Review

Molecular enzymology of carnitine transfer and transport

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Abstract

Carnitine (L-3-hydroxy-4-N-trimethylaminobutyric acid) forms esters with a wide range of acyl groups and functions to transport and excrete these groups. It is found in most cells at millimolar levels after uptake via the sodium-dependent carrier, OCTN2. The acylation state of the mobile carnitine pool is linked to that of the limited and compartmentalised coenzyme A pools by the action of the family of carnitine acyltransferases and the mitochondrial membrane transporter, CACT. The genes and sequences of the carriers and the acyltransferases are reviewed along with mutations that affect activity. After summarising the accepted enzymatic background, recent molecular studies on the carnitine acyltransferases are described to provide a picture of the role and function of these freely reversible enzymes. The kinetic and chemical mechanisms are also discussed in relation to the different inhibitors under study for their potential to control diseases of lipid metabolism. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The cell relies on the carnitine system (Fig. 1) to regulate the localised, limited pools of CoA derivatives. Acyl-CoA pools provide activated substrates for many key metabolic pathways such as the TCA cycle and lipid and cholesterol synthesis, for post-translational modification of proteins and for detoxification mechanisms. The reversible transfer of activated acyl groups from the limited pools of membrane-impermeable CoA to the plentiful, mobile carnitine provides transport between compartments, a considerable reservoir of activated acyl groups and excretion of excess acyl moieties. Transport occurs in the import of fatty acids for energy production in mammalian mitochondria (reviewed in [1]) and in yeast peroxisomes [2]. The reservoir function refers to the acetyl-L-carnitine pool in heart and sperm (reviewed in [1]) and to the long-chain acylcarnitine pool, which cells use for membrane repair when they lack energy to activate fatty acids [3]. Excretion of carnitine derivatives occurs via the urine, where they provide a marker for clinical measurements, and in bile, where it was recently demonstrated that long-chain acyl derivatives accumulate [4]. As a whole, the
carnitine system both connects the various acyl-CoA pools and damps fluctuations in their acylation state that would be detrimental to cell homeostasis.

The carnitine system consists of carrier proteins that transport carnitine across the membranes and enzymes, carnitine acyltransferases, that catalyse the reversible equilibrium:

\[ \text{acyl-CoA} + L\text{-carnitine} \rightleftharpoons \text{CoA} + \text{acyl-L-carnitine} \]

We review here the molecular genetics and enzymology of these proteins that play key roles in activated acyl group homeostasis and transport.

2. Getting it in – the transport of carnitine into cells

2.1. Molecular genetics of the mammalian plasma membrane transporters (OCTN2)

Recent efforts [5–8] provide a clear picture of the plasmalemmal carnitine transporter OCTN2 (Fig. 2). The human gene for OCTN2, SLC22a5 (i.e., member 5 of solute carrier family 22), contains 10 exons and maps to chromosome 5q31, a region that researchers identified using linkage analysis of families with inherited systemic carnitine deficiency [7,9]. The mouse gene, known from the juvenile visceral steatosis (jvs) mutation [10], a missense mutation (Table 1), maps to chromosome 11 [11]. This protein of 557 amino acids probably contains 12 putative transmembrane domains (Figs. 2 and 3); however, some disagree about predictions of these domains [7,12,13]. Identified sequences include three putative N-glycosylation sites in the first extracellular loop, several putative phosphorylation sites in intracellular loops [6,7], a sugar transporter protein signature motif [6], and an ATP/GTP binding motif [7]. Unique among organic cation transporters, OCTN2 needs sodium to transport carnitine but not for other organic cations [6,14]. OCTN2 most closely resembles OCTN1 and OCTN3, the latter only known from mouse (GenBank database accession number BAA78343) [211].

An ancestral OCTN gene must have duplicated as a direct repeat, as the genes for OCTN1, a low-affinity, sodium-independent carnitine transporter [15], and OCTN2 are in tandem [16]. Deletion of both genes causes carnitine deficiency in mice [16], with all characteristics of the jvs mutation [10,17], including cardiomyopathy and fatty liver. Complementation with a genomic fragment that encodes human OCTN2, but not human OCTN1 [16], rescues the phenotype.

OCTN2 sequence analysis of systemic carnitine deficiency in humans, first reported by Nezu et al. [12] and then by others [18–23], represented a breakthrough for understanding of this disease. Fig. 2 and Table 1 present disease-causing amino acid substitutions known in human OCTN2. This compilation includes mutations from a large-scale analysis of a Japanese subpopulation and mutations in heterozygotes that may account for low plasma levels of carnitine [21].

2.2. Functional aspects of and mutations in the plasma membrane transporters

Carnitine and acylcarnitines do cross membranes; so, a non-saturable component to uptake in cells exists (see [24]). However, proteins mediate both the cellular accumulation of carnitine across the plasma membrane (from about 50 μM in plasma to millimolar levels in cells) and the rapid flux across the mitochondrial inner membrane required for β-oxidation (reviewed in [1]).
The discovery of the plasma membrane transport of carnitine dates to the recognition in the 1970s (reviewed in [1]) that liver, the major site of the final step of carnitine synthesis, has a low affinity ($K_M = 5.6 \text{ mM}$ [25]) and other tissues have a high affinity ($K_M = 4^{\pm 2} \text{ mM}$ [26]) for carnitine. OCTN2 presumably is responsible for the high-affinity activities measured in this early work. Variations in transport rates and in levels of expressed mRNA are found in different tissues. Starvation and glucagon increase the carnitine content of liver but not that of heart [1,27,28]. Local regulation may also alter carnitine transport because palmitoyl-CoA and ATP can alter the number of carnitine binding sites [29].

Mutations in human and mouse OCTN2 that cause carnitine deficiency and site-directed mutagenesis in rat OCTN2 reveal some crucial residues in the protein (Table 1). Both individual substitutions Y211F and P478L block carnitine transport but not organic cation transport, e.g., tetraethylammonium (TEA) [14]; these experiments suggest spatially different carnitine and TEA transport sites. The swapping of different parts of human and rat OCTN2 [14] reveal residues 123–239 (Fig. 2) as critical for the species-specific OCTN2 characteristics. Compared to human OCTN2, rat OCTN2 is less effective as a carnitine transporter and more effective as an organic cation transporter [14,30].

Transporter activity in a given cell should influence the intracellular carnitine content. Rat heart contains about twice as much carnitine as rat liver [27]. Seminal fluid contains the most carnitine with levels as high as 60 mM (see [1]). It has been speculated that two transporters, one at the basal membrane and one at the apical, may be necessary to achieve

Fig. 2. Amino acid sequence alignment of human, rat and mouse OCTN2 and related proteins from the solute carrier superfamily 22. Shaded residues are identical to a consensus derived from the sequences shown. Twelve putative transmembrane domains are indicated with roman numbered bars above the sequence alignment. The sugar transporter signature motif (Prosite PDOC00190) is indicated by + symbols, the ATP/GTP binding signature motif (Prosite PDOC00017) by $U$ symbols. The region that is critical for the discussed species-specific OCTN2 characteristics (123–239) is indicated between arrowheads. Mutations that result in decreased OCTN2 function are circled and numbered below the sequences (see Table 1 for details).
this large gradient. However, in the mammary gland, only a basal transporter drives the high flux of carnitine to these cells and, hence, into milk [24]. The expression of the transporter in the mammary gland increases dramatically with the onset of pregnancy and lactation. This increase suggests a temporal, hormonal regulation of expression.

The liver transporter has a high $K_M$ for L-carnitine (5.6 mM in hepatocytes) and a lower $K_M$ for the precursor, butyrobetaine [25]; therefore, it is probably not OCTN2. OCTN2 is not expressed at high levels in liver [6,30]. The liver transporter is sodium-dependent and, therefore, is probably not OCTN1 either. The liver produces the majority of endogenous L-carnitine in mammals; experiments with perfused liver suggest a protein-mediated release of carnitine. Mersalyl inhibits the release; ions or ouabain do not [31]. The observed $V_{max}$ is 2.47 nmol min$^{-1}$ g$^{-1}$ of liver and the $K_M$ is 0.27 mM (intracellular carnitine concentration in liver is about 0.5 mM). Starvation apparently decreases carnitine output from perfused liver, suggesting decreased activity of the protein. If this work is confirmed, the efflux protein would be a target for molecular investigation as it could influence carnitine output to other tissues. Such a protein could be hormonally regulated because glucagon increases the carnitine content of liver, as does starvation [27,28].

Identifying carnitine derivatives in urine and bile implicates carnitine in the export of excess activated acyl groups [3,4]. In particular, urinary output of valproylcarnitine during valproate therapy and of the carnitine esters of β-oxidation intermediates in acyl-CoA dehydrogenase deficiency patients demonstrates a substantial export of intracellular esters. Presumably, this export, down a concentration gradient, could be due to passive diffusion. The slow export from mammary gland cells is not stimulated by reversing the sodium ion gradient; so the uptake system at the basal membrane probably does not reverse [1]. Although one group reports a saturable efflux from perfused rat liver with a $K_M$ of 0.15 mM and a turnover greater than the daily efflux, the passive efflux measured in the same experiment could account for the daily turnover [31]. A volume-activated amino acid channel may also contribute to carnitine efflux from swollen cells [1]. The recently characterised OCTN1 transporter could also modulate intracellular carnitine, providing a pH-regulated path of efflux [15].

Table 1
Mutations in the carnitine proteins

<table>
<thead>
<tr>
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<th>Species</th>
<th>Effect on transport</th>
<th>References</th>
</tr>
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<td>1 R169Q</td>
<td>human</td>
<td>slight decrease in vitro</td>
<td>[200]</td>
</tr>
<tr>
<td>2 M179L</td>
<td>human</td>
<td>slight decrease in vitro</td>
<td>[21]</td>
</tr>
<tr>
<td>3 V211C</td>
<td>human</td>
<td>slight decrease in vitro</td>
<td>[22]</td>
</tr>
<tr>
<td>4 Y211F</td>
<td>rat</td>
<td>carnitine, not organic cations</td>
<td>[14]</td>
</tr>
<tr>
<td>5 Y251F</td>
<td>rat</td>
<td>slight decrease in vitro</td>
<td>[14]</td>
</tr>
<tr>
<td>6 W283R</td>
<td>rat</td>
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<td>[21,201]</td>
</tr>
<tr>
<td>7 L352R</td>
<td>mouse</td>
<td>jvs mutation</td>
<td>[12,13]</td>
</tr>
<tr>
<td>8 L352R</td>
<td>rat</td>
<td>carnitine+organic cations</td>
<td>[14]</td>
</tr>
<tr>
<td>9 M352R</td>
<td>human</td>
<td>carnitine+organic cations</td>
<td>[14]</td>
</tr>
<tr>
<td>10 Y358F</td>
<td>rat</td>
<td>carnitine+organic cations</td>
<td>[14]</td>
</tr>
<tr>
<td>11 Y426</td>
<td>rat</td>
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<td>[14]</td>
</tr>
<tr>
<td>12 E452K</td>
<td>human</td>
<td>reduced $V_{max}$</td>
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<td>14 S467C</td>
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<td>[21]</td>
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<td>[19]</td>
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<td>[14]</td>
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<td>[14]</td>
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<tr>
<td>18 Y486F</td>
<td>rat</td>
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</tr>
<tr>
<td>19 Y492F</td>
<td>rat</td>
<td>slight decrease in vitro</td>
<td>[14]</td>
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3. Rapid internal equilibration – the intracellular transporters

3.1. Molecular genetics of the mitochondrial carnitine transporter (CACT)

Clones for the rat [32] and human [33] mitochondrial inner membrane carnitine/acylcarnitine transporter (CACT, also known as the translocator, translocase, or carrier) are known. CACT is a member of the mitochondrial carrier family, which includes ornithine, ADP/ATP, phosphate, 2-oxoglutarate and citrate carriers as well as the proton carriers (uncoupling proteins). All these transporters are about 30 kDa, including rat CACT at 32.5 kDa [38]. Mammalian CACT proteins contain 301 amino acid residues (Fig. 4). These proteins have a triple repeat structure; each repeat contains two membrane-spanning domains. CACT proteins occupy the mitochondrial inner membrane with the N-terminus, two small hydrophilic loops and the C-terminus facing the intermembrane space; three larger hydrophilic loops face the matrix side (Fig. 3). CACT proteins appear to have a carnitine-specific binding sequence of R(A,S)(V,F)PANAA(T,C)F [85] near the C-terminus (see Fig. 4). All analyses of CACT mutations (deletions, frameshifts) have revealed drastic effects on protein structure. To date, no single-amino acid substitution studies have been published.

Table 1 (continued)

<table>
<thead>
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<td>activity</td>
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<tr>
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<td>R631C</td>
<td>human CPT-II</td>
<td>activity</td>
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</tbody>
</table>
3.2. Functional aspects of the mitochondrial carnitine transporter

Identification and characterisation of mitochondrial carnitine-acylcarnitine exchange carrier function date to the 1970s [39,40]. The same gene product may also provide carnitine transport in peroxisomes as both organelles express a protein reactive with the same CACT peptide antibody [42].

The kinetic parameters of CACT reveal the asymmetric nature of the electrically neutral and pH-independent exchange. In a purified system [34,41], the \( V_{\text{max}} \) is 1.7 \( \mu \text{mol min}^{-1} \text{mg}^{-1} \), with \( K_M \) values of 0.5 mM and 8.7 mM on the internal and external faces, respectively. Acyl-L-carnitine homologues (acyl chain lengths of 12–16 carbons) transport as rapidly as carnitine but with \( K_M \) values of \( \sim 5 \mu \text{M} \). The ping-pong mechanism for the carnitine carrier [41] contrasts with other mitochondrial carriers. This mechanism allows the carrier to function as a uniporter to adjust the organelle concentrations of carnitine. The uniporter functions more slowly at 0.5% of the exchange rate [41,43].

The activity of the carrier requires thiol groups. N-Ethylmaleimide and mersalyl inhibit (IC \(_{50} \) 5 \( \mu \text{M} \) and 0.05 \( \mu \text{M} \), respectively) both the exchange and uniport activities [41]. However, at high concentrations of mercurials, passive efflux of carnitine occurs: the opening of a non-specific channel, as seen for some other mitochondrial carriers, might be the reason. The thiol groups modified to give the loss of specificity seem to differ from those modified for inhibition of exchange [44].

Other inhibitors include acyl-L-carnitine analogues with a charged group at the \( \omega \) end [39]. Acyl-\( \Delta \)-carnitines bind as well as acyl-L-carnitines (the \( K_i \) value for decanoyl-\( \Delta \)-carnitine inhibition of L-carnitine exchange is 12 \( \mu \text{M} \)) but are transported slowly (\( \Delta \)-carnitine) or not at all [207]. A very recent study demonstrates convincingly that acyl-\( \Delta \)-carnitines target CACT for inhibition of fatty acid oxidation [208].

4. Equilibration with CoA pools – the carnitine acyltransferases

4.1. Molecular genetics of the carnitine acyltransferases

4.1.1. Carnitine acetyltransferases (CAT)

CAT activity is found in mammalian mitochondria, peroxisomes and endoplasmic reticulum (ER) [45]. In mammals, at least in rat hearts, CAT is not a cytosolic enzyme [46]. Translated as a precursor of 626 amino acids, human mitochondrial CAT contains a 28- or 29-residue N-terminal mitochondrial targeting signal (MTS), which is clipped off during translocation through the mitochondrial inner membrane [47,48]. Corti et al. [48] have discussed the possibility that cleavage of the MTS occurs between residues 28 and 29 of the precursor. Their experimental data suggest, however, that cleavage probably occurs between residues 29 and 30, where the sequence more closely resembles the well-conserved cleavage site consensus RXY\(_{\Delta}\)S/A [163]. At the C-terminus (AKL), the precursor contains a peroxisomal sorting signal, which functions predominantly when the MTS is not translated. Translation of the
The peroxisomal form of CAT starts at a second start codon and, for human CAT, produces a protein of 605 amino acid residues. The different initial amino acid sequences come from differential splicing that produces two mRNAs with and without the exon for the mitochondrial targeting sequence [48]. The second or third exon contains the start of translation of the peroxisomal form (as the starts of transcription are unknown the exon numbering is preliminary) [63]. The differential splicing might result from the aberrant splice donor dinucleotide GC instead of GU in the ‘mitochondrial’ first intron [48]. In yeast, a single gene for CAT [49] also enables the translation of both a mitochondrial and a peroxisomal isoform [50], but the underlying mechanism does not involve splicing. In both Saccharomyces cerevisiae and Candida tropicalis, the CtCAT gene from the latter is differentially translated because the translational machinery has a differential preference for the first or second start codon [51]. Furthermore, studies in yeast show that CAT has both a C-terminal AKL and an internal peroxisomal targeting signal (PTS). When both the mitochondrial targeting sequence and the C-terminal AKL are deleted, the majority of the gene product is still sorted to the peroxisomes [50]. In addition, or alternatively, the folded state of the protein may contain specific information for peroxisomal sorting [52].

Yeast have another gene, YAT1, which encodes a cytosolic form of CAT that is attached to the mitochondrial outer membrane [53]. In mammals, it is not known whether the ER form of CAT comes from the same gene that encodes mitochondrial and peroxisomal CAT. The sequence KVEL in CAT (pos. 492–495) could possibly function like the ER retention signal KDEL [48], which normally is found at the C-terminus. In pigeon breast CAT [54], the KVEL sequence is absent (KADL in pigeon CAT, pos. 493–496); consequently, the KVEL sequence in human CAT may be irrelevant for sorting.

### 4.1.2. Carnitine octanoyltransferases (COT)

The human [55], rat [56] and bovine [57] cDNA sequences of peroxisomal COT encode proteins of 612 amino acids (about 70 kDa) that contain differ-
ent C-terminal sequences (THL, AHL and PHL, respectively), thought to serve as a PTS. However, as in CAT (see above), other PTSs may be involved or the folded state of the protein may contain specific information for sorting. Genomic sequences from rat [56] reveal 17 exons with the start codon on the second exon. Expression of the rat gene for COT (Crot) is subject to trans-splicing, i.e., splicing of different primary transcripts to one mRNA [58]. In the case of rat liver COT, the different mRNAs that result from trans-splicing contain either a direct repeat of exon 2 or a direct repeat of exons 2 and 3. Therefore, including the normal transcript, the three mRNA sequences result in the expression of two COT proteins: one of normal size, i.e., 69 kDa, and one of 79 kDa [58]. The transcript that contains the repeat of exon 2 does not produce a COT protein due to a frameshift because the number of nucleotides in exon 2 is not a multiple of three. The human gene, CROT, contains an additional intron at the start of the coding region [63].

4.1.3. Carnitine palmitoyltransferases II (CPT-II)

CPT-II, a mitochondrial matrix protein associated with the inner mitochondrial membrane, is translated as a precursor of 658 amino acids in both human [59] and rat [60]. The mature protein is about 70 kDa, as the N-terminal MTS of 25 amino acid residues is removed during mitochondrial import [59,61]. The genomic structure of human CPT2 [62] is similar to the mouse gene [64], which also encodes a precursor of 658 amino acids.

4.1.4. Carnitine palmitoyltransferases I

(CPT-I; L-CPT-I and M-CPT-I)

Different genes, called CPT1A and CPT1B, encode the two known mammalian isoforms of CPT-I, liver-type carnitine palmitoyltransferase (L-CPT-I) and muscle-type carnitine palmitoyltransferase (M-CPT-I), respectively (reviewed in [65]). The L-CPT-I isoforms, proteins of 773 amino acids, have predicted and apparent sizes of about 88 kDa. The M-CPT-I isoforms, proteins of 772 amino acids, have predicted sizes of about 88 kDa, but apparent sizes of about 82 kDa by SDS-PAGE analysis. cDNA sequences of the genes for L-CPT-I are known for rat [66,67], man [68], mouse [69]; van der Leij, unpublished) and sheep ([70]; Price et al., unpublished). Protein sequencing of rat L-CPT-I reveals that the mature enzyme retains the N-terminus [71]. A mitochondrial targeting sequence at residues 123–147 immediately behind the second transmembrane region has also been shown to act as a stop-transfer sequence to anchor CPT-I in the outer membrane [210]. After ATP-dependent integration in the membrane [72], the majority of the enzyme is on the cytosolic face of the mitochondria, anchored by two transmembrane domains (Figs. 3 and 5). The initial biochemical studies [73] for rat L-CPT-I and the recent immunocytological studies on human M-CPT-I with green fluorescent protein [74] support these conclusions.

The rat M-CPT-I cDNA, obtained by screening a brown adipose cDNA library with a white adipose cDNA probe, encodes a protein that was called CPT-I-like protein [75]. The strong cardiac expression of this clone [75,76] and its human orthologue [77] is consistent with the identification of the encoded protein as M-CPT-I [76].

In another approach to clone human CPT1B, part of the cDNA sequence was assembled after database screening of human expressed sequence tags with

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Fig. 5. Amino acid sequence alignment of nine carnitine acyltransferases. The alignment is shown with shaded residues at positions where these are identical to a consensus sequence derived from an alignment of 15 carnitine acyltransferases (including also rat L-CPT-I, rat M-CPT-I, mouse CAT, pigeon CAT, bovine COT and mouse CPT-II). The two CPT-I transmembrane domains are indicated with roman numbered bars above the sequence alignment. Carnitine/choline acyltransferase motifs are indicated by + symbols (Prosite PS00439) and by U symbols (Prosite PS00440). N-terminal signals for mitochondrial targeting and C-terminal signals for peroxisomal targeting are boxed. The cleavage site of the mitochondrial targeting sequence of yeast CAT has not been determined experimentally and is predicted according to [163]. Start methionines of the peroxisomal forms of CAT are underscored. Mutations which result in loss of malonyl-CoA sensitivity (CPT-I) or loss of enzyme activity are circled and numbered below the sequences (see Table 1 for details). The mutations studied in rat L-CPT-I and bovine COT are indicated in the human L-CPT-I and rat COT sequence, respectively. D. melan., Drosophila melanogaster; S. cerev., Saccharomyces cerevisiae.
the rat L-CPT-I cDNA sequence as query [78]. This in silico cloning effort permitted the isolation from human heart and skeletal muscle of several cDNAs.

Analyses of these cDNAs show that sometimes the last intron of human \textit{CPT1B} is not removed from the primary transcript because of competition between splicing and polyadenylation. The first exon is untranslated and alternative first exons exist, which was noted [78] based on data of Zhu et al. [79]. Further analyses of the alternative splicing of the \textit{CPT1B} gene in human [80] and rat [81] reveal that the two first exons (exon 1a and 1b, also called U and M) do not co-exist in one transcript and that two promoters drive the expression of human \textit{CPT1B}. Consequently, the expression of the 5' untranslated mRNA for human M-CPT-I differs from the alternative splicing of the transcript for L-CPT-I in rats where the second untranslated region is sometimes skipped [67].

Human \textit{CPT1B} (for genomic information, see [63]), which was sequenced as the middle gene of seven from a 180-kb BAC clone, is located closely downstream of a choline/ethanolamine kinase gene [78,82]. \textit{CPT1B} is even co-expressed with this upstream gene [83]. The gene syteny is conserved in rats [84] and mice [69]. In \textit{Drosophila}, only one gene for CPT-I appears [86]. The protein is slightly larger than mammalian counterparts (782 amino acids, Fig. 5), and, when expressed in yeast, shows characteristics typical of a CPT-I enzyme.

\subsection*{4.1.5. Carnitine acyltransferases in the endoplasmic reticulum}

A recent proposal, based on the common immunoreactivity, suggests that all the cytoplasmic-facing CPT enzymes are the same [87]. Although kinetic data support this proposal for mitochondrial and peroxisomal enzymes, the overt microsomal activity has subtle kinetic differences [88–90]. The only known candidate gene sequence for a microsomal carnitine acyltransferase, unrelated to the transferase family discussed above, is GRP58 [91,92], also known as Erp57, Erp60 or Erp61. This protein is thought to have various functions; whether it is also a carnitine acyltransferase remains controversial (reviewed in [104]).

\section*{4.2. Mutations in the carnitine acyltransferases}

Mutations at more than 30 positions in the acyltransferase protein family have been diagnosed or made through site-directed mutagenesis. Table 2 and Fig. 5 contain 30 of these mutations that affect enzyme function. Of the naturally occurring human mutations, the most frequent occur in CPT-II. CPT-II deficiency, the most common muscular lipid metabolism disorder [93], can present with different times of onset and with different phenotypes. The adult form with muscular or hepatomuscular presentation and the neonatal form with hepatocardio-muscular presentation represent the most extreme phenotypes in this recessive disorder. Infantile and intermediate phenotypes occur as well (recently reviewed in [65,94,95]).

The S113L mutation, among the first identified, is the most common in CPT-II [93]. Reported in several studies of homozygotic or compound heterozygotic CPT-II deficiency (e.g., [62,94,96–98]), this mutation has an allele frequency of 60%. The affected serine residue at human CPT-II position 113 is conserved within CPT-I, CPT-II, and ‘vertebrate’ CAT (human, mouse, pigeon), but not in any COT proteins nor in yeast CAT (Fig. 5). S113L, a ‘mild’ mutation, is associated only with the adult form of CPT-II deficiency. A strict correlation between severity of each mutation and onset and phenotype, however, cannot be made as clear exceptions exist. For example, a homozygous R631C mutation gives a different expression of the disorder in unrelated patients [94]. Therefore, genotypic differences outside the \textit{CPT2} gene may have major influences in certain cases.

Polymorphisms in CPT-II as single entities do not significantly affect CPT-II enzyme function (reviewed in [65,94]). Like S113L, P50H [95,97] and E174K [99,100], which have mild consequences, are associated with the adult form of CPT-II deficiency. P50H is a change in the first fully conserved proline of an LPXLP motif (pos. 49–53 of CPT-II). The glutamate at position 174 is less conserved, although other transferases share glutamates in the vicinity (Fig. 5). Severe consequences for CPT-II function result from F383Y [100] and Y628S [101]. Both affect residues that are barely conserved outside CPT-II (Fig. 2).
In contrast to CPT-II deficiency, deficiencies in the other transferases are still rare and only known at the sequence level for CPT1A [102,206]. A recent CPT1A mutation analysis [206] has identified G710E, which is adjacent to a GFG pattern that is associated with carnitine binding in rat CPT-II [103].

4.3. Functional properties of the carnitine acyltransferases

Recent reviews have summarised the locations and functions in the cell [104,105]. The native structures of CAT and COT are monomers [106,107]. For CPT-II, only aggregates, such as 660 000 kDa [108], are isolated after detergent solubilisation, although a homotetramer has been suggested [109]. Inactivation of CPT-I by radiation of outer membranes has produced a target molecular weight that is close to the monomer molecular weight, suggesting (but not proving) that the normal form in the membrane could be a monomer [110].

The sigmoidal kinetics often observed for CPT-I (e.g., [111,112]) could indicate multisubunit co-operation. However, artefactual sigmoidicity or non-linear time courses caused by interactions of palmitoyl-CoA with albumin or micelles must first be excluded. Acyl-CoA binding protein (ACBP) prevents these technical problems [90]. Sigmoidicity could still arise via the malonyl-CoA regulatory site, which could also explain the high substrate inhibition observed for palmitoyl-CoA [113]. Sigmoidicity with palmityl-CoA is not changed by malonyl-CoA inhibition [111] but disappears after treatment with the thiol reagent DTNB [112]. Consequently, the sigmoidicity arises from the protein and not from an artefact. This implicates a thiol group in the reversible alteration of sensitivity to malonyl-CoA [114]. These observations may be explained by (a) the flexible interaction between the N-terminal region and the main part of the enzyme or (b) the complex responses of malonyl-CoA inhibition to membrane environment (see below) or (c) both.

The effect of detergents on activity and membrane attachment generated many early controversies about the CPTs. The use of Triton X-100, which inactivates CPT-I completely but activates and solubilises CPT-II, delayed the identification of CPT-I as a separate protein [60]. In one laboratory, octylglucoside solubilised active CPT-I [113], whereas in another, it inhibited and failed to solubilise it [60]. In a third, it failed to solubilise either CPT-I or the peroxisomal malonyl-CoA-sensitive enzyme but, of the five detergents studied, octylglucoside had the least effect on the activity of COT, CPT-I and CPT-II (A.S. Friend and R.R. Ramsay, unpublished data). Others found that octylglucoside produces complex effects on extracted CPT-II [115].

Despite numerous publications on these acyltransferases, the susceptibility of the assay to artefacts, complicated by both high substrate and strong product inhibition, has produced data that are only suitable for internal comparisons. Kinetic studies of carnitine acyltransferases should take into account problem variables: micelle concentrations of the long-chain acyl derivatives (e.g., [116] and references therein), the presence (or absence) of albumin ([117,118] and references therein), the other components (membranes bind palmitoyl-CoA [113,116,119]) and concentrations of lipid or detergent or both ([60,113,115], all of which influence acyl-CoA availability [118]). As mentioned above, the recent use of recombinant ACBP overcomes some problems inherent in experiments with long-chain acyl-CoA substrates [90]. Table 2 gives true kinetic constants for purified CAT, COT and CPT-II [120–123] but it is difficult to discuss the level of saturation of these enzymes with acyl-CoA substrates in the cell. The cytoplasmic content of CoA is low (less than 10% of the cellular pool in heart [124] and variable in liver [125]). The concentration of free acyl-CoA is essentially zero because all the cytoplasmic long-chain acyl-CoA will be bound to ACPB. It has been suggested that CPT-I uses directly the pool of acyl-CoA bound to ACBP. From the earliest study on the first purified transferase, CAT [126], to the more recent studies, all find that V changes little in the range pH 7–8 [1]. Where differences in K_M are noted, at least 85% saturation with carnitine could be maintained at normal cellular concentrations. Kinetic parameters are usually determined within this range. Table 2 gives examples of values obtained for each transferase. The V for acyl-carnitine formation (removal of acyl-CoA, the forward direction) is generally higher than that for the reverse direction. However, as noted above, local
concentrations of the substrates will determine the net flux.

Table 2 also shows information on the different kinetic properties of the isoforms of CPT-I (muscle and liver). They have the same $K_M$ for palmitoyl-CoA but the $K_M$ for carnitine in the muscle form is 10-fold higher than in the liver form. This correlates with the higher carnitine concentration in heart than in liver [27,124]. Indeed, McGarry et al. [120,127], who surveyed a range of tissues and species, have shown an inverse correlation between carnitine $K_M$ and malonyl-CoA IC$_{50}$. Jackson et al. [181] have now demonstrated that the inverse relationship of carnitine $K_M$ and malonyl-CoA sensitivity is caused by modulation of the catalytic domain by the N-terminal residues.

The first structural information on CPT-I and mutation of N-terminal residues make it easier to understand some observations. Interaction of the N-terminal with the large C-terminal domain helps stabilise the catalytic site and modulate malonyl-CoA sensitivity [73,128,129] (see below). As shown by Zammit’s group [130] using a set of six chimeric proteins plus the parental forms, interactions between the two cytosolic parts of the protein (see Fig. 3) contribute to interaction with the first substrate, palmitoyl-CoA. The pairing of the transmembrane domains influences the interaction with carnitine [130] but removal of the N-terminal transmembrane domain still leaves an active protein [181]. From their study of both the M and L forms of the enzyme, we see that the C-terminal portion of the protein gives rise to a 100-fold difference in sensitivity to malonyl-CoA (M more sensitive). What is clear from this work [130,181] is that the catalytic core contains the malonyl-CoA binding site. Modulation of the malonyl-CoA sensitivity depends, however, on the N-terminal section [128–130].

The same studies [130] imply that the membrane anchor helices could transmit the influence of membrane changes. Recent observations ex vivo [131] emphasise microenvironmental influences on the kinetics of this special enzyme. More CPT-I is found in the contact sites between the outer and inner membrane than in the normal outer membrane [131,132], and its kinetic properties are changed dramatically (F. Fraser and V.A. Zammit, personal communication). In the outer membrane, malonyl-CoA inhibition affects $V$ but not $K_M$ for palmitoyl-CoA, whereas in the contact sites, $V$ is unchanged but the apparent $K_M$ for palmitoyl-CoA is greatly increased. Consequently, the mode of inhibition by malonyl-CoA is competitive in the contact sites, where channelling to $\beta$-oxidation seems likely, so that excess long chain acyl-CoA could overcome the inhibition and prevent accumulation. In the outer membrane, uncompetitive inhibition ensures that accumulating acyl-CoA required for other cytosolic functions is not converted into acylcarnitine.

Although the main target for designing inhibitors in this enzymic family is CPT-I because it influences the rate of mitochondrial fatty acid oxidation, the kinetics are the hardest to study because they must be determined in the mitochondrial outer membrane. In the native state, problems include the influence of the membrane fluidity on activity, the sensitivity of malonyl-CoA inhibition to the fluidity, the binding of palmitoyl-CoA and palmitoylcarnitine to the membrane, sensitivity to proteolysis and the differences in mitochondria prepared from animals in dif-

<table>
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<tr>
<th>Gene</th>
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<td>?</td>
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<td>CPTer</td>
<td>&lt;4</td>
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*30–60 μM in the presence of bovine serum albumin.
ferent physiological states (starvation, diabetes, hormonal changes, the presence of proliferators, etc.). The reconstitution of the protein into artificial membranes [79,133,185] and its expression in yeast free from endogenous activity [130] will permit detailed kinetic studies.

5. Acyl group transfer – the kinetic and chemical mechanisms

5.1. Kinetic mechanisms

Kinetic mechanisms for CAT [122], COT and CPT-II [121] are known. From primary plots and product inhibition studies, CAT and COT show rapid equilibrium random order kinetics indicating that all four substrates bind well to the free enzyme. In contrast, CPT-II follows a compulsory order ternary complex mechanism in which CoA must bind first. CPT-II has a very low affinity for l-carnitine, which has a $K_i$ value of 11.7 mM for product inhibition. In contrast, COT has a 100-fold greater affinity for l-carnitine, which has a $K_M$ value (0.1 mM) equivalent to the dissociation constant. The observation that CPT-II binds acylcarnitine analogues whereas COT binds carnitine analogues (see Sections 5.2.4 and 5.2.5 below) reflects this difference.

The ordered mechanism of CPT-II, in which acyl-CoA binds first, ensures that this reversible enzyme responds to the acylation state of the CoA pool in the mitochondrial matrix. The matrix concentrations of both CoA and acyl-CoA [124] exceed the measured $K_M$ values [121] so that most CPT-II will have the first (CoA) substrate bound. Certainly CPT-II in normal mitochondria has never appeared to limit the rate of fatty acid oxidation. The step that does control the access of fatty acyl-CoA to the matrix for $\beta$-oxidation is CPT-I on the mitochondrial outer membrane. Unpublished data (cited in [134]) indicate an ordered mechanism for CPT-I too.

5.2. Chemical mechanism

In the past decade, our understanding of the basic chemistry of carnitine acyltransferases has progressed considerably. Computational studies of acyl transfer equilibria explain why acylcarnitines have a large acyl group transfer potential; sequence studies and site-directed mutagenesis provide further information for elaborating a chemical mechanism; and measurements of $K_M$ and $K_i$ values for substrate and putative transition state analogues provide additional data for developing a picture of the active site. Despite the absence of a crystal structure of a carnitine acyltransferase or detailed chemical mechanistic studies, one can define the mechanistic possibilities for the reaction shown below.

$$\text{CoA-SH} + R_1\text{O} + H\text{C}\text{-O} \rightleftharpoons \text{CoA-S} + \text{Carnitine} + \text{HO-R}_1$$

(1)

5.2.1. Reaction energetics

Acyl-CoA, a thioester, has a high acyl transfer potential. Acylcarnitine, an oxyester, must have a similar potential to avoid coupling the acylcarnitine-to-CoA transfer to an energy-releasing reaction. The equilibria between acetyl-CoA and various oxyesters (see Eq. 1) are calculated from the free energies of hydrolysis ($\Delta G_{\text{hyd}}$ at 25°C) for acetyl-CoA [135,136], acetylcaritnine [137], acetylcarnitine [138] and ethyl acetate [139] of -34.3, -33.0, -27.1 and -24.7 kJ/mol, respectively. Acetylcarnitine has the most favourable equilibrium for transfer to CoA; in fact, acetyl transfer occurs readily in both directions [137]. For acetylcarnitine and ethyl acetate, acetyl transfer to CoA costs energy.

A recent study [209] explains the high acetyl transfer potential of acetylcarnitine using solvation energies. Solvation energies, rather than intrinsic instability, can determine the reactivity of ‘high-energy’ compounds [140–142]. For acetyl oxyesters, the solvation of acetic acid (or acetate) drives the hydrolysis; however, for charged esters and alcohols, the relative solvation energy of the ester compared to the alcohol accounts for the differences in acetyl transfer potential. Calculations of $\Delta H_{\text{solv}}$ between three pairs of charged ester alcohols — the zwitterionic acetylcaritnine–carnitine pair, the cationic acetylcarnitine–carnitine pair, and the anionic 3-acetoxypropanoate–3-hydroxypropanoate pair — give 9.8, 18.5 and 18.7 kJ/mol, respectively [209]. In all cases, the esters are more solvated than the alcohols. The zwitterionic acetylcaritnine–carnitine pair in which both have large dipole moments, has the smallest...
5.2. Proposed mechanism

Most mechanisms for acyl transfer involve a tetrahedral intermediate. No direct evidence supports or disputes this mechanism for carnitine acyltransferases. The role of histidine in the mechanism dates back to Chase and Tubbs [143], who used both chemical modification and substrate analogues to implicate a histidine. Site-directed mutagenesis identifies it as H372 in CPT-II [103] and H327 in COT [144]. The loss of activity of D376A and D464A mutants of CPT-II suggests a role for aspartate, and recent chemical modification studies suggest that a lysine contributes to catalysis [103,145]. A catalytic role for an acyl intermediate with serine is unlikely [57,121]. A direct transfer between carnitine and CoA appears logical. The simplest mechanism has the histidine serving as a general base – removing a proton from carnitine or CoA depending on the direction of transfer – to promote formation of a tetrahedral intermediate. An aspartate may potentiate the histidine function. The protonated histidine donates a proton to the departing group, either carnitine or CoA.

Other residues contribute to binding but not directly to catalysis. An arginine probably forms a strong salt bridge with the carboxylate of carnitine. A cleft similar to the aromatic residue-rich pocket in acetylcholinesterase for recognising the trimethylam-
monio group of choline [146] is likely for carnitine. A serine–threonine–serine triad conserved in all the carnitine acyltransferases (and in choline acyltransferases) contributes, perhaps by providing an aqueous-like microenvironment, to binding of carnitine [57], which is strongly solvated (see above). Substitution of all three residues of the STS triad gives a 1000-fold increase in $K_M$ for carnitine. In CPT-II, three substitutions – V605A, G609A and G611A – resulted in a higher $K_M$ for carnitine [103].

5.2.3. Substrate analogues
A study of a series of cyclohexyl carnitine analogues (Fig. 6a) has probed the preferred conformation for the binding of carnitine. The analogue in Fig. 6a (CC) binds selectively to the active site of CPT-I but is a non-competitive inhibitor of CPT-II [147]. In addition, a study with the tert-butyl analogue of carnitine, HDH, reveals the enhancement provided by a positive charge for carnitine acyltransferase activity [148].

5.2.4. Inhibitors – irreversible
Irreversible inhibitors of CPT (Fig. 6b) have a reactive epoxide with an alkyl chain and a carboxyl group (e.g., TDGA [149], etomoxir [150] and DNP-ET [151]). DNP-ET selectively inhibits L-CPT-I [152]. In a recent study of 12 analogues, SNU-13b [153] shows better hypoglycaemic activity and less acute toxicity than etomoxir in streptozotocin-induced diabetic rats. Presumably, these compounds selectively inhibit L-CPT-I.

5.2.5. Inhibitors – reversible
Reversible inhibitors of CPT (Fig. 6b,d) are either putative transition state analogues (e.g., HPC [134], SDZ-CPI-975 [154]) or acylcarnitine analogues (e.g., N-PAC [155], SDZ-269-456 and AM [184]). N-PAC forms when CPT reacts with palmitoyl-CoA and L-aminocarnitine (emeriamine) [156].

The two putative transition state analogues have similar potency for CPT-I. HPC [134] and SDZ-CPI-975 [157] reversibly inhibit CPT-I activity with $K_i$ values in the low micromolar range as a competitive inhibitor with palmitoyl-CoA and non-competitive inhibitor with carnitine. SDZ-CPI-975 [158] and HPC [121] inhibit partially purified CPT-II in the sub-micromolar range.

Although not as potent as HPC and SDZ-CPI-975, AM stereoisomeric analogues inhibit CPT-I more effectively than CPT-II [184]. Assays of two microsomal CPTs reveal little discrimination among the stereoisomers but rat liver mitochondrial CPT-I and CPT-II show distinct differences. Compound (2R,6S)-AM ($n = 12$), which does not inhibit CPT-II, emerges as a potentially useful compound for the selective inhibition of CPT-I.

6. Regulation
Changes in the activity and expression of CPT-I in response to hormones have been reviewed [65]. CPT-I increases in starvation (e.g., [159]) and decreases in hypothyroidism [162–164]. Insulin regulates CPT-I via the insulin growth factor I receptor [160,161]. The switch from the mixture with L-CPT-I present shortly after birth to the predominant M-CPT-I found in adults has been demonstrated in rat [165] and sheep [70] heart.

CPT-II mRNA and activity is likewise increased in starvation and diabetes and also by peroxisomal proliferators. Peroxisomal COT increases in parallel with CPT-II after induction by peroxisomal proliferators suggesting a parallel mechanism (reviewed in [166]). Fish oil diets also induce peroxisomal COT in rats in keeping with the role of the peroxisomes in shortening very-long-chain fatty acids for transfer as medium-chain acylcarnitines to the mitochondria. In yeast, where β-oxidation occurs only in peroxisomes, COT and the enzymes of fatty acid oxidation are induced during growth on fatty acid.

Now that complete sequence information, specific inhibitors of L and M-CPT-I and antibodies to CPT-I, CPT-II and COT are available, changes in the specific forms of the enzymes can be investigated. For example, epitopes of L-CPT-I are present not only in mitochondria but also in peroxisomes and endoplasmic reticulum [87]. A study with specific inhibitors and antibodies demonstrates [127] that heart contains both M-CPT-I and L-CPT-I (the L form at 2% only). This study suggests that activity measurements are no longer adequate. Most likely, some hormonal effects mentioned above should be re-examined at the molecular level.
6.1. Transcriptional regulation

The tissue-specific and temporal expression of CPT1A and CPT1B as compared to the body-wide and relatively steady expression of CPT2 has been reviewed [65]. CPT1A is expressed in liver and many other tissues, whereas CPT1B is significantly expressed in skeletal and cardiac muscle and in testis. However, adipocytes have species-specific differences (mouse versus man and rat) [167]. Despite this, CPT1B promoter sequences are remarkably conserved among man, mouse, rat and sheep.

The expression of several enzymes needed for fatty acid transport and oxidation is regulated at the transcriptional level via the peroxisome proliferator-activated receptor α (PPARα) [168, 169]. Like many nuclear membrane receptors, PPARα can form heterodimers with related receptors, e.g., the retinoid X receptor [170], to mediate the signalling between ligand and target gene. The promoters of these target genes contain specific sequences that are binding sites for the receptor dimers. The PPARα binding sites are known as peroxisome proliferator response elements (PPRE). Studies with a knockout mouse model provide direct evidence that PPARα participates in transcription of CPT1B, but is less prominent in that of CPT1A and CPT2 [169–173].

The CPT2 gene responds to accumulating intracellular fatty acid intermediates (presumably long-chain acyl-CoAs) via the presence of a FARE in the promoter [170, 174]. Hence, CPT1B is strongly activated when etomoxir inhibits CPT-I [170] or under fasting conditions [173]. A PPARα-mediated response to fasting might also contribute to activating CPT1B transcription in the liver [81]. Furthermore, a mammalian orthologue of a chicken ovalbumin upstream promoter transcription factor (COUP-TF) counteracts the FARE and competes with PPARα [81]. This has special interest because COUP-TF and other transcription factors like SP1 play a role in cardiac development and in hypertrophic responses of the heart associated with metabolic foetal gene re-expression programmes [175].

The CPT1A gene is also induced by fasting and etomoxir treatment. The induction is also significantly when independent of PPARα knockout mice [172, 173], and the CPT1A promoter contains no sequences similar to the FARE of CPT1B. CPT1A promoter studies have revealed roles for transcription factors like SP1 (see above) and SRY [176], but the molecular mechanisms of the responses to fatty acids and cyclic AMP [177] remain unknown. CPT1A is expressed in the heart when cardiac carnitine levels are low [165], and the juvenile steatosis mouse, which lacks a functional OCTN2, shows increased cardiac CPT1A expression [178]. Administering L-carnitine represses and reverses the effect [178], thus bypassing the active step in cardiac carnitine uptake through the action of low-affinity carnitine transporters or through diffusion. Although the effect of carnitine on cardiac CPT1A expression might suggest suppression by carnitine, a direct role for carnitine on gene transcription has not been shown [177], and the relieving effect of carnitine on long-chain acyl-CoA accumulation may well explain the consequences of carnitine supply in the juvenile steatosis mouse.

Intriguingly, the CPT2 gene promoter does not contain a FARE like the known one of CPT1B, but CPT2 does respond to specific fibrate induction through PPARα, at least in the liver [169]. This CPT2 response is only seen with fibrates and not with (long-chain) fatty acids, pointing to activator-specific causes of CPT2 transcription induction.

The transcription of CPT1 and CPT2 has been studied relatively extensively compared to the expression of COT and CAT [177]: no promoter studies of the genes for CAT, CACT and OCTN2 have been reported yet.

6.2. Physiological regulation by malonyl-CoA

Malonyl-CoA, a substrate analogue, is, as would be expected, a competitive inhibitor of CAT, COT and CPT-II. The Ki value of COT is similar to values for other short-chain CoA esters [179]. This inhibition involves interaction of malonyl-CoA presumably in the active site and differs from the regulatory sensitivity of CPT-I to malonyl-CoA.

The observation of the extreme sensitivity of L-CPT-I to malonyl-CoA dates back to 1978 [180]. Malonyl-CoA, a fatty acid synthesis intermediate, enables the inhibition of the fatty acid oxidation under conditions where synthesis is required. We
summarise here, omitting much of the controversy, the molecular basis for inhibition.

- Both the L and M form contain a malonyl-CoA binding site and a catalytic site in one protein [65]. Contrary to earlier conclusions (summarised in [65]), the malonyl-CoA binding site and the active site are on the same side of the membrane [65,73]. Fig. 2 shows the proposed orientation of the domains.
- Isoforms L and M differ in malonyl-CoA sensitivity [112,120].
- The N-terminus, not essential for catalytic activity, modulates the response to malonyl-CoA, but the large C-domain sets the sensitivity [128–130,181,182]. Deleting various portions of the N-terminus both negatively and positively affects the sensitivity [181].
- Malonyl-CoA sensitivity decreases as pH increases (for attenuation of fatty acid oxidation during acidosis [183]).
- Inhibition by malonyl-CoA is non-competitive with palmitoyl-CoA in the outer membrane but is purely competitive in the contact sites (F. Fraser and V.A. Zammit, unpublished).
- Membrane insertion contributes to modulation of malonyl-CoA sensitivity as demonstrated by reconstitution into liposomes at different temperatures ([185] and older references therein). The alterations in the kinetics of L- and M-CPT-I when each transmembrane domain is exchanged demonstrate how these effects are transmitted [130].

The altered kinetics of malonyl-CoA inhibition of L-CPT-I in response to the environment (temperature, lipids, membrane) suggests flexibility in the protein. Arguments for an allosteric site for malonyl-CoA have been summarised [65]. However, protection by malonyl-CoA against inactivation by the covalent suicide substrate etomoxiryl-CoA or the bisubstrate formed in the active site from bromoacetyl-CoA plus carnitine suggests that either the two sites overlap or they affect each other strongly enough to mutually influence binding. The recent revelation [181] that the N-terminus affects both malonyl-CoA inhibition and carnitine binding demonstrates that malonyl-CoA acts at the active site.

Recently, Guzmán et al. [186] proposed that, in concert with malonyl-CoA, cytoskeletal components regulate CPT-I. This novel model of regulation needs further exploration.

6.3. Pharmaceutical regulation – drug development

One approach to formulating an effective drug for type II (non-insulin-dependent) diabetes mellitus (NIDDM) is to design selective inhibitors of L-CPT-I. Anderson [187] points out that L-CPT-I is a better target than M-CPT-I or CPT-II. L-CPT-I catalyses a key step in supplying fatty acyl groups to fatty acid oxidation. Controlling fatty acid oxidation can regulate blood glucose levels and ameliorate some symptoms of NIDDM, a condition that accounts for over 90% of the cases of diabetes [188]. Inhibitors of L-CPT-I can decrease fatty acid oxidation and, therefore, serve as adjuvant therapeutic agents to help manage NIDDM. [187]. Isozyme-selective inhibitors offer the potential of minimising undesirable side effects.

In order to treat NIDDM, these inhibitors must overcome the challenges described in Anderson’s trenchant review [187]. In cynomolgus monkeys, SDZ-CPI-975, a reversible inhibitor, lowers blood glucose without inducing the cardiac hypertrophy caused by etomoxir, an irreversible inhibitor [189]. SDZ-CPI-975 causes ‘hepatic mitochondrial aberrations’ and development of this drug has slowed [187]. Anderson rightfully concludes, “major issues would need to be more critically examined before committing to full development” of an L-CPT-I inhibitor as a drug.

Irreversible inhibitors are being developed cautiously because of myocardial hypertrophy. Etomoxir has been used in two recent studies on humans. In a dietary study, etomoxir stimulated appetite in subjects who had a high dietary intake of fat [190]. Inhibiting fatty acid oxidation signals a desire to eat in those subjects who consume a lot of fat. In a limited study with patients who suffered from chronic heart failure, etomoxir improved the clinical status of the patients and showed no side effects of long-term (3 months) administration [191]. In congestive heart failure, calcium homeostasis is impaired and certain contractile proteins are altered. Through gene expression, etomoxir enhances the levels of sarcoplasmic
reticulum Ca\(^{2+}\)-ATPase and \(\alpha\)-myosin heavy chain protein. These clinical observations may encourage clinical studies of other CPT-I inhibitors.

Developing isozyme-selective inhibitors of CPT remains a viable goal for the following reasons: (1) CPTs have functions beyond the liver and (2) acylcarnitines can modulate the activity of other enzymes. For example, L- and M-CPT-I have roles in sperm maturation [192], so a selective CPT inhibitor might serve as a male contraceptive agent.

Palmitoylcarnitine inhibits protein kinase C in neuroblastoma NB-2a cells [193]; a palmitoylcarnitine analogue might inhibit tumour cell proliferation. Inhibition of CPT-I produces palmitoyl-CoA, which is fuel for de novo ceramide synthesis that in turn leads to apoptosis. Palmitoylcarnitine, a lysophospholipase transacylase inhibitor [194], interferes with \textit{Candida} adherence to lysophospholipids and the HEp-2 cell line [195]; a palmitoylcarnitine analogue might serve as an antimicrobial agent [196]. Consequently, continued development of acylcarnitine analogues offers benefits beyond controlling NIDDM.

### 7. Future

The carnitine system is being studied in species other than mammals. For example, metabolic studies in fish indicate that the relationship between fatty acid oxidation and CPT levels and the sensitivity of CPT-I to malonyl-CoA is similar to mammalian ones [197]. Plants have carnitine and carnitine acyltransferase activities, but the proteins are unidentified. Although CPT activity exists on either side of the pea leaf chloroplast inner membrane, only a 20-kDa protein cross-reacted with antisera to beef heart CPT-II [198]. A sequence from the \textit{Arabidopsis thaliana} genome sequencing project is annotated as homologous to the transporter, CACT, but phylogenetic analyses point to a closer relationship with ornithine translocators (F.R. van der Leij, unpublished). Some dispute the physiological significance of the carnitine system in plants, yet plants, like animals, use limited pools of CoA. Carnitine in plants most likely facilitates the transport of activated fatty acids during desaturation, elongation and lipid synthesis – e.g., during periods of rapid membrane synthesis – but also during lipid mobilisation and transport to the glyoxysome.

Sequencing of genomes and transcripts of various organisms continues to reveal genes and proteins that belong to the family of carnitine/choline acyltransferases. Gene phylogeny studies have now been carried out on about 50 genes of this family (F.R. van der Leij et al., unpublished). The first results of maximum likelihood and parsimony analyses of human and yeast genes, known to encode active transferases, are given in [63]. For \textit{CPT1A} and \textit{CPT1B} the general picture is that an ancestral \textit{CPT1} gene duplicated probably when or even before vertebrates evolved. \textit{CPT1} genes are the closest relatives, a conclusion supported by conservation of their exon junction positions. Human genes for COT, CAT and CPT-II are more closely related to each other than to the genes for CPT-I. Apparently, CPT-I and CPT-II are the most distant members of the family. This distance between CPT-I and CPT-II is independent of the differences in the C- and N-termini. The relation of the human genes to the yeast \textit{CAT2} gene for mitochondrial/peroxisomal CAT (Cat2p) and the \textit{YAT1} gene for Yat1p points to separate branches for human CAT and Cat2p. However, these branches are at central positions and the distance between CAT and Cat2p is less than the intraspecies distances between Cat2p and Yat1p or between CPT-I and CPT-II. Therefore, although the root of the tree has not been defined, it is likely that a common ancestor of the carnitine/choline acyltransferase family is a CAT-like enzyme. Furthermore, \textit{YAT1} shares an ancestral gene with \textit{CPT2} that is not shared by the other genes, suggesting the possible conservation of protein functions despite their differences in subcellular localisation and substrate specificity.

The sequence and molecular genetics information generated in the last 10 years has opened up the molecular studies of carnitine proteins and facilitated dissection of their multiple roles and intracellular locations. Still awaiting elucidation are microsomal CPTs and further insight into their role in lipoprotein synthesis (see [89]). Molecular probing of CPT-I has begun to give a picture of CPT-I and its physiological regulation by malonyl-CoA for the control of fatty acid oxidation. The next big advance will be the crystal structures, both as an aid to
understanding the mechanism and as a tool in drug design.

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