well as the cysteine residue in the C-terminal domain involved in the sulpho-KMUS-mediated cross-linking, should yield valuable future information about the folding of native L-CPT1 *in vivo*.

We thank Dr M. A. Ventura (Paris, France) for her help for the statistical analysis. We are grateful to N. Marchand for taking care of the animals. This work was supported by a grant from the Alliance Program (Egide and the British Council) and by Diabetes UK (V. N. J.). A. F. and S. G. were recipients of a doctoral fellowship from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie, and from the Fondation pour la Recherche Médicale respectively. K. B., N. T. P. and V. A. Z. were supported by the Scottish Executive (SEERAD).

REFERENCES

- McGarry, J. D. and Brown, N. F. (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur. J. Biochem.* **244**, 1–14
- Ramsay, R. R., Gandour, R. D. and van der Leij, F. R. (2001) Molecular enzymology of carnitine transfer and transport. *Biochim. Biophys. Acta* **1546**, 21–43
- McGarry, J. D., Mannaerts, G. P. and Foster, D. W. (1977) A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J. Clin. Invest.* **60**, 265–270
- Prentki, M. and Corkey, B. E. (1996) Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes* **45**, 273–283
- Zammit, V. A. (1999) The malonyl-CoA-long-chain acyl-CoA axis in the maintenance of mammalian cell function. *Biochem. J.* **343**, 505–515
- Price, N., van der Leij, F., Jackson, V., Corstorphine, C., Thomson, R., Sorensen, A. and Zammit, V. (2002) A novel brain-expressed protein related to carnitine palmitoyltransferase I. *Genomics* **80**, 433–442
- Ontko, J. A. and Johns, M. L. (1980) Evaluation of malonyl-CoA in the regulation of long-chain fatty acid oxidation in the liver. Evidence for an unidentified regulatory component of the system. *Biochem. J.* **192**, 959–962
- Bremer, J. (1981) The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA. *Biochim. Biophys. Acta* **665**, 628–631
- Graham, B. D. and Zammit, V. A. (1986) Restoration of the properties of carnitine palmitoyltransferase I in liver mitochondria during re-feeding of starved rats. *Biochem. J.* **239**, 485–488
- Graham, B. D. and Zammit, V. A. (1988) Role of carnitine palmitoyltransferase I in the regulation of hepatic ketogenesis during the onset and reversal of chronic diabetes. *Biochem. J.* **249**, 409–414
- Prip-Buus, C., Pegorier, J. P., Duee, P. H., Kohl, C. and Girard, J. (1990) Evidence that the sensitivity of carnitine palmitoyltransferase I to inhibition by malonyl-CoA is an important site of regulation of hepatic fatty acid oxidation in the fetal and newborn rabbit. Perinatal development and effects of pancreatic hormones in cultured rabbit hepatocytes. *Biochem. J.* **269**, 409–415
- Kerner, J., Distler, A. M., Minkler, P. E., Parland, W., Peterman, S. M. and Hoppel, C. L. (2004) Phosphorylation of rat liver mitochondrial carnitine palmitoyltransferase-I: effect on the kinetic properties of the enzyme. *J. Biol. Chem.* **279**, 41104–41113

- 13 Zammit, V. A., Corstorphine, C. G., Kolodziej, M. P. and Fraser, F. (1998) Lipid molecular order in liver mitochondrial outer membranes, and sensitivity of carnitine palmitoyltransferase I to malonyl-CoA. *Lipids* **33**, 371–376
- 14 Kolodziej, M. P. and Zammit, V. A. (1990) Sensitivity of inhibition of rat liver mitochondrial outer-membrane carnitine palmitoyltransferase by malonyl-CoA to chemical- and temperature-induced changes in membrane fluidity. *Biochem. J.* **272**, 421–425
- 15 McGarry, J. D. and Brown, N. F. (2000) Reconstitution of purified, active and malonyl-CoA-sensitive rat liver carnitine palmitoyltransferase I: relationship between membrane environment and malonyl-CoA sensitivity. *Biochem. J.* **349**, 179–187
- 16 Fraser, F., Corstorphine, C. G. and Zammit, V. A. (1997) Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane. *Biochem. J.* **323**, 711–718
- 17 Cohen, I., Kohl, C., McGarry, J. D., Girard, J. and Prip-Buus, C. (1998) The N-terminal domain of rat liver carnitine palmitoyltransferase 1 mediates import into the outer mitochondrial membrane and is essential for activity and malonyl-CoA sensitivity. *J. Biol. Chem.* **273**, 29896–29904
- 18 Cohen, I., Guillerault, F., Girard, J. and Prip-Buus, C. (2001) The N-terminal domain of rat liver carnitine palmitoyltransferase 1 contains an internal mitochondrial import signal and residues essential for folding of its C-terminal catalytic domain. *J. Biol. Chem.* **276**, 5403–5411
- 19 Shi, J., Zhu, H., Arvidson, D. N., Cregg, J. M. and Woldegiorgis, G. (1998) Deletion of the conserved first 18 N-terminal amino acid residues in rat liver carnitine palmitoyltransferase I abolishes malonyl-CoA sensitivity and binding. *Biochemistry* **37**, 11033–11038
- 20 Shi, J., Zhu, H., Arvidson, D. N. and Woldegiorgis, G. (1999) A single amino acid change (substitution of glutamate 3 with alanine) in the N-terminal region of rat liver carnitine palmitoyltransferase I abolishes malonyl-CoA inhibition and high affinity binding. *J. Biol. Chem.* **274**, 9421–9426
- 21 Jackson, V. N., Zammit, V. A. and Price, N. T. (2000) Identification of positive and negative determinants of malonyl-CoA sensitivity and carnitine affinity within the amino termini of rat liver- and muscle-type carnitine palmitoyltransferase I. *J. Biol. Chem.* **275**, 38410–38416
- 22 Jackson, V. N., Price, N. T. and Zammit, V. A. (2001) Specificity of the interactions between Glu-3, Ser-24, and Gln-30 within the N-terminal segment of rat liver mitochondrial overt carnitine palmitoyltransferase (L-CPT I) in determining the malonyl-CoA sensitivity of the enzyme. *Biochemistry* **40**, 14629–14634
- 23 Pan, Y., Cohen, I., Guillerault, F., Feve, B., Girard, J. and Prip-Buus, C. (2002) The extreme C terminus of rat liver carnitine palmitoyltransferase I is not involved in malonyl-CoA sensitivity but in initial protein folding. *J. Biol. Chem.* **277**, 47184–47189
- 24 Jackson, V. N., Cameron, J. M., Fraser, F., Zammit, V. A. and Price, N. T. (2000) Use of six chimeric proteins to investigate the role of intramolecular interactions in determining the kinetics of carnitine palmitoyltransferase I isoforms. *J. Biol. Chem.* **275**, 19560–19566
- 25 Jogl, G. and Tong, L. (2003) Crystal structure of carnitine acetyltransferase and implications for the catalytic mechanism and fatty acid transport. *Cell* **112**, 113–122
- 26 Wu, D., Govindasamy, L., Lian, W., Gu, Y., Kukar, T., Agbandje-McKenna, M. and McKenna, R. (2003) Structure of human carnitine acetyltransferase. Molecular basis for fatty acyl transfer. *J. Biol. Chem.* **278**, 13159–13165
- 27 Young, M. M., Tang, N., Hempel, J. C., Oshiro, C. M., Taylor, E. W., Kuntz, I. D., Gibson, B. W. and Dollinger, G. (2000) High throughput protein fold identification by using experimental constraints derived from intramolecular cross-links and mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5802–5806
- 28 Prip-Buus, C., Cohen, I., Kohl, C., Esser, V., McGarry, J. D. and Girard, J. (1998) Topological and functional analysis of the rat liver carnitine palmitoyltransferase 1 expressed in *Saccharomyces cerevisiae*. *FEBS Lett.* **429**, 173–178
- 29 Prip-Buus, C., Thuillier, L., Abadi, N., Prasad, C., Dilling, L., Klasing, J., Demaugre, F., Greenberg, C. R., Haworth, J. C., Droin, V. et al. (2001) Molecular and enzymatic characterization of a unique carnitine palmitoyltransferase 1A mutation in the Hutterite community. *Mol. Genet. Metab.* **73**, 46–54
- 30 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 31 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
- 32 Brown, N. F., Esser, V., Foster, D. W. and McGarry, J. D. (1994) Expression of a cDNA for rat liver carnitine palmitoyltransferase I in yeast establishes that catalytic activity and malonyl-CoA sensitivity reside in a single polypeptide. *J. Biol. Chem.* **269**, 26438–26442
- 33 Thumelin, S., Esser, V., Charvy, D., Kolodziej, M., Zammit, V. A., McGarry, D., Girard, J. and Pegorier, J. P. (1994) Expression of liver carnitine palmitoyltransferase I and II genes during development in the rat. *Biochem. J.* **300**, 583–587
- 34 Mynatt, R. L. and Cook, G. A. (1994) Modulation of the malonyl-CoA sensitivity of hepatic carnitine palmitoyltransferase (CPT) by phospholipids. *Biochem. Soc. Trans.* **22**, 113S
- 35 Zammit, V. A. and Corstorphine, C. G. (1985) Altered release of carnitine palmitoyltransferase activity by digitonin from liver mitochondria of rats in different physiological states. *Biochem. J.* **230**, 389–394
- 36 Fraser, F. and Zammit, V. A. (1998) Enrichment of carnitine palmitoyltransferases I and II in the contact sites of rat liver mitochondria. *Biochem. J.* **329**, 225–229
- 37 Hoppel, C. L., Kerner, J., Turkaly, P., Turkaly, J. and Tandler, B. (1998) The malonyl-CoA-sensitive form of carnitine palmitoyltransferase is not localized exclusively in the outer membrane of rat liver mitochondria. *J. Biol. Chem.* **273**, 23495–23503
- 38 Hoppel, C., Kerner, J., Turkaly, P. and Tandler, B. (2001) Rat liver mitochondrial contact sites and carnitine palmitoyltransferase-I. *Arch. Biochem. Biophys.* **392**, 321–325
- 39 Fraser, F., Padovese, R. and Zammit, V. A. (2001) Distinct kinetics of carnitine palmitoyltransferase i in contact sites and outer membranes of rat liver mitochondria. *J. Biol. Chem.* **276**, 20182–20185

Received 7 September 2004; accepted 21 October 2004

Published as BJ Immediate Publication 21 October 2004, DOI 10.1042/BJ20041533