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Pig Liver Carnitine Palmitoyltransferase

CHIMERA STUDIES SHOW THAT BOTH THE N- AND C-TERMINAL REGIONS OF THE ENZYME ARE IMPORTANT FOR THE UNUSUAL HIGH MALONYL-CoA SENSITIVITY*

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Pig and rat liver carnitine palmitoyltransferase I (L-CPTI) share common K_m values for palmitoyl-CoA and carnitine. However, they differ widely in their sensitivity to malonyl-CoA inhibition. Thus, pig L-CPTI has an IC_{50} for malonyl-CoA of 141 nM, while that of rat L-CPTI is 2 μM. Using chimeras between rat L-CPTI and pig L-CPTI, we show that the entire C-terminal region behaves as a single domain, which dictates the overall malonyl-CoA sensitivity of this enzyme. The degree of malonyl-CoA sensitivity is determined by the structure adopted by this domain. Using deletion mutation analysis, we show that malonyl-CoA sensitivity also depends on the interaction of this single domain with the first 18 N-terminal amino acid residues. We conclude that pig and rat L-CPTI have different malonyl-CoA sensitivity, because the first 18 N-terminal amino acid residues interact differently with the C-terminal domain. This is the first study that describes how interactions between the C- and N-terminal regions can determine the malonyl-CoA sensitivity of L-CPTI enzymes using active C-terminal chimeras.

The outer mitochondrial membrane enzyme carnitine palmitoyltransferase I (CPTI)[1] catalyzes the conversion of long-chain acyl-CoAs to acylcarnitines in the presence of L-carnitine. This is the initial step in the entry of long-chain fatty acids into the mitochondrial matrix, where they undergo β oxidation. This is also the first rate-limiting step in fatty acid oxidation in all tissues and is highly regulated by its physiological inhibitor, malonyl-CoA (1). Inhibition of CPTI by malonyl-CoA, the first intermediate in fatty acid synthesis, allows the cell to coordinate fatty acid oxidation and synthesis (2). Understanding the regulation of CPTI by malonyl-CoA is important since this enzyme is a potential pharmacological target in type 2 diabetes, where excessive fatty acid oxidation is undesirable and must be controlled.

There are two isotypes of CPTI: a liver (L-CPTI) and a muscle (M-CPTI) isotype. Both genes encode for proteins, which share a high degree of identity but that differ markedly in their kinetic characteristics. L-CPTI has a much lower K_m for carnitine and a higher IC_{50} for malonyl-CoA inhibition than the muscle isotype (1). These kinetic characteristics of the liver and muscle enzymes are also retained when the cDNAs are expressed in a heterologous system (3–6). CPTI is an integral membrane protein that has two α-helical hydrophobic transmembrane domains, the amino and most of the carboxyl regions being exposed to the cytosolic face of the membrane (7).

It has been shown that the first 18 N-terminal amino acids of L-CPTI play a critical role in malonyl-CoA sensitivity since deletion of this region leads to loss of inhibition by malonyl-CoA (8). Further work demonstrated that this region acts as a positive determinant of malonyl-CoA sensitivity, while amino acids 19–30 act as a negative determinant (9). Interestingly, although the first 18 amino acids of rat L-CPTI and M-CPTI are identical, the role of the M-CPTI N-terminal residues in determining malonyl-CoA sensitivity is different (10). Pig L-CPTI encodes for a 772-amino acid protein that shares 86 and 63% identity with rat L-CPTI and human M-CPTI, respectively. When expressed in the yeast *Pichia pastoris*, the pig L-CPTI enzyme shows kinetic characteristics similar to human or rat L-CPTI (11). However, the pig enzyme, unlike the rat liver enzyme, shows a much higher sensitivity to malonyl-CoA inhibition that is characteristic of human or rat M-CPTI enzymes (10, 11). Therefore, pig L-CPTI behaves like a natural chimera of the L- and M-CPTI isotypes, which makes it a useful model to study the structure-function relationships of the CPTI enzymes (11). Because the sequence identity between the pig and rat L-CPTIs is high, we predicted that chimeric proteins that combine segments of the C-terminal domain from pig and rat L-CPTIs would be active. Thus, in this study, we focused our attention on changes in malonyl-CoA sensitivity observed in chimeric pig and rat L-CPTI enzymes. In addition, we also studied the presence of positive and negative determinants of malonyl-CoA sensitivity in the N-terminal region of pig L-CPTI and the putative role of such determinants in the observed aberrant kinetic behavior of this enzyme.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids—Pig L-CPTI and rat L-CPTI were subcloned into pHW010, generating PLCPTI/pHW010 and RLCPTI/pHW010 as previously described (11, 12). To generate the chimeras, a *Hin*dIII site (shown in italics in the primer sequence below) was introduced into the cDNAs of both enzymes by overlap extension. Both cDNAs were modified using the same strategy: two independent PCRs (15 cycles each) were performed with primers A (5′-CTCCCTG*AAGCTT*CTGCTCTCCTAC-3′) and B (5′-CGTAGTTGCTGTTCACC-3′), and primers C (5′-GCCTTTCAGTTCACGGTCAC-3′) and D (5′-GTAGGAGAGCAG*AAGCTT*CAGGGAG-3′) using the pig and rat L-CPTI cDNAs as templates. In the subsequent overlap extension reaction, 200 ng of each PCR product was annealed and reamplified for a further 15

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[1] The abbreviations used are: CPTI, carnitine palmitoyltransferase I; L-CPTI, liver isotype of CPTI; M-CPTI, muscle isotype of CPTI; GAP, glyceraldehyde-3-phosphate dehydrogenase.

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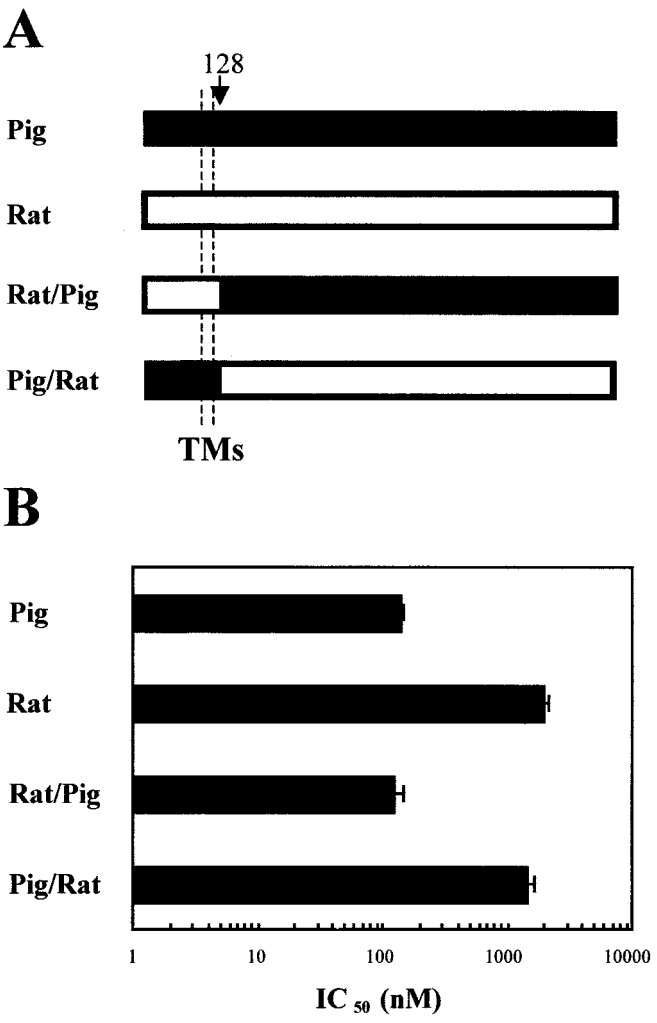

FIG. 1. **Role of the C-terminal region in determining the IC_{50} for malonyl-CoA.** *A*, schema of pig and rat L-CPTI chimeras. The *number* over the *vertical arrow* indicates the amino acid number at which both proteins were recombined. *TMs* indicates the position of the two transmembrane domains**.** *B*, isolated mitochondria were assayed for CPT activity in the presence of increasing concentrations of malonyl-CoA, as described under "Experimental Procedures." Each construct was assayed at least three times with at least two independent mitochondrial preparations.

cycles of PCR using primers A and D. The resulting PCR products were digested with *Bgl*II and cloned into either *Bgl*II-cut PLCPTI/pHW010 or *Bgl*II-cut RLCPTI/pHW010, generating Pig- and Rat-V128L/pHW010. This resulted in the introduction of a point mutation, valine to leucine, at position 128. This mutation did not affect the kinetic characteristics of the enzymes (results not shown).

To construct the pig/rat and rat/pig L-CPTI chimeras, Pig- and Rat-V128L/pHW010 were digested with *Eco*RI to remove the inserts that were subcloned into *Eco*RI-cut $BSSK^+$. The resulting plasmids, Pig- and Rat-V128L/$BSSK^+$ were digested with *Hin*dIII. The restriction enzyme *Hin*dIII cuts Pig- and Rat-V128L/pHW010 before the ATG start codon of the insert, and the pig and rat cDNA inserts at position 384 after the ATG start codon. The insert from the digested Pig-V128L/$BSSK^+$ was ligated into *Hin*dIII-cut Rat-V128L/$BSSK^+$ and vice versa, resulting in Pig/Rat and Rat/Pig inserts in $BSSK^+$. The two inserts were then released and subcloned into *Eco*RI-cut pHW010.

A Rat/Pig-A L-CPTI chimera was generated from Pig-V128L/$BSSK^+$. Pig-V128L/$BSSK^+$ was digested with *Kpn*I that cuts before the ATG start codon of the insert and the pig L-CPTI cDNA at position 660. This insert was subcloned into *Kpn*I-cut Rat-V128L/$BSSK^+$, resulting in the plasmid Pig/Rat3/$BSSK^+$. Rat-V128L/$BSSK^+$ was cut with *Hin*dIII. *Hin*dIII cuts Rat-V128L/$BSSK^+$ before the ATG start codon of the insert and the rat L-CPTI cDNA at position 384. This insert was purified and subcloned in the correct orientation into *Kpn*I-cut Pig/Rat3/$BSSK^+$, resulting in Rat/PigA/$BSSK^+$. The insert from Rat/PigA/$BSSK^+$ was excised by digestion with *Eco*RI and ligated in the correct orientation into *Eco*RI-cut pHW010, resulting in Rat/Pig-A L-CPTI in pHW010.

Rat/Pig-B was generated from Pig-V128L/$BSSK^+$. Pig-V128L/$BSSK^+$ was cut with *Xho*I and *Stu*I. *Xho*I cuts Pig-V128L/$BSSK^+$ before the ATG start codon, and *Stu*I cuts the insert at position 1770 after the start codon. This insert was purified and cloned in the correct orientation into *Xho*I/*Stu*I-cut Rat-V128L/$BSSK^+$, resulting in Pig/Rat2/$BSSK^+$. Rat-V128L/$BSSK^+$ was cut with *Kpn*I that cuts Rat-V128L/$BSSK^+$ before the ATG start codon of the insert and the rat cDNA at position 660. This insert was purified and cloned in the correct orientation into *Kpn*I-cut Pig/Rat2/$BSSK^+$, generating Rat/PigB in $BSSK^+$. The insert from Rat/PigB/$BSSK^+$ was excised with *Eco*RI and ligated in the correct orientation into *Eco*RI-cut pHW010, resulting in Rat/Pig-B L-CPTI cDNA in pHW010.

Rat/Pig-C was generated from Rat-V128L/$BSSK^+$. Rat-V128L/$BSSK^+$ was cut with *Xho*I and *Stu*I. *Xho*I cuts Rat-V128L/$BSSK^+$ before the ATG start codon, and *Stu*I cuts the insert at position 1770 after the start codon. This insert was purified and subcloned in the correct orientation into *Xho*I/*Stu*I-cut Pig-V128L/$BSSK^+$, resulting in Rat/Pig-C in $BSSK^+$. The insert from Rat/Pig-C in $BSSK^+$ was excised with *Eco*RI and ligated in the correct orientation into *Eco*RI-cut pHW010, resulting in Rat/Pig-C in pHW010.

To generate Δ18Pig, the pig L-CPTI cDNA was amplified with the primers Δ18fwd (5′-GACATC*GAATTC***ATG**ATCGACCTTCGAATGAG-C-3′) and primer D. An ATG start codon (shown in bold) was added immediately after the *Eco*RI site (shown in italics). The PCR product was cloned into *Eco*RV-cut $BSSK^+$, resulting in the plasmid Δ18/$BSSK^+$. Pig-V128L/$BSSK^+$ was cut with *Aat*II and *Not*I. *Aat*II cuts the insert at position 508, and *Not*I cuts Pig-V128L/$BSSK^+$ after the stop codon. The resulting plasmid, Δ18Pig/$BSSK^+$, was digested with *Eco*RI, and the insert was cloned into *Eco*RI-cut pHW010, generating Δ18Pig/pHW010. Δ28Pig was generated in the same way but using the primer Δ28fwd (5′-GACATC*GAATTC***ATG**AGGCAGATCTATCTGTCT-3′) instead of Δ18fwd. Δ18Pig/Rat and Δ28Pig/Rat were generated following the same strategy and using as template Δ18Pig/$BSSK^+$ and Δ28Pig/$BSSK^+$, respectively. Both plasmids were amplified with the primers T7 and primer D. The resulting PCR products were digested with *Hin*dIII and *Eco*RV and cloned into *Eco*RV/*Hin*dIII-cut Pig/Rat/$BSSK^+$, resulting in Δ18 Pig/Rat and Δ28 Pig/Rat in $BSSK^+$. The insert from Δ18 Pig/Rat and Δ28 Pig/Rat in $BSSK^+$ were excised with *Eco*RI and ligated in the correct orientation into *Eco*RI-cut pHW010, resulting in Δ18Pig/Rat and Δ28Pig/Rat in pHW010.

P. pastoris Transformation—All constructs were linearized in the glyceraldehyde-3-phosphate dehydrogenase (GAP) gene promoter by digestion with *Pag*I and integrated into the GAP gene promoter locus of *P. pastoris* GS115 by electroporation (13). Histidine prototrophic transformants were selected on YND (0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories, Inc., Detroit, MI)) plates and grown on YND medium. Mitochondria were isolated by disrupting the yeast cells with glass beads as previously described (12, 14).

CPT Assay—CPT activity was assayed by the forward exchange method using L-[^{3}H]carnitine as previously described (12). The standard assay reaction mixture contained in a total volume of 0.5 ml: 200 mM L-[^{3}H]carnitine (~10,000 dpm/nmol), 80 μM palmitoyl-CoA, 20 mM HEPES (pH 7.0), 1% fatty acid-free albumin, and 40–75 mM KCl with or without malonyl-CoA as indicated. Incubations were performed for 3 min at 30 °C. The K_m for palmitoyl-CoA was determined by varying the palmitoyl-CoA concentration in the presence of a fixed molar ratio (6.1:1) of palmitoyl-CoA:albumin as previously described (13).

Western Blot Analysis and DNA Sequencing—Proteins were separated by SDS-PAGE in a 6% gel and transferred onto nitrocellulose membranes as previously described (12). Pig L-CPTI-specific antibody was obtained as previously described (11) and used at a 1:1000 dilution. Proteins were detected using the ECL chemiluminescence system (Amersham Biosciences). DNA sequencing was performed using the Big Dye™ kit (Applied Biosystems, PerkinElmer Life Sciences) according to the manufacturer's instructions.

RESULTS

Generation of Mutant CPT Proteins for Expression in P. pastoris—All constructions were performed as described in "Experimental Procedures." Sequences were confirmed by DNA sequencing. *P. pastoris* was chosen as an expression system because it does not have endogenous CPT activity and because other CPT proteins have been successfully expressed using this system (4, 11, 12). All constructs were subcloned in the vector

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TABLE I

Activity and malonyl-CoA sensitivity for yeast-expressed wild type and mutant L-CPTI constructs

Mitochondria (100 μg) from the yeast strains expressing wild-type or L-CPTI chimeras were assayed for CPT activity and kinetic parameters as described under "Experimental Procedures." Each construct was assayed at least three times with at least two independent mitochondrial preparations.

Strain	Activity	Carnitine	Palmitoyl-CoA
	nmol/min/mg	K_m (μM)	K_m (μM)
Wild-type			
Pig L-CPTI	5.2 ± 0.6	126.3 ± 4.3	35.4 ± 2.0
Rat L-CPTI	7.8 ± 0.5	100.3	43.0
Mutants			
Pig/Rat	7.1 ± 1.2	147.6 ± 33.6	31.5 ± 4.1
Rat/Pig	18.2 ± 1.9	104.6 ± 7.3	52.0 ± 7.0
Rat/Pig-A	5.5 ± 05	107.0 ± 35.5	60.0 ± 12.0
Rat/Pig-B	4.8 ± 05	111.0 ± 29.0	43.3 ± 8.0
Rat/Pig-C	6.5 ± 0.7	110.7 ± 28.0	45.1 ± 9.0
Δ18	12.3 ± 1.3	82.7 ± 8.1	63.0 ± 5.4
Δ28	12.7 ± 0.7	97.0 ± 10.3	61.8 ± 14.2
Δ18 Pig/Rat	8.5 ± 0.6	123.6 ± 9.3	49.7 ± 6.2
Δ28 Pig/Rat	8.7 ± 1.2	101.7 ± 8.7	47.4 ± 5.3

pHW010 and expressed under the control of the GAP gene promoter (6). Yeast transformants were grown as previously described (12). No CPT activity was found in the control yeast strain with the empty vector (data not shown).

Chimeric Proteins—Pig/Rat and Rat/Pig L-CPTI were constructed as shown in Fig. 1*A* by ligating pig and rat L-CPTI together just after the second transmembrane domain. These two chimeras have similar affinity for carnitine and palmitoyl-CoA as the parental wild-type enzymes (Table I). The IC_{50} of the Pig/Rat L-CPTI construct was similar to that of rat L-CPTI, while the IC_{50} of the Rat/Pig construct was similar to that of the wild-type pig L-CPTI (Fig. 1*B*). We previously showed that pig L-CPTI migrates anomalously faster than its predicted molecular weight on SDS-PAGE. Western blot analysis of Pig/Rat and Rat/Pig L-CPTI shows that it is the C-terminal region that dictates the migration pattern of L-CPTI proteins (Fig. 2).

To construct carboxyl-end chimeras, the C-terminal region was divided into three discrete regions: region A, from the second transmembrane domain (amino acid 128) to amino acid 220; region B, from amino acid 220–590; and region C, from amino acid 590 to the end. The three carboxyl-end chimeras were based on rat L-CPTI in which regions A, B, or C were substituted by the corresponding region of pig L-CPTI, generating Rat/Pig-A, Rat/Pig-B and Rat/Pig-C (Fig. 3*A*). Rat/Pig-A, -B, and -C were expressed in *P. pastoris*, and mitochondria were isolated from the yeast strains expressing each construct and tested for activity and malonyl CoA sensitivity. The chimeras were all active, with similar specific CPT activities and similar affinity for carnitine and palmitoyl-CoA as the parental wild-type enzymes (Table I). Fig. 3*B* shows that Rat/Pig-A, -B, and -C were all sensitive to malonyl-CoA inhibition, indicating that all these chimeras have a functional malonyl-CoA binding site. Fig. 3*B* also shows that, surprisingly, the three C-terminal region chimeras had malonyl-CoA sensitivity similar to that of wild-type pig L-CPTI. Statistical analysis showed that the difference in malonyl-CoA sensitivity between pig L-CPTI, Rat/Pig-A, Rat/Pig-B, and Rat/Pig-C were not statistically different ($p = 0.118$).

Fig. 4 shows that two of the C-terminal region chimeras, Rat/Pig-A and -C, migrated on SDS-PAGE gels according to their molecular weights, while Rat/Pig-B chimera migrated fasted than expected form the predicted molecular weight. Thus, in our hands, Rat/Pig-A and -C are the first CPTI analyzed chimeras in which there is not a direct correlation between an anomalous protein migration pattern on SDS-PAGE with a high degree of enzyme sensibility to malonyl-CoA inhibition (Fig. 3*B*).

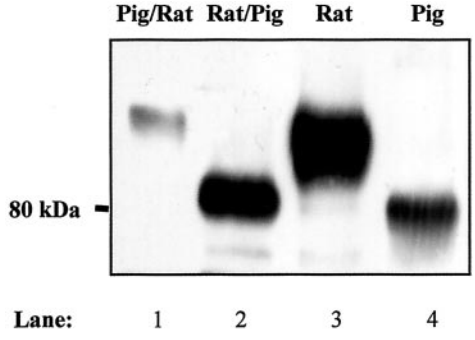

FIG. 2. **Immunoblot showing expression of chimeras and wild-type L-CPTI enzymes in the yeast *P. pastoris*.** Mitochondria (10 μg of protein) were separated on a 6% SDS-PAGE. The immunoblot was performed as described under "Experimental Procedures." *Lane 1*, Pig/Rat chimera; *lane 2*, Rat/Pig chimera; *lane 3*, wild-type rat L-CPTI; and *lane 4*, wild-type pig L-CPTI.

N-terminal Deletion Mutants—Four N-terminal deletion mutants, Δ18 Pig, Δ18 Pig/Rat, Δ28 Pig, and Δ28 Pig/Rat, were expressed in *P. pastoris*. Mitochondria isolated from the yeast strains expressing each of the deletion mutants (Fig. 5*A*) had specific activity of 12.3 ± 1.3, 8.5 ± 0.6, 12.7 ± 0.7, and 8.7 ± 1.2 nmol/min/mg of protein, respectively. The four deletion mutants were highly insensitive to malonyl-CoA inhibition (Fig. 5*B*), but statistical difference was observed in regards to malonyl-CoA sensitivity between Δ18 or Δ18 Pig/Rat *versus* Δ28 or Δ28 Pig/Rat (Fig. 5). They migrated on SDS-PAGE gels according to their molecular weight (Fig. 6), taking into account the anomalous migration pattern of the parental protein in the case of Δ18 Pig and Δ28 Pig (Fig. 6).

DISCUSSION

The C-terminal Region of L-CPTI Behaves As a Single Domain—Pig L-CPTI and rat L-CPTI have the same K_m values for carnitine and palmitoyl-CoA but differ markedly in regards to their malonyl-CoA sensitivity: pig L-CPTI has an IC_{50} value of 141 nM (10), while that of rat L-CPTI is 2 μM (8). To date all studies using chimeric proteins have been performed using combinations of L-CPTI and M-CPTI (9, 10, 15). Interpretation of such studies has often been difficult because the liver and muscle isotype have only 62% sequence identity, and they differ in both their affinity for carnitine and sensitivity to malonyl-CoA inhibition. Moreover, chimeric proteins of the 197 N-terminal amino acid residues between rat L-CPTI and human M-CPTI were reported to be inactive (10), probably because of the low degree of identity (62%) between the L-CPTI and M-CPTI isotypes.

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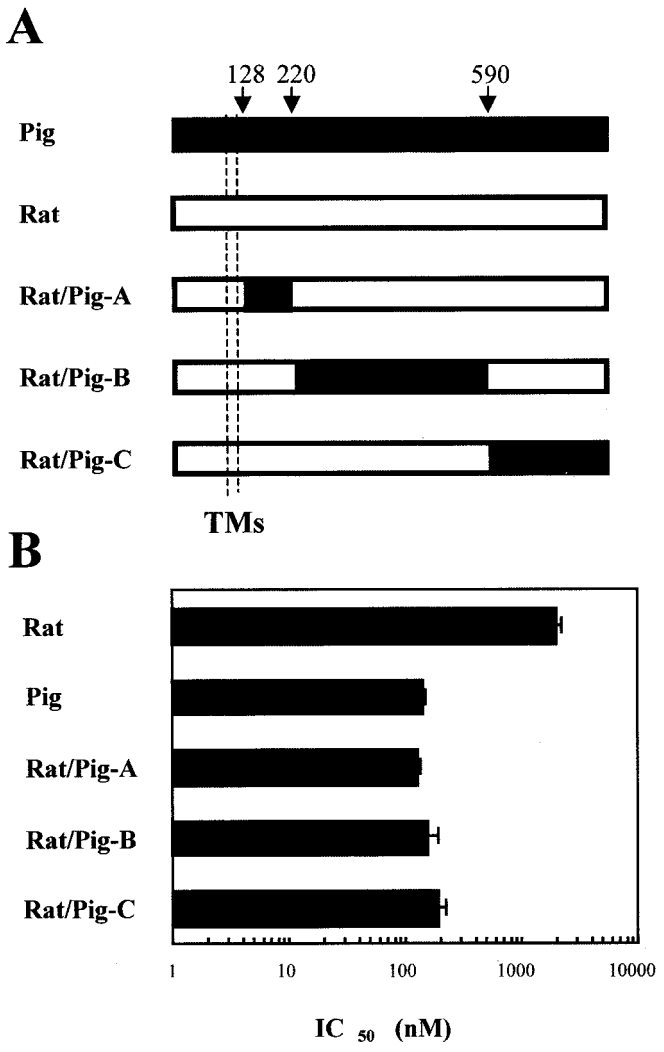

FIG. 3. **The C-terminal region behaves as a single domain with respect to the sensitivity to malonyl-CoA inhibition.** *A*, schema of pig and rat L-CPTI chimeras. The *numbers* over the *vertical arrows* indicate the amino acid number at which the proteins were recombined. *TMs* indicate the position of the two transmembrane domains**.** *B*, isolated mitochondria were assayed for CPT activity in the presence of increasing concentrations of malonyl-CoA, as described under "Experimental Procedures." Each construct was assayed at least three times with at least two independent mitochondrial preparations.

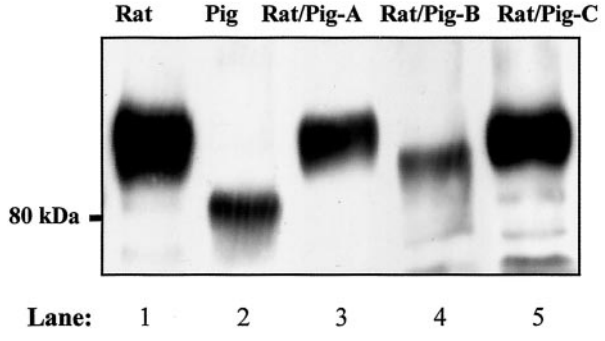

FIG. 4. **Immunoblot showing expression of wild-type and chimeric L-CPTI enzymes in the yeast *P. pastoris*.** Mitochondria (10 μg of protein) were separated on a 6% SDS-PAGE. The immunoblot was performed as described under "Experimental Procedures." *Lane 1*, wild-type rat L-CPTI; *lane 2*, wild-type pig L-CPTI; *lanes 2*, *3*, and *4* represent Rat/Pig-A, Rat/Pig-B, and Rat/Pig-C chimeras, respectively.

To perform a functional study of the C-terminal domain of L-CPTI proteins, we constructed chimeric proteins between pig and rat L-CPTI proteins. We predicted these chimeras would

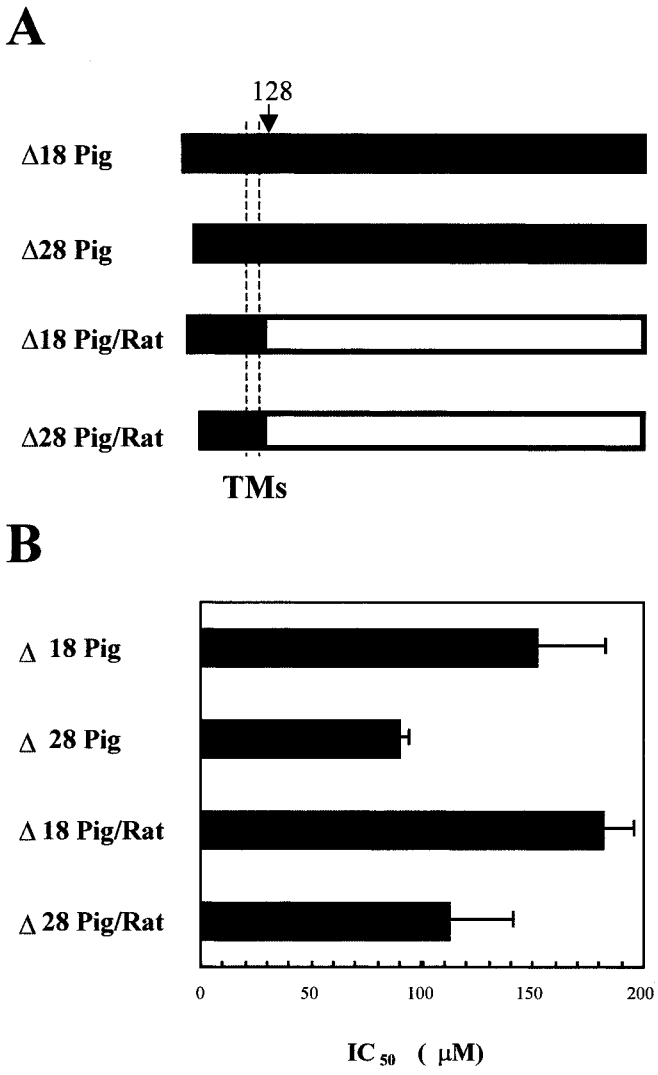

FIG. 5. **The C-terminal domain determines the degree of sensitivity to malonyl-CoA inhibition through its interaction with the N-terminal end.** *A*, schema of N-terminal deletion mutants obtained from pig or pig/rat L-CPTI. The *number* over the *vertical arrow* indicates the amino acid number at which both proteins were recombined. *TMs* indicates the position of the two transmembrane domains. *B*, isolated mitochondria were assayed for CPT activity in the presence of increasing concentrations of malonyl-CoA, as described under "Experimental Procedures." Each construct was assayed at least three times with at least two independent mitochondrial preparations. Statistical analysis, using one-way analysis of variance, indicated that the average IC_{50} value of the Δ18 deletion mutant was significantly different from that of the Δ28 deletion mutants ($p > 0.05$). The average IC_{50} value of the construct Δ18 Pig/Rat was also significantly different from that of Δ28 Pig/Rat ($p > 0.05$). However, there was no significant difference between the average IC_{50} values of Δ18 *versus* Δ18 Pig/Rat and Δ28 *versus* Δ28 Pig/Rat ($p > 0.05$).

be active because pig and rat L-CPTI share a high degree of identity (86%) compared with 62% identity between rat L-CPTI and human/rat M-CPTI, making it more likely that the resulting C-terminal chimeras would be active. Moreover, pig and rat L-CPTI only differ in their degree of malonyl-CoA sensitivity, which makes a chimeric protein between the two proteins a good tool to study the regulation of L-CPTI by malonyl-CoA.

Pig/Rat and Rat/Pig L-CPTI chimeras have the same K_m values for carnitine and palmitoyl-CoA as the parental proteins, which predicts that the active site of these chimeric proteins is similar to that of the parental proteins (Table I), thus suggesting that they adopt a similar active site conformation as the corresponding wild-type enzymes. The IC_{50} values

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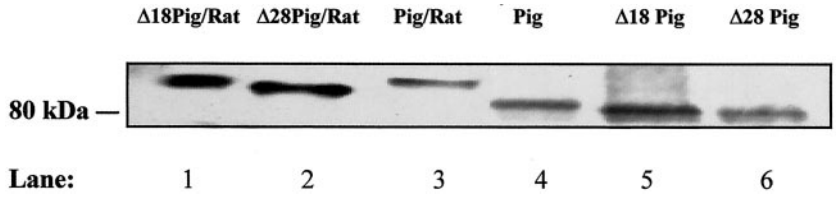

FIG. 6. **Immunoblots showing expression of wild-type and deletion mutants L-CPTI enzymes in the yeast *P. pastoris*.** Mitochondria (10 μg of protein) were separated on a 6% SDS-PAGE. The immunoblot was performed as described under "Experimental Procedures." *Lanes 1* and *2*, Δ18 Pig/Rat and Δ28 Pig/Rat, respectively; *lane 3*, Pig/Rat chimera; *lane 4*, wild-type pig L-CPTI; and *lanes 5* and *6* represent Δ18 Pig and Δ28 Pig deletion mutants, respectively.

for malonyl CoA inhibition of these two constructs shows that the C-terminal region dictates the degree of malonyl-CoA sensitivity of the overall enzyme (Fig. 1*B*). This is in agreement with the results obtained from chimeric proteins between rat L-CPTI and human/rat M-CPTI (9, 10, 15).

Human/rat M-CPTI (4, 16) and pig L-CPTI (11) migrate faster on SDS gel electrophoresis than their predicted molecular weight. Since, these proteins maintain their anomalous migration pattern when produced *in vitro*, it is predicted that this phenomenon stems from intrinsic differences in the primary sequence of the two proteins. Western blot analysis of the Pig/Rat and Rat/Pig chimeras showed that the C-terminal region dictates the migration pattern of CPTI proteins, since they migrate according to their C-terminal region (Fig. 2). We predict that differences in the primary sequence of the C-terminal region of these proteins makes them adopt a conformation resistant to denaturation by SDS and thus causes the proteins to migrate faster than their predicted molecular weight.

The three carboxyl end chimeric proteins, Rat/Pig-A, Rat/Pig-B, and Rat/Pig-C, were active, allowing kinetic determination of C-terminal region chimeric proteins. Surprisingly, substitution of small rat L-CPTI fragments with the corresponding pig L-CPTI fragment (92, 370, and 183 amino acid residues for Rat/Pig-A, -B, and -C, respectively) was sufficient to confer high malonyl-CoA sensitivity to the rat L-CPTI (Fig. 3*B*). This result suggests the following. (i) None of the A, B, or C region is by itself uniquely responsible for the degree of malonyl-CoA sensitivity of the enzyme. Substitution of any of these regions of pig L-CPTI with the corresponding region of rat L-CPTI generates enzymes with malonyl-CoA sensitivity similar to that of the wild-type pig L-CPTI enzyme. This indicates that the C-terminal region of L-CPTI proteins behave as a single domain, which dictates the malonyl-CoA sensitivity of the enzyme. (ii) None of the chimeric proteins studied had a malonyl-CoA sensitivity value intermediate between that of wild-type pig and rat L-CPTI. All of them behaved like one of the parental (pig or rat) L-CPTI proteins, suggesting that the L-CPTI C-terminal region adopts two distinct structures: a low and a high malonyl-CoA sensitivity state. The high malonyl-CoA sensitivity state seems to be more susceptible to change into the low malonyl-CoA sensitivity state but not vice versa.

The finding that the entire C-terminal region of L-CPTI behaves as a single domain is in agreement with previous proteolytic studies with intact rat liver mitochondria. Early studies (17) showed that trypsin/chymotrypsin treatment of membrane-bound L-CPTI lead to a rapid decrease of activity but that this effect was partially overcome by malonyl-CoA binding. When L-CPTI was subjected to trypsin/chymotrypsin treatment in the presence of malonyl-CoA, the enzyme remained active, although a single 8-kDa fragment was liberated. The authors concluded that this 8-kDa fragment was not essential for catalytic function; however, at that stage further interpretation of these results was not possible. Using the current knowledge on L-CPTI structure and our results, we predict that the 8-kDa fragment liberated upon trypsin/chymotrypsin treatment is probably the extreme N-terminal region of L-CPTI (the first ~70 N-terminal amino acid residues) and that binding of malonyl-CoA to the enzyme may further tighten the highly folded state of the C-terminal domain, thus rendering it more inaccessible to trypsin/chymotrypsin treatment. Other studies showed that L-CPTI is highly resistant to proteolytic digestion at low, non-membrane disrupting concentrations of the proteases, suggesting that CPTI, and more specifically the C-terminal region, is highly folded (7, 18).

Previous reports by others and us correlated high sensitivity to malonyl-CoA inhibition of M-CPTI and pig L-CPTI with the anomalous SDS migration pattern of the enzyme (4, 11, 16). However, chimeras that share the carboxyl end of pig or rat L-CPTI (Rat/Pig-A and Rat/Pig-C) show a high degree of sensitivity to malonyl-CoA inhibition with a normal SDS migration pattern (Figs. 3*B* and 4). These results suggest that the primary structure of the enzyme plays a role in determining the sensitivity to malonyl-CoA inhibition independently of its role in the overall structure.

The C-terminal Domain Determines the Degree of Sensitivity to Malonyl-CoA Inhibition through Its Interaction with the N-terminal End—The fact that Δ18 Pig is more sensitive to malonyl-CoA inhibition than the Δ28 Pig provides evidence that the extreme N-terminal region of pig L-CPTI contains positive (amino acids 1–18) and negative (amino acids 19–28) determinants of malonyl-CoA sensitivity, like rat L-CPTI (19). Hence, although pig L-CPTI is highly sensitive to malonyl-CoA inhibition like rat/human M-CPTI that do not have the negative determinant of malonyl-CoA sensitivity, its N-terminal region appears to have the same function as the low malonyl-CoA-sensitive rat L-CPTI.

Interestingly, while Pig and Pig/Rat L-CPTI have different malonyl-CoA sensitivity, deletion of the first 28 N-terminal amino acid residues from these two constructs generates enzymes, Δ28 Pig and Δ28 Pig/Rat L-CPTI, which have similar malonyl-CoA sensitivity (Fig. 5*B*). This suggests that the first 28 amino acid residues determine the difference in malonyl-CoA sensitivity between pig and rat L-CPTIs. The same result was obtained when only the first 18 N-terminal amino acids were deleted (Δ18 Pig and Δ18 Pig/Rat L-CPTI), which suggests that the first 18 N-terminal amino acids are sufficient to determine the difference in malonyl-CoA sensitivity between pig and rat L-CPTIs.

In summary, our data demonstrate that (i) the C-terminal end of L-CPTI is a single domain that dictates the degree of malonyl-CoA sensitivity of L-CPTI enzymes and (ii) deletion of the first 18 N-terminal amino acid residues of pig and Pig/Rat L-CPTIs generates enzymes with similar IC_{50} values. This suggests that the first 18 N-terminal amino acid residues and the C-terminal domain interact and that it is this interaction that determines the degree of malonyl-CoA sensitivity of L-CPTI. Since the first 18 N-terminal amino acid residues are identical in pig and rat L-CPTIs, what determines the nature of this interaction is the primary and secondary structure of the C-terminal domain.

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Pig Liver Carnitine Palmitoyltransferase: CHIMERA STUDIES SHOW THAT BOTH THE N- AND C-TERMINAL REGIONS OF THE ENZYME ARE IMPORTANT FOR THE UNUSUAL HIGH MALONYL-CoA SENSITIVITY

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