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Identification of Conserved Amino Acid Residues in Rat Liver Carnitine Palmitoyltransferase I Critical for Malonyl-CoA Inhibition

MUTATION OF METHIONINE 593 ABOLISHES MALONYL-CoA INHIBITION*

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Carnitine palmitoyltransferase (CPT) I, which catalyzes the conversion of palmitoyl-CoA to palmitoylcarnitine facilitating its transport through the mitochondrial membranes, is inhibited by malonyl-CoA. By using the SequenceSpace algorithm program to identify amino acids that participate in malonyl-CoA inhibition in all carnitine acyltransferases, we found 5 conserved amino acids (Thr^{314}, Asn^{464}, Ala^{478}, Met^{593}, and Cys^{608}, rat liver CPT I coordinates) common to inhibitable malonyl-CoA acyltransferases (carnitine octanoyltransferase and CPT I), and absent in noninhibitable malonyl-CoA acyltransferases (CPT II, carnitine acetyltransferase (CAT) and choline acetyltransferase (ChAT)). To determine the role of these amino acid residues in malonyl-CoA inhibition, we prepared the quintuple mutant CPT I T314S/N464D/A478G/M593S/C608A as well as five single mutants CPT I T314S, N464D, A478G, M593S, and C608A. In each case the CPT I amino acid selected was mutated to that present in the same homologous position in CPT II, CAT, and ChAT. Because mutant M593S nearly abolished the sensitivity to malonyl-CoA, two other Met^{593} mutants were prepared: M593A and M593E. The catalytic efficiency (V_{max}/K_m) of CPT I in mutants A478G and C608A and all Met^{593} mutants toward carnitine as substrate was clearly increased. In those CPT I proteins in which Met^{593} had been mutated, the malonyl-CoA sensitivity was nearly abolished. Mutations in Ala^{478}, Cys^{608}, and Thr^{314} to their homologous amino acid residues in CPT II, CAT, and ChAT caused various decreases in malonyl-CoA sensitivity. Ala^{478} is located in the structural model of CPT I near the catalytic site and participates in the binding of malonyl-CoA in the low affinity site (Morillas, M., Gómez-Puertas, P., Rubí, B., Clotet, J., Ariño, J., Valencia, A., Hegardt, F. G., Serra, D., and Asins, G. (2002) *J. Biol. Chem.* 277, 11473–11480). Met^{593} may participate in the interaction of malonyl-CoA in the second affinity site, whose location has not been reported.

The enzyme carnitine palmitoyltransferase (CPT)[1] I catalyzes the conversion of long chain fatty acyl-CoAs to acylcarnitines, which is the first step in the transport of fatty acyl-CoA groups from the cytosol to mitochondria where they undergo β-oxidation. This reaction is inhibited by malonyl-CoA, and so this enzyme could be the most physiologically important regulatory step in mitochondrial fatty acid oxidation (1). This process allows the cell to signal the relative availability of lipid and carbohydrate fuels in liver, heart, skeletal muscle, and pancreatic β-cell (2). The mechanism of malonyl-CoA inhibition can be potentially mimicked by pharmacological malonyl-CoA-related agents for the treatment of metabolic disorders such as diabetes, insulin resistance, and coronary heart disease (3).

Mammals express two isoforms of CPT I, a liver isoform (L-CPT I) and a heart/skeletal muscle isoform (M-CPT I), which are the products of two different genes (4, 5). The identity in amino acids residues is high (62%) but they are differentially regulated by malonyl-CoA. The L-CPT I isoform is inhibited by malonyl-CoA to a much lesser extent than the M-CPT I isoform (the IC_{50} value for M-CPT I is about 2 orders of magnitude lower than for L-CPT I) (6). This property is probably involved in the finer regulation of fatty acid oxidation in heart and skeletal muscle in comparison to liver.

From studies on the pH dependence of the affinity of CPT I for its substrate and from the ability of palmitoyl-CoA to displace [^{14}C]malonyl-CoA bound to skeletal muscle mitochondria it was hypothesized (7) that the palmitoyl-CoA and malonyl-CoA bind at different sites. A number of studies have shown that in rat liver CPT I there are two malonyl-CoA binding sites: one with greater capacity for binding and regulation of the inhibitor and not susceptible to competition from acyl-CoA, which behaves as an allosteric component (8–12); and a second acyl-CoA binding site, which is located near the catalytic site (13).

Various groups have attempted to establish the basis of the L-CPT I/malonyl-CoA interactions. The probable binding sites of malonyl-CoA in L-CPT I were deduced to be at the C terminus after preparation of several L-CPT I chimeras whose IC_{50} values for malonyl-CoA corresponded to the C-terminal region (14) of the chimera. However, the N terminus of L-CPT I was also shown to influence the enzyme/inhibitor interaction. Mutation of Glu^3, His^5, or His^{140} produced a loss of malonyl-CoA

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

sensitivity (15, 16). In addition, the removal of the segment comprised between amino acids 1 and 18 in L-CPT I and 1–28 in M-CPT I produced a decrease in malonyl-CoA sensitivity, which emphasizes the importance of the N terminus before the first transmembrane region as a modulator of the malonyl-CoA inhibition (17, 18). On the basis of these results, it was proposed that the two malonyl-CoA inhibitable domains might be located at the C terminus as suggested by several kinetic studies. The development of a CPT I catalytic core model (19) allowed us to assign the low affinity binding site to a domain near the catalytic channel in which palmitoyl-CoA is bound containing the catalytic acyl-CoA binding domain (20)

Here we used the SequenceSpace algorithm program to identify five amino acid residues (Thr^{314}, Asn^{464}, Ala^{478}, Met^{593}, and Cys^{608}), which may contribute to the sensitivity of CPT I to malonyl-CoA. The proposal is based on the finding that they are present in malonyl-CoA-inhibitable CPT I ((isoforms L- and M-) and COT from various organisms and absent in noninhibitable acyltransferases (CPT II, CAT, and ChAT). Mutation of these amino acids to their counterparts in CPT II showed that mutation of Met^{593} by itself, M593S, or the quintuple mutant containing the M593S mutation, T314S/N464D/A478G/M593S/C608A, or other Met^{593} point mutants such as M593A and M593E nearly abolished malonyl-CoA sensitivity of L-CPT I. The remaining mutated amino acids showed slight, varied sensitivity to malonyl-CoA inhibition.

EXPERIMENTAL PROCEDURES

Tree-determinants Analysis—Sequences of proteins from the carnitine-choline acyltransferase family were obtained using BLAST (21). Multiple alignment was performed using ClustalW (22). The analysis of conserved differences (tree-determinants) between malonyl-CoA-regulated (L-CPT I, M-CPT I, and COT) and nonregulated (CPT II, CAT, and ChAT) acyltransferases, using multivariate statistics for low-dimensional representation, was done using the SequenceSpace algorithm (23, 24). Graphics of vectors representing protein sequences and individual residues from the multiple alignment were performed using the Sequence Space Java-based viewer (www.industry.ebi.ac.uk/SeqSpace).

Construction of Site-directed Mutants—Plasmids $pYESLCPTI^{wt}$ and $pYESLCPT^{A478G}$ were obtained as previously described (20). Plasmids $pYESLCPT^{T314S}$, $pYESLCPT^{N464D}$, $pYESLCPT^{M593S}$, $pYESLCPT^{M593A}$, $pYESLCPT^{M593E}$, and $pYESLCPT^{C608A}$ were constructed using the QuikChange polymerase chain reaction-based mutagenesis procedure (Stratagene) with the $pYESLCPT^{wt}$ plasmid as template. The following primers were used: primer T314S.for 5′-GGGAGCGACTCTTCAATAG-TTCCCGGATCCCTGGG-3′, primer T314A.rev 5′-CCCAGGGATCCG-GGAA*C*TATTGAAGAGTCGCTCCC-3′, primer N464D.for 5′-CACCTT-TGTTGTCTTCAAAGACAGCAAGATAGGC-3′, primer N464D.rev 5′-GCCTATCTTGCTGTCTTTGAAGACACCAAAGGTG-3′, primer M593S.for 5′-CCTCACATATGAGGCCTCCAGTACCCGGCTCTTCCG AGAAGG-3′, primer M593S.rev 5′-CCTTCTCGGAAGAGCCGGGTAC-TGGAGGCCTCATATGTGAGG-3′, primer M593A.for 5′-CCTCAC-ATATGAGGCCTCCGCGACCCGGCTCTTCCGAGAAGG-3′, primer M593A.rev 5′-CCTTCTCGGAAGAGCCGGGTCGCGGAGGCCTCATA TGTGAGG-3′, primer M593E.for 5′-CCTCACATATGAGGCCTCCGA-GACCCGGCTCTTCCGAGAAGG-3′, primer M593E.rev 5′-CCTTCTC-GGAAGAGCCGGGTCTCGGAGGCCTCATATGTGAGG-3′, primer C608A.for 5′-GAGACTGTACGCTCCGCCACTATGGAGTCCTGC-3′, and C608A.rev 5′-GCAGGACTCCATAGTGGCGGAGCGTACAGTCT-C-3′ (the mutated nucleotides are underlined). The plasmid $pYESLCPTI^{T314S/N464D/A478G/M593S/C608A}$ was obtained by the same method, but performing each new mutation stepwise starting on plasmid $pYESLCPT^{T314S}$. 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 L-CPT I in Saccharomyces cerevisiae—The expression of the constructs containing L-CPT I wild type and mutants (see above) in yeast cells and the preparation of the cell extracts were performed as described in Ref. 19. *S. cerevisiae* was chosen as an expression system for L-CPT I wild type and the mutants because it does not have endogenous CPT I activity.

Determination of Carnitine Acyltransferase Activity—Carnitine palmitoyltransferase activity was determined by the radiometric method as described in Ref. 19 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 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 palmitoyl-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 Excel software. All protein concentrations were determined using the Bio-Rad protein assay with bovine albumin as standard.

Immunological Techniques—Western blot analysis was performed as described (19). 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 C-terminal domain.

RESULTS

Residues Conserved in Malonyl-CoA Inhibited Versus Noninhibited Carnitine-Choline Acyltransferases—An exhaustive analysis of the presence of residues shared by all the malonyl-CoA-regulated enzymes of the carnitine-choline acyltransferase family *versus* the malonyl-CoA nonregulated members of the same family was performed using the algorithm SequenceSpace (23, 24). This method uses a vectorial representation of each protein sequence as a point in a multidimensional space (SequenceSpace) and multivariate statistics, principal component analysis, to allow reduction of the number of dimensions. This representation allows us not only to define clusters of proteins according to specific properties by choosing the appropriate axes defined by the highest corresponding eigenvalues (also known as proper values), but also to project the individual residues on the same axes, and thus trace the positions conserved in the subfamilies defined. The main advantage of this method is the possibility of predicting which residues may be responsible for the specific characteristics of each protein subfamily or group of subfamilies as has been reported previously for short- and medium-long substrate specificity for the carnitine-choline acyltransferases protein family (19, 20) or effector recognition by some members of the Ras superfamily (25).

The two-dimensional projection of sequence vectors on the plane defined by the axes corresponding to eigenvalues 2 and 4 showed clustering of the enzyme subfamilies according to their malonyl-CoA inhibition properties (Fig. 1*A*). Proteins whose activity is not regulated by malonyl-CoA (CPT II, CAT, and ChAT subfamilies) were grouped, whereas the sequences of the proteins regulated by malonyl-CoA (COT, L-CPT I, and M-CPT I) occupy separate, and opposite, zones. The projection of the individual amino acid residues on the same plane (Fig. 1*B*) revealed the amino acids responsible for this segregation might be responsible for the susceptibility to malonyl-CoA of the corresponding enzymes. Five of these amino acids (Thr^{314}, Asn^{464}, Ala^{478}, Met^{593}, and Cys^{608}) were present in all malonyl-CoA inhibitable carnitine acyltransferases and absent in the nonmalonyl-CoA inhibitable acyltransferases (CPT II, CAT, and ChAT from several species). Fig. 2 shows the sequence alignment of three fragments of the C-terminal region of various acyltransferases. We can also observe that those enzymes that are not inhibitable by malonyl-CoA (CPT II, CAT, and ChAT) show the same amino acids in these positions, which are different from those observed in inhibitable malonyl-CoA acylcarnitines. As an example the positions and amino acids of CPT II are given: Ser^{223}, Asp^{363}, Gly^{377}, Ser^{490}, and Ala^{505} (Fig. 2).

Expression of Wild Type and Mutants in S. cerevisiae—We

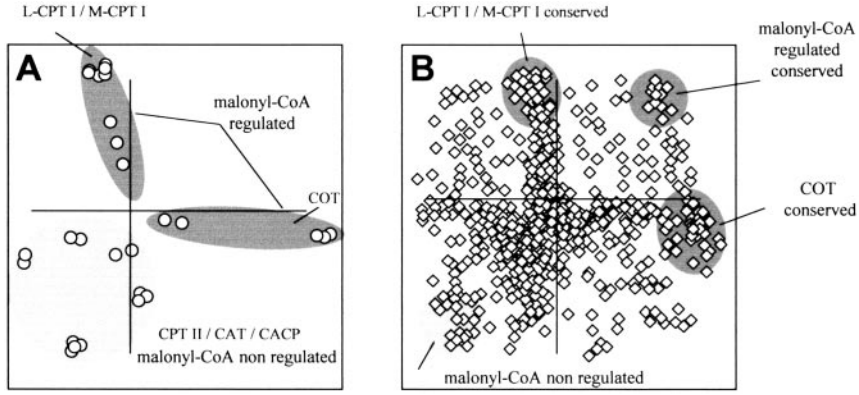

FIG. 1. **Sequence space analysis of the carnitine-choline acyltransferase family.** *A*, protein sequences projected onto the plane defined by principle axes 2 and 4. This two-dimensional space allows separation of protein subfamilies according to their malonyl-CoA regulatory characteristics; CPT II, CAT, and ChAT (*CACP*) enzymes (malonyl-CoA insensitive) are clustered to the *lower left corner* of the panel, whereas CPT I (L- and M-isoforms) and COT (malonyl-CoA inhibitable enzymes) are projected on the *upper* and *right* areas of the vertical and horizontal axes, respectively. *B*, the sequence of each subfamily is represented as a vector point in a multidimensional space (sequence space), with residue positions and types as the basic dimensions. Single residues completely conserved in CPT I or COT subfamilies are projected in the same position as their corresponding protein sequences. Residues conserved in both groups of malonyl-CoA-regulated enzymes occupy the *upper right corner*, whereas the residues conserved in the nonregulated cluster of acyltransferases (CPT II, CAT, and ChAT) occupy the *opposite* one. Residues located in alignment positions present in both opposite corners of the two-dimensional plot are responsible for protein cluster segregation and are predicted to be involved in malonyl-CoA sensitivity.

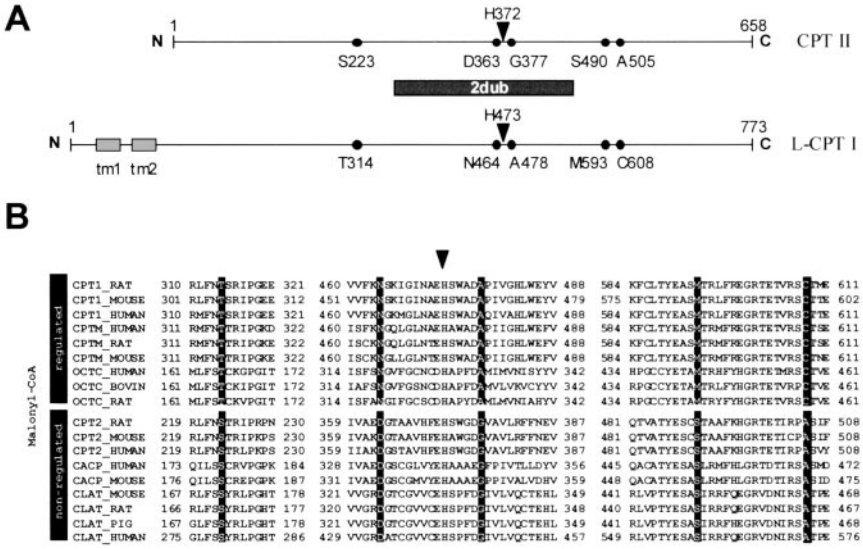

FIG. 2. **Alignment of representative sequences of mammalian carnitine-choline acyltransferases.** Amino acid sequence of 18 representative members of the malonyl-CoA-insensitive enzymes, CPT II (CPT2) from rat, mouse, and human; CAT (*CACP*) from human and mouse; ChAT (*CLAT*) from human, pig, rat, and mouse; and malonyl-CoA inhibitable enzymes L-CPT I (CPT1) from rat, mouse, and human; M-CPT I (CPTM) from human, rat, and mouse; and COT (*OCTC*) from human, rat, and bovine, were obtained from the SwissProt data bank and aligned using ClustalW (22). *A*, schematic representation of the position of the tree-determinant residues obtained using the SequenceSpace algorithm (23, 24) on the rat CPT II and L-CPTI proteins: Ser^{223}/Thr^{314}, Asp^{363}/Asn^{464}, Gly^{377}/Ala^{478}, Ser^{490}/Met^{593}, Ala^{505}/Cys^{608}. Transmembrane regions of L-CPT I are also represented (*tm1* and *tm2*). Position of the catalytic histidine (His^{372}/His^{473}) as well as the previously three-dimensional modeled core of the proteins, 2dub, (amino acids 368–567 of L-CPT I) (19, 20), are indicated. *B*, selected regions of the multiple alignment of the protein family. Subfamily conserved residues according to malonyl-CoA regulation are *shadowed*. Position of catalytic histidine (*arrowhead*) is also indicated.

prepared a quintuple mutant, T314S/N464D/A478G/M593S/C608A, and separately, the point mutants T314S, N464D, A478G, M593S, and C608A and all were expressed in *S. cerevisiae*. After we observed that mutant M593S nearly abolished the sensitivity to malonyl-CoA (see below), new point Met mutants were prepared: M593A and M593E. All transformed yeast cells expressed a protein with the same molecular mass (88 kDa) and the mutant enzymes were expressed in roughly the same proportion per milligram of protein as the wild type L-CPT I as deduced from immunoblot analysis (data not shown).

Kinetic Properties of CPT I Wild Type and Mutants—L-CPT I activities of the wild type, quintuple mutant variant T314S/N464D/A478G/M593S/C608S, and point mutants were similar (values ranged between 14 and 20 nmol min^{-1} mg $protein^{-1}$) when the protein was overexpressed 20 h after galactose induction, showing that the various mutations assayed produce small changes in L-CPT I activity (Table I).

All mutants exhibited standard saturation kinetics when the carnitine concentration was varied relative to a constant concentration of the second substrate, palmitoyl-CoA, and when palmitoyl-CoA concentration was varied relative to a constant carnitine concentration, a property identical to that of the wild type L-CPT I (Fig. 3). The quintuple mutant produced small changes in the kinetic constants for carnitine and palmitoyl-CoA as substrates (Table I). Catalytic efficiency (V_{max}/K_m) was increased by a factor of 2.6 (carnitine) and 2.2 (palmitoyl-CoA). The catalytic efficiency for carnitine as substrate of those point

TABLE I

Enzyme activity, malonyl-CoA sensitivity and kinetic parameters of carnitine palmitoyltransferase I in Saccharomyces cerevisiac cells expressing CPI I wild type and point mutants, T314S, N464D, A478G, C608A, M593S, M593A, M593E and quintuple mutant T314S/N464D/A478G/M593S/C608A (QM)

Extracts from yeast expressing wild type and several mutants of L-CPT I were assayed for activity, malonyl-CoA sensitivity, and kinetics as described under "Experimental Procedures." The results are the mean ± S.D. of at least three independent experiments with different preparations. In parentheses are shown the increase (in-fold number) of the catalytic efficiency (V_{max}/K_m) versus to that of the wild type.

L-CPT I	Activity	IC_{50} malonyl-CoA	K_m		V_{max}		Catalytic efficiency	
			Carnitine	Palmitoyl-CoA	Carnitine	Palmitoyl-CoA	Carnitine	Palmitoyl-CoA
	nmol min^{-1} mg protein^{-1}		*μm*		*nmol min^{-1} mg protein^{-1}*		V_{max}/K_m	
Wild-type	17.7 ± 0.9	12.3	127 ± 4.5	4.9 ± 0.3	6.6 ± 0.8	6.3 ± 0.4	0.05 (× 1)	1.28 (× 1)
T314S	14.4 ± 2.1	15.0	88.2 ± 2.4	1.7 ± 0.5	12.8 ± 0.1	6.8 ± 0.1	0.15 (× 2.8)	3.98 (× 3.1)
N464D	20.1 ± 3.1	8.7	69.5 ± 8.2	4.1 ± 0.4	19.4 ± 1.4	18.9 ± 3.6	0.28 (× 5.6)	4.63 (× 3.6)
A478G	16.7 ± 0.7	39.5	327 ± 41	15.1 ± 4.0	69.8 ± 9.3	50.4 ± 17	0.21 (× 4.1)	3.34 (× 2.6)
C608A	17.3 ± 1.7	27.5	51.6 ± 4.0	24.3 ± 2.0	23.7 ± 5.0	67.5 ± 9.0	0.46 (× 8.8)	2.78 (× 2.2)
M593S	17.0 ± 0.8	319	124 ± 0.8	7.4 ± 1.2	133 ± 18	20.9 ± 1.6	1.07 (× 21)	2.84 (× 2.2)
M593A	17.2 ± 0.9	155	56.3 ± 2.1	6.1 ± 0.2	32.5 ± 4.6	30.3 ± 4.7	0.58 (× 12)	4.81 (× 3.7)
M593E	14.1 ± 1.8	220	150 ± 3.4	6.3 ± 0.5	31.3 ± 2.6	27.5 ± 1.8	0.21 (× 4.2)	4.37 (× 3.4)
QM	13.6 ± 1.6	258	95.7 ± 2.8	4.6 ± 1.5	13.1 ± 4.7	13.0 ± 6.3	0.14 (× 2.6)	2.84 (× 2.2)

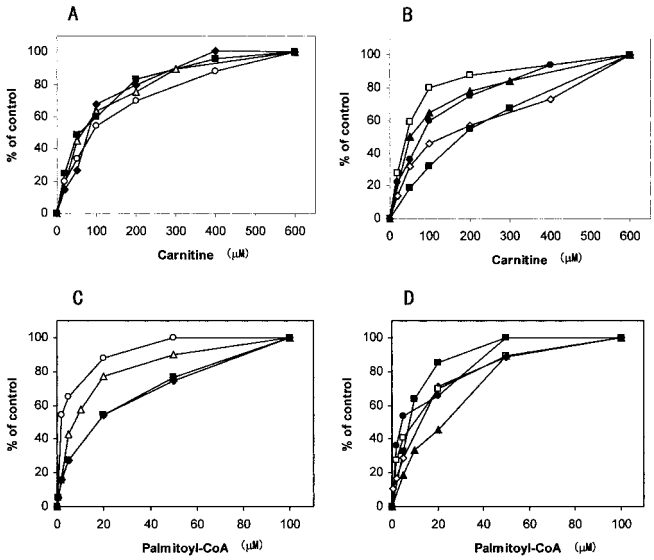

FIG. 3. **Kinetic analysis of wild type and different mutants of L-CPT I.** Yeast extracts (10 μg of protein) of (*A* and *C*) wild type (*open circles*) and mutants M593S (*open triangles*), M593A (*black rhombus*), M593E (*black squares*), and (*C* and *D*) T314S (*open rhombus*), N464D (*open squares*), A478G (*black squares*), C608A (*black triangles*), and quintuple mutant T314S/N464D/A478G/M593S/C608A (*black circles*) were incubated at increasing concentrations of carnitine (*A* and *B*) and palmitoyl-CoA (*C* and *D*).

mutants that altered the sensitivity to malonyl-CoA increased (see below). The catalytic efficiency of the methionine mutants increased between 4.2- and 21-fold, C608A increased 8.8-fold, and A478G increased 4.1-fold. T314S, which produced a small change in malonyl-CoA sensitivity (see below), increased the V_{max}/K_m value by only 2.8, whereas in N464D, in which the sensitivity to malonyl-CoA was unchanged (see below), the catalytic efficiency was modified by a factor of 5.6.

An analogous tendency was also observed in K_m for palmitoyl-CoA but the changes were smaller. K_m values for palmitoyl-CoA were 24.3, 15.1, 7.4, 6.1, and 6.3 for mutants C608A, A478G, M593S, M593A, and M593E, respectively (K_m value for the wild type was 4.9) (Table I). Catalytic efficiencies for palmitoyl-CoA as substrate increased in all mutants, the values ranging between 2.78 and 4.81 (Table I).

Inhibition of CPT I Wild Type and Mutants by Malonyl-CoA—When inhibitory kinetics *versus* increasing concentrations of malonyl-CoA was performed, the quintuple mutant practically abolished the sensitivity toward malonyl-CoA (IC_{50} of 258 *versus* 12.3 μM of the wild type) (Fig. 4*B* and Table I). Even at concentrations as high as 100 μM malonyl-CoA the CPT I quintuple mutant maintained 80% of the activity of the control without malonyl-CoA.

We then addressed the individual responsibility of the separate CPT I mutants for the malonyl-CoA sensitivity. Mutants T314S, N464D, M593S, and C608A expressed in *S. cerevisiae* were incubated with increasing amounts of malonyl-CoA, and CPT I activity was determined. Mutant A478G had been previously studied in Ref. 20 and showed decreased sensitivity to malonyl-CoA (IC_{50} of 39.5 *versus* 12.3 μM of the wild type).

The kinetics of inhibition by malonyl-CoA depended on the mutant considered. Whereas mutant M593S (Fig. 4*A*) showed very low sensitivity at malonyl-CoA inhibition (IC_{50} of 319 μM), the other mutations produced varied changes in malonyl-CoA sensitivity. L-CPT I C608A slightly modified the sensitivity to malonyl-CoA (IC_{50} is 27.5 μM), the change in IC_{50} of mutant T314S was small, whereas N464D showed similar sensitivity to malonyl-CoA to the wild type (Fig. 4*B* and Table I). Because the highest changes in sensitivity to malonyl-CoA and K_m values for carnitine were observed in the methionine mutants (point and quintuple mutants), we additionally prepared two new mutants: M593A and M593E to examine whether Met^{593} was essential to the malonyl-CoA interaction in L-CPT I. Results show that the sensitivity to malonyl-CoA was also nearly abolished in these mutants (Fig. 4*A*) (IC_{50} values of 155 and 220 μM, respectively) as in the M593S mutant, confirming the essential role of Met^{593} in this interaction.

DISCUSSION

We attempted to identify the amino acids in the C-terminal domain of L-CPT I that are responsible for the inhibition of the catalytic activity by malonyl-CoA. Over many years much work has been done to identify the domains in L-CPT I that may bind malonyl-CoA. Different groups have tested different empirical hypotheses and mutated amino acids, mostly in the amino-terminal region of L-CPT I. The results have shown that this domain plays a role in the regulation of CPT I by malonyl-CoA, because in some cases the sensitivity to the inhibitor is impaired.

A different approach was employed by our group very recently. This was based on the conservation of two histidine residues, which are present in the inhibitable malonyl-CoA carnitine acyltransferases (CPT I and COT) and absent in noninhibitable enzymes (CPT II and CAT). Mutation of both histidines resulted in the abolition of malonyl-CoA sensitivity in COT (26). Analogous results were observed in CPT I when its concentration at the mitochondrial membranes was not high. Mutation of other amino acids in the domain proximal to the catalytic site (Ala^{478} and Pro^{479}) indicated that a malonyl-CoA-

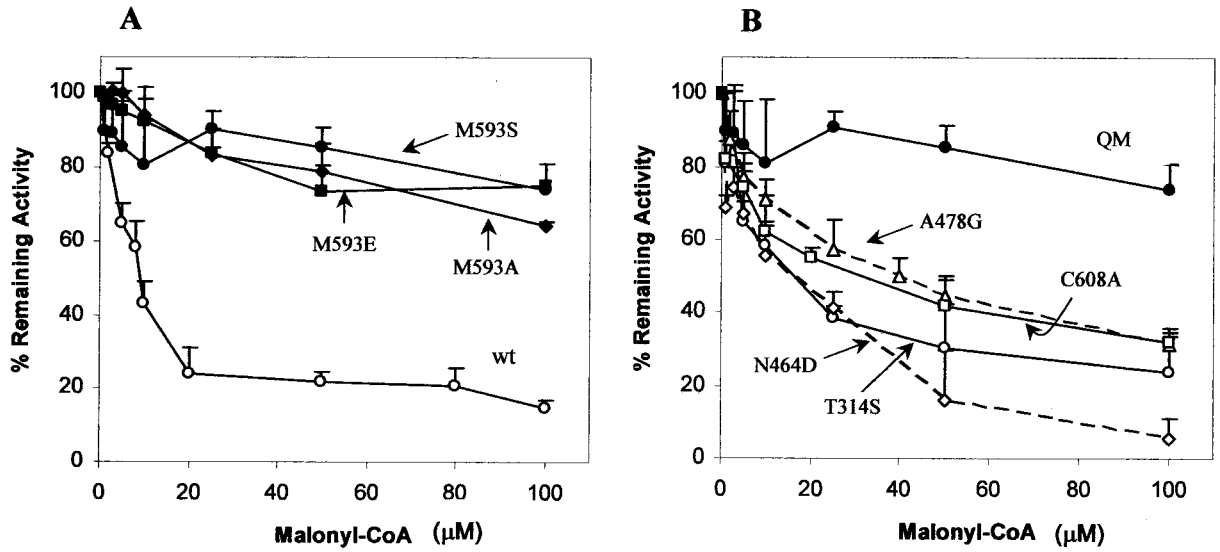

FIG. 4. **Effect of malonyl-CoA on the activity of yeast overexpressed L-CPT I (wild type) and several mutants.** *A*, L-CPT I wild type (*open circles*) and point methionine mutants M593S (*black circles*), M593A (*black rhombus*), M593E (*black squares*), and *B*, quintuple mutant (*QM*) (*black circles*) and point mutants T314S (*open circles*), N464D (*open rhombus*, *broken line*), A478G (*open triangles*, *broken line*), and C608A (*open squares*) overexpressed in yeast were incubated with increasing concentrations of malonyl-CoA and the enzyme activity was measured. Data are expressed relative to control values in the absence of inhibitor (100%) as the mean of three independent measurements.

inhibitable domain was probably the low-affinity malonyl-CoA binding site. Our previous studies showed that the location of malonyl-CoA in the structural model was compatible with competition of the inhibitor *versus* the substrate in the malonyl-CoA low affinity binding site (20).

The site-directed mutagenesis study used here to identify amino acids responsible for malonyl-CoA inhibition is based on the comparison of the sequences in a range of carnitine and choline acyltransferases, taking the positive or negative sensitivity to malonyl-CoA as a discriminatory criterion. The biocomputing study has shown that five amino acids are present in all CPT I (isoforms L- and M-) and in COT from various organisms and that they are absent not only in other nonmalonyl-CoA-inhibitable carnitine acyltransferases but also in ChAT. In rat L-CPT I these amino acids are Thr^{314}, Asn^{464}, Ala^{478}, Met^{593}, and Cys^{608}. The corresponding positional amino acids in CPT II, CAT, and ChAT are Ser^{223}, Asp^{363}, Gly^{377}, Ser^{490}, and Ala^{505}, respectively (coordinates of rat CPT II). Therefore, we considered it highly probable that these amino acids were involved in the interaction of malonyl-CoA. Results confirmed in part this supposition. The quintuple mutant reduced malonyl-CoA sensitivity almost completely (80% activity at 100 μM malonyl-CoA (which is outside the physiological range)), supporting the initial hypothesis. The results obtained using separate single mutants indicate that not all of these amino acids have the same role in malonyl-CoA inhibition. Whereas M593S nearly abolished the sensitivity to malonyl-CoA like the quintuple mutant, A478G increased the IC_{50} from 12 to 39.5 μM (20). The other amino acids are less responsible for the inhibition.

The relevance of Met^{593} as a critical amino acid for malonyl-CoA sensitivity was confirmed by the results of mutation to other two amino acids, Ala and Glu. The mutants equally showed diminished sensitivity to malonyl-CoA like mutant M593S. Met^{593}, when mutated to Ser as it appears in CPT II and CAT, decreased the sensitivity to malonyl-CoA in a stronger fashion than when it was mutated to other amino acids like Ala and Glu, which were unrelated to this position in other carnitine acyltransferases. Therefore, we conclude that the occurrence of Ser in this position has probably been evolutionary conserved in nonmalonyl-CoA-sensitive carnitine acyltransferases because it prevents sensitivity to malonyl-CoA. In any case, it appears that Met^{593} is critical in the interaction of malonyl-CoA with L-CPT I.

It was of interest to measure the kinetic constants of all CPT I mutants. Several authors reported the competition between malonyl-CoA and carnitine (27, 28). The tissues in which the sensitivity of CPT I to malonyl-CoA is highest are those that require the highest concentration of carnitine to drive the reaction and the requirement for carnitine and sensitivity to malonyl-CoA appears to be inversely related. The authors concluded that the sites to which the two metabolites bind are closely associated (27, 7). Studies by Bird and Saggerson (28) showed on the one hand that malonyl-CoA reduced the effectiveness of carnitine as substrate, and on the other hand, that carnitine might diminish the regulatory effect of malonyl-CoA (29). Although a clear mechanism for this competition could not be established, the data strongly supported this idea. In the present study the various CPT I mutants have altered K_m or V_{max} for carnitine. Whereas the K_m for C608A was half of the wild type, its V_{max} was 3.6-fold higher. The mutant M593S had the same K_m value for carnitine as the wild type but its V_{max} increased 20-fold. The mutant A478G increased both the K_m value and the V_{max} with respect to the wild type values. The relationship between these values and catalysis is best revealed in the term catalytic efficiency. This term as calculated by the V_{max}/K_m ratio varies considerably among different mutants. Carnitine catalytic efficiencies for mutants M593S, M593A, M593E, C608A, and A478G increased 21-, 12-, 4.2-, 8.8-, and 4.1-fold with respect to the wild type. This means that mutations designed to decrease malonyl-CoA sensitivity strongly modified the catalytic efficiency of CPT I mutants measured in the absence of malonyl-CoA. Interestingly, the increase in catalytic efficiency appears to be roughly proportional to the extent of the alteration in malonyl-CoA sensitivity. The IC_{50} values for malonyl-CoA run in the same direction to the catalytic efficiency of the mutants. This indicates that those mutants that can locate carnitine better at the catalytic site might displace malonyl-CoA from its site, preventing the binding of the metabolite and thus the inhibition of CPT I.

Because L-CPT I has not been crystallized, we do not know the proximity of Met^{593} to the site of carnitine binding to perform the catalytic event. However, Met^{593} is very near the tripeptide $TET^{602-604}$, which has been reported to play an

important role in the accommodation of carnitine in catalysis. Cronin (30) showed that mutation of the homologous tripeptide VDN in choline acetyltransferases to TET greatly increased the catalytic efficiency of the reaction (137-fold) using carnitine as substrate. This proximity between Met^{593} and $TET^{602-604}$ would explain the inverse correlation observed between the catalytic efficiency for carnitine and the IC_{50} for malonyl-CoA values of the mutants assayed. A new scenario appears in the mutual interaction between carnitine and malonyl-CoA in CPT I. The domain comprised, at least, between amino acid residues 593 and 604 is probably the site of interaction between carnitine and malonyl-CoA, which exclude each other. Higher catalytic efficiencies for carnitine in the mutants are followed by decreases in the inhibitory sensitivity to malonyl-CoA.

It is equally interesting to note that all mutants tested show higher catalytic efficiency for palmitoyl-CoA as substrate than the wild type. The increase in V_{max}/K_m ranges from 2- to 3-fold. Previous work with a partially purified preparation of CPT I had indicated that the kinetics of the reaction with respect to carnitine concentration could be highly dependent on the concentration of the second substrate, palmitoyl-CoA (29). Experiments carried out by Bird and Saggerson (28) showed that in fasted animals, in which carnitine concentration was decreased, the IC_{50} values for malonyl-CoA increased up to 17-fold and the binding of [2-^{14}-C]malonyl-CoA was reduced by 35% at 50 μM palmitoyl-CoA and to even lower values at increasing palmitoyl-CoA concentrations.

Only two of these mutated amino acids are located in the three-dimensional CPT I structural model. Ala^{478} is one of the amino acids present in the low affinity site of malonyl-CoA interaction. This amino acid together with Pro^{479} and His^{483} conform a domain to which malonyl-CoA appears to bind (20). Mutation of this amino acid would explain a decrease in sensitivity to malonyl-CoA, and therefore it would also explain the increase in catalytic efficiency. On the other hand, Asn^{464} is also present in the catalytic core of the structural model of CPT I (20), but its location does not permit any conclusions about a participation in the malonyl-CoA inhibitory effect. In fact it is located on the opposite site to malonyl-CoA (data not shown). Therefore, it is not surprising that its mutation from Asn^{464} to Asp^{464} does not alter sensitivity to the inhibitor. As a corollary of this study, we conclude that the occurrence of the five other amino acids (Ser^{223}, Asp^{363}, Gly^{377}, Ser^{490}, and Ala^{505}) at the positions, respectively, identical to those amino acids seen in CPT I may be sufficient to prevent the sensitivity to malonyl-CoA not only to carnitine acyltransferases such as CPT II and CAT but also to ChAT.

The use of either the quintuple mutant or the methionine point mutants may allow studies on the influence of these negative dominant CPT Is, which are expected to be independent of malonyl-CoA concentration in a range of tissues such as liver, muscle, and the β-cell, in which the metabolism of fatty acids plays important roles in ketone body synthesis, resistance to insulin, and glucose-stimulated insulin secretion, respectively. Some of these topics are the subject of current investigations in our laboratory.

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