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REVIEW

Structural insight into function and regulation of carnitine palmitoyltransferase

Arne C. Rufer · Ralf Thoma · Michael Hennig

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Abstract The control of fatty acid translocation across the mitochondrial membrane is mediated by the carnitine palmitoyltransferase (CPT) system. Modulation of its functionality has simultaneous effects on fatty acid and glucose metabolism. This encourages use of the CPT system as drug target for reduction of gluconeogenesis and restoration of lipid homeostasis, which are beneficial in the treatment of type 2 diabetes mellitus and obesity. Recently, crystal structures of CPT-2 were determined in uninhibited forms and in complexes with inhibitory substrate-analogs with anti-diabetic properties in animal models and in clinical studies. The CPT-2 crystal structures have advanced understanding of CPT structure–function relationships and will facilitate discovery of novel inhibitors by structure-based drug design. However, a number of unresolved questions regarding the biochemistry and pharmacology of CPT enzymes remain and are addressed in this review.

Keywords Type 2 diabetes mellitus · Carnitine palmitoyltransferase · Enzyme isoforms · Malonyl-CoA · Drug discovery

Roles of CPTs in metabolic disease mechanisms

Type 2 diabetes mellitus (T2D) is a metabolic disorder characterized by resistance of peripheral target tissues, especially skeletal muscle, adipose tissue, and liver, to insulin and concomitant relative insulin deficiency. The occurrence of T2D is highly correlated with an increased risk of cardiovascular disease (atherosclerosis, diabetic retinopathy), neuropathy (sensory loss, diabetic foot disease), and nephropathy (kidney failure rendering patients dependent on hemodialysis). These complications emerge because of chronic hyperglycemia, which causes tissue damage by non-enzymatic glycation of proteins, lipids, and DNA [1]. The vastly growing incidence of T2D worldwide (diabetes epidemic) [2] and its severe health consequences have to be met by pharmacologic intervention.

Today several treatments are established for T2D that are applied depending on disease progression. Clinically mild presentations of T2D can be treated by established anti-diabetic drugs such as insulin secretagogs, exemplified by the more classic sulfonylurea compounds, the recently developed DPP-IV inhibitors, and insulin sensitizing thiazolidinediones (TZDs), which are agonists of the nuclear hormone receptor PPARγ, and metformin [3]. Advanced T2D with pancreatic failure requires administration of exogenous insulin. Several members of the TZD class of compounds have been approved for treatment of T2D. However, some TZDs have had to be withdrawn from clinical development and the use of approved substances had to be restricted because of hepatotoxicity and potential cardiotoxicity [3, 4]. In addition, it seems that weight gain is a common side effect of treatment with TZDs.

Strategies for the development of an improved treatment of T2D include the search for novel pathways and molecular targets involved in the control of glucose homeostasis. Innovative drug molecules should be highly efficacious and administered orally with simple dose regimen. As monotherapy or in combination with established therapies, they should effectively ameliorate the symptoms of T2D and, therefore, delay or avoid the use of insulin treatment. One

A. C. Rufer · R. Thoma · M. Hennig (✉)
F. Hoffmann-La Roche AG,
Pharma Research Discovery Technologies,
4070 Basel, Switzerland
e-mail: michael.hennig@roche.com

such novel strategy of T2D treatment—with no drug molecule currently on the market—is inhibition of the carnitine palmitoyltransferase (CPT) system (EC 2.3.1.21).

The CPT system consists of several components:

1. CPT-1, an integral transmembrane protein of the mitochondrial outer membrane that catalyzes the transfer of acyl moieties from co-enzyme A (CoA) to carnitine;
2. the carnitine–acylcarnitine translocase (CACT) transporter located in the mitochondrial inner membrane; and
3. CPT-2, a monotopic membrane protein on the matrix side of the mitochondrial inner membrane that reverses the reaction catalyzed by CPT-1.

These consecutive transesterifications enable the import of long-chain fatty acids (LCFA) into mitochondria (Fig. 1).

The acyl-CoA released by CPT-2 is channeled into β-oxidation and ultimately yields acetyl-CoA. In mitochondria of hepatocytes the acetyl-CoA derived from β-oxidation is a potent allosteric activator of pyruvate carboxylase, which catalyzes the first step of gluconeogenesis. In diabetic patients whose insulin-sensitive tissues are deprived of glucose, lipogenesis in the adipose tissue and β-oxidation in the liver occur at increased rates. The increased substrate flux through the CPT system together with prevailing glucagonergic effects in insulin-resistant

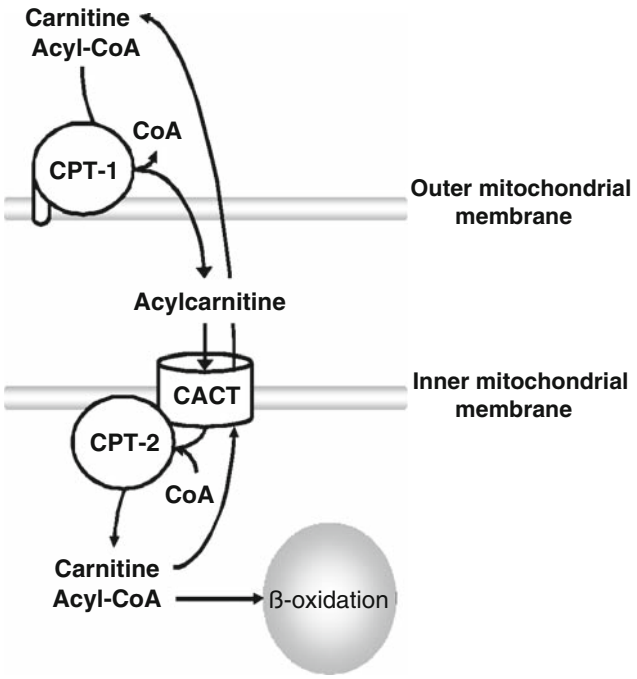

Fig. 1 Model of acyl translocation by the CPT system. CPT-1 is located on the outer mitochondrial membrane and delivers acylcarnitine to CPT-2 via the transporter CACT, which are both located on the inner mitochondrial membrane

T2D further suppress glycolysis and provide redox equivalents needed for gluconeogenesis.

To effectively treat T2D by exploiting the CPT system, two presumably synergistic approaches have been outlined:

1. inhibition of the CPT system in the liver with subsequent reduction of the gluconeogenesis rate; and
2. mobilization of ectopic lipid depots by stimulating the activity of the CPT system in skeletal muscle of obese patients, which would lead to increased insulin sensitivity [5].

For drug discovery the second approach is challenging because of the low feasibility of finding and optimizing compounds that activate the catalysis of an enzyme compared with active-site inhibitors, but progress in tools and methods for drug discovery might render such mechanisms tractable. The existence of organ-specific isoforms of CPT-1 expressed in liver (L-CPT-1) and muscle (M-CPT-1) might enable tackling of both mechanisms. A third isoform of CPT-1 expressed predominantly in brain tissue (B-CPT-1) was discovered in 2002 by Price et al. [6], but initially no catalytic activity could be detected in yeast extracts with heterologously expressed B-CPT-1. Later it was shown that B-CPT-1 KO mice show reduced food intake and body weight but have an increased susceptibility to becoming obese on a high-fat diet when compared with wild-type mice [7]. Recently, Sierra et al. [8] demonstrated that B-CPT-1 has, in fact, catalytic activity and is located in the endoplasmic reticulum. Because B-CPT-1 is co-expressed with L-CPT-1 in the retina [9], the latter is most likely the relevant mitochondrial isoform in brain neurons; this has also been shown by expression tests with northern blotting in rat hypothalamus [10]. In contrast, CPT-2 exists only as a single isoform that is ubiquitously expressed (Fig. 2).

With the characterization of four different CPT isoforms and the identification of the CPT system as drug target, the next challenge is the discovery and development of molecules that can selectively modify the individual enzymatic activities of the CPT system. An important step was the isolation of emeriamine (i.e., aminocarnitine) derivatives, fungal metabolites that were shown to inhibit CPT-1 and have hypoglycemic activity [11, 12]. The development of a series of generic, aminocarnitine-based CPT inhibitors with anti-diabetic potential [13–16] provided proof of concept in respect of reduction of glucose levels in diabetic animal models. A recent study demonstrated that L-aminocarnitine can also inhibit CPT-2 in cultured cells [17]. On the basis of this result it has to be assumed that aminocarnitine derivatives can freely equilibrate over all components of the CPT system. Such unselective inhibition would clearly provoke undesired side-effects and, therefore, new chemical entities with improved specificity profiles need to be found. In this review we will focus on biochemical, structural, and

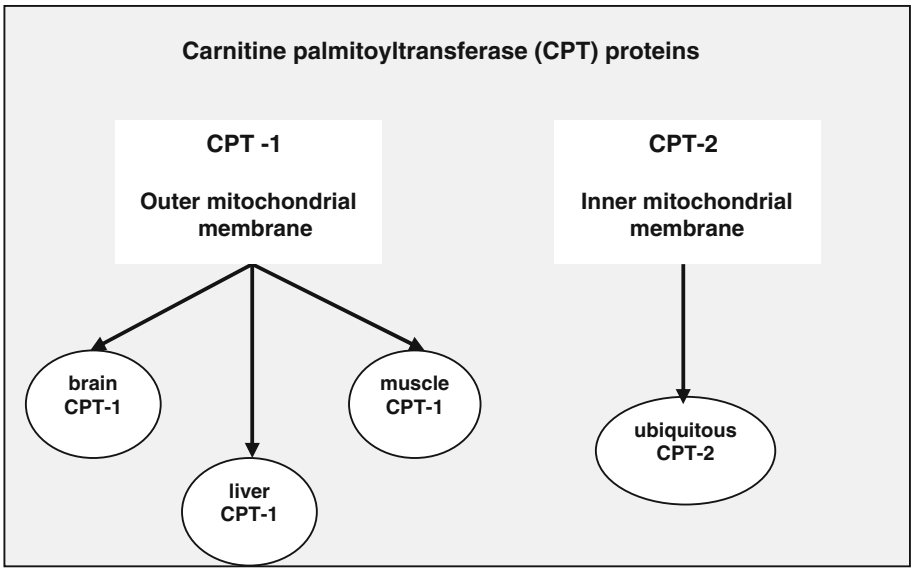

Fig. 2 Overview of CPT proteins and known isoforms. In contrast with the ubiquitous CPT-2 isoform, CPT-1 is differentially expressed in different tissues. More than one CPT-1 isoform may be present in one tissue (see text for details)

pharmacological data that may contribute to the development of specific modulators of the various CPT isoforms.

Biochemical and biophysical data of CPT isoforms

CPT sources and reconstitution

The existence of separate CPT-1 and CPT-2 activities on either side of mitochondrial membranes was proposed as early as 1963 [18], but it took three decades to confirm this proposal by cloning and characterization of the individual enzymes [19, 20]. Considerable research effort was required to distinguish biochemically between CPT-1 and CPT-2 [21] and their (partial) purification and characterization as individual proteins [22–25].

CPT-2 can be purified by detergent extraction from native tissues and expressed with high yield in *Escherichia coli* [26]. In contrast, CPT-1 needs to be reconstituted in liposomes in order to recover pure and active enzyme from expression in *Pichia pastoris* [27]. Mitoplast preparations from the yeast *Saccharomyces cerevisiae* expressing CPT-1 also contain enzyme that has properties similar to that derived from native sources with regard to malonyl sensitivity and, importantly, its correct membrane topology [28, 29]. The yeast-expression systems have the advantage that they do not have any endogenous CPT activity, which enables investigation of mutant forms of CPT. In a recent review Zammit [30] summarized data demonstrating that in diabetic animal models the lipid composition and, as a result of changes in cholesterol contents, the fluidity of the mitochondrial outer membrane affects CPT-1 activity. This emphasizes the difficulty in characterizing the activity and inhibitor-sensitivity of CPT isoforms in vitro, because the choice of lipid or detergent for reconstitution (see below) may lock the enzyme in a state that is not representative of the metabolic condition of interest.

Quaternary structures

Comprehensive data on the degree of oligomerization of CPT enzymes in preparations from various native tissues and expression systems have been published. Interpretation of the results has been complicated by the need to use detergents. The enhanced quality of protein preparations and the refinement of preparative sizing technologies have improved the precision and accuracy of the investigation of CPT quaternary structures. A historic overview of the published data is given in Table 1.

From the data compiled in Table 1 we conclude that the solution molecular weights reported for CPT-1 and CPT-2 are inconsistent, but can be explained by the use of different detergent conditions for characterization of the individual preparations. While predominantly gel filtration has been used as the analytical method for size determination, the experimental conditions, including detergent concentrations and the methods used for enzyme purification, differ substantially.

CPT-2 solubilized with the detergent Triton X-100 below and above its critical micelle-forming concentration showed formation of CPT-2 oligomers. This could indicate the presence of aggregates in preparations with low detergent contents, whereas at high detergent concentrations the dominant species are CPT-2 molecules incorporated into micelles. The average molecular weight of a Triton X-100 micelle is reported to be 90,000 Da, which may lead to a significant overestimation of the molecular weight in solution.

Table 1 Solution molecular weights of CPT-1 and CPT-2

CPT source	Extraction/ solubilization detergent	Assay/detergent (*v*/*v*)	App. molecular weight [Da]	Ref.
CPT-1				
NA	NA	Gel filtration/0.15% Tween 20	150,000[a]	[25]
Rat liver	0.1% Tween 80	Gel filtration/0.1% Tween 80	430,000	[106]
Recombinant rat L-CPT-1 from *S. cerevisiae*	1% Triton X-100 or 1% Digitonin	Gel filtration/1% Triton X-100 or native PAGE/ (1) 1% Triton X-100 (2) 2% Digitonin	275,000 (trimer) or 550,000–620,000 (hexamer = dimer of trimers)	[40]
Recombinant human L-CPT-1 (aa 168-778) from *E. coli*	0.1% Fos12	Analytical ultracentrifugation/ 0.1% Fos12	97% monomer[b]	Roche, unpublished results
Recombinant human M-CPT-1 (aa 168–778) from *E. coli*	0.1% DDM	Analytical ultracentrifugation/ 0.1% DDM	70% monomer and 25% dimer[b]	Roche, unpublished results
CPT-2				
Bovine liver	Endogenous lipids	Gel filtration/endogenous lipids	68,000	[24]
NA	NA	Gel filtration/0.15% Tween 20	76,000[a]	[25]
Rat liver	0.1% Tween 80	Gel filtration/0.1% Tween 80	430,000	[106]
Bovine heart	6% Triton X-100/2% Triton X-100	Gel filtration/1% Triton X-100	510,000	[35]
Rat liver	0.5% Triton X-100	Gel filtration/0.1% Triton X-100 or 0.5% Tween 20	280,000–300,000	[107]
Bovine liver	None	Gel filtration/none	63,500 ± 800	[108]
Bovine heart	6–0.002% Triton X-100, 25–12 mM βOG	Gel filtration, 12 mM βOG = 0.35%	660,000	[36]
Human liver	0.5% Tween 20	Gel filtration/0.1% Tween 20	274,000	[109]
Recombinant rat CPT-2 from *E. coli*	0.1% Triton X-100/ 1% βOG	Analytical ultracentrifugation/ 0.1–1% βOG	(1) 73,500, 1% βOG (2) 305,000 =80% tetramer, 0.1% βOG	[32]
Recombinant rat CPT-2 from *E. coli*	(1) 1% βOG (2) 1.5 mM DDM	Gel filtration, static light scattering/ (1) 1% βOG (2) 1.5 mM DDM	(1) 88,000, 1% βOG (2) 117,900, 1,5 mM DDM	Roche, unpublished results
Recombinant rat CPT-2 from *E. coli*	None	Gel filtration, light scattering/none	Hexamer–octamer	[31]

[a] Presumably CPT-2, because SDS was found to dissociate the 150-kDa entity [25] and CPT-1 is considered non-extractable by Tween 20 [110]

[b] Different from 100% because of sedimentation loss of aggregates

In the authors' laboratory rat CPT-2 eluted as pure monomer from preparative size-exclusion chromatography at peak concentrations exceeding 10 mg/ml in the presence of 1% (*v*/*v*) β-OG or 0.1% (*v*/*v*) Triton X-100, while preparations with *n*-dodecyl-β-D-maltoside or CHAPS still contained oligomers and aggregates. These findings were corroborated by analytical ultracentrifugation and clearly demonstrated that the choice of detergent has a profound effect on the quaternary structure of CPT-2. The CPT-2 construct used for these studies comprised amino acids 27–658, i.e., the mitochondrial leader sequence was removed and replaced by an amino-terminal His-tag

comprising 20 residues. Obviously, wild-type CPT-2 is devoid of this amino-terminal (NT) modification when purified from native tissues or expressed with a different tag configuration [31] and we cannot exclude the possibility that this might affect the oligomerization and the membrane attachment of CPT-2. The crystal structures of CPT-2 [31, 32] and our recent structure-based computational model of its membrane association [33] further support that CPT-2 is a monomeric protein. In addition, a query on the PISA server [34] including all deposited CPT-2 crystal structures does not predict the formation of any homo-oligomeric complexes (our unpublished results). Based on the abovementioned data, we propose a monomeric structure for CPT-2.

Besides the oligomerization state, the choice of detergent and the formation of mixed micelles with substrate molecules may also have a pronounced effect on CPT-2 activity [35, 36]. The availability of the substrates palmitoylcarnitine (forward reaction, acyl-import into mitochondria) or palmitoyl-CoA (reverse reaction, acyl-export) is affected by the detergent used for solubilization of CPT-2. Assays with submicellar concentrations of detergents have been described [36], and a well defined lipid environment is likely to be important for maximum activity. The membrane anchor of CPT-2 inserts into the inner leaflet of the inner mitochondrial membrane, approximately one tenth of which consists of the lipid cardiolipin [37], a lipid required for the activity and stability of the transporter component of the CPT system, CACT [38, 39]. Analysis of the crystal structures of CPT-2 and its orientation relative to the membrane based on our previous computational model [33] identified a number of basic residues that could potentially interact with the negatively charged cardiolipin. It would be a great extension of our understanding of the CPT system if structural and enzyme kinetic data of CPT-2 preparations containing cardiolipin became available.

The only systematic study aimed at revealing the quaternary structure of L-CPT-1 was published recently by Faye et al. [40]. This study describes the existence of CPT-1 trimers and their potential to assemble into hexamers. Interestingly, the authors allocate the interaction surface to the NT first 150 residues preceding the catalytic core of CPT-1. This is in agreement with unpublished results from the authors' laboratory which demonstrated the catalytic cores of both L-CPT-1 and M-CPT-1 to be monomeric in the absence of the first 167 amino acids (Table 1). The NT domain is important for mitochondrial targeting and the malonyl-CoA sensitivity of CPT-1 isoforms [41, 42] and also comprises the two transmembrane helices. The sequence that is necessary and sufficient for the oligomerization of CPT-1 was further mapped to residues 97–147 of the NT domain, as opposed to residues 1–47, which are capable of interacting with the catalytic domain in an intramolecular manner and mediate malonyl-CoA sensitivity [43]. The current model of CPT-1 membrane topology predicts that residues 48–122 cover two transmembrane helices and a connecting loop situated in the mitochondrial intermembrane space. This membrane topology is evolutionarily conserved, because similar positions of the transmembrane helices were proposed on the basis of the sequence of a CPT-1-like protein from the fruit fly *Drosophila melanogaster* [44].

Faye et al. [40] hypothesized that the cooperativity of CPT-1 with regard to palmitoyl-CoA turnover and malonyl-CoA inhibition may be attributed to the oligomerization of CPT-1.

Overall, investigation of the oligomerization of the isoforms of CPT-1 is currently less complete, but oligomerization could be an important mechanism for regulation of their biological function. Electron microscopy on preparations of intact mitochondrial membranes, including contact sites that are enriched with CPT molecules [45–47], might help to elucidate how the CPT-1 isoforms are assembled.

The crystal structures of CPT-2

The crystal structure of rat CPT-2 was recently elucidated and published independently by two groups [31, 32]. Although solved in different crystallographic space groups and at medium to high resolution (2.6–1.6 Å), the structures are essentially identical as judged from values of 0.23–0.47 Å for root-mean-square-deviations of equivalent Cα-positions [33]. CPT-2 has the same fold as other carnitine acyltransferases with known crystal structures, namely carnitine acetyltransferase (CrAT) [48–52] and carnitine octanoyltransferase [53].

A structurally intriguing aspect of CrAT is its conversion into a "pseudo CPT" by introduction of just two point mutations, D356A and M564D [54]. In addition, the combined mutations Ala106Met/Thr465Val/Thr467Asn/Arg518Asn were found to shift substrate preference from carnitine to choline. These findings emphasize the high degree of structural similarity among carnitine acyltransferases and their similarity to the vital enzyme choline acetyltransferase [55, 56], and need to be exploited for the development of selective inhibitors.

CPT-2, like other carnitine acyltransferases, consists of NT and CT domains, each of which is made up of a six-stranded, central anti-parallel β-sheet surrounded by α-helices (Fig. 3). A common feature of the carnitine acyltransferases is that their NT and CT domains show a significant degree of structural homology within a given enzyme and with chloramphenicol acetyltransferase (CAT) and dihydrolipoyl transacetylase (E2pCD) [48]. CAT and

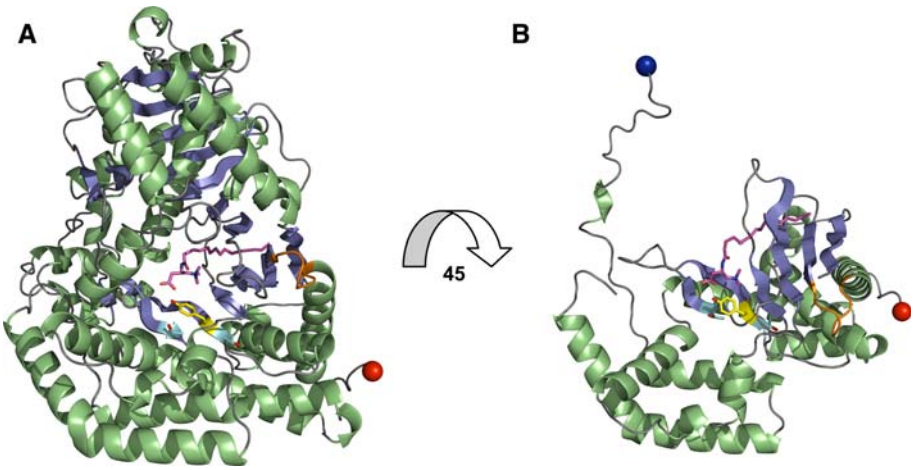

Fig. 3 Positions of post-translational modifications of CPT-1 mapped on the crystal structure of rCPT-2. **a** Complete view of the structure that shows the modifications to lie in vicinity of the catalytic site tunnel, except for Tyr282 (Tyr162 in rCPT-2). Color scheme: *orange* loop containing Ser741 and Ser 747 of CPT-1 (627-633 in rCPT-2); *cyan*: Thr588 and Thr604 of CPT-1 (Thr485 and Thr501 in rCPT-2); *yellow* Tyr589 of CPT-1 (Tyr486 of rCPT-1), *magenta* compound 16, *green* helices, *blue* sheets, *gray* loops, NT *blue sphere* CT *red sphere*. **b** Zoom rotated 45° towards point of view and with NT-domain removed for clarity

E2pCD are functional trimers with their catalytic sites situated at protomer interfaces, and the active site tunnel of carnitine acyltransferases penetrates these enzymes at the interface of their NT and CT domains. This finding was attributed to a potential gene duplication event and it was hypothesized that a once developed (oligomeric) acetyltransferase activity could have been transferred to a single polypeptide chain.

As yet there is no experimental structure of any of the three known CPT-1 isoforms, but a high degree of sequence homology in the core catalytic domain suggests that their structures are similar to that of CPT-2. Various homology models of CPT-1 have been reported [57–61] and it is certain that the CPT-2 structures have had a significant impact on better understanding of the structural and functional properties of CPT-1.

Mechanism of acylcarnitine and carnitine shuttle in mitochondria

The crystal structures of CPT-2 [31, 32], the location of detergent molecules within these structures, and computational models of the membrane association [33] suggest a membrane association mediated by residues Asn179–Asn208. This insertion is not found in any other type of carnitine acyltransferase with known sequence and contains a pair of anti-parallel helices. The distribution of hydrophobic and polar amino acids renders this insert a likely candidate for the membrane anchor of CPT-2.

Acylcarnitine moieties imported into the mitochondrial matrix by CACT do not equilibrate with the pool of acylcarnitine in the mitochondrial lumen [62]. On the basis of this finding it is intriguing to hypothesize that CPT-2 could be localized on the inner mitochondrial membrane by direct contact with CACT. With the CPT-2-specific insert submerged into the membrane or bound to CACT, the large entrance to the active site via the CoA site of CPT-2 would also be oriented towards the membrane. In this arrangement channeling of acylcarnitine substrates could occur from the transporter CACT into the receiving enzyme CPT-2. A tight juxtaposition of CACT and CPT-2 would generate a microenvironment from which the carnitine that emerges after transesterification could be transported back into the cytosol by CACT.

Another point of interest is the biochemical link of CrAT and CACT. Most hepatic CrAT activity is located in the mitochondrial lumen, where it functions to buffer the concentration of activated acetyl-CoA by equilibration with acetylcarnitine [63]. Excess amounts of acetyl-CoA derived from a high rate of β-oxidation can be transported into the cytosol via CACT. Transfer of the acetyl group from CoA on to carnitine by CrAT is a prerequisite for this process, and it would be interesting to know whether CPT-2 is significantly involved. If it is, then the association of CPT-2 with the membrane and/or its putative interaction with CACT needs to be established in such a way that bidirectional transport of acyl-CoA to and from CPT-2 is possible.

In order to improve our understanding of the stimulation of gluconeogenesis by acetyl-CoA it would be interesting to investigate the import of palmitoylcarnitine and the export of acetylcarnitine. Evidence of simultaneous operation of these processes and information about whether they occur at similar pools of CACT (contact sites vs. bulk membrane) would add significant knowledge.

Post-translational modification of CPT-1 and its correlation with the structure of CPT-2

Hoppel et al. [64–66] identified Ser741 and Ser747, and Thr588 and Thr604, as in-vitro and in-vivo phosphorylation sites, respectively, on L-CPT-1. Phosphorylation of L-CPT-1 on the two carboxy-terminal sites Ser741 and Ser747 was associated with increased catalytic activity and modulation of the response to the physiological inhibitor malonyl-CoA [64].

Residues Ser741 and Ser747 are not conserved between L-CPT-1 and CPT-2. Based on sequence alignments and the structure of rCPT-2 they would presumably be located in an exposed loop, which connects strand β18 with the CT helix α27. As judged from the corresponding region in the rCPT-2 structure, i.e., residues 627–633, this loop would be readily accessible for post-translational modification (Fig. 3).

In contrast, residues Thr588 and Thr604 are homologous with Thr485 and Thr501 in rCPT-2. The likely effect of phosphorylation of these residues can be discussed on the basis of the high sequence homology in the residues surrounding the putative phosphorylation sites. The rCPT-2 crystal structure shows these residues to lie within strands β13 and β14 (with nearly 60% amino acid identity to equivalent residues in L-CPT-1) as part of the extended anti-parallel β-sheet in the core of CPT-2. For steric reasons, this position is difficult to reconcile with their phosphorylation by a conventional protein kinase. One possible explanation was provided in a recent review by Zammit [30], who proposed that phosphorylation of L-CPT-1 may already occur during translation. In contrast with Thr485 of rCPT-2, residue Thr501 has two arginine residues (Arg503 and Arg554) in its vicinity, both of which are fully conserved in the CPT enzyme family and could compensate the two negative charges introduced by phosphorylation. In addition, phosphorylation of Thr501 would certainly change the position of the side chain of Tyr486 (Tyr589 in L-CPT-1), that directly interacts with the carboxyl group of carnitine. It is, therefore, likely that phosphorylation on the equivalent Thr604 of L-CPT-1 would have an effect on affinity for the substrate and catalysis. Obviously, phosphorylation of Thr485 (Thr588 in L-CPT-1) would also change the orientation of the side chain of Tyr486 and, accordingly, the interaction of Tyr589 of L-CPT-1 with the aminocarnitine head-group of substrate or inhibitor molecules would be altered.

If L-CPT-1 is constitutively phosphorylated it would be interesting to know the effect of this modification on the structural integrity of the enzyme, and how L-CPT-1 from native tissue relates to recombinant material in that regard. In a scenario where pools of differentially phosphorylated or otherwise modified (see below) L-CPT-1 exist, it would be essential to examine whether the modifications affect the response of L-CPT-1 to inhibitors.

In addition to phosphorylation, it has been demonstrated that both liver and muscle isoforms of CPT-1 can be nitrated [67–69]. This modification occurs on Tyr282 and on the highly conserved Tyr589 (Tyr486 in rCPT-2; Fig. 3). Nitration of Tyr589 would be likely to affect substrate binding, as discussed above for phosphorylation of neighboring Thr588. The degree of M-CPT-1 nitration in cardiac muscle is increased during sepsis and inflammation; this was hypothesized to underlie the concomitant decrease in M-CPT-1 activity observed for these conditions [69].

L-CPT-1 isolated from rat liver has also been shown to be acetylated on amino-terminal Ala2 [66]. The modifications mentioned above may be part of allosteric or pathological mechanisms for regulating CPT-1 activity and stability.

Substances that modify CPT-activity

Pharmacological modulation of the CPT system has been discussed for treatment of multiple diseases, including T2D, obesity [5], cardiac reperfusion injury (i.e., ischemic crisis) [70] and psoriasis [71]. Treatment of T2D by inhibition of L-CPT-1 has raised special attention, has been examined by several investigators, and was recently reviewed [72–75]. Here, we will focus the discussion on recently published biochemical data and their interpretation in the light of the available crystal structures of CPT-2.

The physiological inhibitor of L-CPT-1 is malonyl-CoA and understanding the details of its interaction with L-CPT-1 may reveal how the regulatory NT-domain interferes with the activity of the catalytic domain.

Interestingly, the mutation of the active site residue Met593 to Ser, Ala and Glu, almost abrogate inhibition by malonyl-CoA [76]. The importance of this region is confirmed by the finding that the intrinsically less malonyl-CoA-sensitive isoform L-CPT-1 displays a marked increase in inhibition by malonyl-CoA on introduction of a single point mutation, Glu590Ala [77]. Both Glu590 and Met593 are part of strand β13, which lines the entrance to the active site via the CoA site. Mutation of Glu590 also destroys an internal salt-bridge to the highly conserved His640 of helix α23 (His539 in rat CPT2), which might affect the structural integrity of this region of the enzymatic core.

These results are corroborated by mutation analysis of residues located in the amino-terminal regulatory domain. The point mutations Glu3Ala, Ser24Ala, and Gln30Ala diminish the interaction with the catalytic core of L-CPT-1 [43]. In addition, an increase in membrane fluidity of the

mitochondrial outer membrane—which was demonstrated to occur because of a change in lipid composition in diabetic rats—disrupts the interaction of the NT and CT domains [43]. The available data imply a high degree of conformational flexibility and complex mutual interactions for the NT and CT domains, which would certainly complicate drug-discovery programs targeting the allosteric malonyl-CoA site. This view is supported by attempts in the authors' laboratory to co-express the soluble parts of the NT and CT domains of CPT-1, which did not yield a stable complex (unpublished results). Nevertheless, mutagenesis studies in combination with in-silico modeling have been instrumental in identifying the contact sites of the regulatory amino-terminal and carboxy-terminal catalytic domains of CPT-1, both with regard to each other and to the binding site of malonyl-CoA [43, 61, 76, 78, 79].

So far, the α-ketoacid compound Ro25-0187 is the only inhibitor whose binding site has been mapped to the regulatory malonyl-CoA site by competition experiments [80, 81]. Despite the presence of an allosteric mechanism of inhibition for CPT-1 by malonyl-CoA [82], drug-discovery programs have devoted their effort to the catalytic site. This may turn out to be the more feasible route, because crystal structures of CPT-2 have become available to guide compound development whereas no experimental structural information is yet available about the regulatory domain of CPT-1 enzymes. Moreover, it is assumed that the deep tripartite active site tunnel with pronounced hydrophobic and polar features has higher drugability than the allosteric site, which was modeled to lie at a rather shallow interface of the regulatory and catalytic domains [61].

With the isolation of L-aminocarnitine derivatives, for example the emericedins and similar substrate analogs, potent catalytic site inhibitors of CPT enzymes became available [11–17]. These studies demonstrated the antidiabetic effects of some acylaminocarnitine compounds and their metabolically more stable and non-hydrolyzable urea derivatives [15, 16]. The strongest hypoglycemic and anti-ketogenic effects were observed with palmitoyl-substituted compounds, which were most similar to the preferred in vivo CPT substrate, palmitoylcarnitine. These compounds do not show isoform specificity and L-aminocarnitine is able to inhibit the ubiquitous CPT-2 in intact mitochondria [83]. However, substrate analogs are valuable research tools for early target validation and proof of concept investigations.

The compound class glycidic acid derivatives [84, 85] has been characterized as covalent and irreversible CPT inhibitors, as shown for the best studied members of this class, tetradecyl glycidic acid (TDGA) and etomoxir. Cardiotoxic side effects of glycidic acid derivatives have been attributed to high reactivity of their epoxide moiety [74]. It has been stated that etomoxir (and 2-oxiranecarboxylic acids in general) have to be converted to CoA esters to become potent inhibitors of CPT-1 [86]. In this sense etomoxir is a precursor of an inhibitor.

Phenylalkyl oxirane carboxylates (POCA) exert their full efficacy as CPT-1 inhibitors when converted to POCA-CoA [87–89]. Besides inhibiting CPT-1, POCA stimulates peroxisome proliferation similar to fibrates and thereby increases lipid catabolism via peroxisomal oxidation.

The best investigated small-molecule inhibitors of CPT enzymes are ST1326 and etomoxir (Fig. 4), and a rich source of literature is available on the in-vitro and in-vivo pharmacology of these compounds. ST1326 is a non-cleavable analog of palmitoylcarnitine, the physiological substrate of CPT-2. The crystal structure of CPT-2 with ST1326 [32] located the binding interface to the acyl and carnitine tunnels of the active site of CPT-2 and furnished a detailed view of the binding mode. Hydrophobic interaction of the aliphatic 14 carbon atom chain contributes substantially to the binding affinity of ST1326; this is further supported by detection of electron density in the apo-structure that can be interpreted as an aliphatic C16 chain at this location [31]. The more hydrophilic aminocarnitine head group contributes three direct hydrogen bonds to the amino acid side chains of His372, Ser590, and Tyr486 and one bond to a water molecule, whereas no covalent interaction could be observed. In contrast, no crystal structure of the irreversible covalent inhibitor etomoxir has yet been determined. However, the current data support the view that the activated Ser590 is positioned well to provide the covalent bond. Similarities in the structure (Fig. 4) with regard to the aliphatic carbon chain suggest a similar position of this part of the molecule compared with ST1326.

Assessment of the CPT system as drug target

The biological mechanism of the CPT system is an attractive target for treatment of T2D, on the basis of its key role in maintaining fatty acid metabolism and its concomitant effect on glucose homeostasis. The proof of concept for triglyceride-lowering and hypoglycemic effects of the CPT inhibitors etomoxir and ST1326 have been demonstrated in humans and add this enzyme to the targets for treatment of T2D.

In addition, tools for a successful drug-discovery program are now available, such as high quality protein preparations, robust assays, and, recently, the structure of CPT-2. A number of potent inhibitors of CPT enzymes and crystal structures of complexes with these compounds show the good chemical tractability of the modulation of CPT activity by small molecules. The availability of

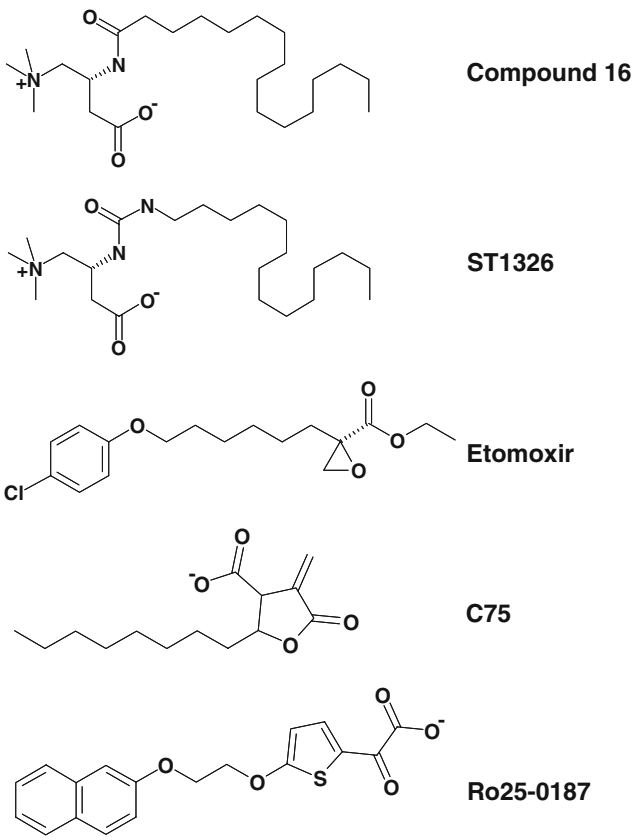

Fig. 4 Ligands that modify the activity of CPT enzymes. The active form of etomoxir is assumed to be its CoA derivative (see text for details)

compounds for the modulation of enzyme activity together with biochemical and in-vivo data provides an excellent basis for discussing of the consequences of the drug-mediated modulation of the CPT system.

The key open point that must be addressed is the optimal isoform specificity of inhibitors of the CPT system. For example, examination of rat cardiac myocytes is difficult in that they express both M-CPT-1 and L-CPT-1 isoforms, until an isoform switch occurs during the early postnatal phase, after which M-CPT-1 remains the predominant isoform in the rat heart [90–92]. The cardiac expression profile of CPT-1 isoforms in humans needs further investigation in order to improve the characterization of cardiotoxic site effects.

The detrimental effects of a non-specific inhibitor are exemplified by accumulation of ectopic lipids in skeletal muscle of rats after prolonged oral administration of etomoxir, which inhibits both L-CPT-1 and M-CPT-1 irreversibly [111]. Merrill et al. [93] described high-dose (1 mM) in-vitro studies with etomoxir that demonstrated induction of acute oxidative stress in HepG2 cells, as exemplified by upregulation of glutathione reductase and thioredoxin reductase mRNA after 6 h of acute treatment. Reduced levels of glutathione as a consequence of oxidative stress induced by etomoxir and the reversible CPT-1 inhibitor CPI975 were also observed for tissue slices of rat and human livers [94]. Besides these changes in cellular redox-status, abnormalities in gene expression profiles and mitochondrial morphology were reported. Although even high doses of CPI975 do not induce cardiac hypertrophy as observed with etomoxir, a significant body of evidence emphasizes the hepatotoxic effect of CPI975 [74]. The exact mechanism that couples L-CPT-1 inhibition with the occurrence of mitochondrial lesions remains to be elucidated. The side effects of etomoxir and related CPT inhibitors derived from glycidic acid may not be mechanism-based but mediated by the reactivity of the epoxide moiety of these compounds. Complete inhibition, as in the case of irreversible inhibitors such as etomoxir, should clearly be avoided in long-term pharmacological treatment because this could provoke the accumulation of lipids in the cytosol of cells other than adipocytes and deplete the mitochondrial matrix of precursors for membrane lipids.

When substrate flux through the CPT system to β-oxidation is reduced by inhibition of mitochondrial CPT-1, metabolism is shifted toward utilization of glucose, as described by Randle [95, 96]. Besides being an advantage for ameliorating the symptoms of T2D, this metabolic switch can be exploited for treatment of stable angina pectoris and myocardial crisis. Carbohydrate oxidation needs less oxygen than oxidation of fatty acids for equivalent energy supplies. Two excellent and recent reviews [97, 98] summarize use of the CPT inhibitor perhexiline as antianginal drug. The occurrence of hepatotoxic and neurotoxic side effects under prolonged perhexiline treatment has been attributed to its lack of CPT isoform specificity.

However, two compounds have been described that have some isoform selectivity, although these inhibitors achieve their specificity by different means. In cardiac myocytes, and to a lesser extend in hepatocytes, the compound oxfenicine (*S*-2-(4-hydroxyphenyl)glycine) is converted to hydroxyphenylglyoxylate, which is an inhibitor of CPT-1 with lower IC50 for M-CPT-1 [99]. The conversion is catalyzed by a mitochondrial aminotransferase that has higher activity in heart muscle than in the liver. Together these two effects result in marked tissue specificity. Another example is a dinitrophenol derivative of etomoxir (DNP–etomoxir) which is a potent and specific inhibitor of L-CPT-1 [68, 91].

Several reports have documented the effect of CPT-1 inhibitors in the central nervous system, especially the hypothalamus, and have pointed out the exquisite role of the hypothalamus–pituitary axis and its interaction with the periphery via peptide hormones in whole-body energy homeostasis [10, 100–102].

Knock-down and pharmacological inhibition of CPT-1 activity in rat hypothalamus has been shown to result in reduced food intake and reduction of endogenous glucose production [10]. Interestingly, in this study genetic abrogation of L-CPT-1 activity was sufficient to enable observation of these effects. Therefore, the brain-specific isoform B-CPT-1, although expressed in hypothalamic neurons [6], does not seem to be involved in this process, even though B-CPT-1 KO mice show a similar phenotype of hypophagia [7].

Therefore, the currently available data suggest that a drug molecule targeted at the CPT system ought to be isoform-specific and be distributed non-systemically to avoid mechanism-related side effects. This leaves the individual CPT-1 isoforms that are differentially expressed in the relevant tissues as drug targets, because CACT and CPT-2 are ubiquitously expressed enzymes. An alternative option might be a compound lacking isoform selectivity but with pronounced organ-specificity, e.g., because of specific transport processes. Drug–drug interactions must be considered, because of the frequent clinical coherence of T2D and obesity or dyslipidemia as symptoms of the metabolic syndrome. As a very special case, the concomitant administration of CPT inhibitors and statins was investigated with regard to aggravation of the very rare sub-symptomatic CPT-2 deficiency into an overt form [103–105].

With all the research tools of modern drug discovery in hand, the discovery and development of chemical entities that modulate the activity of the CPT, safely and with high efficacy, for treatment of patients, is within reach.

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