Pig Liver Carnitine Palmitoyltransferase I, with Low K_m for Carnitine and High Sensitivity to Malonyl-CoA Inhibition, Is a Natural Chimera of Rat Liver and Muscle Enzymes†

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ABSTRACT: The outer mitochondrial membrane enzyme carnitine palmitoyltransferase I (CPTI) catalyzes the initial and regulatory step in the β-oxidation of fatty acids. The genes for the two isoforms of CPTI-liver (L-CPTI) and muscle (M-CPTI) have been cloned and expressed, and the genes encode for enzymes with very different kinetic properties and sensitivity to malonyl-CoA inhibition. Pig L-CPTI encodes for a 772 amino acid protein that shares 86 and 62% identity, respectively, with rat L- and M-CPTI. When expressed in *Pichia pastoris*, the pig L-CPTI enzyme shows kinetic characteristics (carnitine, $K_m = 126$ μM; palmitoyl-CoA, $K_m = 35$ μM) similar to human or rat L-CPTI. However, the pig enzyme, unlike the rat liver enzyme, shows a much higher sensitivity to malonyl-CoA inhibition ($IC_{50} = 141$ nM) that is characteristic of human or rat M-CPTI enzymes. 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.

Carnitine palmitoyltransferase I (CPTI),[1] an integral outer mitochondrial enzyme, catalyzes the first step in the entry of long-chain fatty acids into the mitochondrial matrix, where they undergo β-oxidation. CPTI catalyzes the conversion of long-chain acyl-CoAs to acylcarnitines in the presence of L-carnitine. The reaction catalyzed by CPTI is the rate-limiting step in fatty acid oxidation and is highly regulated by its physiological inhibitor, malonyl-CoA (*1*). 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 needs to be controlled.

CPTI is encoded by at least two structural genes referred to as L-CPTI and M-CPTI based upon the tissues of origin, liver (L) and muscle (M), in which the expression of these genes was first studied. However, L-CPTI is widely expressed with the highest level of expression in liver, kidney, ovary, spleen, pancreatic islets, intestine, and to a lesser extent, in heart. M-CPTI is only expressed in muscle, adipose tissue, heart, and testis. The two isotypes of CPTI differ markedly in their kinetic characteristics, namely the K_m for carnitine and the sensitivity to malonyl-CoA inhibition (*1*). These kinetic characteristics are retained when the cDNAs are expressed in a heterologous system (*2*−*6*, *17*). Thus, recombinant L-CPTI shows a lower K_m for carnitine and decreased sensitivity to malonyl-CoA inhibition (higher IC_{50}), while recombinant M-CPTI shows a higher K_m for carnitine and increased sensitivity to inhibition by malonyl-CoA (lower IC_{50}). Site-directed mutagenesis of conserved amino acid residues (*5*, *7*, *8*), N-terminal deletion analysis (*8*−*11*) and generation of chimeras between the amino and carboxyl end of L- and M-CPTI (*5*, *10*, *12*) have shown that the catalytic site resides in the carboxyl end of the enzyme and that the N-terminal region plays a critical role in malonyl-CoA sensitivity, since its deletion leads to loss of of malonyl-CoA inhibition.

The finding that pig L-CPTI is more than 20 times more sensitive to malonyl-CoA inhibition than rat L-CPTI (*13*, *14*) drew our attention to the CPTI isotype that is expressed in pig liver. Therefore, we isolated a full-length cDNA encoding pig L-CPTI and studied the kinetic characteristics of the *Pichia pastori* expressed enzyme.

EXPERIMENTAL PROCEDURES

Northern Blot Analysis. Pig tissues were obtained and subjected to Northern blot analysis as previously described (*15*). The pig L-CPTI probe consisted of a 1731-bp fragment (nucleotides 707−2438) obtained by reverse transcription-polymerase chain reaction (RT-PCR) with the forward primer

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[1] Abbreviations: EST, expressed sequence tags; CPTI, carnitine palmitoyltransferase I; L-CPTI, liver isoform of CPTI; M-CPTI, heart/skeletal muscle isoform of CPTI; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

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5′-GGGAAGAGTACATCTACCTCC-3′ and the reverse primer 5′-GAACAGTTTTTCATCCTGAAC-3′. The rat M-CPTI probe consisted of a *Xho*I−*Pst*I fragment (nucleotides 1078−1725) of the rat M-CPTI cDNA.

RT-PCR. Total liver RNA obtained from 2-week-old pigs starved for 48 h was used as the template for cDNA synthesis. All reactions were performed with M-MuLV polymerase and with 0.1 μg of random hexamers. The primers used were PigR1, 5′-GAACAGTTTTTCAT CCTGAAC-3′; PigR2, 5′-CGTAGTAGTTGCTGTTCACC-3′; GuessF1, 5′-AAGTTAAAATCCTGGTGGGC-3′; and GuessF2, 5′-GCCTTTCAGTTCACGGTCAC-3′.

cDNA Extension by RACE (Rapid Amplification of cDNA Ends). Poly(A)$^+$ mRNA from pig liver (2 μg) was the template in the first-strand synthesis, using a pig-specific reverse primer (RaceR1, 5′-CTCGGCGAACATCCAGC-CGTGGTAG-3′). After RNA degradation, an anchor sequence (5′-CACGAATTCACTATCGATTCTGGAACCT-TCAGAGGTGGGGCC-3′) was ligated to the 3′ end of the cDNA first strand with T4 RNA ligase. The 5′ end of pig L-CPTI was subsequently amplified by PCR with an anchor primer (RaceR1, 5′-GGCCCCACCTCTG AAGGTTC-3′) and a pig-specific reverse primer (RaceR2, 5′-CTTGG-CGTACATTGTCGAC ATGAC-3′) located upstream of RaceR1. The PCR products were cloned in the pGEM-T vector (Promega). The inserts of the resulting plasmids were subsequently sequenced. The SMART RACE cDNA amplification kit was from Clontech.

Cloning of the Pig L-CPTI Open Reading Frame. PCR primers were designed to amplify by RT-PCR the open reading frame (ORF) of pig L-CPTI using the pig liver cDNA library as the template. The forward primer was designed to generate an *Eco*RI restriction site 5′ of the ATG start codon. The reverse primer hybridized to a site 3′ of a unique *Xba*I site in pig L-CPTI. PCR primers were the following: forward, 5′-GAATTCACCATGGCAGAGGCTCAC-3′, and reverse, 5′-GCAGCTATGAACTGTTAACAC-3′. The PCR product was ligated as an *Eco*RI−*Xba*I fragment into BSSK$^+$ to produce pBS-PLCPTI. This plasmid contains the complete pig L-CPTI ORF under control of the T7 promoter. The ORF was sequenced to ensure that no mutation had been introduced by PCR.

Construction of Plasmids for CPT Expression in P. pastoris. An *Eco*RI site was introduced by PCR immediately 5′ of the ATG start codon of the pig L-CPTI cDNA to enable cloning into the unique *Eco*RI site located 3′ of the glyceraldehyde-3-phosphate dehydrogenase gene promoter (*GAPp*) in plasmid pHW010 (*16*, *17*). PCR primers were designed to generate a 228-bp fragment with a *Hin*dIII and an *Eco*RI restriction site immediately 5′ of the ATG start codon in the pig L-CPTI cDNA. The reverse primer was designed to introduce a *Hin*dIII restriction site. The PCR product was digested by *Hin*dIII and ligated into *Hin*dIII-cut pUC119 to produce pUC-5′PLCPTI. pUC-5′PLCPTI was then digested with *Bsu*36I and *Xba*I to introduce the remainder of the pig L-CPTI cDNA and excised from pBS-PLCPTI as a *Bsu*36I−*Xba*I fragment, producing pPUC-PLCPTI. This vector was digested with *Eco*RI to release a full-length CPTI fragment of 2489 bp, which was then ligated into *Eco*RI-cut pHW010 to produce pGAP-PLCPTI. pGAP-PLCPTI was linearized in the GAP gene promoter by digestion with *Pag*I and integrated into the *GAPp* locus of *P. pastoris* GS115 by electroporation (*8*). Histidine prototrophic transformants were selected on YND plates and grown on YND medium. Mitochondria were isolated by disrupting the yeast cells with glass beads as previously described (*7*, *17*).

CPT Assay. CPT activity was assayed by the forward exchange method using L-[^{3}H]carnitine as previously described (*17*). The standard assay reaction mixture contained in a total volume of 0.5 mL: 1 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, except for the determination of the IC_{50} where the incubation time was 5 min. 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 (*8*).

Generation of Anti-Pig L-CPTI Antibody. Antibody against amino acid residues 303−441 of pig L-CPTI was raised as follows: Two primers were used to amplify a 414-bp fragment. PCR primers were the following: forward, 5′-CAAATTGAATTCTGCTCCGCCCAGTGGG-3′, and reverse, 5′-ATTGAACTCGACGGACTTGGCGTAGCCG-3′. The forward primer introduced an *Eco*RI restriction site and the reverse primer introduced a *Xho*I restriction site. The PCR fragment was digested with *Eco*RI and *Xho*I and cloned into *Eco*RI−*Xho*I-cut pGEX-4T-1 to produce pGEX-PLCPTI. This plasmid was transformed into the *E. coli* strain DH5α. The fusion protein was induced for 3 h with 0.1 mM isopropyl-β-D-thiogalactoside and purified on glutathione Sepharose beads. The purified protein (200 μg) was used to immunize New Zealand white rabbits (*18*).

Western Blot Analysis. Proteins were separated by SDS−PAGE in a 7.5% gel and transferred onto nitrocellulose membranes as previously described (*17*). A 1:2000 dilution of the pig L-CPTI specific antibody was used. Proteins were detected using the ECL chemiluminescence system (Amersham Pharmacia Biotech).

In Vitro Transcription and Translation. The pBS−PLCPTI plasmid was used to produce pig L-CPTI proteins. To obtain the rat L-CPTI protein, the rat cDNA (*17*) was excised from the pHW010 plasmid as an *Eco*RI fragment and cloned into *Eco*RI-cut BSSK$^+$ under the control of the T7 promoter. Transcription and translation in the presence of [^{35}S]-methionine was performed with 1 μg of each plasmid using a commercially available kit according to the manufacturer's instructions (Promega).

DNA Sequencing. DNA sequencing was performed using the Big Dye kit (Applied Biosystems, Perkin-Elmer) according to the manufacturer's instructions.

RESULTS

The cDNA for pig L-CPTI was obtained by PCR-derived methods (RT-PCR and RACE) starting from a previously isolated truncated EST clone (*19*). The strategy employed to obtain the sequence of pig L-CPTI is outlined in Figure 1. The partial EST cDNA clone of pig L-CPTI contained 123 nucleotides from the ORF. On the basis of this sequence, a specific reverse primer (PigR1) annealing with nucleotides +2418 to +2438 of the L-CPTI cDNA [using the numeration

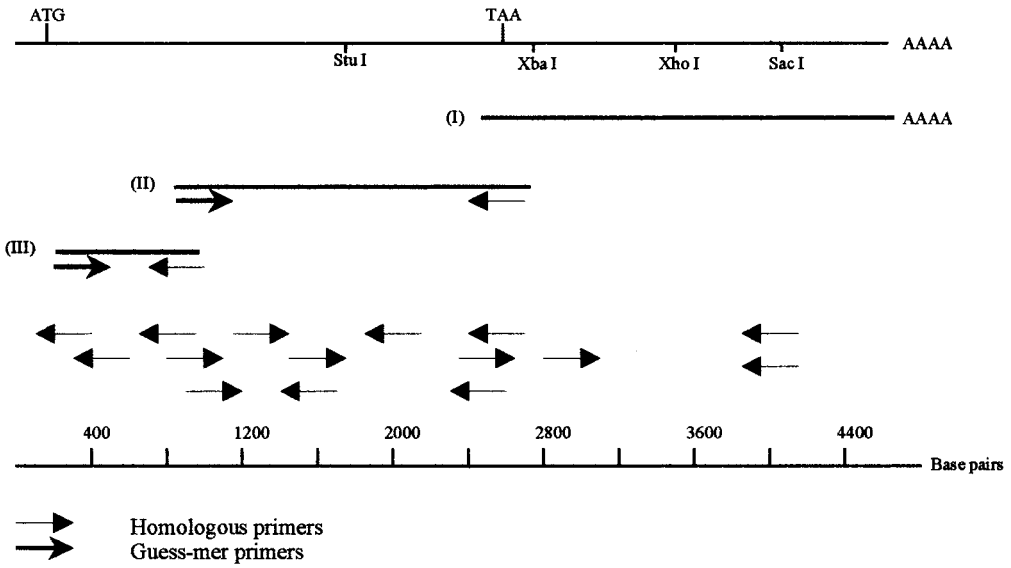

FIGURE 1: Sequencing strategy for pig L-CPTI. (I) represents the cDNA clone 11D1. (II) and (III) are the two PCR products which were obtained using the "Guess-mer" approach. The cDNA clone and at least two independent PCR products of each type (II and III) were fully sequenced.

of Esser et al. (*6*)] was designed. A "Guess-mer" forward primer, GuessF1, corresponding to nucleotides +664 to +685, was designed based on sequence alignment of the rat (*6*), mouse (*20*), and human (*21*) L-CPTI cDNAs. PigR1 and GuessF1 were used to PCR amplify a 1.7-kb fragment of the pig L-CPTI cDNA. This PCR product was sequenced and, based on the sequence obtained, a second pig L-CPTI-specific reverse primer, PigR2, annealing to nucleotides +747 to +767, was designed. A "Guess-mer" forward primer, GuessF2, annealing to nucleotides +25 to +45, was designed as previously described for the primer GuessF1. PigR2 and GuessF2 were used to PCR amplify an overlapping 742-bp fragment of the pig L-CPTI cDNA. This PCR product was sequenced.

Since PCR products and not isolated clones were sequenced, the risk of taking into account any mutation introduced by the PCR was negligible. Moreover, several overlapping PCR products were independently generated and sequenced to confirm the sequence.

5′-RACE was carried out to determine the nucleotide sequence of the 5′ region of the pig cDNA (Figure 2A). Two independent RACE experiments were performed. Both revealed the existence of two 5′-cDNA products. These two products are different at the 5′ end but share a common sequence from position −14. The largest extended to position −155 and the other to position −107. Dot blot analysis indicated a similar abundance of both RACE products in the RACE reaction (data not shown). Of the 25 RACE products analyzed, none contained both exons; hence, we deduced that these two exons are alternatively transcribed. The existence of the two 5′ alternative ends was confirmed by RT-PCR (Figure 2B). Thus, like human M-CPTI (*22*), pig L-CPTI has two alternative noncoding exons. Attempts to quantify the relative abundance of the two isotypes in vivo by primer extension experiments were unsuccessful, since the extension stopped at position −75 because of the high GC content of the sequence (data not shown).

Both the "Guess-mer" approach and the RACE method allowed determination of the sequence of full-length pig L-CPTI (GenBank accession number AF288789). The pig L-CPTI cDNA has an ORF of 2316 nucleotides and a 3′ untranslated region of 1955 nucleotides. A putative adenylation signal (AATAAA) was detected in the 3′ untranslated region at position +4247. The cDNA encodes for a protein of 772 amino acids with a predicted molecular mass of 88 kDa. The amino acid sequence shares extensive identity with rat (85.9%) and human (89.2%) L-CPTI, while the degree of identity with human and rat M-CPTI is much lower (62.3 and 61.7%, respectively).

Figure 3 shows that the pig L-CPTI transcript is 4.7 kb, the same size previously reported for the rat L-CPTI mRNAs (*6*). M-CPTI was detected in pig tissues using a heterologous rat probe. The pig M-CPTI mRNA is 3 kb, similar to that of M-CPTI from other mammals (*22*).

In 2-week-old piglets, L-CPTI is expressed in kidney, duodenum, liver, heart, and testis but not in muscle. The muscle isotype is expressed at high levels in heart and at lower levels in muscle but not in testis. To see if the absence of M-CPTI in testis was due to the age of the pig, Northern blot experiments were performed with 1-day-old to adult pig testis. Irrespective of the age of the animal, the liver isotype but not the muscle isotype was expressed in pig testis (results not shown).

The pig L-CPTI antibody strongly cross-reacted with yeast-expressed pig L-CPTI. The antibody also cross-reacted with rat L-CPTI and more weakly with human M-CPTI (Figure 4A). Despite the predicted size of 88 kDa, pig L-CPTI migrated as an 82-kDa protein, similar to that of human and rat M-CPTI (Figure 4; ref *23*). Thus, the anomalous behavior of the muscle isotype is also observed with pig L-CPTI. The in vitro transcribed and translated protein (Figure 4B) also migrated in a manner similar to that of the protein isolated from yeast mitochondria, suggesting as with M-CPTI (*23*) that this anomalous migration pattern is an intrinsic characteristic of the protein. It is interesting to note that all CPTs that have been reported to be highly sensitive to malonyl-CoA inhibition [i.e., rat (*23*) and human (Figure 4, ref *3*) M-CPTI] migrate faster than the CPTI protein that is less sensitive to malonyl-CoA.

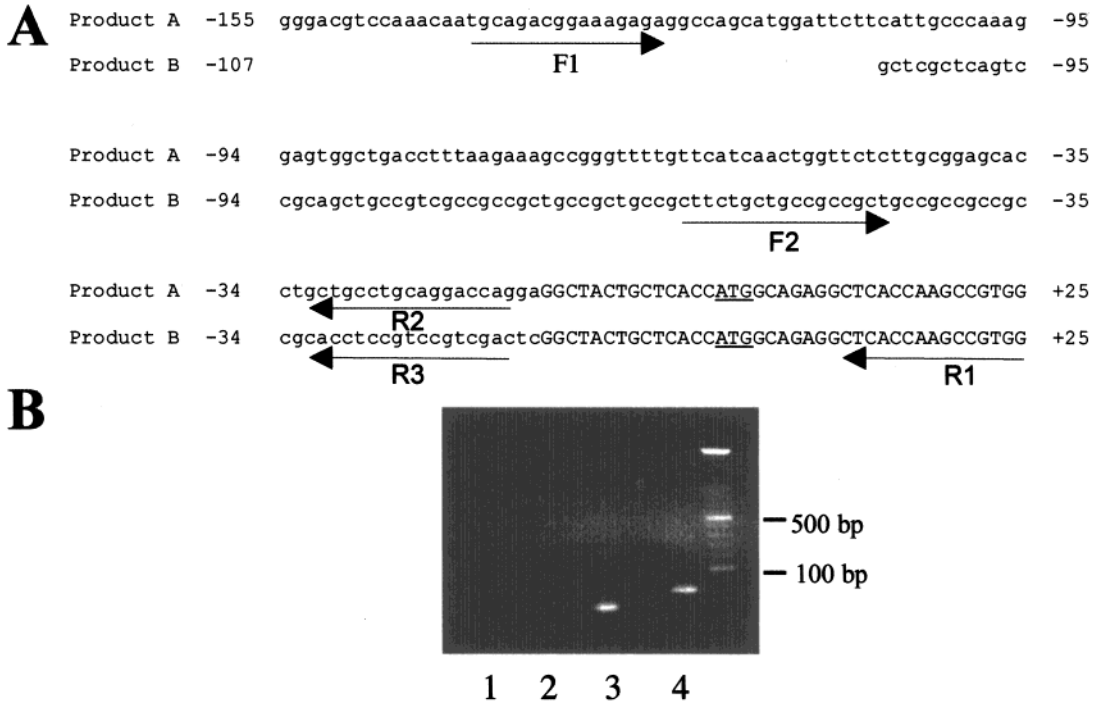

FIGURE 2: 5′ RACE products. (A) Nucleotide sequence of the two RACE products. The ATG initiation codon translation is underlined. The longest product extends to position −155, while the shortest extends to position −107. The exon containing the start codon starts at position −14. The arrows indicate the position of the primers. (B) Gel electrophoresis of the RT-PCR products. The reactions were performed using primers F1 and R3 (lane 1), F2 and R2 (lane 2), F2 and R1 (lane 3), and F1 and R1 (lane 4). The molecular weight standards of 500−100 base pairs (bp) are shown.

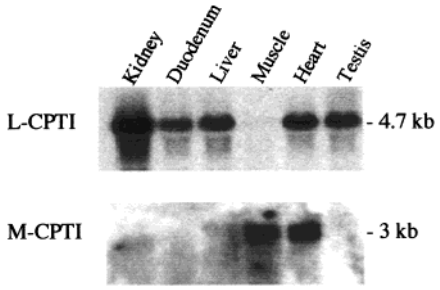

FIGURE 3: Northern blot showing tissue-specific expression of pig liver and muscle CPTI. Total RNA samples obtained from 3-week-old pigs were analyzed for L-CPTI and M-CPTI mRNA abundance. Equal loading of each lane was confirmed by the use of a β-actin probe (results not shown).

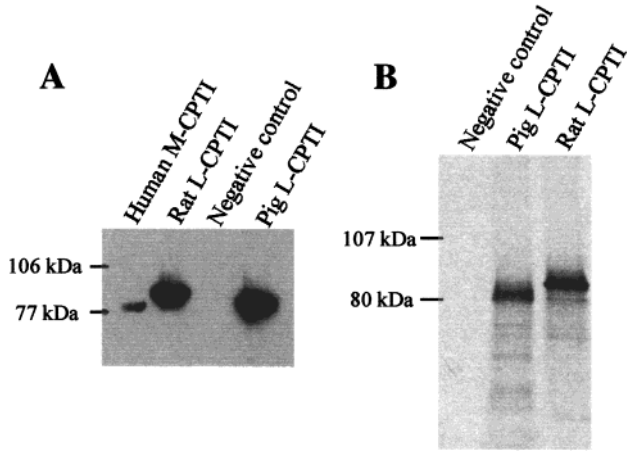

FIGURE 4: Mobility of CPTI proteins. (A) Immunoblot showing the expression and migration pattern of yeast-expressed CPTI proteins. Approximately 10 μg of protein were applied in each lane. The negative control was mitochondria from the yeast strain with the pHW010 vector but without the insert. (B) Mobility of in vitro synthesized ^{35}S-labeled pig and rat L-CPTI proteins. As a control, the transcription and transduction product of the plasmid without insert was used.

P. pastoris was chosen as a heterologous system to express pig L-CPTI, because other CPT proteins have been successfully expressed in this system (*3*, *17*). The pig L-CPTI cDNA was subcloned in the vector pHW010 and expressed under the control of the glyceraldehyde-3-phosphate dehydrogenase gene promoter (*16*). Yeast transformants were grown as previously described (*17*). No CPT activity was found in the control yeast strain with the vector but without the CPTI cDNA insert.

Isolated mitochondria from the CPTI expression strain had a high level of malonyl-CoA-sensitive CPT activity (5.24 ± 0.59 nmol/min/mg of protein). Yeast-expressed pig L-CPTI exhibited normal saturation kinetics with respect to its two substrates, palmitoyl-CoA and carnitine, as shown in Figure 5, panels A and B. The K_m for palmitoyl-CoA was 35.44 ± 1.96 μM. The K_m for carnitine was 126.34 ± 4.32 μM. Thus, pig L-CPTI exhibited kinetic characteristics very similar to those of rat L-CPTI (*7*, *24*) with respect to palmitoyl-CoA and carnitine. However, pig L-CPTI was much more sensitive to malonyl-CoA inhibition than rat L-CPTI, since the IC_{50} for malonyl-CoA was 141.50 ± 6.36 nM, thus behaving with respect to malonyl-CoA sensitivity more like the muscle isotype (Figure 5C, Table 1). Therefore, pig L-CPTI behaves like a natural chimera of the L-CPTI and M-CPTI isotypes.

Pig L-CPTI exhibits a high degree of homology with CPTs from other mammals (Figure 6). Interestingly, the N-terminal end and the transmembrane domains (TM1 and TM2) are highly homologous to other liver CPTIs. The sequence alignment also shows the presence of an arginine residue at

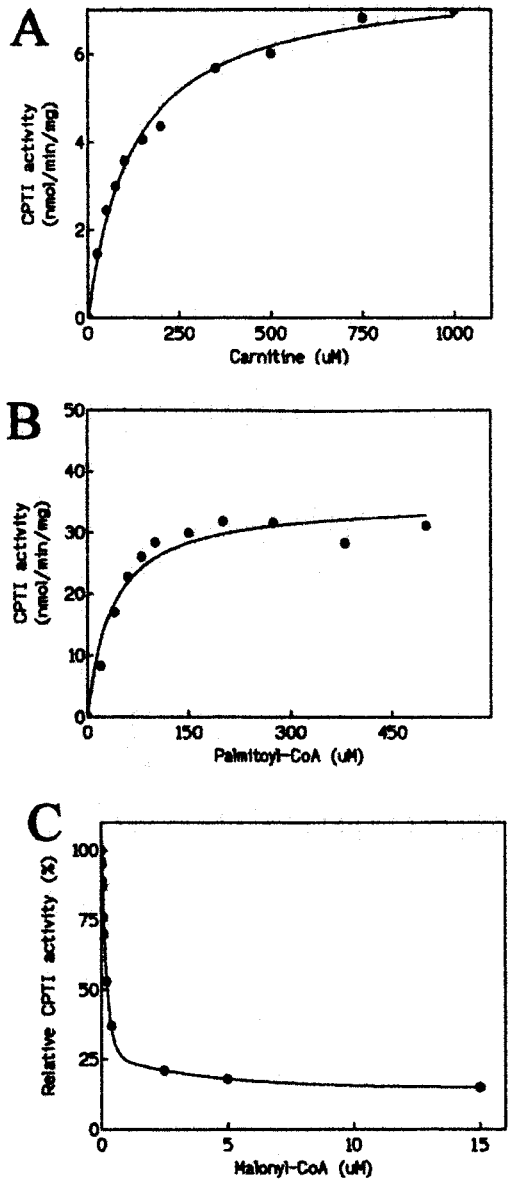

FIGURE 5: Kinetic analysis of pig L-CPTI. Isolated mitochondria (150 μg of protein) were assayed for CPTI activity in the presence of increasing concentrations of carnitine (A), palmitoyl-CoA (B), and malonyl-CoA (C), as described in the Experimental Procedures.

Table 1: Kinetic Characteristics of Pig, Rat, and Human CPTIs[a]

	IC_{50} for malonyl-CoA (nM)	K_m for carnitine (μM)	K_m for palmitoyl-CoA (μM)
pig L-CPTI	141	126	35
rat L-CPTI	1900	100	43
human M-CPTI	69	666	42

[a] Mitochondria (150 μg) from the yeast strains expressing pig L-CPTI, rat L-CPTI, and human M-CPTI were assayed for CPT activity and malonyl-CoA sensitivity as described in the Experimental Procedures. IC_{50} is the concentration of malonyl-CoA needed to inhibit 50% of the activity of the yeast-expressed CPTIs.

position 297, which is present in all liver isotypes with low malonyl-CoA sensitivity but absent in pig L-CPTI and the muscle isotypes. To investigate the role of this arginine residue on malonyl-CoA sensitivity, we generated a human M-CPTI mutant with an arginine residue inserted at position 297. This mutant behaved like the wild-type human M-CPTI in terms of its malonyl-CoA sensitivity (results not shown).

DISCUSSION

Studies reporting a very low rate of β-oxidation (*13*) and ketogenesis (*13*, *25*−*27*) in newborn pig liver clearly indicate that perinatal changes in lipid metabolism in pigs differ markedly from those described in other mammals. Previous studies with isolated mitochondria showed that pig L-CPTI was >20 times more sensitive to malonyl-CoA inhibition than rat L-CPTI (*13*, *14*). Hence, it can be postulated that, in newborn pigs, the high sensitivity to malonyl-CoA inhibition of pig L-CPTI could be due to the expression of a L-CPTI gene with different kinetic characteristics than the previously studied rat L-CPTI.

The full-length pig L-CPTI cDNA spans 4271 nucleotides and encodes a protein of 772 amino acids with a predicted molecular mass of 88 kDa. The predicted amino acid sequence shares extensive homology with rat (85.9%) and human (89.2%) L-CPTI, while the degree of identity with human (62.3%) and rat (61.7%) M-CPTI is much lower. The pig L-CPTI mRNA is of the same length (4.7 kb) and exhibits a tissue expression pattern similar to those of the human and rat L-CPTI mRNAs.

Pig L-CPTI has two exons 5′ to the exon containing the ATG translation initiation codon. These two exons are transcribed in an alternative fashion, thus suggesting the possibility of alternative gene promoters or of an alternative splicing after initiation of transcription from an unidentified exon(s). The two transcripts that are generated by this alternative splicing encode for the same protein. Attempts to determine the transcription initiation by primer extension or by ribonuclease protection assay were unsuccessful, probably because of the high GC content of the pig L-CPTI 5′-untranslated regions. Alternative splicing has also been observed with rat L-CPTI (*28*).

The kinetic characteristics of yeast-expressed pig L-CPTI, namely the K_ms for carnitine and palmitoyl-CoA, were found to be similar to those of human and rat L-CPTI (Table 1). However, with respect to malonyl-CoA sensitivity, pig L-CPTI behaved like the M-CPTI enzyme. The low IC_{50} value obtained with the yeast-expressed enzyme is similar to the IC_{50} values observed in isolated pig liver mitochondria (*13*, *14*). Thus, isolated mitochondria from the yeast strain expressing the pig L-CPTI cDNA exhibited a low K_m for carnitine and a low IC_{50} for malonyl-CoA, characteristics similar to those of rat L-CPTI and M-CPTI, respectively. In adult rat liver mitochondria, the total carnitine concentration is low (*29*); consequently, L-CPTI has a low K_m for carnitine, which also appears to be the case for pig L-CPTI. The structural basis for the differences in malonyl-CoA sensitivity between L-and M-CPTI is not known, but chimera studies suggest that this may be an intrinsic property of the CPTI isotypes. This is the first report of a CPT protein behaving like a natural chimera between the liver and muscle isotypes as previously described (Table 1). Therefore, pig L-CPTI is an ideal model for structure−function studies of the substrate and malonyl-CoA binding sites. Since these observations were made with yeast-expressed CPTI, our results clearly establish that the kinetic characteristics obtained are an intrinsic property of the protein.

Previous experiments suggest that the interaction between the two putative trans-membrane domains of CPTI enzymes (TM1 and TM2) is important in determining the K_m for

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          1               15 16              30 31              45 46              60 61              75 76              90
RATM      MAEAHQAVAFQFTVT PDGVDFRLSREALRH IYLSGINSWKKRLIR IKNGILRGVYPGSPT SWLVVVMATVGSNYC KVDISMGLVHCIQRC  90
MOUSEM    ----HQAVAFQFTVT PDGVDFRLSREALRH IYLSGINSWKKRLIR IKNGILRGVYPGSPT SWLVVVMATVGSNYC KVDISMGLVDCIQRC  86
HUMANM    MAEAHQAVAFQFTVT PDGVDFRLSREALKH VYLSGINSWKKRLIR IKNGILRGVYPGSPT SWLVVIMATVGSSFC NVDISLGLVSCIQRC  90
PIGL      MAEAHQAVAFQFTVT PDGIDLRMSHEALRQ IYLSGLHSWKKKFIR FKNGIITGVFPASPS SWLIVVVGVMSTMYA KIDPSLGVIAKINRT  90
HUMANL    MAEAHQAVAFQFTVT PDGIDLRLSHEALRQ IYLSGLHSWKKKFIR FKNGIITGVYPASPS SWLIVVVGVMTTMYA KIDPSLGIIAKINRT  90
RATL      MAEAHQAVAFQFTVT PDGIDLRLSHEALKQ ICLSGLHSWKKKFIR FKNGIITGVFPANPS SWLIVVVGVISSMHA KVDPSLGMIAKISRT  90

                                                                                  TM1

          91          105 106         120 121         135 136         150 151         165 166         180
RATM      LPTRYGSYGTPQTET LLSMVIFSTGVWATG IFLFRQTLKLLLSYH GWMFEMHSKTSHATK IWAICVRLLSSRRPM LYSFQTSLPKLPVPS 180
MOUSEM    LPERYGHFGTPQTEA LLSMVIFSTGVWATG IFFFRQTLKLLLSYH GWMFEMHSKTSHATK IWAICVRLLSSRRPM LYSFQTSLPKLPVPS 176
HUMANM    LPQGCGPYQTPQTRA LLSMAIFSTGVWVTG IFFFRQTLKLLLCYH GWMFEMHGKTSNLTR IWAMCIRLLSSRHPM LYSFQTSLPKLPVPR 180
PIGL      LDATG--YLSSRTQN VVSGVLFGTGLWVAL IVTMRYSLKVLLSYH GWMFAEHSKMSRATK IWMMMVRVFSGRKTM LYSFQTSLPRLPVPA 178
HUMANL    LETAN--CMSSQTKN VVSGVLFGTGLWVAL IVTMRYSLKVLLSYH GWMFTEHGKMSRATK IWMGMVKIFSGRKPM LYSFQTSLPRLPVPA 178
RATL      LDTTG--RMSSQTKN IVSGVLFGTGLWVAV IMTMRYSLKVLLSYH GWMFAEHGKMSRSTK IWMAMVKVLSGRKPM LYSFQTSLPRLPVPA 178

                    TM2

          181         195 196         210 211         225 226         240 241         255 256         270
RATM      VPATIHRYLDSVRPL LDDEAYFRMESLAKE FQDKIAPRLQKYLVL KSWWATNYVSDWWEE YVYLRGRSPIMVNSN YYAMDFVLIKNTSQQ 270
MOUSEM    VPATIHRYLDSVRPL LDDEAYYRMETLAKE FQDKTAPRLQKYLVL KSWWATNYVSDWWEE YVYLRSRSPLMVNSN YYAMDFVLIKNTNVQ 266
HUMANM    VSATIQRYLESVRPL LDDEEYYRMELLAKE FQDKTAPRLQKYLVL KSWWASNYVSDWWEE YIYLRGRSPLMVNSN YYVMDLVLIKNTDVQ 270
PIGL      VQDTVSRYLESVKPL MKEAEFKRMTALAQD FAVSLGPRLQWYLKL KSWWATNYVSDWWEE YIYLRGRGPLMVNSN YYAMDLLYITPTHIQ 268
HUMANL    VKDTVNRYLQSVRPL MKEEDFKRMTALAQD FAVGLGPRLQWYLKL KSWWATNYVSDWWEE YIYLRGRGPLMVNSN YYAMDLLYILPTHIQ 268
RATL      VKDTVSRYLESVRPL MKEEDFQRMTALAQD FAVNLGPKLQWYLKL KSWWATNYVSDWWEE YIYLRGRGPLMVNSN YYAMEMLYITPTHIQ 268

          271         285 286         300 301         315 316         330 331         345 346         360
RATM      AARLGNTVHAMIMYR RKLDREEIKPV-MAL GMVPMCSYQMERMFN TTRIPGKETDLLQHL SESRHVAVYHKGRFF KVWLYEGSCLLKPRD 359
MOUSEM    AARLGNAVHAMIMYR RKLDREEIKPV-MAL GMVPMCSYQMERMFN TTRIPGKETD----- --------------- --------------- 320
HUMANM    AARLGNIIHAMIMYR RKLDREEIKPV-MAL GIVPMCSYQMERMFN TTRIPGKDTDVLQHL SDSRHVAVYHKGRFF KLWLYEGARLLKPQD 359
PIGL      AARAGNGIHAILLYR RKLDREEIKPI-LLG STVPLCSAQWERMFN TSRIPGEETDTIQHL RDSKHIVVFHRGRYF KVWLYYDGRLLKPRE 357
HUMANL    AARAGNAIHAILLYR RKLDREEIKPIRLLG STIPLCSAQWERMFN TSRIPGEETDTIQHM RDSKHIVVYHRGRYF KVWLYHDGRLLKPRE 358
RATL      AARAGNTIHAILLYR RTLDREELKPIRLLG STIPLCSAQWERLFN TSRIPGEETDTIQHI KDSRHIVVYHRGRYF KVWLYHDGRLLRPRE 358

          361         375 376         390 391         405 406         420 421         435 436         450
RATM      LEMQFQRILDDTSPP QPGEEKLAALTAGGR VEWAEARQKFFSSGK NKMSLDTIERAAFFV ALDEDSHCYNPDDEA S-LSLYGKSLLHGNC 448
MOUSEM    --------------- --------------- --------------- --------------- --------------- --------------- 320
HUMANM    LEMQFQRILDDPSPP QPGEEKLAALTAGGR VEWAQARQAFFSSGK NKAALEAIERAAFFV ALDEESYSYDPEDEA S-LSLYGKALLHGNC 448
PIGL      IEQQMQRILDDPSEP QPGEAKLAALTAGDR VPWANCPQAYFGHGK NKQSLDAVEKAAFFV TLDETEQGYRKEDPD RSMDGYAKSLLHGQC 447
HUMANL    MEQQMQRILDNTSEP QPGEARLAALTAGDR VPWARCRQAYFGRGK NKQSLDAVEKAAFFV TLDETEEGYRSEDPD TSMDSYAKSLLHGRC 448
RATL      LEQQMQQILDDPSEP QPGEAKLAALTAADR VPWAKCRQTYFARGK NKQSLDAVEKAAFFV TLDESEQGYREEDPE ASIDSYAKSLLHGRC 448

          451         465 466         480 481         495 496         510 511         525 526         540
RATM      YNRWFDKSFTLISCK NGQLGLNTEHSWADA PIIGHLWEFVLATDT FHLGYTETGHCVGEP NTKLPPPQRMQWDIP EQCQTAIENSYQVAK 538
MOUSEM    --------------- --------------- --------------- --------------- --------------- --------------- 320
HUMANM    YNRWFDKSFTLISFK NGQLGLNAEHAWADA PIIGHLWEFVLGTDS FHLGYTETGHCLGKP NPALAPPTRLQWDIP KQCQAVIESSYQVAK 538
PIGL      FDRWFDKSFTFIVFK NGKMGMNAEHSWADA PIVAHLWEYVMSIDS FQLGYEEDGHCKGDT NPNIPYPTRLQWDIP EECQEVIETSLSCAN 537
HUMANL    YDRWFDKSFTFVVFK NGKMGLNAEHSWADA QIVAHLWEYVMSIDS LQLGYAEDGHCKGDI NPNIPYPTRLQWDIP GECQEVIETSLNTAN 538
RATL      FDRWFDKSITFVVFK NSKIGINAEHSWADA PIVGHLWEYVMATDV FQLGYSEDGHCKGDT NPNIPKPTRLQWDIP GECQEVIDASLSSAS 538

          541         555 556         570 571         585 586         600 601         615 616         630
RATM      ALADDVELYCFQFLP FGKGLIKKCRTSPDA FVQIALQLAHFRDKG KFCLTYEASMTRMFR EGRTETVRSCTSEST AFVRAMMTGSHKKQD 628
MOUSEM    --------------- --------------- --------------- --------------- --------------- --------------- 320
HUMANM    ALADDVELYCFQFLP FGKGLIKKCRTSPDA FVQIALQLAHFRDRG KFCLTYEASMTRMFR EGRTETVRSCTSEST AFVQAMMEGSHTKAD 628
PIGL      LLADDVDFHSFPFTT FGKGLIKKCRTSPDA FVQLALQLAHYKDMG KFSLTYEASMTRLFR EGRTETVRSCTTESC NFVLAMVDPTQPVEQ 627
HUMANL    LLANDVDFHSFPFVA FGKGIIKKCRTSPDT FVQLALQLAHYKDMG KFCLTYEASMTRLFR EGRTETVRSCTTESC DFVRAMVDPAQTVEQ 628
RATL      LLANDVDLHSFPFDS FGKGLIKKCRTSPDA FIQLALQLAHYKDMG KFCLTYEASMTRLFR EGRTETVRSCTMESC NFVQAMMDPKSTAEQ 628

          631         645 646         660 661         675 676         690 691         705 706         720
RATM      LQDLFRKASEKHQNM YRLAMTGAGIDRHLF CLYIVSKYLGVRSPF LDEVLSEPWSLSTSQ IPQFQICMFDPKQYP NHLGAGGGFGPVADH 718
MOUSEM    --------------- --------------- --------------- --------------- --------------- --------------- 320
HUMANM    LRDLFQKAAKKHQNM YRLAMTGAGIDRHLF CLYLVSKYLGVSSPF LAEVLSEPWRLSTSQ IPQSQIRMFDPEQHP NHLGAGGGFGPVADD 718
PIGL      KLKLFKIAAEKHQHL YRLAMTGSGVDRHLF CLYVVSKYLAVDSPF LKEVLFEPWRLSTSQ TPRQQVELFDLEKNP EYVSSGGGFGPVADD 717
HUMANL    RLKLFKLASEKHQHM YRLAMTGSGIDRHLF CLYVVSKYLAVESPF LKEVLSEPWRLSTSQ TPQQQVELFDLENNP EYVSSGGGFGPVADD 718
RATL      RLKLFKIACEKHQHL YRLAMTGAGIDRHLF CLYVVSKYLAVDSPF LKEVLSEPWRLSTSQ TPQQQVELFDFEKNP DYVSCGGGFGPVADD 718

          721         735 736         750 751         765 766
RATM      GYGVSYMIAGENTMF FHVSSKLSSSETNAL RFGNHIRQALLDIAD LFKISKTDS- 772
MOUSEM    --------------- --------------- --------------- ---------- 320
HUMANM    GYGVSYMIAGENTIF FHISSKFSSSETNAQ RFGNHIRKALLDIAD LFQVPKAYS- 772
PIGL      GYGVSYIIVGDHLIS FHISSKFSCPETDSH RFGKHLKQAMTDIIG LFSFCPNSKK 772
HUMANL    GYGVSYILVGENLIN FHISSKFSCPETDSH RFGRHLKEAMTDIIT LFGLSSNSKK 773
RATL      GYGVSYIIVGENFIH FHISSKFSSPETDSH RFGKHLRQAMMDIIT LFGLTINSKK 773
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FIGURE 6: Sequence alignments of CPTI proteins. Shaded areas show the two transmembrane domains (TM1 and TM2). Sequences were obtained from the following sources: rat M-CPTI (RATM) (*23*); mouse M-CPTI (MOUSEM) (*20*) human M-CPTI (HUMANM) (*32*); human L-CPTI (HUMANL) (*21*); and rat L-CPTI (RATL) (*6*); pig L-CPTI (PIGL) (this paper).

carnitine (*10*, *12*). The behavior of pig L-CPTI, with a low carnitine K_m, agrees with this hypothesis, since its transmembrane domains line up better with L-CPTI than M-CPTI isotypes (see Figure 6). It was also suggested that a basic residue (Arg-52) present within TM1 could be responsible for the different behavior of M- and L-isotypes (*12*).

However, our data does not support this hypothesis since Pig L-CPTI, that in terms of malonyl-CoA sensitivity behaves as a M-CPT I, has a tyrosine and not an arginine residue at this position. The discovery that a liver isotype of CPTI behaves like a muscle isotype with respect to malonyl-CoA sensitivity but retains the other kinetic characteristics of the liver isotypes suggests the presence of separate, independent, nonoverlapping malonyl-CoA and carnitine binding sites. However, since the K_m for palmitoyl-CoA is similar for both the liver and muscle isotypes, suggesting a common or overlapping site, our pig L-CPTI natural chimera must also retain a similar palmitoyl-CoA binding site.

During this study, we became aware of some published reports of yeast-expressed CPTs with extremely low sensitivity to malonyl-CoA inhibition (*12*, *30*). IC_{50}s of 55 μM for L-CPTI and 3 μM for M-CPTI were reported, using the expression vector pGAPZ (Invitrogen, San Diego, CA) and the *P. pastoris* strain X-33. These values are extremely high, since the IC_{50} calculated for isolated rat liver or muscle mitochondria are 2.7 μM and 34 nM, respectively (*31*). The authors attributed the high IC_{50} values to differences between CPTI environments in *P. pastoris* and mammalian mitochondria and/or differences in assay conditions. When we first tried to express pig L-CPTI in *P. pastoris* we used the same system: the cDNA and 5′ nucleotides upstream of the start codon [Kozac consensus sequence (*32*)] were cloned into pGAPZ. Our yeast-expressed pig L-CPTI was active, but the enzyme was highly insensitive to malonyl-CoA inhibition (IC_{50} = 10 μM). We then subcloned the cDNA without the 5′ noncoding region into the pHW010 vector, and the IC_{50} of yeast-expressed pig L-CPTI was very similar to that which had been calculated for isolated pig liver mitochondria (*13*, *14*). We had previously observed the same phenomenon with human M-CPTI where the IC_{50} changed from 500 μM with the multicopy vector pPICZ-B system to 69 nM with the single copy vector pHW010 (*3*, *4*). The underlying mechanisms for the change in IC_{50} for malonyl-CoA inhibition of the CPTs with the different vectors are under investigation in our laboratory. Nevertheless, to avoid expressing CPT proteins with anomalous behavior we believe it is preferable to use the published procedures described in the Experimental Procedures for expressing the CPTI isotype in *P. pastoris* (*3*, *16*, *17*).

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