Structural model of carnitine palmitoyltransferase I based on the carnitine acetyltransferase crystal

Montserrat MORILLAS*, Eduardo LÓPEZ-VIÑAS†, Alfonso VALENCIA‡, Dolors SERRA*, Paulino GÓMEZ-PUERTAS†, Fausto G. HEGARDT*[1] and Guillermina ASINS*

*Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Barcelona, E-08028 Barcelona, Spain, †Bioinformatics Laboratory, National Astrobiology Center, Consejo Superior de Investigaciones Científicas, E-28850 Torrejón de Ardoz, Madrid, Spain, and ‡Protein Design Group, National Center for Biotechnology, Consejo Superior de Investigaciones Científicas, E-28049 Cantoblanco, Madrid, Spain

CPT I (carnitine palmitoyltransferase I) catalyses the conversion of palmitoyl-CoA into palmitoylcarnitine in the presence of L-carnitine, facilitating the entry of fatty acids into mitochondria. We propose a 3-D (three-dimensional) structural model for L-CPT I (liver CPT I), based on the similarity of this enzyme to the recently crystallized mouse carnitine acetyltransferase. The model includes 607 of the 773 amino acids of L-CPT I, and the positions of carnitine, CoA and the palmitoyl group were assigned by superposition and docking analysis. Functional analysis of this 3-D model included the mutagenesis of several amino acids in order to identify putative catalytic residues. Mutants D477A, D567A and E590D showed reduced L-CPT I activity. In addition, individual mutation of amino acids forming the conserved Ser^{685}-Thr^{686}-Ser^{687} motif abolished enzyme activity in mutants T686A and S687A and altered K_m and the catalytic efficiency for carnitine in mutant S685A. We conclude that the catalytic residues are His^{473} and Asp^{477}, while Ser^{687} probably stabilizes the transition state. Several conserved lysines, i.e. Lys^{455}, Lys^{505}, Lys^{560} and Lys^{561}, were also mutated. Only mutants K455A and K560A showed decreases in activity of 50 %. The model rationalizes the finding of nine natural mutations in patients with hereditary L-CPT I deficiencies.

Key words: carnitine acetyltransferase, carnitine palmitoyltransferase I, docking analysis, fatty acid β-oxidation; site-directed mutagenesis, three-dimensional structural model.

INTRODUCTION

CPT I (carnitine palmitoyltransferase I) catalyses the conversion of long-chain fatty acyl-CoAs into acylcarnitines in the presence of L-carnitine. This is the first step in the transport of long-chain fatty acids from the cytoplasm to the mitochondrial matrix, and it is the rate-limiting step in β-oxidation, as CPT I is tightly regulated by its physiological inhibitor malonyl-CoA, thereby conferring on CPT I the ability to signal the availability of lipid and carbohydrate fuels [1]. CPT I has a critical role in general metabolism in the heart, liver and pancreatic β-cells, and is a potential target for the treatment of metabolic disorders, involving diabetes and coronary heart disease [2]. Mammalian tissues express two isoforms of CPT I, a liver isoform (L-CPT I) and a muscle isoform (M-CPT I), which are the products of two different genes [3,4]. Other members of this family are CRAT (carnitine acetyltransferase) and COT (carnitine octanoyltransferase), which use short- and medium-chain acyl-CoAs respectively as substrates. In particular, CRAT may serve as a buffering system to maintain the appropriate levels of acyl-CoA and CoA in the various cellular compartments [5].

The crystal structure of mouse and human CRAT, alone and complexed with their substrates carnitine and CoA, has recently been reported [6,7]. This information has provided critical insights into the molecular basis for acyl-chain transfer and a possible common mechanism among the wide range of carnitine acyltransferases. The mechanism proposed for catalysis by carnitine acyltransferases is similar for a series of enzyme-transferring groups, such as lipases, proteases, acetylcholinesterases and other similar enzymes [8–10]. In all of these enzymes, a histidine residue is known to interact with an aspartate residue in catalysis, constituting what is referred to as a catalytic triad, which also involves either a serine or a threonine residue. The histidine residue is critical in catalysis and acts as a general base [9]. In CRAT, the position of this histidine at the centre of the catalytic tunnel allows access to both substrates, carnitine and acetyl-CoA, which lie on opposite sides of the tunnel [7]. The activated 3-hydroxy group of carnitine, probably acting instead of the hydroxy group of serine or threonine, can then directly attack the carbonyl carbon in acyl-CoA or acylcarnitine, and the reaction can proceed without the formation of an acyl-enzyme intermediate. Previous site-directed mutagenesis experiments have demonstrated an essential catalytic role for the analogous catalytic histidine in L-CPT I, COT and CPT II [11–13].

Early site-directed mutagenesis studies by McGarry's team showed that two aspartic residues in CPT II (Asp^{477} and Asp^{567} in L-CPT I) are essential for activity [13]. In the human CRAT crystal it was proposed that, in place of an aspartic residue, Glu^{326} could play the role of negative charge on the sole basis that it is very close to the catalytic histidine [6,7]. Asp^{477} in L-CPT I is orthologous to Glu^{326} in human CRAT.

In the present paper we propose a 3-D (three-dimensional) model for L-CPT I based on the CRAT crystal. The model is validated by site-directed mutagenesis of amino acids putatively involved in catalysis. Our study demonstrates that mutation of either one of the conserved residues Asp^{477} and Asp^{567}, which are located near carnitine and CoA respectively in the model, nearly abolishes enzyme activity. In addition, we also explored the role of the conserved motif Ser^{685}-Thr^{686}-Ser^{687} [14]. Thr^{686} and Ser^{687} are critical, since their mutation to alanine abolishes enzyme

Abbreviations used: CPT, carnitine palmitoyltransferase; 3-D, three-dimensional; L-CPT I, liver carnitine palmitoyltransferase I; M-CPT I, muscle carnitine palmitoyltransferase I; COT, carnitine octanoyltransferase; CRAT, carnitine acetyltransferase; RMSD, root-mean-square displacement.

[1] To whom correspondence should be addressed (e-mail hegardt@farmacia.far.ub.es).

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activity. Ser^{685} may be involved in carnitine binding, as K_m and V_{max} are modified in the mutant S685A. Ser^{687} might contribute to stabilization of the transition state, and Asp^{477}, together with His^{473}, could be involved directly in catalysis. Mutation of residues Lys^{455}, Lys^{505}, Lys^{560} and Lys^{561}, which have been suggested to play a role in catalysis because of their reactivity to chemical modification, affects activity to a lesser extent, and their role could be mainly one of structural maintenance. We have also localized in the model some mutations described for human L-CPT I deficiency. The model could explain the changes in activity produced by these natural mutations.

EXPERIMENTAL

Construction of the model

The structural model for CPT I was made using homology modelling procedures based on multiple alignment of the members of the carnitine/choline acyltransferase family of proteins, including the known 3-D structure of mouse CRAT (Protein Data Bank accession numbers: free enzyme structure, 1NDB; carnitine complex, 1NDF; CoA complex, 1NDI [6]). The model for rat CPT I, residues 166–772, was built using the program Swiss-Pdb Viewer and the SWISS-MODEL server facilities [15–18] (http://www.expasy.org/swissmod/SWISS-MODEL.html).

The RMSD (root-mean-square displacement) between backbone α-carbons of the template 1NDB and the CPT I structural model was 0.65 Å (1 Å = 0.1 nm). No further energy minimization procedures were applied to the model, and its structural quality was checked using the WHAT CHECK routines [19] from the WHAT IF program [20] and the PROCHECK validation program from the SWISS-MODEL server facilities [21]; briefly, the quality values of the model were within the expected region for protein structural models.

Positioning of the palmitoyl group in the L-CPT I model by docking

The putative palmitoyl-CoA binding site was modelled based on information from docking procedures using the AutoDock [22,23] and Hex [24] docking programs. Protein target and ligands (palmitoyl-CoA and carnitine) were prepared using the AutoDock algorithms Addsol and AutoTors, and a global search was conducted using AutoGrid and AutoDock (100 cycles). Ligand structures close to the original position for the CoA molecule in the PDB 1NDI structure were selected. The macromolecular docking program Hex was used to manually refine the position for the acyl extension of the ligand molecule. Ribbon plots were drawn using RASMOL [25] and the Swiss-Pdb Viewer.

Construction of site-directed mutants

Plasmids $pYESLCPTI^{wt}$ and $pYESLCPTI^{H473A}$ were obtained as described previously [11]. Plasmids $pYESLCPTI^{K455A}$, $pYESLCPTI^{D477A}$, $pYESLCPTI^{K505A}$, $pYESLCPTI^{K560A}$, $pYESLCPTI^{K561A}$, $pYESLCPTI^{D567A}$, $pYESLCPTI^{E590A}$, $pYESLCPTI^{S685A}$, $pYESLCPTI^{T686A}$ and $pYESLCPTI^{S687A}$ were constructed using the Quick Change PCR-based mutagenesis procedure (Stratagene) with the $pYESLCPTI^{wt}$ plasmid as template. The appropriate substitutions and the absence of unwanted mutations were confirmed by sequencing the inserts in both directions using an Applied Biosystems 373 automated DNA sequencer.

Expression of L-CPT I in *Saccharomyces cerevisiae*

The expression of constructs containing L-CPT I wild type and mutants in yeast cells and the preparation of cell extracts were performed as described in [11]. *S. cerevisiae* was chosen as an expression system for L-CPT I wild type and mutants because it does not have endogenous L-CPT I activity.

Determination of carnitine acyltransferase activity

CPT activity was determined by the radiometric method, as described in [11], with minor modifications. The substrates were L-[*methyl*-^{3}H]carnitine and palmitoyl-CoA. Enzyme activity was assayed for 4 min at 30 °C in a total volume of 200 μl.

For determination of the K_m for carnitine, palmitoyl-CoA was fixed at 135 μM. For determination of the K_m for acyl-CoA, the carnitine concentration was fixed at 400 μM. K_m values were estimated by analysing the data from three experiments using the program Enzifit (Biosoft).

Values reported in the text are the means and S.D.s of 3–5 determinations. All protein concentrations were determined using the Bio-Rad protein assay with BSA as standard.

Immunological techniques

Western blot analysis was performed as described [11]. The antibody for rat L-CPT I was kindly provided by Dr C. Prip-Buus (Université René Descartes, Paris, France) and was directed against a peptide comprising residues 317–430, in the cytosolic catalytic C-terminal domain. In order to quantify the level of expression, ECF™ Western blotting (using fluorescence detection) was performed, and analysis and quantification were carried out using the fluorescence scanning instrument Molecular Dynamics Storm 840™.

RESULTS

Structural model of L-CPT I

The first structural model for L-CPT I, proposed by our group, was constructed by threading or 'remote homology design' [11], and was based on the 3-D structure of rat enoyl-CoA hydratase, in the absence of an appropriate reference crystal. Since the crystals of two members of the family, murine and human CRAT [6,7], have recently been reported, we were able to design a more accurate structural model of L-CPT I by homology modelling, including most of the protein sequence. The 3-D L-CPT I model was built from residues 166 to 772. The N-terminus cannot be modelled, since it is absent from CRAT.

The 3-D model of rat L-CPT I is based on the structure of 1NDB, the free enzyme structure of mouse CRAT. A multiple structural alignment of several M-CPT I and L-CPT I enzymes and the amino acid sequence of 1NDB is shown in Figure 1. A ribbon picture of the final model of L-CPT I, including the putative location of the carnitine and palmitoyl-CoA ligands, is shown in Figure 2. Although the scaffold structure of the modelled L-CPT I is essentially similar to the one used as the template (1NDB), some differences can be observed. First, between α helix no. 12 and β sheet no. 9 in the 1NDB structure, a loop was built *de novo* for rat L-CPT I due to the presence of some extra residues in the sequences of the latter protein and of the other member of the family, M-CPT I. An addition can also be observed between β strands 13 and 14, where two extra β strands were built into the loop in the new model. The structure of these two loops should be considered to be less accurate than the rest of the model, as they do not have a counterpart in the template structure. Numbering of the α helices and β strands in the proposed model was performed without changing the labels of the template.

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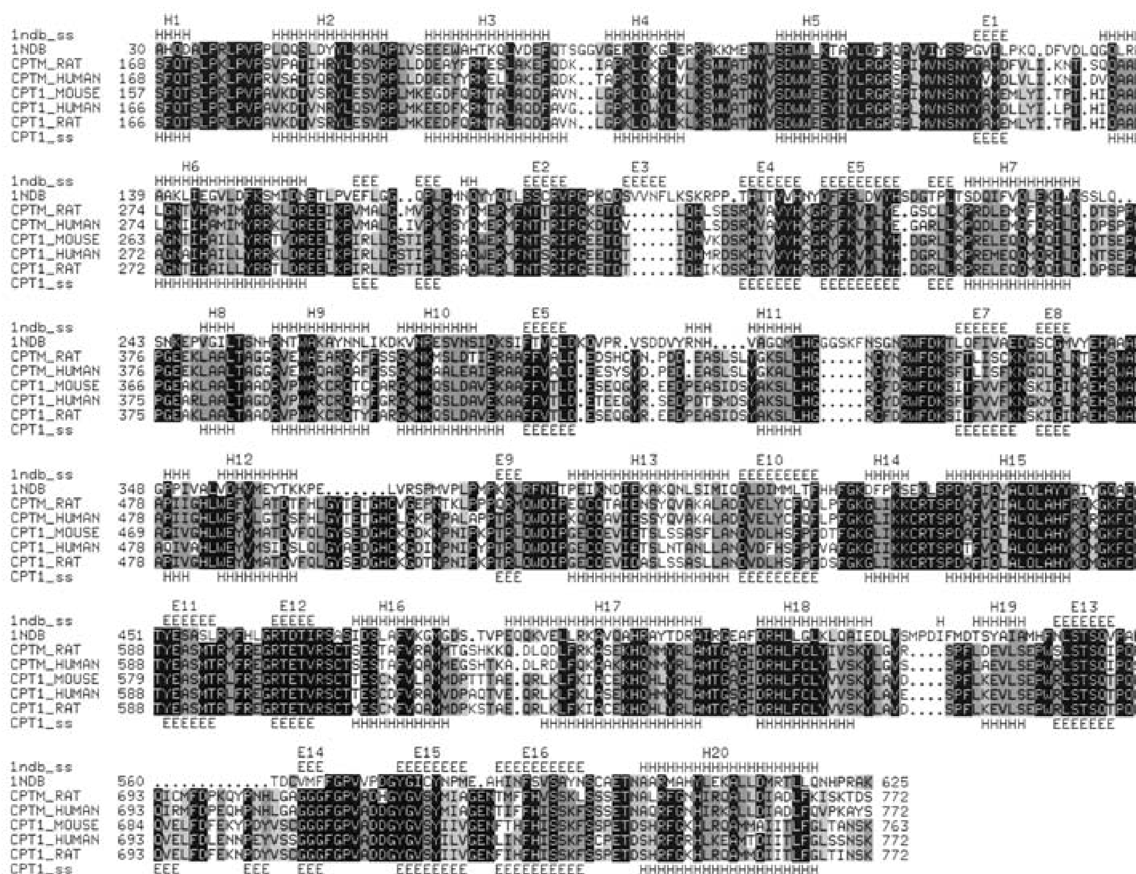

Figure 1 Multiple sequence alignment of several carnitine acyltransferases

The amino acid sequences of rat and human M-CPT I (CPTM_RAT, CPTM_HUMAN), mouse, rat and human L-CPT I (CPT1_MOUSE, CPT1_RAT, CPT1_HUMAN) and the Protein Data bank structure 1NDB (the mouse CRAT enzyme) were aligned as described in the Experimental section. The secondary structure (ss) elements of 1NDB are numbered as described previously [6]. The modelled secondary elements of rat L-CPT I are also indicated (H, α helix; E, β strand).

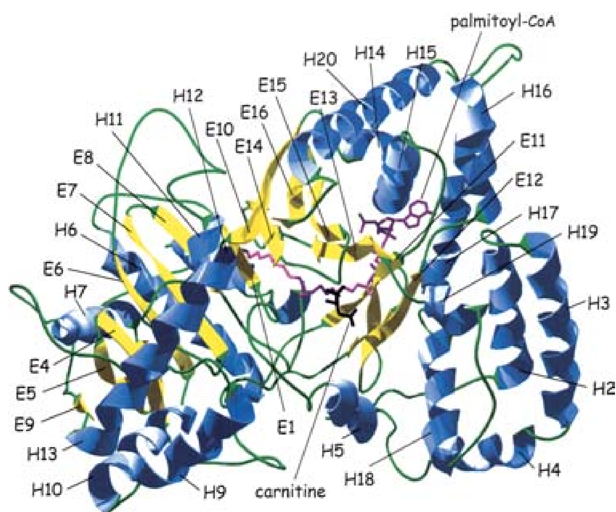

Figure 2 Structural model of rat L-CPT I

A proposed model for L-CPT I is shown using the ribbon plot representation. Secondary structure elements are numbered according to the 1NDB structure, as in Figure 1. β Strands are in yellow and α helices are in blue. The putative sites for carnitine (black) and palmitoyl-CoA (magenta) are indicated.

Proposed sites for carnitine, CoA and the palmitoyl group in the model of L-CPT I

The structural locations of carnitine and CoA were obtained previously for mouse CRAT [6] and were included in the PDB structures 1NDI and 1NDF. In the structural model for rat L-CPT I, the site for carnitine has been conserved without variation, including the 3-D co-ordinates of the ligand in the new model (Figure 2). The location of the CoA group of the palmitoyl-CoA molecule has also been maintained (Figure 2). A schematic drawing of the interactions between CoA, carnitine and the proximal amino acids of L-CPT I that lie within a radius of less than 5 Å with respect to either CoA or carnitine is shown in Figure 3 (upper panel). The catalytic His^{473} establishes a hydrogen-bonding interaction with the hydroxy group of carnitine. Asp^{477}, which presumably stabilizes the positive charge of His^{473}, is in close proximity. The positive charge of Arg^{655} probably interacts with the negative charge of the carboxylate group of carnitine. Thr^{602} and Tyr^{589} are close to carnitine, and they probably form hydrogen bonds with it, as reported for mouse CRAT. The amino acids of the tripeptide Ser^{685}-Thr^{686}-Ser^{687} also are very close to carnitine, and may participate in catalysis. Several other amino acid residues that are less than 5 Å from CoA are shown in Figure 3 (upper panel). These include Glu^{590}, Asp^{567},

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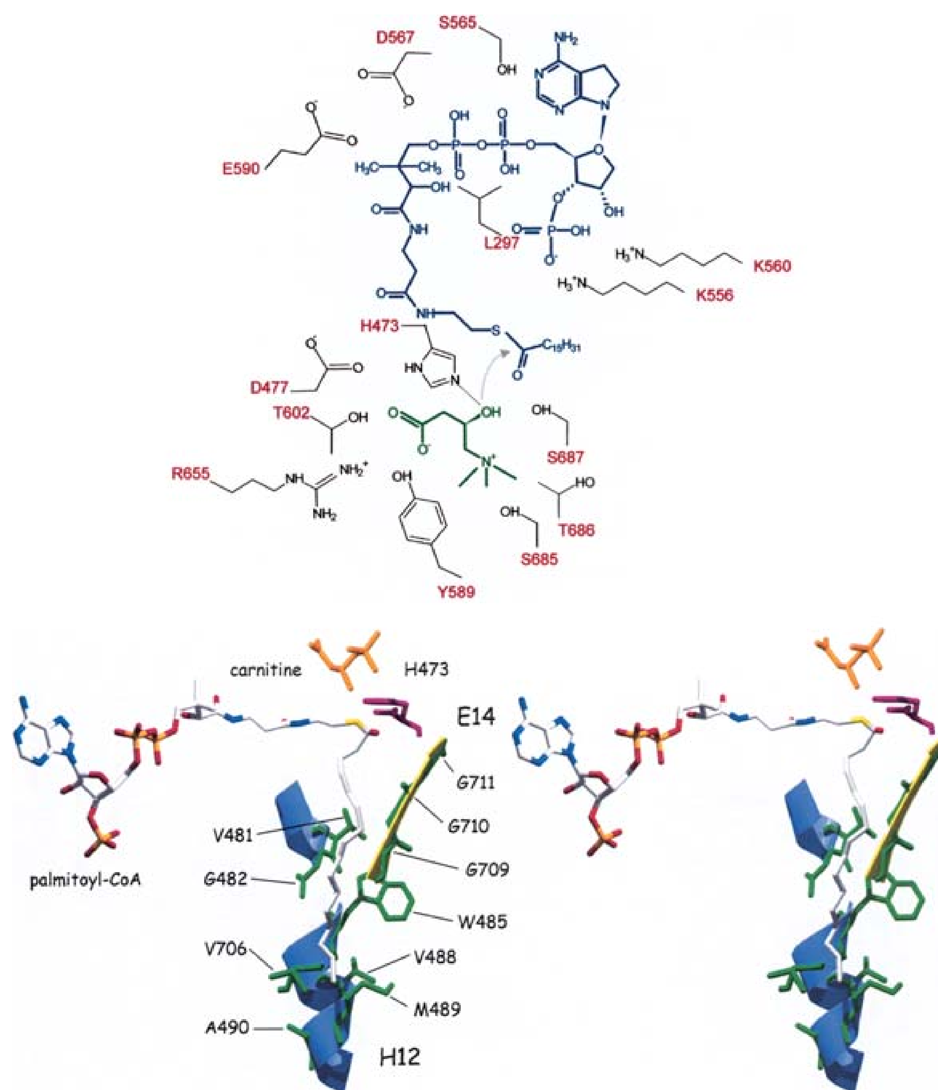

Figure 3 Proposed model of the location of amino acids close to carnitine and CoA (upper panel), and to the palmitoyl group of palmitoyl-CoA (lower panel), in rat L-CPT I

Upper panel: amino acid residues in a radius of 5 Å from CoA (blue) and carnitine (green) and probably participating in the catalytic event are shown. Lower panel: stereo diagram showing hydrophobic residues in a radius of 4.0 Å from the atoms in the fatty acid tail, which are depicted in green and labelled. The locations of α helix 12 and β strand 14, surrounding the hydrophobic tunnel, are indicated. The CoA and carnitine molecules, as well as the catalytic residue His^{473}, are also represented.

Leu^{297}, Ser^{565}, Lys^{556} and Lys^{560}, whose interactions with several atoms of CoA have been suggested from data recorded for CRAT [6,7].

A model is proposed for the positioning of the palmitoyl part of palmitoyl-CoA in a hydrophobic pocket of L-CPT I (Figure 3, lower panel). This is of greater interest than interactions described for CoA and carnitine, since positioning of the palmitoyl group has not been described previously. The pocket seen in CRAT is not valid, since the palmitoyl group is much longer than the acetyl group and requires a different accommodation from that observed in CRAT. To adequately define the new binding pocket for this long substrate, a docking procedure using the simulation docking algorithms AutoDock [22,23] and Hex [24] was used. The result of the docking analysis indicates that the fatty acid extension of the palmitoyl-CoA molecule is included in a hydrophobic pocket enclosed by α helix 12 and β strand 14. Figure 3 (lower panel) shows the amino acid residues located within a radius of 4 Å around the palmitoyl group. Hydrophobic residues in helix 12 are located on the same face of the helix, and point towards the three consecutive glycine residues in strand 14. These three residues (Gly^{709}-Gly^{710}-Gly^{711}) are present in all CPT I enzymes (L- and M-isoforms), but not in CRAT, in which they are modified to Val-Met-Phe. It seems that these three small amino acids facilitate access of the long-chain palmitoyl-CoA to the pocket.

Search for putative catalytic amino acids by site-directed mutagenesis

It has been suggested that a triad of amino acids, comprising a histidine, an aspartate and a serine, is present in the catalytic domains of several hydrolases and transferases whose mechanisms of reaction are similar [9]. The putative catalytic histidine in L-CPT I was assigned as His^{473}, since this residue is fully conserved in all carnitine acyltransferases and its mutation to alanine

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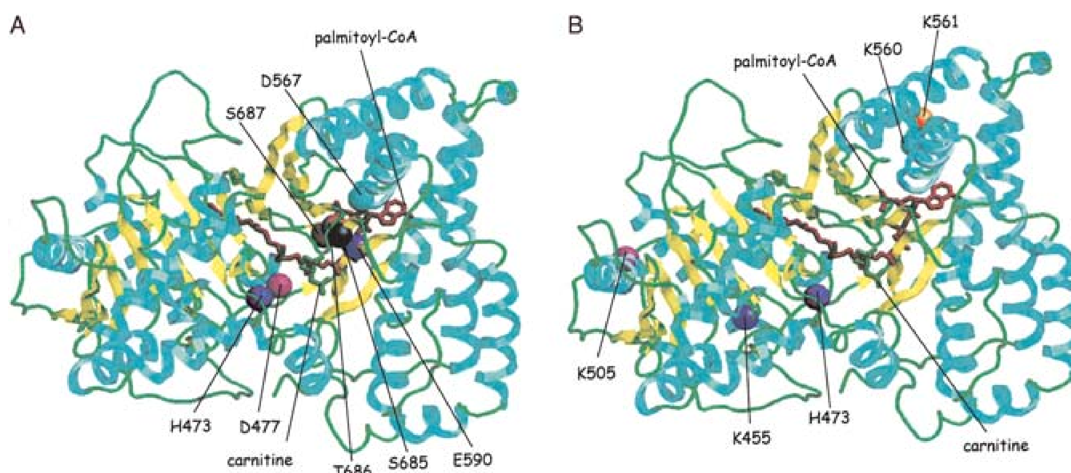

Figure 4 Localization of mutated amino acids in the L-CPT I structural model

The model is depicted in approximately the same orientation as in Figure 2. β Strands are in yellow and α helices are in blue. Stick representations of a molecule of palmitoyl-CoA (red) or carnitine (green) are included, suggesting their putative locations at the active site. (**A**) The positions of L-CPT I amino acids possibly involved in catalysis: His^{473} (blue), Asp^{477} (magenta), Asp^{567} (cyan), Glu^{590} (purple), Ser^{685} (black), Thr^{686} (red) and Ser^{687} (grey). (**B**) The positions of several L-CPT I lysine residues less closely involved in catalysis: Lys^{455} (purple), Lys^{505} (magenta), Lys^{560} (cyan) and Lys^{561} (orange). The catalytic His^{473} (blue) is also shown in (**B**) as a reference.

Table 1 Kinetic characteristics of wild-type L-CPT I and mutants

Extracts from yeast expressing wild-type and several mutants of L-CPT I were assayed for activity and kinetics as described in the Experimental section. The results are means ± S.D. of at least three independent experiments using different preparations. In parentheses are shown the variation (fold) in the catalytic efficiency (V_{max}/K_m) compared with the wild type. n.d., not determined.

Enzyme	Activity (nmol · min^{-1} · mg of $protein^{-1}$)	Expression (%)	Corrected activity (%)	K_m (μM)		V_{max} (nmol · min^{-1} · mg of $protein^{-1}$)		Catalytic efficiency	
				Carnitine	Palmitoyl-CoA	Carnitine	Palmitoyl-CoA	Carnitine	Palmitoyl-CoA
Wild type	36.1 ± 2.9	100	100	101	5.7	39.7	47.6	0.39 (× 1)	8.35 (× 1)
D477A	0.72 ± 0.04	102	1.96	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
D567A	1.26 ± 0.3	123	2.85	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S685A	17.6 ± 2.4	98	48	262	8.7	29.6	33.3	0.11 (× 0.3)	3.83 (× 0.5)
T686A	0	110	0	–	–	–	–	–	–
S687A	0	106	0	–	–	–	–	–	–
E590D	18.9 ± 0.9	140	37.4	137	5.7	29.8	28.6	0.22 (× 0.6)	5.02 (× 0.6)
K455A	14.7 ± 2.1	70	58.1	147	6.1	23.8	29.8	0.16 (× 0.4)	4.89 (× 0.6)
K505A	35.2 ± 1.2	142	68.7	105	2.4	35.7	33.3	0.34 (× 0.9)	13.88 (× 1.7)
K560A	19.9 ± 2.3	104	52.9	110	6.3	19.8	14.9	0.18 (× 0.5)	2.36 (× 0.3)
K561A	39.3 ± 1.8	129	84.5	130	5.9	53.6	47.6	0.41 (× 1.1)	8.07 (× 1)

abolished enzyme activity [11]. Moreover, His^{473} is located close (2.8 Å) to the S atom of CoA in our L-CPT I model. Therefore we explored the amino acid residues surrounding the carbonyl of the palmitoyl group in the 3-D model of L-CPT I. We located two aspartates, Asp^{477} and Asp^{567} (Figure 4), that could participate in the catalytic event, since they are well conserved in all carnitine transferases, COT, CRAT and CPT I (L- and M-), as well as in choline acetyltransferases. Experiments carried out with CPT II showed that mutations in orthologous amino acids abolished enzyme activity [13]. To explore the role of these residues in the acyltransferase reaction, we prepared expression plasmids encoding the mutant variants L-CPT I D477A and D567A. The constructs were expressed in *S. cerevisiae* and the mitochondrial fractions were assayed for CPT I activity. This was nearly abolished in both cases (2 % of residual activity for mutant D477A and 2.9 % for mutant D567A) (Table 1 and Figure 5A). Since only Asp^{477} is close (4.1 Å) to the catalytic His^{473} (Asp^{567} is 13.1 Å from His^{473}), we conclude that Asp^{477} probably participates in catalysis.

We also explored a possible role for Glu^{590} of L-CPT I, since mutation of the orthologous Glu to Asp in CPT II abolished enzyme activity [26]. Therefore mutant E590D was expressed in *S. cerevisiae*, and revealed 37 % residual activity (Table 1 and Figure 5A). Since Glu^{590} is located 9.5 Å from carnitine and 12.0 Å from the sulphur atom of CoA in the model (Figure 4A), farther away than Asp^{477} (4.1 and 4.4 Å respectively), we conclude that Glu^{590} does not participate directly in the catalytic event, but probably stabilizes, together with Asp^{567}, the positioning of CoA on its site (Figure 3, upper panel).

In the search for other residues that play a role in catalysis, we examined conserved serine residues in all carnitine acyltransferases and choline acetyltransferases, and found a conserved Ser^{685}-Thr^{686}-Ser^{687} motif near the C-termini. Localization of these residues in the L-CPT I 3-D model near the catalytic site and near both substrates, i.e. carnitine (Ser^{685} and Thr^{686}) and palmitoyl-CoA (Ser^{687}) (Figure 4A), suggested that one of them could participate in the catalytic process. To address this, individual point mutants S685A, T686A and S687A were

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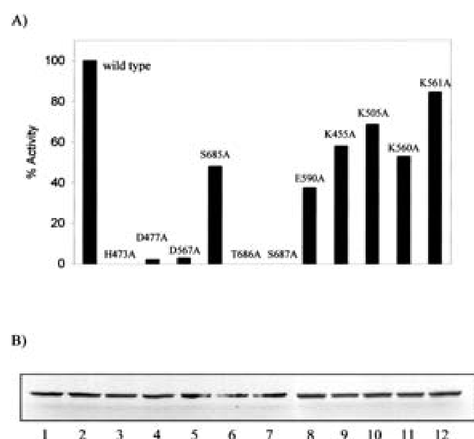

Figure 5 (A) Relative activity and (B) immunoblots showing expression of wild-type and mutant L-CPT I enzymes

(**A**)Yeast extracts (10 μg of protein) of wild type and mutant proteins were assayed for activity. The activity was corrected for the level of protein expression of each mutant. (**B**) *S. cerevisiae* extracts (60 μg) were separated by SDS/PAGE and subjected to immunoblotting using specific antibodies. Proteins used were L-CPT I wild type (wt) (lane 1), H473A (lane 2), D477A (lane 3), D567A (lane 4), S685A (lane 5), T686A (lane 6), S687A (lane 7), E590D (lane 8), K455A (lane 9), K505A (lane 10), K560A (lane 11) and K561A (lane 12).

prepared and expressed in *S. cerevisiae*. Mutant S685A showed a decrease in activity of 48 %; its K_m for carnitine increased 3-fold, while its V_{max} decreased by 20 %. With the T686A and S687A mutants, enzyme activity was abolished and the kinetic constants could not be measured (Table 1 and Figure 5A). Since the side chain of Thr^{686} points away from the active site, the OH group is unlikely to contribute to catalysis. It may, however, have a role in positioning of the substrate on the two neighbouring serines, and its mutation might disrupt the conformation of the enzyme in this area, as was observed for CRAT [6].

Several other residues have been implicated previously in catalysis in other members of the carnitine acyltransferase family. We explored the putative roles of several lysine residues. Lys^{455} is a conserved amino acid 18 residues upstream of the catalytic His^{473} and it was located proximal to the catalytic histidine in the former L-CPT I model. Lys^{505} had been implicated in catalysis in COT [27]. Lys^{560} and Lys^{561} are located in the proposed model at 3.7 Å and 11.0 Å respectively from the 5′ phosphate group of CoA (Figure 4B). Although only the first lysine of these two is fully conserved in the carnitine acyltransferase family, we mutated both residues independently, as resolution of the model does not allow us to identify the best candidate. Mutant K455A showed altered activity accompanied by 70 % protein expression. Thus the activity of the mutant was 58 % of wild type (Table 1 and Figure 5A). Mutant K505A retained 69 % activity. Lys^{505} is far away from the substrates in the model. With regard to the other two lysines (Lys^{560}-Lys^{561}) proximal to the 5′ phosphate group of CoA, mutation of the residue closer to CoA had the greatest effect on activity. Mutant K560A had 53 % of wild-type activity, whereas mutant K561A showed 85 % activity (Figure 5A).

The mutants generated in this study which conserved measurable enzyme activity were assayed for determination of kinetic constants. The K_m values for carnitine of the lysine mutants K455A, K505A, K560A, K561A differed little from that of the wild type, but their V_{max} values for carnitine ranged between 50 % and 135 % of wild type. K_m and V_{max} for palmitoyl-CoA were similar to wild type. Only the K560A mutant showed a decreased V_{max} (30 %) (Table 1). It appears that those mutants with altered catalytic efficiency affect the V_{max} more than the K_m for both carnitine and palmitoyl-CoA. Overall, the lysines studied here contributed less to catalytic activity than the other amino acids assayed, such as Asp^{477}, Asp^{567}, Glu^{590}, Ser^{685}, Thr^{686} and Ser^{687}.

Western blot analysis of wild-type L-CPT I and the mutants using polyclonal antibodies is shown in Figure 5(B). For both the wild type and mutants, proteins of the predicted sizes were synthesized with similar levels of expression.

DISCUSSION

The structural difference between L-CPT I and other carnitine acyltransferases is that the former contains an additional N-terminal domain of about 160 amino acid residues that is not present in CPT II, COT or CRAT. This N-terminal region comprises a cytosolic segment of 47 amino acids, and two transmembrane regions. Both the N- and C-terminal regions are projected into the cytosol. The C-terminal region contains both the catalytic and the malonyl-CoA binding site, although there might be some interaction between the N-terminus and the malonyl-CoA site, since mutation of specific N-terminal amino acids results in the abolition of malonyl-CoA sensitivity. The proposed 3-D model includes all C-terminal domain amino acids from Ser^{166} to Lys^{772}, analogous to that present in CRAT.

Comparison between the 3-D model based on similarity to CRAT and the model based on similarity to a fragment of enoyl-CoA hydratase

In a previous study [11] we proposed a 3-D structural model of the catalytic core of the L-CPT I and COT proteins, designed on a threading procedure or 'remote homology design', taking the crystal of rat enoyl-CoA hydratase as a template. This model was partial, since it comprised only 200 amino acids (co-ordinates 368–567) and could not show the carnitine binding site. In this model, His^{473} of L-CPT I was located very close to the sulphur atom of the acyl-CoA substrate, a result that supported the model and offered a structural explanation for the catalytic activity in which this histidine residue has been implicated.

Using the data from the former model to find candidate residues comprising the catalytic site, we chose two amino acids, Asp^{421} and Ser^{423}, both of which were modelled close to the acyl-CoA molecule [11]. Mutation of either of these residues to alanine did not affect catalytic activity (results not shown). These results suggested that the previous model was inaccurate and that it should be modified when new data were available to improve the assignments of interactions between the various amino acids. A careful measurement of distances of these amino acids now reveals that they are far away from the catalytic site.

The present model improves on the previous one, since it was constructed by direct homology: the template used was obtained after crystallization of another member of the carnitine acyltransferase family, in this case mouse CRAT. The general organization of the new model coincides closely with that of CRAT, but it contains specific arrangements, particularly those referring related to the binding of the substrate palmitoyl-CoA, which is much longer than acetyl-CoA. As shown in Figures 2 and 3 (lower panel), the L-CPT I model contains a pocket near the catalytic site, which could accommodate the fatty long-chain palmitoyl-CoA. Docking analyses using specialized algorithms confirmed this possibility.

In addition to structural features, the 3-D L-CPT I model shows several functional properties that support its structure. Mutation of Asp^{477}, Asp^{567}, Thr^{686} or Ser^{687} practically abolished enzyme activity. Mutation of other amino acid residues produced

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partial decreases in enzyme activity, e.g. Glu[590], Ser[685] and the lysines studied in the present paper. These residues probably play a secondary role, maintaining the conformation of the positive charge distribution of β sheets and α helices throughout the molecule. Ramsay and co-workers [27] had suggested that Lys[358] of COT might be involved in the COT catalytic process. Localization of the analogous lysine (Lys[505]) in the L-CPT I model and determination of enzyme activity of its mutant have ruled out a probable role in catalysis.

Proposed binding sites for carnitine and palmitoyl-CoA

Figure 3 shows a general arrangement of the amino acids involved in the interactions with carnitine and the palmitoyl group. We chose these residues on the basis of four criteria: (1) the effects of their mutation on enzyme activity; (2) docking analysis using the specialized AutoDock algorithms; (3) their distance to the S atom of CoA in some cases, to several groups forming the palmitoyl molecule or to the various atoms of carnitine; and (4) the general positions of similar residues in the mouse or human CRAT crystal.

Arg[655], whose distance to the carboxylate group of carnitine is 3.7 Å, probably forms a salt bridge with this group at the active site (Figure 3, upper panel). It was shown previously [28] that mutation of the equivalent residue in COT to asparagine increased the apparent K_m for carnitine by 1650-fold. Ser[687] (whose distance to the OH group of carnitine is only 4.3 Å), mutation of which to alanine abolished enzyme activity, is probably involved in maintaining the proper conformation of the enzyme in catalysis. It is noteworthy that the hydroxylated side chain of Thr[686] points away from the active site, as is the case in mouse CRAT. As the Ser[685]-Thr[686]-Ser[687] motif has been observed in all carnitine acyltransferases, it is possible that Thr[686] may help to maintain the proper conformation of the enzyme in this area, particularly that of Ser[687], which may stabilize the transition state, as has been pointed out for COT [14]. This effect is not observed for the first member of the motif (Ser[685]), because its mutation only decreased enzyme activity by 50 %. Other amino acid residues (Thr[602], Tyr[589]) might also participate in positioning of carnitine in the catalytic channel, in view of their small distances to this molecule.

His[473] is critical for catalysis, acting as a general base. Its distance to the hydroxy group of carnitine is 3.1 Å. Since Asp[477] is close to His[473] in the model, and its mutation abolishes enzyme activity, its role in catalysis is probably the stabilization of the positive charge that develops after deprotonation of the substrate. Asp[567] and Glu[590] might stabilize the position of the pantothenic group of CoA. Lys[556] and Lys[560], which are located only 4.14 and 3.7 Å respectively from the 3′ phosphate group of the CoA ribose ring, probably interact with this group. Other amino acid residues might also participate in the positioning of CoA in the catalytic channel, as suggested for CRAT [6], including Leu[297] and Ser[565].

Figure 3 (lower panel) shows how the palmitoyl group is accommodated in a hydrophobic pocket. The palmitoyl group does not form a putative straight line with the CoA group, but it bends within the catalytic His[473] into the direction of a hydrophobic region. This is possible because at the beginning of the bend there are three glycines (residues 709–711), which are small enough to allow the hydrocarbon chain to enter the pocket. It is noteworthy that CRAT does not contain this tripeptide; in contrast, CRAT contains three voluminous amino acids (Val, Met and Phe) within this area. The hydrophobic pocket is located between amino acids of helix H12 and the glycine residues of β sheet 14. It should be emphasized that immediately before Val[706] there is an extra loop that is present in all isoforms of CPT I (L- and M-) (residues 693–705) but is absent from CRAT. All of these circumstances favour localization of the palmitoyl group in L-CPT I and not in CRAT, whose natural substrate is the CoA derivative of the small two-carbon acid, the acetyl group. Figure 3 (lower panel) also shows that amino acids involved in the palmitoyl pocket are hydrophobic residues, i.e. Val[481], Trp[485], Val[488], Met[489] and Ala[490] of helix 12 and Val[706] of the coiled turn, and that all are with 4 Å of the palmitoyl group.

Small differences in catalytic mechanism among the various carnitine acyltransferases may occur as a consequence of (1) the different lengths of the hydrocarbon chains of the fatty acyl-CoA substrates; (2) the fact that the reaction of L-CPT I is inhibited by malonyl-CoA; and (3) the fact that L-CPT I is bound to the mitochondrial outer membrane, unlike mitochondrial CPT II, which is close to the inner membrane, peroxisomal COT or mitochondrial CRAT.

Table 2 Missense mutations causing human L-CPT I deficiency

n.e., not expressed; n.d., not determined.

Mutant	Distance to sulphur atom of CoA (Å)	Instability	Expression system	Activity (% of wild type)	Reference
C304W	16	++	COS cells	1.7	[29]
R357W	38	+	COS cells	14.5	[29]
E360G	33	+++	Fibroblasts	0	[30]
delR395	25	+	COS cells	0.1	[29]
A414V	23	+++	n.e.	n.d.	[31]
D454G	18	+++	*S. cerevisiae*	2	[32]
L484P	14	++	COS cells	23.9	[29]
Y498C	42	+++	n.e.	n.d.	[31]
G710E	8.2	Stable	*S. cerevisiae*	Undetectable	[33]

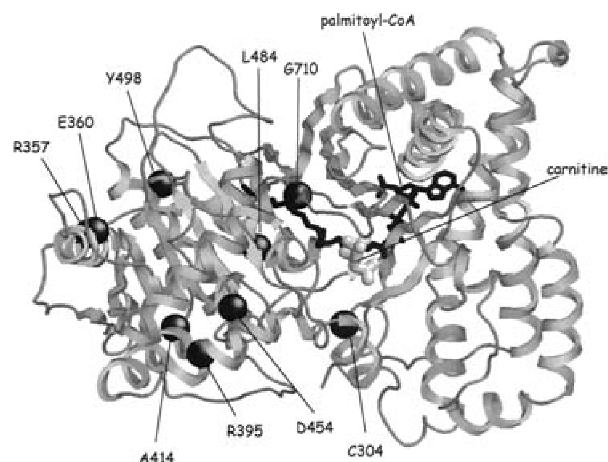

Figure 6 Localization in the L-CPT I structural model of amino acids found to be mutated in hereditary CPT I deficiencies described previously

Ribbon plot representation of the proposed model for L-CPT I, as in Figure 4. The positions of L-CPT I amino acids are indicated as spheres. See Table 2 for further details.

Localization of amino acid residues involved in human CPT I deficiency

The present model also provides a molecular basis for understanding the effects of human missense mutations in L-CPT I (Table 2 and Figure 6). The natural missense mutations have been validated by heterologous expression in mammalian or yeast cells. These mutations can be roughly divided into two categories, depending on whether they affect the activity directly or indirectly.

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The only known natural mutation (G710E) that decreases the activity without changing the stability of the protein is localized very close to the catalytic His[473], and the introduction of a negative charge may impair the catalytic process. Alternatively, mutation of Gly[710], which is one of the amino acids of the tripeptide putatively involved in the opening of the palmitoyl hydrophobic pocket, to the more voluminous Glu[710] residue impedes the positioning of palmitoyl-CoA. The other natural mutations described in the literature produce unstable proteins, and involve amino acids located far from the catalytic pocket, such as Cys[304], Arg[395], Glu[360], Ala[414], Asp[454], Leu[484] and Tyr[498]. The loss of enzyme activity which causes the hereditary diseases is thus explained by a loss of stability (Table 2).

No natural mutation has been described in M-CPT I. Thus M-CPT I deficiency, as a disease, has not been identified hitherto. However, more that 150 families have been reported with deficiencies in CPT II, with a prevalent S113L mutation found in 50 % of mutant alleles. Another 10 families with an infantile-type deficiency of CPT II have been identified; this deficiency is lethal during the first month of life [34]. Preparation of a 3-D CPT II model based on the CRAT model is expected to explain the molecular effects of these natural mutations.

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