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Carnitine/acylcarnitine translocase and carnitine palmitoyltransferase 2 form a complex in the inner mitochondrial membrane

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Abstract Carnitine/acylcarnitine translocase and carnitine palmitoyltransferase 2 are members of the carnitine system, which are responsible of the regulation of the mitochondrial CoA/acyl-CoA ratio and of supplying substrates for the ß-oxidation to mitochondria. This study, using cross-Linking reagent, Blue native electrophoresis and immunoprecipitation followed by detection with immunoblotting, shows conclusive evidence about the interaction between carnitine palmitoyltransferase 2 and carnitine/acylcarnitine translocase supporting the channeling of acylcarnitines and carnitine at level of the inner mitochondrial membrane.

Keywords Protein–protein interaction · ß-oxidation · Carnitine · Mitochondria · Channeling

Abbreviations used

DTE	Dithioerythritol
NEM	*N*–Ethylmaleimide
CACT	Carnitine/acylcarnitine translocase
CPT-1a	Carnitine palmitoyltransferase 1a
CPT-2	Carnitine palmitoyltransferase 2
DDM	*n*-Dodecyl-ß-D-maltopyranoside
ACSL	Long-chain acyl-CoA synthetase
VDAC	Voltage-dependent anion channel
BNE	Blue native electrophoresis

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Introduction

Fatty acids represent metabolic energy source for many tissues in particular muscle ones [1, 2]; moreover, several evidences support that fatty acids oxidation is also performed in nervous system [3, 4]. Fatty acids are oxidized by the β-oxidation pathway, which is realized by enzymes localized into the mitochondrial matrix or in peroxisomes [5, 6]. As first step of mitochondrial fatty acids catabolism they must be converted to acyl-CoA thioesters by action of the long-chain acyl-CoA synthetase (ACSL) which is localized in the cytosol [7]. Then, the fatty acyl-CoA can cross the outer mitochondrial membrane through the voltage-dependent anion channel (VDAC) but not the inner membrane due to the lack of a specific transporter. The carnitine palmitoyltransferase-1a (CPT-1a), an integral outer mitochondrial membrane protein, catalyzes the transesterification of the fatty acyl groups from CoA to carnitine with production of acylcarnitines that, from the cytosol, are released into the intermembrane space [2]. It has been recently demonstrated that CPT-1a, ACSL, and VDAC form an outer membrane complex that channels fatty acyl groups through the outer mitochondrial membrane [8]. Acylcarnitines, are then translocated by the carnitine/acylcarnitine translocase (CACT), localized on the inner mitochondrial membrane, into the mitochondrial matrix where carnitine palmitoyltransferase 2 (CPT-2) catalyzes the reverse reaction transferring the acyl groups from

carnitine to mitochondrial CoA, thereby providing substrates for ß-oxidation. Thus the carnitine shuttle system operates the fatty acids transfer from cytosolic to matrix CoA. The CACT belongs to the mitochondrial carrier family, sharing all the distinctive properties of this protein family and catalyzes both acylcarnitines exchange with free carnitine according to an antiport mechanism and unidirectional transport of acylcarnitines [9–12]. This translocase has been well characterized concerning its function and structure/function relationships using the proteoliposome experimental model together with site-directed mutagenesis, chemical labeling and bioinformatics [13–15]. CPT-2 has been studied at molecular level since a 1.6 Å resolution structure of the full length enzyme has been obtained and a structure of CPT-2 in complex with the generic CPT inhibitor ST1326, a substrate analogue mimicking palmitoylcarnitine, was solved at 2.5 Å resolution [16, 17]. Interestingly, CPT-2 and CACT share some structural features such as charged residues and hydrophobic domains involved in the substrate binding, [15, 18, 19], suggesting that very similar energy of interaction may occur among the two proteins and acylcarnitines. This common structure relationships suggest the idea that acylcarnitines could be channeled from the translocase to the enzyme thus facilitating the sequences of reactions essential for accomplishing the oxidation of the fatty acids coming from the cytosol. Very recently, a novel function was proposed for CPT-2 and CACT, a reverse activity of the carnitine shuttle responsible of acylcarnitines export under condition of acyl-CoA excess [20] so that acylcarnitines channeling has to be considered also on the opposite direction. Using combined experimental approaches, we have demonstrated that CPT-2 and CACT interact forming a supramolecular complex in the inner mitochondrial membrane with the obvious function of channeling acylcarnitines.

Materials and methods

SDS-PAGE and western blot analysis

SDS-PAGE electrophoresis was performed in the presence of 0.1 % SDS according to Laemmli [21]. Minigel system, sizes 8 cm × 10 cm × 0.75 mm, was used. Stacking gel and separation gel 5 and 17.5 % acrylamide, respectively, (acrylamide/bisacrylamide ratio 30:0.2) were used. Proteins were transferred to nitrocellulose membrane (Schleicher and Schuell, PROTRAN BA 85 cellulosenitrat). Residual binding sites on the membrane were blocked by incubation with 3 % BSA in buffer composed of 150 mM NaCl, 50 mM Tris–HCl, 0.05 % Tween20 pH 7.5, for 10 min and then incubated with a rabbit polyclonal anti-

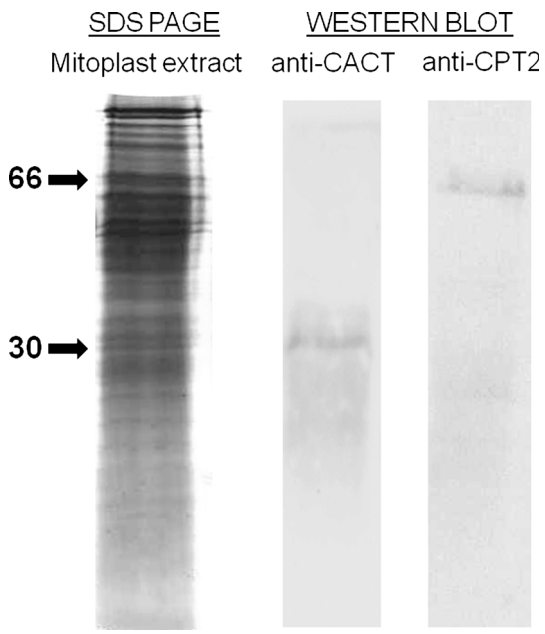

Fig. 1 Anti-CACT and anti-CPT2 antibodies specificity. Mitoplasts solubilized as described in 2.3 and loaded onto SDS-PAGE were immunodecorated with anti-CACT or anti-CPT2 as indicated

CACT (dilution 1:1.000) or anti-CPT-2 from Abnova (dilution 1:1.000)in 0.5 % BSA solution for 1 h at room temperature (RT). The anti-CACT antibodies were produced in our laboratory resulting specific both against rat and human CACT [4, 22]. The specificity of both antibodies in the experimental condition used has been tested, Fig. 1.

Blue native electrophoresis (BNE) and second-dimension electrophoresis

BNE was performed as references [23, 24] and is briefly summarized below. Mitoplasts (0.4 mg) were washed with BNE buffer containing 0.75 mM aminocaproic acid, 50 mM Bis–Tris (Sigma-Aldrich). The pellet was resuspended in BNE buffer with digitonin or DDM (Sigma-Aldrich) at detergent/protein ratio of six, kept on ice for 30 min, mixed every 5 min, and centrifuged at 16.000 × g for 30 min. Proteases inhibitor, 1 mM PMSF (Sigma-Aldrich), and Coomassie blue G-250 (BIO-RAD) in a detergent/dye ratio of 8 were added to the supernatant. The protein complexes were separated on a 6–13 % gradient acrylamide gel. Each lane was cut out of the BNE gel and incubated in SDS denaturation buffer. The lanes were turned 90° and loaded onto the top of SDS-PAGE acrylamide gel to perform a second-dimension electrophoresis. Proteins were transferred onto PVDF membrane (Immobilon-P PVDF Transfer Membrane EMD Millipore) and immunodecorated with anti-CACT antibodies. The spot

corresponding to CACT was detected as in Sect. *2.1*. PVDF membrane was then subjected to stripping, i.e., the removal of primary and secondary antibodies from the western blot membrane by incubation with 1 % SDS, 25 mM Glycine at pH 2 for 25 min. The membrane was then washed 3 times with 150 mM NaCl, 50 mM Tris–HCl pH 7.5 and immune-decorated with anti-CPT-2 antibodies. The corresponding spots were detected as previous described.

Immunoprecipitation with anti-CACT

CACT antibodies (diluted in the PBS buffer 1:500) was incubated with Protein G agarose beads (KPL) for 2 h at 4 °C. Mitoplasts were solubilized with PBS/digitonin buffer at detergent/protein ratio of 6 and centrifuged at $16.000\times g$ for 30 min. The supernatant was incubated overnight at 4 °C on gently rock with the pre-conjugated Protein G agarose/anti-CACT. Beads were then collected by centrifugation and washed five times with solubilization buffer. To take the immuno-precipitated proteins off the Protein G, the beads were resuspended in 2X Laemmli buffer and boiled for 5 min. SDS-PAGE and western blot analysis were then performed.

Formaldehyde cross-linking

Mitoplasts were obtained from freshly prepared rat liver mitochondria at a concentration of 10 mg/ml [25]. Aliquots of 0.4 mg mitoplasts preparation were pelleted in 1.5 ml reaction tube and resuspended in PBS. Mitoplasts were then centrifuged and resuspended in PBS (control) or PBS and formaldehyde (Sigma-Aldrich) at a final concentration of 0.5 or 1 %. The incubation was performed at RT for 7 min. Mitoplasts were then centrifuged at $1.800\times g$ for 3 min. The supernatant was removed and the formaldehyde reaction quenched mixing the pellet with 0.25 mM glycine. Mitoplasts were then washed with PBS and solubilized in Laemmli buffer. After SDS-PAGE, onto a 8 % acrylamide gel (acrylamide/bisacrylamide ratio 30:0.2), western blot analysis was performed.

Results

Protein complex revealed by BNE

Rat liver mitoplasts were treated either with digitonin or DDM, with at a detergent/protein ratio, respectively, of 6 and 2.5, to achieve membrane protein complex extraction. The protein extracts were subjected to BNE, which is mild enough to keep intact protein–protein interactions. The lanes containing the mitoplasts extracts obtained either with digitonin or DDM were subjected to a second electrophoresis separation under denaturing conditions in the presence of SDS (Figs. 2a, 3a). After transferring onto a PVDF membrane, immunostaining of both CACT and CPT-2 has been performed. The membrane was first immunodecorated with anti-CACT, Figs. 2b and 3b, and after stripping, with anti-CPT-2 antibodies, Figs. 2c and 3c. Figures 2d and 3d show the superimposition of the immunostaining with the two different antibodies. After treatment of the mitoplasts with digitonin (Fig. 2d) only spots located in the same vertical lane, related to CACT or CPT-2, appeared indicating that in the first BNE run these proteins were totally associated. After solubilization of mitoplasts with DDM, some spots were observed which were not located in the same vertical line, indicating that a fraction of the protein complex was dissociated by DDM treatment.

Co-immunoprecipitation of CPT-2 and CACT

The two approaches described above, i.e.,formaldehyde cross-linking and BNE, suggested that CPT-2 and CACT are functionally associated at the inner mitochondrial membrane level. Antibody against CACT protein was used to test whether CPT-2 can co-immunoprecipitate with CACT. In Fig. 4a, an immunodecorated band was detected at about 65 kDa after immunoprecipitation (IP) corresponding to the CPT-2 identified in the solubilized mitoplasts. No immunostaining was observed in the flow-through or in the beads supernatant not incubated with solubilized mitoplasts. Analogously, after stripping and immunostaining with the anti-CACT an immunostained band at about 30 kDa was observed after immunoprecipitation in Fig. 4b, which corresponded to the CACT immunostained in the mitoplast extract. Also in this case no immunostaining was observed in the control lines. As a negative control, the presence in the immunoprecipitate of oxoglutarate carrier, that is a member of the mitochondrial carrier proteins and has similar molecular weight of CACT, was tested. No reaction was detected using anti-oxoglutarate carrier antibody from Sigma (not shown). The co-immunoprecipitation approach confirmed the association of CACT and CPT-2.

Formaldehyde cross-link of complex forming proteins

Mitoplasts, obtained from rat liver mitochondria, were treated with formaldehyde which is known to cross-link close proteins [26]. Immunodecoration of the treated mitoplasts with anti-CACT (Fig. 5a) revealed, besides a band at about 30 kDa corresponding to CACT monomer, also a band at 93 kDa which was more evident at higher formaldehyde concentration. Similarly after staining with the anti CPT-2 (Fig. 5b), the same band at 93 kDa was

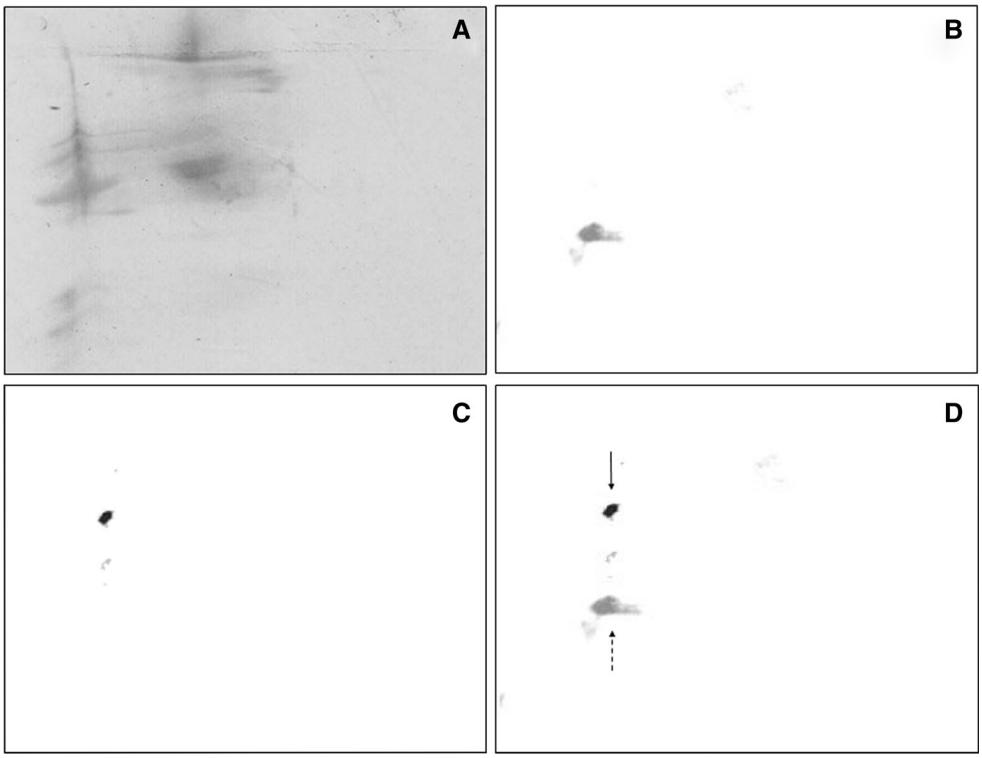

Fig. 2 Two-dimensional SDS-PAGE. Digitonin extract of rat liver mitoplasts separated under native conditions in a first-dimension BN-PAGE and denatured by SDS in the gel strip. The strip was applied onto a second-dimension SDS-PAGE gel. **a** Proteins visualized by Comassie staining; **b** Western blot analysis with anti-CACT antibodies and **c** Western blot analysis with anti-CPT-2 antibodies was performed as described in Sect. *2.1*; **d** superimposition of (**b**) and (**c**). *Solid arrow* indicates anti-CPT-2 spot, *dashed arrow* indicates anti-CACT spot

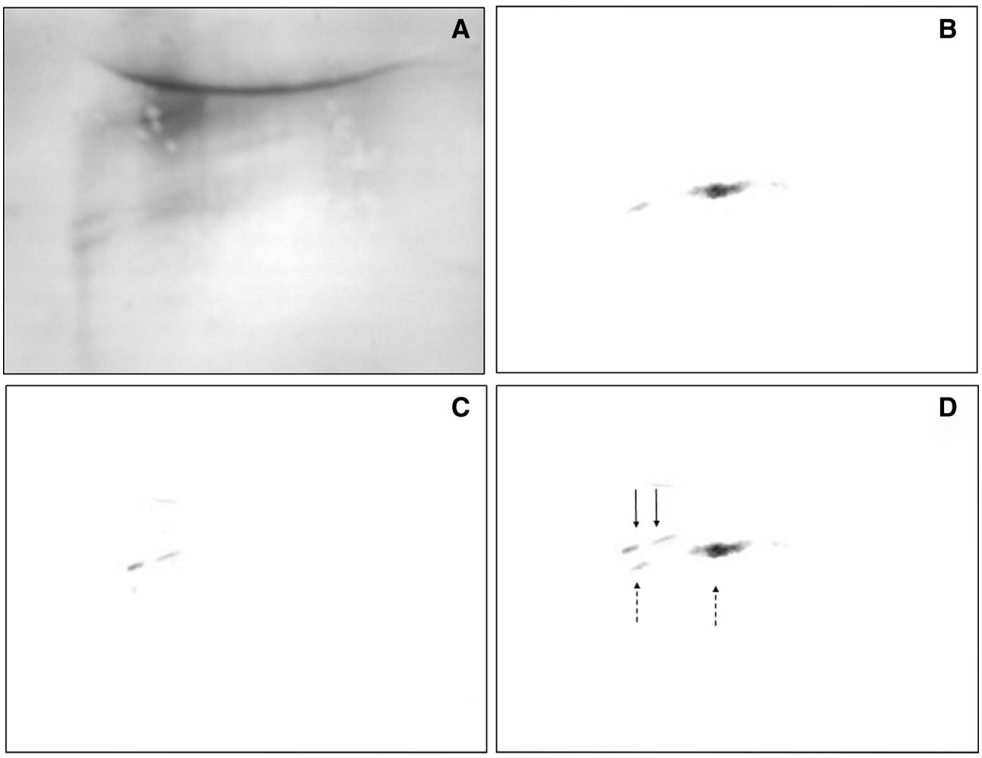

Fig. 3 Two-dimensional SDS-PAGE. DDM extract of rat liver mitoplasts separated under native conditions in a first-dimension BN-PAGE and denatured by SDS in the gel strip. The strip was applied onto a second-dimension SDS-PAGE gel. **a** Proteins visualized by Comassie staining; **b** Western blot analysis with anti-CACT antibodies; **c** Western blot analysis with anti-CPT-2 antibodies was performed as described in Sect. *2.1*; **d** superimposition of (**b**) and (**c**). *Solid arrows* indicate anti-CPT-2 spots, *dashed arrows* indicate anti-CACT spots

revealed, besides the band at 66 kDa corresponding to CPT-2 monomer. The untreated sample showed only the single bands, respectively, at about 30 and 66 kDa (Fig. 5a, b).

Discussion

In this study, an interaction between CACT and CPT-2, devoted to the formation of a supramolecular complex, has

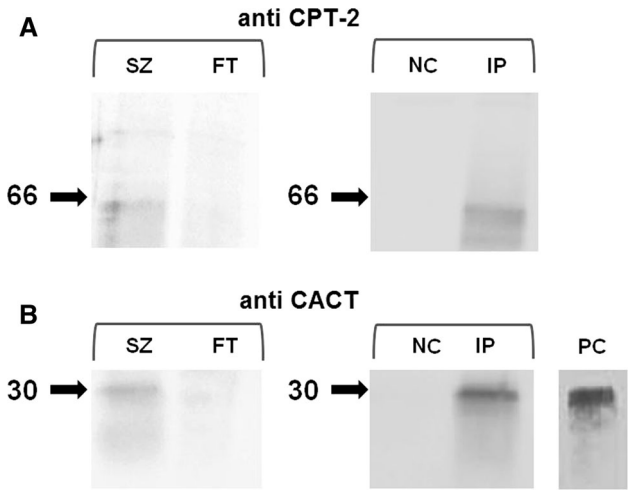

Fig. 4 Co-immunoprecipitation of CACT and CPT-2. Co-immunoprecipitation experiments were carried out as described in Immunoprecipitation with anti-CACT section. Using CACT antibody, bound to Protein G agarose beads. Western blot was performed and the nitrocellulose membrane was cut and immunodecorated with: **a** anti-CACT or **b** anti-CPT-2. SZ: solubilized mitoplasts; FT: flow through; NC: negative control in which the pre-conjugated protein G agarose/anti-CACT beads were incubated overnight at 4 °C without solubilized mitoplasts; *IP* immunoprecipitated protein, *PC* positive control, immunoprecipitated purified recombinant CACT [13]

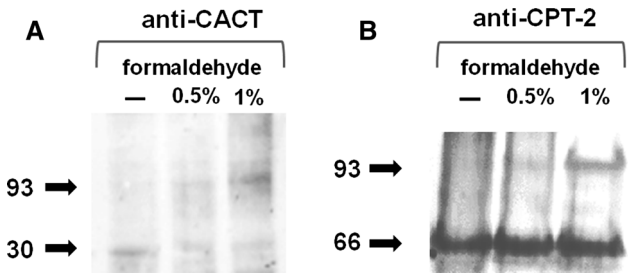

Fig. 5 Mitoplasts were treated with different concentrations of formaldehyde, lysed, and analyzed by western blot using: **a** anti-CACT or **b** anti-CPT-2 antibodies. Molecular weight was indicated by *arrows*

been demonstrated. A protein complex has also been revealed in BNE experiments performed either with digitonin or with DDM. Digitonin has been previously used to study mitochondrial electron transport chain complexes and super-complexes [27, 28] and the subunits composition of outer membrane mitochondrial protein translocase TOM 22 [29, 30] such as DDM to isolate mitochondrial respiratory supercomplex I-III [31]. CACT and CPT-2 were observed exclusively as complex after mitoplasts digitonin solubilization while, after treatment with DDM, also CACT and CPT-2 monomers were observed (Fig. 2, 3). These data indicate that in native membranes CACT and CPT-2 form a supramolecular complex. After treatment with DDM part of the complex was disrupted probably due

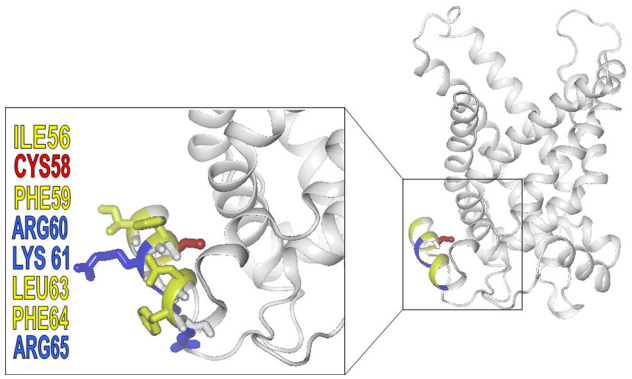

Fig. 6 Structural model of rat CACT. Ribbon diagrams viewing the carrier from the lateral side. Detail evidences hydrophobic (yellow), basic (blue) and cysteine 58 (red) in CACT h12 matrix loop connecting transmembrane alpha helixes 1 and 2. The homology model has been represented using the molecular visualization program VMD

to DDM stronger delipidating properties, suggesting a possible involvement of lipids in the protein–protein interaction. It is well documented that CACT has an essential requirement for cardiolipin, which is a specific phospholipid of the inner mitochondrial membrane [9, 13, 32, 33]. Moreover, it has been previously suggested that cardiolipin interacts with Cys 58 of the CACT [34] located in the first hydrophilic loop (loop h1-2) of the translocator, protruding toward the matrix [19]. It is not possible to establish the molecular nature of CACT/CPT-2 association, i.e., if there is a direct protein–protein interaction or a connection through a lipid adaptor or both of them, indeed we argue a possible role of cardiolipin in the complex forming. Cardiolipin is crucial for substrate channeling in supercomplex of the mitochondrial respiratory chain [35, 36] and for defining ADT/ATP carrier interactome [37]. In the previously proposed model of membrane association of CPT-2, the 30 amino acids loop (Asn179-Asn208) binds the IMM both by hydrophobic residues, that submerge into the core of the membrane, and by positively charged residues, that facilitate association with the polar hydrophilic head-groups of cardiolipin molecules of IMM [17]. CACT matrix loop h12, that contains Cys58, is constituted by several hydrophobic and basic amino acids disposed along all the loop as evident in Fig. 6. We postulate that the CPT-2 binding membrane loop and the CACT loop h12 do interact with IMM in a similar way binding vicinal cardiolipin molecules and membrane core [16, 17, 34, 38].

The interaction between the two proteins has been further demonstrated by the immuno-precipitation procedure that confirmed the existence of the CACT/CPT-2 complex as evident in Fig. 4. Formaldehyde, which is one of the shortest available cross-linkers with a spacer arm length of 2.3–2.7 Å and is highly permeable toward cell membranes, allowed the stabilization, from rat liver mitoplasts, of a

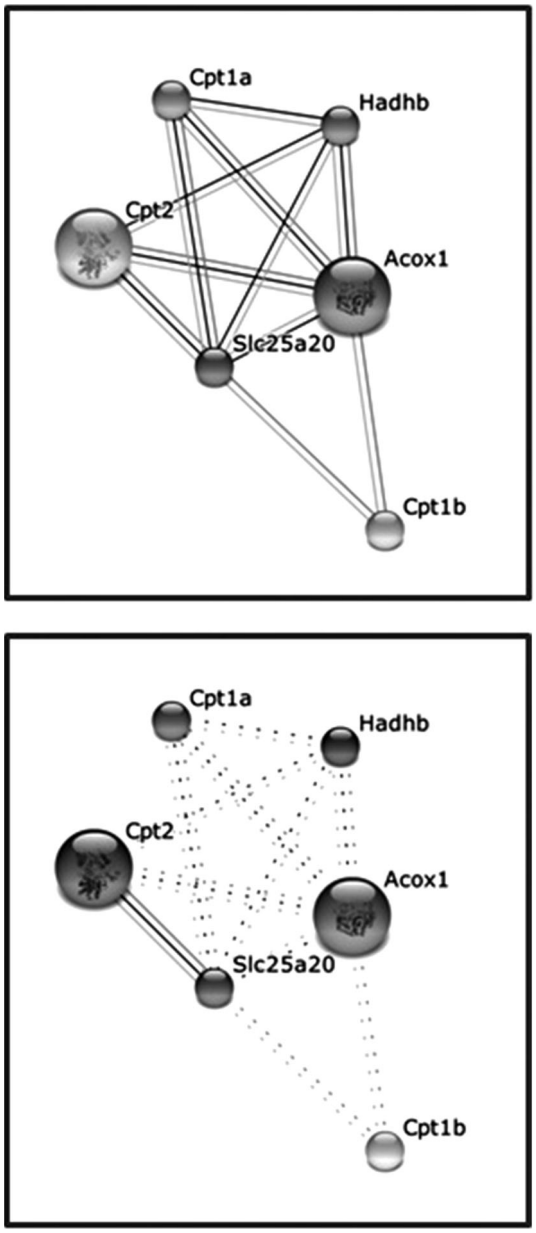

Fig. 7 Protein–protein interaction network displayed by EMBL STRING. Screenshots illustrate the STRING interactions using CACT as a query (**a**) reports a network consisting of the highest scoring interaction partners. This network has been then rearranged and clustered revealing tightly connected functional modules in (**b**). *Black lines* represent protein co-expression; *gray lines* represent significant protein interactions extracted from the abstracts of scientific literature (textmining); *light gray lines* represent significant interactions gathered from curated databases. Slc25a20: mitochondrial carnitine/acylcarnitine translocase (CACT); Acox1: peroxisomal acyl-coenzyme A oxidase; Hadhb: trifunctional enzyme subunit beta, mitochondrial Precursor (TP-beta); CPT1a: Carnitine O-palmitoyltransferase 1, liver isoform (CPT1-L); CPT1b: carnitine O-palmitoyltransferase 1, muscle isoform (CPT1-M); CPT-2: carnitine O-palmitoyltransferase 2

protein complex containing both CACT and CPT-2, as revealed by immunodecoration with anti-CPT-2 and anti-CACT (Fig. 5). The demonstrated interaction between CACT and CPT-2 devoted to the fatty acids translocation through the inner mitochondrial membrane is in line with the grooving evidences of substrates channeling involved in several metabolic pathways [39–42] and in particular is consistent with Lee et al. [8] who established strong protein–protein interaction between CPT1a, ACSL, and VDAC that forms a supramolecular complex engaged in activated fatty acids transfer through the mitochondrial outer membrane. Channeling of acylcarnitines substrates from CACT into the active site of the receiving enzyme CPT-2 has been hypothesized by Rufer et al. [43]. Fraser and Zammit evidence that CACT and CPT-2 are located both at the contact sites level and in the inner mitochondrial membrane [44] and, later, also Hoppel et al. [45] demonstrated that CACT and CPT-2 are co-expressed in both contact sites and IMM . We argue that the acylcarnitine channeling can function also in the opposite direction since CACT catalyzes acylcarnitines exit from mitochondria according to Violante et al. [20] and to Madiraju et al. [46], who, respectively, evidenced that CACT deficiency compromises mitochondrial export of acylcarnitines, and that acetylcarnitine export from mitochondria supplies acetyl groups for histone acetylation. Our conclusions are also supported by Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database of physical and functional interactions [47]. As evident in Fig. 7a, the predicted protein interactions of CACT (Slc25a20) show that it interacts mostly with CPT1 and CPT-2 (three lines connecting the enzymes and in particular the black one that represents the co-expression of the proteins). When the clustering algorithm KMEANS is used with more stringent values, the cluster CACT/CPT-2 still remains, Fig. 7b, thus confirming that this protein–protein interaction is the most confident among the ones in which CACT is involved. [http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm (KMEANS implementation)] [48]. The results here reported give new information on a crucial step of fatty acids oxidation highlighting the existence of a supramolecular complex involved in the carnitine network [49] aimed at evident acylcarnitine channeling.

Declaration of interest The authors report no conflicts of interest.

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