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Structural Model of a Malonyl-CoA-binding Site of Carnitine Octanoyltransferase and Carnitine Palmitoyltransferase I

MUTATIONAL ANALYSIS OF A MALONYL-CoA AFFINITY DOMAIN*

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Carnitine octanoyltransferase (COT) and carnitine palmitoyltransferase (CPT) I, which facilitate the transport of medium- and long-chain fatty acids through the peroxisomal and mitochondrial membranes, are physiologically inhibited by malonyl-CoA. Using an "*in silico*" macromolecular docking approach, we built a model in which malonyl-CoA could be attached near the catalytic core. This disrupts the positioning of the acyl-CoA substrate in the channel in the model reported for both proteins (Morillas, M., Gómez-Puertas, P., Roca, R., Serra, D., Asins, G., Valencia, A., and Hegardt, F. G. (2001) *J. Biol. Chem.* 276, 45001–45008). The putative malonyl-CoA domain contained His^{340}, implicated together with His^{131} in COT malonyl-CoA sensitivity (Morillas, M., Clotet, J., Rubí, B., Serra, D., Asins, G., Ariño, J., and Hegardt F. G. (2000) *FEBS Lett.* 466, 183–186). When we mutated COT His^{131} the IC_{50} increased, and malonyl-CoA competed with the substrate decanoyl-CoA. Mutation of COT Ala^{332}, present in the domain 8 amino acids away from His^{340}, decreased the malonyl-CoA sensitivity of COT. The homologous histidine and alanine residues of L-CPT I, His^{277}, His^{483}, and Ala^{478} were also mutated, which decreased malonyl-CoA sensitivity. Natural mutation of Pro^{479}, which is also located in the malonyl-CoA predicted site, to Leu in a patient with human L-CPT I hereditary deficiency, modified malonyl-CoA sensitivity. We conclude that this malonyl-CoA domain is present in both COT and L-CPT I proteins and might be the site at which malonyl-CoA interacts with the substrate acyl-CoA. Other malonyl-CoA non-inhibitable members of the family, CPT II and carnitine acetyltransferase, do not contain this domain.

Interest in malonyl-CoA as a metabolic regulator has increased in recent years, as its function is not restricted to liver regulation of fatty acid oxidation and synthesis. Its contribution to the β-oxidation of long-chain fatty acids in other tissues, such as heart, skeletal muscle, and the β-cell has been widely studied (1). In liver, a low insulin/glucagon ratio decreases malonyl-CoA and shifts the fatty acid traffic throughout the oxidative process. The opposite is also true: when the insulin/glucagon ratio increases, malonyl-CoA concentration also rises, followed by a decrease in β-oxidation (2, 3). The situation is similar in rat heart: incubation of perfused rat heart with glucose and insulin raises malonyl-CoA concentrations with concomitant suppression of palmitate oxidation (4). Furthermore, when the glucose concentration in β-cell is raised, the malonyl-CoA concentration also increases and the resulting elevation of cytosolic long-chain fatty acyl-CoAs concentration stimulates exocytosis of insulin granules (5, 6).

Malonyl-CoA acquires its significant regulatory role by virtue of its inhibition of a class of carnitine acyltransferases, carnitine octanoyltransferase (COT)[1] and carnitine palmitoyltransferase I (CPT) I. There are two isoforms of CPT I, produced by different genes: the L (liver) type and the M (muscle) type. In mammals, muscle CPT I has a much lower IC_{50} for malonyl-CoA than the liver form, whereas the affinity for long chain acyl-CoA is similar (7, 8). This differential response to malonyl-CoA has been associated with the distinct interaction between the NH_2- and COOH-terminal domains of the protein, which are exposed to the cytosolic side of the mitochondrial membrane (9). In contrast, other members of the family, such as carnitine palmitoyltransferase II (CPT II) and carnitine acetyltransferase (CAT) are not regulated by malonyl-CoA (10).

Pioneer studies showed that malonyl-CoA does not bind to the CPT I active site, although there is a competition behavior between malonyl-CoA and palmitoyl-CoA (11–16). Inhibition by malonyl-CoA is produced by the occurrence of two binding sites, present in both L- and M- types (17, 18). One site is the low affinity site, near the catalytic acyl-CoA-binding domain, in which the inhibitory effect could be exerted by malonyl-CoA and, a lesser extent, other compounds with the CoA moiety, such as acetyl-CoA, glutaryl-CoA, hydroxymethylglutaryl-CoA, methylmalonyl-CoA or free CoA itself (19, 20). The second, high

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[1] The abbreviations used are: COT, carnitine octanoyltransferase; CPT I, carnitine palmitoyltransferase I; L-CPT I, liver isoform of CPT I; M-CPT I, muscle isoform of CPT I; CPT II, carnitine palmitoyltransferase II; CAT, carnitine acetyltransferase; CoA, coenzyme A.

affinity site is separated from the active site and does not compete with acyl-CoA (21–25). Compounds analog to malonyl-CoA, like 4-hydroxyphenylglyoxylate or its derivative Ro 25–0187, could inhibit CPT I without the presence of the CoA moiety (23).

The probable locations of the malonyl-CoA-binding sites in L-CPT I were predicted to be at the cytosolic COOH-terminal domain, after the preparation of several L-CPT I chimeras (26, 27). However, the NH_2 terminus of L-CPT I also influences the enzyme-inhibitor interaction, since mutation of either His^5 or Glu^3 reduced malonyl-CoA sensitivity (17, 26). Moreover, modification of the interaction between the cytosolic NH_2-terminal domain and the cytosolic catalytic COOH-terminal domain of CPT I alters the IC_{50} values for malonyl-CoA (9). In addition, the removal of the segment comprised between amino acids 19 and 40 increases malonyl-CoA sensitivity in L-CPT I, which emphasizes the importance of the NH_2 terminus before the first transmembrane region as a modulator of malonyl-CoA inhibition (27, 28). The possible influence on malonyl-CoA inhibition of amino acids in the NH_2 terminus does not occur in COT protein because the first amino acid of COT corresponds to position 152 of the L-CPT I, and therefore COT does not have the same amino terminus nor the transmembrane domains.

Histidine residues are implicated in the malonyl-CoA interaction by the finding that a decrease in pH (associated with the protonation of the imidazol group of histidine) increases the affinity for malonyl-CoA (12, 29). Our previous work showed that COT-malonyl-CoA interactions implicated two histidine residues, His^{131} and His^{340}, which could involve two domains of interaction and binding of malonyl-CoA (30). The proposal of a structural model of the catalytic core of the COT protein (31), in which one of these histidines, His^{340}, is located has facilitated the study of one of these malonyl-CoA affinity domains.

In this study we identified a putative binding domain of malonyl-CoA located in the model of COT and L-CPT I. From bioinformatic and mutagenic analysis, we define a domain containing amino acids from Ala^{332} to His^{340} in COT, and from Ala^{478} to His^{483} in L-CPT I (its homologous domain) as the site at which malonyl-CoA could bind to with either protein and induce inhibition. The structural models of COT and L-CPT I locate malonyl-CoA near the catalytic channel. The presence of malonyl-CoA in the groove of ligand-protein interaction would interfere with the positioning of the substrate decanoyl-CoA (in COT) or palmitoyl-CoA (in L-CPT I). To confirm this model, we expressed the cDNA for the wild type and different single and double mutants of COT and L-CPT I in *Saccharomyces cerevisiae*.

A similar picture to that proposed for COT and L-CPT I is impossible with the non-inhibitable malonyl-CoA enzymes CPT II or CAT, although they have the same three-dimensional structure (which is analogous to the 2dubE domain described for COT and L-CPT I), since when we use a docking program, malonyl-CoA cannot be located in the model.

EXPERIMENTAL PROCEDURES

Structural Model Building—Structural models of rat CPT I and COT were obtained as previously described (31). Models of the homologous proteins CAT and CPT II were built using a similar procedure. Briefly, sequences homologous to rat CPT II and mouse CAT were obtained using BLAST (32) and aligned using ClustalW (33). Secondary structure analysis of the sequences was performed using the public servers PHD (34), Jpred (35), and Psi-Pred (36). Protein folds were recognized using the threading programs Threader2 (37), based on solvatation and pair potentials, and FUGUE (38), which uses environment-specific substitution tables and a structure-dependent gap penalty to detect remote structural homologues. The structural templates obtained were analyzed using information from the structure classification data bases CATH (39), FSSP (40), and SCOP (41). Using the three-dimensioinal structure of rat enoyl-CoA hydratase (PDB entry 2dub chain E), as selected previously for the homologous proteins CPT I and COT (31), the program Swiss-Pdb Viewer and the SWISS-MODEL server facilities (42–45) were used to build the coordinates for the three-dimensional model of the active site-surrounding regions of CAT and CPT II. Models were validated using ProsaII (46), WHAT-CHECK (47), from WHAT-IF (48), and PROCHECK (49). Calculation and representation of electrostatic potentials of the obtained models were performed using GRASP (50).

Macromolecular Docking—Docking calculations to obtain a molecular model of the interaction between the inhibitor (ligand) malonyl-CoA and the putative receptor proteins CPT I, COT, CPT II, and CAT were performed using the *spherical polar Fourier correlations* based program "Hex" (51). The coordinates of the malonyl-CoA molecule were obtained from the PDB entry 1hnj (three-dimensional structure of the β-ketoacyl-acyl carrier protein synthase III). Program parameters were adjusted to evaluate the docking score at more than 40,000 distinct rotational orientations at each of 10 different intermolecular distances (at increments of ± 1 Å) between the inhibitor molecule and the molecular models of the four acyltransferases indicated above. The 128 highest scoring (lowest energy) orientations were retained for viewing and evaluation.

Tree-determinants Analysis—Conserved differences (tree-determinants) between malonyl-inhibitable (L-CPT I, M-CPT I, COT) and non-inhibitable (CPT II, CAT) carnitine acyltransferases were analyzed with the SequenceSpace algorithm (52, 53), using the multiple alignment of the carnitine-choline acyltransferase family of proteins as input.

Construction of Site-directed Mutants—Plasmids $pYESCOT^{wt}$, $pYESCOT^{H131A}$, $pYESCOT^{H340A}$, and $pYESCOT^{H131A/H340A}$ were obtained as previously described (54). Plasmid $pYESCOT^{A332G}$ was constructed using the QuikChange polymerase chain reaction-based mutagenesis procedure (Stratagene) with the $pYESCOT^{wt}$ plasmid as template and the primer 5′-GCTGTGATCATGCTCCTTATGATGGAATGCTTATGGTGAAC-3′ (the mutated nucleotide is underlined).

Plasmid $pYESLCPTI^{wt}$, which contained nucleotides 103–2701 including the coding region of L-CPT was constructed as described (31). Plasmid $pYESLCPTI^{wt}$ was used for site-directed mutagenesis of His^{277} to Ala^{277} by the asymmetric PCR method (55). The following primers were used: the mutated megaprimer fragment, obtained with the forward primer CPT583.for: 5′-AGCCCATGTTGTACAGCTTCC-3′ and the reverse primer H277A.rev: 5′-AGTATGGCGGCGATGGTGTTGCCAGC-3′ (the mutated nucleotides are underlined), was used with the reverse primer CPT1607.rev: 5′-CCATCCTCTGAGTAACCCAGC-3′. The fragment obtained was digested with *Aat*II (nucleotides 610–1580) and subcloned into the plasmid $pYESLCPTI^{wt}$, obtaining plasmid $pYESLCPTI^{H277A}$. Plasmid $pBSLCPTI^{wt}$, which contained nucleotides 54–2701 of rat L-CPT I subcloned in the *Cla*I and *Eco*RI sites of pBluescript SK^+ vector (Stratagene), was used for construction of plasmid $pYESLCPTI^{H483A}$. The mutated megaprimer fragment was obtained with the forward primer H483A.for: 5′-GTGGGCGCTTTGTGGGAGTATGTCATGGC-3′ (the mutated nucleotides are underlined) and the reverse primer CPT1878.rev: 5′-GGCCTCATATGTGAGGC-3′, which was used with the primer CPT1182.for: 5′-GCAGCAGATGCAGCAGATCC-3′ to obtain a fragment which, after digestion with *Pst*I, was subcloned into the $pBSLCPTI^{wt}$, obtaining $pBSLCPTI^{H483A}$. To obtain the plasmid $pYESCPTI^{H483A}$, the fragment *Kpn*I-*Eco*RI was digested and subcloned into the $pYESCPTI^{wt}$. Plasmid $pYESLCPTI^{H277A/H483A}$ was generated by mutagenesis of His^{277} to Ala using the plasmid $pYESLCPTI^{H483A}$ as template, and the primers described above. Mutant L-CPT I A478G was constructed using the "QuikChange" polymerase chain reaction-based mutagenesis procedure (Stratagene) with $pYESLCPTI^{wt}$ plasmid as template and the primer 5′-CACTCCTGCGCGGACGGGCCCATCGTGGGCCATTTG-3′(mutated nucleotide is underlined). The appropriate substitutions, as well as the absence of unwanted mutations were confirmed by sequencing the inserts in both directions with an Applied Biosystems 373 automated DNA sequencer.

Expression of COT and L-CPT I in S. cerevisiae—The expression of the constructs containing COT and L-CPT I wild type and mutants (see above) in yeast cells and the obtaining of the cell extracts were performed as described in Refs. 30 and 31. Depending on the experiment the time to induce expression of L-CPT I by galactose was varied between 1 and 20 h.

Determination of Carnitine Acyltransferase Activity—Carnitine acyltransferase activity was determined by the radiometric method as described in Ref. 26 with minor modifications. The substrates were L-[*methyl*-^{3}H]carnitine and, decanoyl-CoA for COT and palmitoyl-CoA for L-CPT I. 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, decanoyl-CoA was fixed at 50 μM (for COT) and palmitoyl-CoA was fixed at 135 μM (for L-CPT I). For determination of the K_m for acyl-CoA, carnitine concentration was fixed at 400 μM. When malonyl-CoA inhibition was assayed, increasing concentrations of malonyl-CoA were included. The IC_{50}, defined as the malonyl-CoA concentration that produces 50% inhibition of enzyme activity, was determined using 50 μM acyl-CoA and 400 μM carnitine. K_m was estimated by analyzing the data from three experiments using the program Enzifit (Biosoft) and IC_{50} was calculated by Excel software using linear regression analysis.

Values reported in the text are the means and standard deviations of three to five determinations. Curve fitting was carried out using Sigma plot software. All protein concentrations were determined using the Bio-Rad protein assay with bovine albumin as standard.

Immunological Techniques—Western blot analysis for COT and L-CPT I were performed as described (31). The specific antibody used for rat COT was directed against peptide 344–360. The antibody for rat L-CPT I was kindly given by Dr. V. A. Zammit (Hannah Research Institute, Ayr, Scotland, United Kingdom) and was directed against peptide 428–441, in the cytosolic catalytic COOH-terminal domain.

Isolation of Mitochondria—Rat liver mitochondria were isolated as described elsewhere (56). Mitochondria were dispersed in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA to a final concentration of 40 mg/ml and stored at −70 °C.

RESULTS

Positioning of Malonyl-CoA in the COT Structural Model—Once the structural models of COT and L-CPT I catalytic core, including the putative substrate-binding site, active site, were established (31), we performed exhaustive *in silico* molecular docking analysis to find clues to the putative binding site of malonyl-CoA.

We used the computational system Hex (51) that uses spherical polar Fourier correlations. Each molecule was modeled using three-dimensional parametric functions which encode both surface shape and electrostatic charge and electrostatic potential distributions. By using an expression for the overlap of pairs of parametric functions, an expression for a docking score can be derived as a function of the six degrees of freedom in a rigid body docking search. With the appropriate scaling factors, the docking score can be interpreted as an interaction energy that ranks possible docking solutions according to the minimum value.

After analysis of the best (minimum energy) solutions given by the program for the interaction between the malonyl-CoA molecule and the structural model for the catalytic core of rat COT, a common position was observed for the five best predicted solutions in terms of low energy of the complex and lowest macromolecular distance. The lowest distance solution is shown in Fig. 1*A*. The inhibitor molecule fits into a second cavity in the three-dimensional model, which is distinct from the cavity occupied by the substrate. The simultaneous representation of both molecules (decanoyl-CoA (substrate) and malonyl-CoA (inhibitor), (Fig. 1*B*) offers an explanation for the inhibition process: both molecules compete for a portion of the same spatial groove, despite the different binding site proposed in the model.

To analyze the fitting of malonyl-CoA on the surface of the protein, and to improve our definition of this domain, we have performed a site-directed mutagenesis in some of the amino acids involved in this domain. If site-directed mutants show decreased sensitivity to malonyl-CoA, the precise location of this molecule in the COT model would be supported.

Analysis of the Amino Acids in the Catalytic Core Potentially Involved in the Inhibition of COT by Malonyl-CoA—We expressed COT wild type in *S. cerevisiae*, an organism devoid of endogenous COT activity, and determined the influence of the concentration of the acyl-CoA substrate, decanoyl-CoA, on the inhibitory pattern produced by malonyl-CoA. As shown in Fig. 2*A*, there was a competition in COT wild type between the

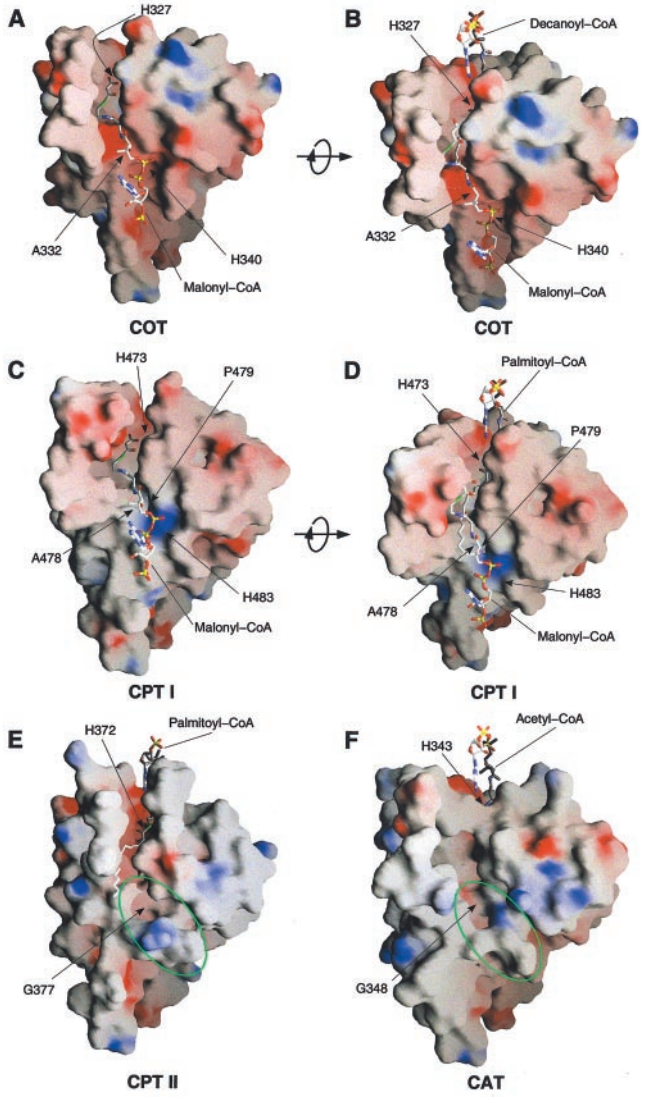

FIG. 1. **Solvent-accessible surface representation of the model for COT, L-CPT I, CPT II, and CAT.** *A*, the lowest distance model among the lower energy score models obtained for malonyl-CoA molecule: the three-dimensional model of the catalytic core of COT has been drawn after the location given by the molecular docking calculations. *B*, both the malonyl-CoA and substrate decanoyl-CoA are represented. The structure has been rotated to show better the relative positions of the two metabolites. The surface is colored according to the calculated electrostatic potential (negative, *red*, to positive, *blue*). On *C* and *D* the same is represented for CPT I. The approximate locations of rat COT Ala^{332}, His^{340} (involved in malonyl-CoA inhibition), and His^{327} (catalytic) and of rat L-CPT I Ala^{478}, Pro^{479}, and His^{483} (involved in malonyl-CoA inhibition) and His^{473} (catalytic) are indicated by *arrows*. The surface of both CPT II and CAT models are represented in *E* and *F*. Approximate locations of catalytic histidines His^{372} (CPT II), His^{343} (CAT), Gly^{377} (CPT II), and Gly^{348} (CAT) are indicated. Molecules of malonyl-CoA (inhibitor), and of decanoyl-CoA (COT), palmitoyl-CoA (CPT I and CPT II), and acetyl-CoA (CAT) as substrates, are represented as *sticks*. The position of the structurally equivalent sites to those in CPT I and COT that were proposed for malonyl-CoA binding are indicated by *green ellipses*.

acyl-CoA and the inhibitor, since when the concentration of decanoyl-CoA increased from 5 to 50 or 200 μM, the curves are similar, but the IC_{50} values rose from 3.3 to 76 or 581 μM. We then expressed the COT H131A mutant and compared the kinetics of malonyl-CoA inhibition of this mutant with that of the double mutant H131A/H340A (Fig. 2*B*). The result of these comparisons shows the particular responsibility of COT His^{340} in the inhibition. Whereas the double mutant is not inhibited at any malonyl-CoA concentration (1–200 μM), the inhibition of

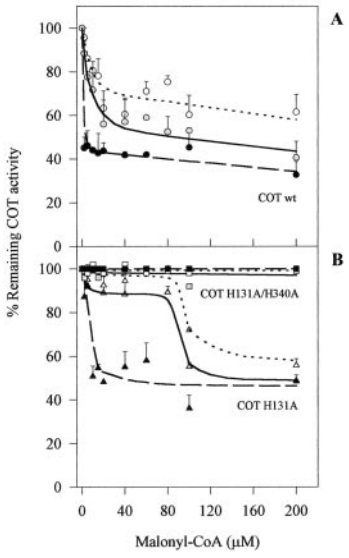

FIG. 2. **Effect of decanoyl-CoA, in the presence of increasing concentrations of malonyl-CoA on the activity of expressed COT wild type and COT mutants H131A and H131A/H340A.** 2 μg of extracts from *S. cerevisiae* expressing COT wild type (*circles*), *panel A*, and mutants H131A (*triangles*) and H131A/H340A (*squares*), *panel B*, after 20 h of galactose induction were assayed for COT activity in the presence of increasing concentrations of malonyl-CoA (1–200 μM), and at different concentrations of decanoyl-CoA as substrate, 5 μM (*dashed lines, black symbols*), 50 μM (*solid lines, gray symbols*) and 200 μM (*dotted lines, white symbols*). The mean data relative to control values in the absence of malonyl-CoA (100%) from three curves obtained from separates yeast expressions are shown.

mutant COT H131A was dependent on the concentration of the substrate. A low decanoyl-CoA concentration (5 μM) gives low IC_{50} values for malonyl-CoA (36 μM). When the substrate concentration is increased to 50 μM or 200 μM the IC_{50} values increase in a sigmoid kinetic, so that the IC_{50} values were not calculated. These results implicate His^{340} in malonyl-CoA inhibition, which was competitive between substrate and inhibitor. They are also consistent with the COT structural model, in which decanoyl-CoA and malonyl-CoA exclude each other.

To confirm the model presented in Fig. 1, we mutated another amino acid, in the COT malonyl-CoA domain, to see whether the sensitivity to the inhibitor was modified. COT Ala^{332} is 8 amino acids away from His^{340} and therefore could participate in the inhibitory process. Two considerations supported this choice. On the one hand, Ala^{332} is the first amino acid after the acidic residue (aspartic or glutamic) common to carnitine octanoyl- and palmitoyltransferases (Fig. 3, *right*). On the other hand, as shown by the tree-determinant analysis of the multiple alignment of the acyltransferase family, alanines at this position are present in all malonyl-CoA inhibitable carnitine acyltransferases (COT and L-CPT I) but absent in non-malonyl-CoA inhibitable carnitine acyltransferases (CPT II and CAT), where they are substituted by glycine. Therefore, we prepared the mutant COT A332G and expressed it in *S. cerevisiae*. The mutated protein had the same molecular mass as the wild type and was expressed at the same levels (Fig. 4). Moreover, the kinetics toward both carnitine and decanoyl-CoA, were similar to wild type, with normal saturation behavior (Table I). The mutant was measured for malonyl-CoA sensitivity and the inhibition results were compared with those of wild type (Fig. 4). At the highest concentration of malonyl-CoA assayed (200 μM), 80% COT activity still remained, while the remaining COT wild type activity was 40%. The IC_{50} increased from 76 μM (wild type) to 492 μM (A332G). This result strongly supports the role of this alanine, which starts a domain from Ala^{332} to at least His^{340} in COT, in the positioning of malonyl-CoA in the model.

Positioning of Malonyl-CoA in the Structural Model of L-CPT I—As indicated for the malonyl-CoA-COT docking model, the best CPT I solutions in terms of low energy of the complex and lowest macromolecular distance were analyzed. The results obtained for the best 10 solutions indicates a common location of the inhibitor molecule in the equivalent site to that described by the COT calculations (Fig. 1*C*). Again, the simultaneous representation of both the substrate (palmitoyl-CoA) and the inhibitor (malonyl-CoA) molecules on the surface of CPT I proposes a model for the competition of both molecules for the same locus but suggesting two different binding sites (Fig. 1*D*).

Analysis of the Amino Acids in the Catalytic Core Potentially Involved in the Malonyl-CoA Inhibition of L-CPT I—We used the same strategy to determine the responsibility of the domain in the malonyl-CoA inhibition of L-CPT I. First, as a control, plasmid $pYESLCPTI^{wt}$ (wild type rat mitochondrial L-CPT I) was transformed in *S. cerevisiae*, an organism devoid of this activity. We obtained an L-CPT I protein with higher activity than that in rat liver mitochondria (17 *versus* 3.5 nmol min^{-1} mg^{-1} protein, respectively). The malonyl-CoA inhibition assay showed a decrease in the activity similar to that observed in rat liver mitochondria (IC_{50} of 12.3 *versus* 6.4 μM) (Fig. 5) indicating that *S. cerevisiae* is a suitable organism in which to study malonyl-CoA inhibition of CPT I.

To perform the inhibitory experiments, we first selected Ala^{478}, which is homologous to COT Ala^{332}, and was also mutated to glycine. The mutant L-CPT I A478G was expressed in *S. cerevisiae* and, as seen in COT A332G, the kinetic constants of the expressed protein were similar to that observed for L-CPT I wild type (Table I). Extracts were assayed for malonyl-CoA inhibition (Fig. 5) and the IC_{50} of the mutant was increased from 12.3 μM (wild type) to 39.5 μM. The residual activity at 100 μM malonyl-CoA in the mutated L-CPT I was 32% whereas in wild type it was 11%. These results show that Ala^{478} is involved in L-CPT I malonyl-CoA sensitivity.

We mutated, first independently and then together, L-CPT I His^{277} and His^{483}, homologous to those previously studied (His^{131} and His^{340}) in COT (Fig. 3). Although L-CPT I His^{277} lies outside the structural model (31) we decided to mutate it, to examine the effect of both histidines on malonyl-CoA inhibition.

To assess whether malonyl-CoA inhibition of L-CPT I depends on the protein concentration in the yeast mitochondrial membranes, as observed at contact sites and in the outer membranes of rat mitochondria (57), we tested the malonyl-CoA inhibition in the L-CPT I wild type and the double mutant H277A/H483A at several times of induction by galactose (Fig. 6). When the induction time increases, which reflects the amount of L-CPT I in the mitochondria, malonyl-CoA appears to inhibit the doubly mutated enzyme similarly to the wild type. However, when the amount of induced L-CPT I is small, malonyl-CoA hardly inhibits the double mutant. Analogous experiments were carried out with the mutant L-CPT I A478G (data not shown), and no change in the malonyl-CoA sensitivity was detected at a range of induction times. Therefore, this anomalous behavior of expressed protein as a function of the enzyme concentration is only observed in histidine mutants.

The kinetics of inhibition by malonyl-CoA were measured at different concentrations of the inhibitor (1–100 μM), with the wild type, the single mutants H277A and H483A, and with the double mutant H277A/H483A at times of induction of 1 and 20 h. After 20 h of induction by galactose, the single or double mutant L-CPT I was not affected (Fig. 7). However, at 1 h of induction the expressed double mutant L-CPT I H277A/H483A

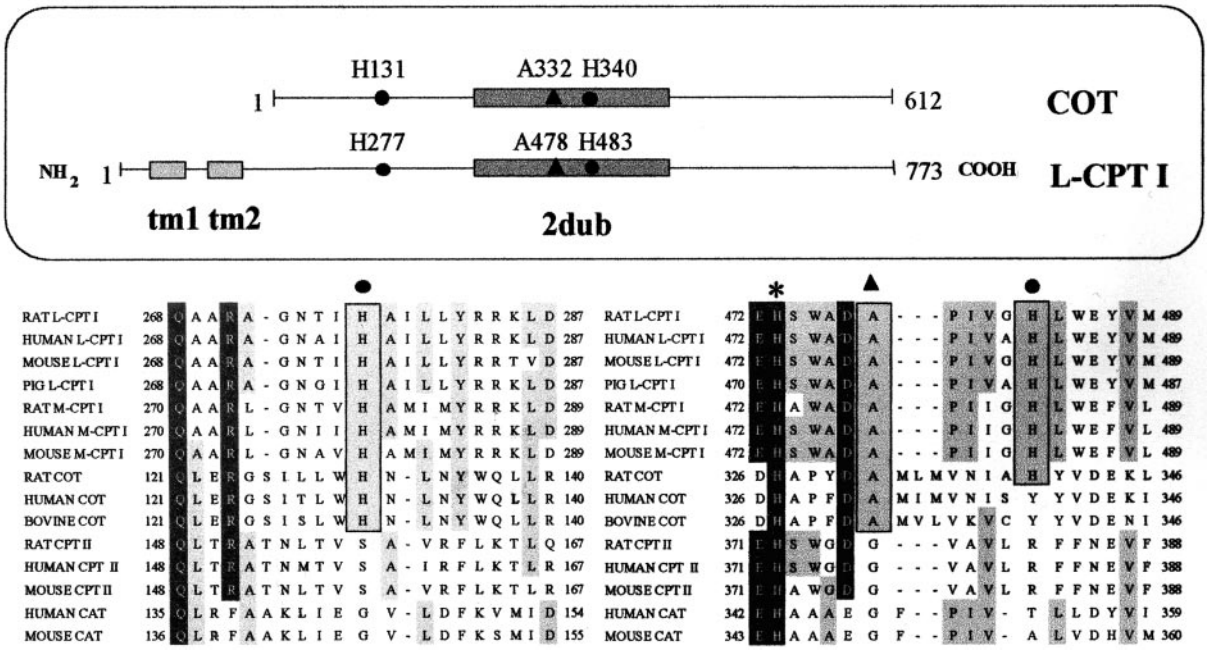

FIG. 3. **Alignment of 15 mammalian carnitine acyltransferases.** Sequences were obtained from the GenBank™ sequence data bank: rat L-CPT I (L07736), human L-CPT I (L39211), mouse L-CPT I (AF017175), pig L-CPT I (AF288789), rat M-CPT I (D43623), human M-CPT I (D87812), mouse M-CPT I (NM009948), rat COT (U26033), human COT (AF168793), bovine COT (U65745), rat CPT II (J05470), human CPT II (U09648), mouse CPT II (NM009949), human CAT (X78706), and mouse CAT (NM007760). Alignment of sequences was obtained with Pileup and Pretty programs from Wisconsin Package version 9.1, Genetics Computer Group (GCG), Madison WI. Amino acids with an identity higher than 80% are shown in *black*. Amino acids with an identity comprised between 50 and 80% are *shadowed*. The amino acids conserved in the malonyl-CoA-sensitive enzymes are framed and the *asterisk* indicates the conserved catalytic histidine in all carnitine acyltransferases. At the *top*, a schematic representation of the rat COT and L-CPT I proteins. The amino acids conserved in the carnitine acyltransferases sensitive to malonyl-CoA are marked: COT Ala^{332} (▲), His^{131} (●), and His^{340} (●), and L-CPT I Ala^{478} (▲), His^{277} (●) and His^{483} (●). Transmembrane domains of L-CPT I, tm1 and tm2, are also represented. *2dub* means the homologous domain of the catalytic core of carnitine acyltransferases to enoyl-CoA hydratase (31).

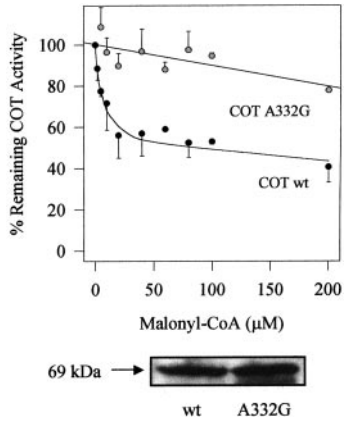

FIG. 4. **Effect of malonyl-CoA on the activity of the expressed wild type and mutant A332G of rat COT.** 2 μg of extracts from yeast expressing COT wild type (●) and mutant A332G (◎) obtained after 20 h of galactose induction were assayed for COT activity in the presence of increasing concentrations of malonyl-CoA (1–200 μM). The mean data relative to control values in the absence of malonyl-CoA (100%) from three curves obtained from separated yeast expressions are shown. At the *bottom*, a representative immunoblot of recombinant COT variants is shown. Samples (COT wild type and COT A332G) were prepared and analyzed as described under "Experimental Procedures." 10 μg of protein were separated by SDS-PAGE and subjected to immunoblotting using specific antibodies for COT. The *arrow* indicates the migration position and the molecular mass of rat liver COT.

was hardly inhibited by malonyl-CoA in the range 1–100 μM (IC_{50} higher than 800 μM). Fig. 7 also shows the pattern of inhibition by malonyl-CoA of the individual expressed mutants L-CPT I H277A and H483A. Mutant H483A (which leaves His^{277} intact) increases the IC_{50} from 9.5 μM (wild type) to 23.1 μM, a small increase showing the role of His^{277} in malonyl-CoA inhibition, probably because it is the high affinity allosteric

TABLE I

Kinetic parameters of carnitine acyltransferase activity in yeast strains expressing wild types and mutants A478G of L-CPT I and A332G of COT

Extracts from yeast expressing wild type and alanine mutations of L-CPT I and COT were assayed for activity and kinetics as described under "Experimental Procedures." The acyl-CoA substrate was palmitoyl-CoA for L-CPT I and decanoyl-CoA for COT. The results are the mean ± S.D. of at least three independent experiments with different preparations.

	Activity	IC_{50} malonyl-CoA	K_m carnitine	K_m decanoyl-CoA
	(nmol/min/mg)	*(μM)*		
COT				
Wild-type	226 ± 9	76	172 ± 46	2.0 ± 0.2
A332G	361 ± 12.8	492	106 ± 2	10.7 ± 2.7
L-CPTI				
Wild-type	17.7 ± 0.9	12	127 ± 4	4.9 ± 0.3
A478G	16.7 ± 7	39	327 ± 41	15.1 ± 4

site. Mutant H277A (which leaves His^{483} intact) produced an enzyme that was less sensitive to malonyl-CoA (IC_{50} of 186 μM). An immunoblot analysis of the L-CPT I-expressed mutants and wild type at 20 and 1 h of induction shows similar expression (Fig. 7).

The dependence of L-CPT I, on enzyme concentration is not associated with marked changes in the kinetic constants (Table II). K_m values for carnitine ranged from 84 μM (wild type) to 179 μM (double mutant). K_m values for palmitoyl-CoA ranged from 5 μM (wild type) to 14.8 μM (double mutant). The induction of the expression for 1 h of the different forms of L-CPT I was different from that observed at 20 h of induction: the K_m values ranged from 121 to 250 μM for carnitine and from 11 to 31 μM for palmitoyl-CoA (Table II). For the purposes of this study, these changes in the K_m of the mutated enzymes can be con-

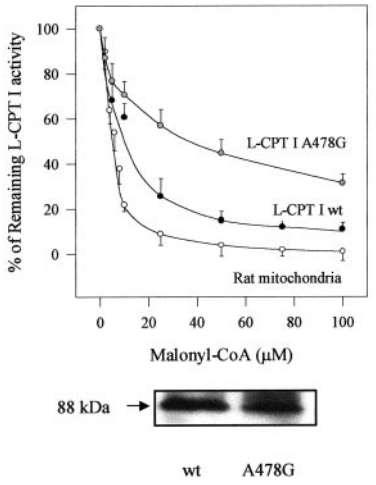

FIG. 5. **Effect of malonyl-CoA on the activity of CPT I of rat liver mitochondria and the expressed wild type and mutant A478G of L-CPT I.** 8 μg of rat liver mitochondria (○), and 10 μg of mitochondria from yeast expressing L-CPT I wild type (●) and mutant A478G (◎) obtained after 20 h of galactose induction were assayed for CPT I activity in the presence of increasing concentrations of malonyl-CoA (1–100 μM). The mean data relative to control values in the absence of malonyl-CoA (100%) from three curves obtained from separated yeast expressions are shown. At the *bottom*, a representative immunoblot of recombinant L-CPT I variants is shown. Samples (L-CPT I wild type and L-CPT I A478G) were prepared and analyzed as described under "Experimental Procedures." 100 μg of protein was separated by SDS-PAGE and subjected to immunoblotting using specific antibodies for L-CPT I. The *arrow* indicates the migration position and the molecular mass of rat liver L-CPT I.

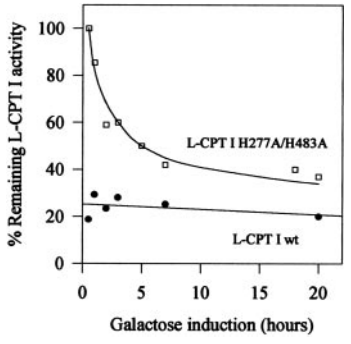

FIG. 6. **Effect of malonyl-CoA on the activity of the expressed L-CPT I wild type and H277A/H483A mutant in function of the hours of galactose induction.** *S. cerevisiae* transformed cells with plasmids pYESCPTI^{wt} (●) and pYESCPTI$^{H277A/H483A}$ (□) were induced for expression by galactose at several times (30 min to 20 h) as described under "Experimental Procedures." 10 μg of mitochondrial preparations was incubated at a concentration of 50 μM malonyl-CoA, the residual L-CPT I activity was measured and represented as percentage of activity without malonyl-CoA incubation.

sidered of secondary importance.

Structural Model of CPT II and CAT—To offer a possible explanation (at least "*in silico*") for the absence of inhibition by malonyl-CoA in the closely related enzymes CPT II and CAT, we constructed a structural model of the catalytic core of both molecules with the same methods as those used for CPT I and COT (31). The central domains of both molecules (residues 278–464, rat CPT II; residues 239–431, mouse CAT), as well as the equivalent positions of members of the other subfamilies were used to perform an extensive fold search using threading procedures. The search identified a clear candidate for all the sequences used: the enoyl-CoA hydratase fold (PDB code 2dub, chain E). Molecular models of the core of CPT II and CAT showing the putative binding sites of their respective acyl-CoA substrates (palmitoyl-CoA and acetyl-CoA) were obtained (Fig.

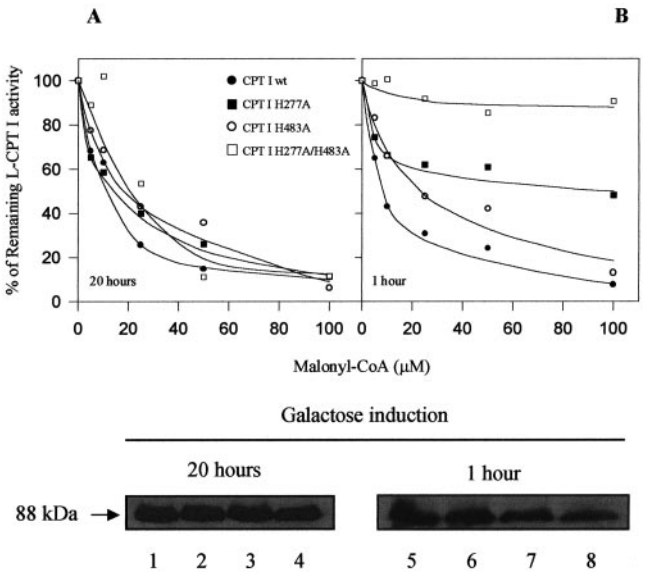

FIG. 7. **Effect of malonyl-CoA on the activity of wild type and mutants H277A, H483A, and H277A/H483A of rat L-CPT I.** *A*, 10 μg of mitochondria from *S. cerevisiae* expressing L-CPT I wild type (●) and H277A (■), H483A (○), and H277A/H483A (□) mutants after 20 h of galactose induction were assayed for L-CPT I activity in the presence of increasing concentrations of malonyl-CoA (1–100 μM). *B*, same as *A* after 1 h of galactose induction. The mean data relative to control values in the absence of malonyl-CoA (100%) from three to four curves obtained from separate yeast expressions are shown. At the *bottom*, representative immunoblots of recombinant L-CPT I variants are shown. *Left*, 20 h, and *right*, 1 h, of galactose induction. The time of the contact of the film was 10 s and 5 min, respectively. Samples of L-CPT I wild type (*lanes 1* and 5), and mutants H277A (*lanes 2* and 6), H483A (*lanes 3* and 7), and H277A/H483A (*lanes 4* and 8) were prepared and analyzed as described under "Experimental Procedures." 100 μg of transformed *S. cerevisiae* mitochondria was separated by SDS-PAGE and subjected to immunoblotting by using specific antibodies for rat L-CPT I. The *arrow* indicates the migration position and the molecular mass of rat mitochondrial L-CPT I.

1, *E* and *F*). The similarity to COT (Fig. 1, *A* and *B*) and CPT I (Fig. 1, *C* and *D*) in terms of shape and substrate-binding sites is not surprising because the four carnitine acyltransferases contain a high proportion of identical amino acid residues.

The same docking calculations for malonyl-CoA performed for CPT I and COT were then done for these two new molecular models. As expected, no similar solutions to that obtained for CPT I or COT was provided by the new models. In addition, no clear accumulation of equivalent docking models was observed among the first structures resulting from the calculations (not shown), indicating that there is no obvious model to propose for the fitting of the malonyl-CoA molecule to the structural models of CPT II or CAT.

The analysis of the sites in the models for CPT II and CAT that are structurally equivalent to those in CPT I and COT (Fig. 1, *E* and *F, green elipses*), indicates that in the former, the lateral chain of a group of residues surrounding positions 382–384 (CPT II) or 352–354 (CAT) occupy the putative inhibitor groove, so that the positioning of malonyl-CoA is prevented. The structural and sequential proximity of these groups of amino acids to residues Gly^{377} (CPT II) or Gly^{348} (CAT) seems to conform a structural cluster implicated in the non-inhibition properties of both enzymes.

DISCUSSION

The structural model of the catalytic core of COT and CPT I, deduced from a threading analysis and confirmed by site-directed mutagenesis and natural human mutants (31), allowed us to locate one of the two histidines involved in malonyl-CoA sensitivity, His^{340} of COT, and the homologous His^{483} of CPT I

TABLE II

Kinetic parameters of carnitine palmitoyltransferase activity in yeast strains expressing wild type and mutants H277A, H483A, and H277A/H483A of L-CPT I

Mitochondria from yeast expressing wild type and histidine mutations of L-CPT I were assayed for activity and kinetics as described under "Experimental Procedures." A, mitochondria were obtained after 20 h of galactose induction of transformed *S. cerevisiae*; *B*, same as *A*, but after 1 h of galactose induction. The results are the mean ± S.D. of at least three independent experiments with different preparations.

L-CPTI	Activity	IC_{50} malonyl-CoA	K_m carnitine	K_m palmitoyl-CoA
	nmol/min/mg	(μM)		
A. 20 h of galactose induction				
Wild-type	17.7 ± 0.9	12.3	127 ± 4	4.9 ± 0.3
H277A	21.4 ± 5.9	13.5	137 ± 22	12.6 ± 5
H483A	18.4 ± 3.7	19.5	84 ± 7.9	9.8 ± 0.7
H277A/H483A	13.7 ± 2.3	20.4	179 ± 20	14.8 ± 2.3
B. 1 h of galactose induction				
Wild-type	1.10 ± 0.32	10	150 ± 33	31 ± 7
H277A	0.96 ± 0.12	186	121 ± 19	30.9 ± 11
H483A	1.17 ± 0.27	23	251 ± 28	11.1 ± 3.8
H277A/H483A	0.76 ± 0.10	> 800	172 ± 22	35 ± 9

in the model. COT His^{131}, and its homologous His^{277} of L-CPT I, could not be located in the model, which comprises amino acids 226–417 of COT and 368–567 of CPT I, obtained using the molecule backbone of enoyl-CoA hydratase (2dub, chain E) as template. When we looked at the amino acids that were in the proximity of COT His^{340} and that could participate in the binding to malonyl-CoA, we focused on Ala^{332}, which is common to all carnitine acyltransferases inhibitable by malonyl-CoA, and which is not present in any of carnitine acyltransferases not inhibitable by malonyl-CoA (Fig. 3).

Thereafter, we applied the *in silico* macromolecular docking program Hex (51) and found that malonyl-CoA fits well into a domain near the catalytic site on the structural model, not only in COT but also in CPT I. As expected, amino acids previously deduced to be related with the interaction with malonyl-CoA are located in the pocket for the inhibitor. We hypothesized that mutagenesis of these selected amino acids would not produce dramatic changes in the IC_{50} values for malonyl-CoA, since it had often been reported that this domain, near or within the catalytic site was the low affinity site, which other CoA derivatives, such as free CoA itself, acetyl-CoA, succinyl-CoA, and hydroxymethylglutaryl-CoA produced a slight inhibition of COT and L-CPT I without affecting the functionality of the high affinity site.

The COT double mutant H131A/H340A did not allow us to measure the contribution of each domain to the competition between substrate and inhibitor, as it is totally insensitive to malonyl-CoA. However, mutant COT H131A, which probably affects the high affinity site, reflected the inhibitory kinetics of the domain containing COT His^{340}. The response of this mutant to varying concentrations of the substrate decanoyl-CoA showed sigmoidal kinetics, which suggested that malonyl-CoA inhibition was competitive *versus* the substrate. The models in Fig. 1, show a steric hindrance, since there is no room for both decanoyl-CoA and malonyl-CoA. At least four methylene carbons of decanoyl-CoA and the carboxylic group of malonyl-CoA compete for the same space. The kinetics of the His^{131} mutant (which maintains intact His^{340}) clearly show that decanoyl-CoA is more accessible than malonyl-CoA, in agreement with the suggestion that this malonyl-CoA site (containing His^{340}) near the catalytic channel is the low affinity site (16, 19, 20), where several acyl-CoA molecules may also bind. So malonyl-CoA could inhibit decanoyl-CoA by binding to this domain, which is close but not coincident with the catalytic site. All these data are consistent with the malonyl-CoA low-affinity site previously described (16).

The mutation of COT Ala^{332}, which affects the domain presented in Fig. 1, shows a high loss of malonyl-CoA sensitivity (the IC_{50} value increases to 492 μM), despite the minimal change in volume and charge entailed by the mutation of an alanine to glycine. This residue might be important since it is conserved in all malonyl-CoA-sensitive carnitine acyltransferases and not present in non-malonyl-CoA-sensitive carnitine acyltransferases. These findings strongly suggest that the domain involving COT amino acids from Ala^{332} to His^{340} is the binding site for malonyl-CoA, as indicated by the model.

The same reasoning was applied to rat L-CPT I. As in the model of COT, malonyl-CoA fits well in a cavity of the CPT I structural model near the catalytic site (Fig. 1*C*). It can be deduced that there is no room for both palmitoyl-CoA and malonyl-CoA, one preventing the location of the other.

Four L-CPT I different mutants were constructed to examine their response to malonyl-CoA, A478G, H277A, H483A, and double mutant H277A/H483A. Mutation of L-CPT I Ala^{478}, which is homologous to COT Ala^{332}, and which is five amino acids away from the L-CPT I His^{473} essential for the catalysis, modified the IC_{50} for malonyl-CoA, without modifying the catalytic activity. This showed that Ala^{478} could participate in the binding of malonyl-CoA, as was observed in COT.

It was surprising that the double mutant H277A/H483A showed different sensitivity to malonyl-CoA depending on the time of galactose induction. It appears that both His^{277} and His^{483} are essential for malonyl-CoA inhibition but not for catalysis, since the kinetic properties of the mutant enzyme are practically indistinguishable from those of the wild type. Their role is only evident when the concentration of the enzyme is low. The results of inhibition by malonyl-CoA indicate that His^{277} is probably located in the malonyl-CoA high affinity site and that His^{483} may participate in the malonyl-CoA affinity site deduced from the structural model (Fig. 1, *C* and *D*). It seems that His^{483}, near the catalytic His^{473}, is less important than His^{277}, which could be the putative allosteric site of inhibition by malonyl-CoA.

The influence of the concentration of single and double histidine mutants of L-CPT I in the yeast mitochondrial membranes on their sensitivity to malonyl-CoA is intriguing, since neither expressed wild type nor mutant A478G shows such dependence. A comparable effect was observed in L-CPT I in its natural environment, rat mitochondria (57). The differences in environments, in outer membranes (where L-CPT I is at low levels) and contact sites (where L-CPT I is enriched) resulted in altered conformation of L-CPT I, which specifically affected the malonyl-CoA sensitivity. Malonyl-CoA inhibition of L-CPT I is extremely sensitive to the physiological and nutritional state, which in turn is correlated with changes in the composition of the membrane (1). High concentrations of the enzyme may

alter the conformation of its cytosolic catalytic COOH-terminal domain. This might modulate the extent to which these histidine residues contribute to the proclivity of rat L-CPT I to be inhibited by malonyl-CoA.

Pro^{479}, whose mutation to Leu not only causes a human L-CPT I deficiency but which also alter sensitivity toward malonyl-CoA inhibition, with a residual activity of 47% at 100 μM malonyl-CoA (58), was located close to His^{483} in the model. This is the only natural mutant reported to affect malonyl-CoA sensitivity, without modifying the catalytic activity dramatically. Amino acids comprised between Ala^{478} and His^{483} seem to conform a malonyl-CoA coupling domain in L-CPT I.

In a previous study (31) we mutated COT Ala^{238} and L-CPT I Ala^{381}, which are far from the catalytic histidines (COT His^{327} and CPT I His^{473}) in the primary structure of the proteins, but close to them in the structural model. The mutation caused a decrease in catalytic activity of 80–85%. However, malonyl-CoA sensitivity was not affected. This shows that the catalytic site was not the low-affinity malonyl-CoA site. In contrast, the mutations performed in the present study had no effect on the catalytic activity, but the sensitivity to malonyl-CoA decreased. This corroborates the hypothesis that the domain, although close to the catalytic channel, is a different site (16).

CPT II and CAT are not inhibited by malonyl-CoA (10). The model proposed by our group identifies a domain in COT and CPT I that can bind malonyl-CoA with low free energy. However, the homologous domain in CPT II and CAT cannot bind malonyl-CoA, as deduced from the results of the docking program, which shows that such a location would entail so much free energy that binding would be impossible.

Identification of residues responsible for the inhibition of carnitine acyltransferases by malonyl-CoA is an important step in elucidating the key to the control of β-oxidation. These results open the way to study whether specific mutations in either carnitine acyltransferases alter the metabolism of fatty acids. Understanding of malonyl-CoA interaction sites in CPT I is important in the design of drugs to control an excess of fatty acid oxidation in the pancreatic β-cell, and also to prevent the development of diabetes mellitus (59–62). Such a study is also important in heart, where myocardial ischemia has been associated with accumulation of long chain acyl-CoA (63, 64).

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