Pig Muscle Carnitine Palmitoyltransferase I (CPTIβ), with Low K_m for Carnitine and Low Sensitivity to Malonyl-CoA Inhibition, Has Kinetic Characteristics Similar to Those of the Rat Liver (CPTIα) Enzyme[†]

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Received June 3, 2004; Revised Manuscript Received July 22, 2004

ABSTRACT: The outer mitochondrial membrane enzyme carnitine palmitoyltransferase I (CPTI) catalyzes the initial and regulatory step in the β-oxidation of long-chain fatty acids. There are two well-characterized isotypes of CPTI: CPTIα (also known as L-CPTI) and CPTIβ (also known as M-CPTI) that in human and rat encode for enzymes with very different kinetic properties and sensitivity to malonyl-CoA inhibition. Kinetic hallmarks of the CPTIα are high affinity for carnitine and low sensitivity to malonyl-CoA inhibition, while the opposite characteristics, low affinity for carnitine and high sensitivity to malonyl-CoA, are intrinsic to the CPTIβ isotype. We have isolated the pig CPTIβ cDNA which encodes for a protein of 772 amino acids that shares extensive sequence identity with human (88%), rat (85%), and mouse (86%) CPTIβ, while the degree of homology with the CPTIα from human (61%), rat (62%), and mouse (60%) is much lower. However, when expressed in the yeast *Pichia pastoris*, pig CPTIβ shows kinetic characteristics similar to those of the CPTIα isotype. Thus, the pig CPTIβ, unlike the corresponding human or rat enzyme, has a high affinity for carnitine (K_m = 197 μM) and low sensitive to malonyl-CoA inhibition (IC_{50} = 906 nM). Therefore, the recombinant pig CPTIβ has unique kinetic characteristics, which makes it a useful model to study the structure−function relationship of the CPTI enzymes.

Short- and medium-chain fatty acids freely diffuse from the cytosol to the mitochondrial matrix. However, the mitochondrial inner membrane is impermeable to long-chain acyl-CoAs. Translocation of long-chain acyl-CoAs across the mitochondrial membrane requires a specific transport system, referred to as the carnitine palmitoyltransferase (CPT) shuttle system, composed of three enzymes, namely, CPTI,[1] carnitine/acylcarnitine translocase, and CPTII. CPTI catalyzes the initial step of this process by converting long-chain acyl-CoAs into acylcarnitine esters in the presence of L-carnitine. Acylcarnitine esters are transported across the inner mitochondrial membrane in exchange for carnitine by the carnitine/acylcarnitine translocase. Finally, CPTII catalyzes the transfer of the acyl group from acylcarnitine to CoA and regenerates the long-chain acyl-CoAs, thus providing substrate for β-oxidation. Because CPTI catalyzes the first rate-limiting step in fatty acid oxidation, it is tightly regulated by its physiological inhibitor, malonyl-CoA (*1*).

CPTI is encoded by at least three structural genes referred to as CPTIα, CPTIβ, and CPTI-C. The expression of CPTI-C seems to be restricted to the central nervous system, and to date, expression of the cDNA for CPTI-C in yeast has resulted in no enzyme activity (*2*). While CPTIα is widely expressed in most tissues, CPTIβ is only expressed in muscle, adipose tissue, heart, and testis (*1*). Studies performed with cDNAs isolated from a variety of mammals (*3*−*9*) have shown that both enzymes differ markedly in their kinetic characteristics, namely, the K_m for carnitine and the sensitivity to malonyl-CoA inhibition. CPTIα exhibits a low K_m for carnitine and decreased sensitivity to malonyl-CoA inhibition (higher IC_{50}), while CPTIβ exhibits a high K_m for carnitine and increased sensitivity to inhibition by malonyl-CoA (lower IC_{50}). Splice variation in the coding region of the CPTIβ gene was proposed to play a major role in the expression of CPTI enzymes with altered malonyl-CoA sensitivity, but the involvement of splice variation in the modulation of malonyl-CoA sensitivity of CPTIβ has now been ruled out (*10*). However, the cloning of pig CPTIα (*11*, *12*) clearly provided evidence that not all CPTIα have a high IC_{50} for malonyl-CoA inhibition, because pig CPTIα was found to be extremely sensitive to malonyl-CoA inhibition (IC_{50} 141 nM) when compared with rat CPTIα (IC_{50} 1900 nM). The high malonyl-CoA sensitivity observed could be due to the low

[†] This project was supported by grants from the Ministerio de Ciencia y Tecnología (SAF2001-2923, Programa Nacional de Biomedicina), from the FIS of the Instituto de Salud Carlos III, Red de Centros RCMN from the Ministerio de Sanidad (C03/08), and from the Ajut de Suport als Grups de Recerca de Catalunya (2001SGR 00432) and by NIH Grant HL52571 (to G.W.).

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

10.1021/bi0488597 CCC: $27.50
Published on Web 09/04/2004

malonyl-CoA levels in this tissue as a result of low rates of hepatic lipogenesis (*13*).

There is very little information about porcine CPTIβ. We previously showed using heterologous probes that CPTIβ is expressed in muscle and heart of pig (*11*). Studies performed with mitochondria isolated from pig muscle showed that, in this tissue, CPTI activity has an affinity for carnitine intermediate between that of rat CPTIα and human CPTIβ described to date (*14*) and that this activity is not very sensitive to malonyl-CoA inhibition (*15*). Hence, we decided to clone the pig CPTIβ and determine its kinetic characteristics to get a better understanding of the structure−function relationships of the CPTI enzymes and of the peculiar porcine lipid metabolism.

EXPERIMENTAL PROCEDURES

Amplification of a Pig CPTIβ cDNA Probe by RT-PCR. Total muscle RNA obtained from 2-week-old pigs was used as a template in a reverse transcriptase reaction performed with M-MulV polymerase and with 0.1 μg of random hexamers. A cDNA fragment, from nucleotide +21 to +726 (using adenine of the start codon as nucleotide +1), of pig CPTIβ was then amplified by PCR from the RT reaction products by using two degenerated primers: OCD1 (5′-GCNGTNGCNTTYCAGTTCAC) and OCD2 (5′-AGGTAGAYRTAYTCYTCCCACCAGTC). These primers were kindly provided by Dr. Feike R. Van Der Leij (Department of Pediatrics, University of Groningen, The Netherlands) and were previously useful to amplify ovine CPTI (*9*). The PCR product was cloned in the pGEM-T vector (Promega), and the resulting plasmid was sequenced. BLAST analysis showed that this cDNA shares extensive identity with human (83%) or rat (84%) CPTIβ, while the degree of identity with human (55%) or rat (53%) CPTIα is much lower. In addition, subsequent database search showed this sequence to have 99.8% identity with a yet unpublished clone (gi: 27414164) characterized as pig M-CPTI. This sequence was used as a probe in the northern blot analysis and also for primer design in the PCR cloning of the ORF of pig CPTIβ.

Northern Blotting. Total RNA was isolated from testis and heart of piglets from different ages and subjected to northern blot analysis as previously described (*16*). The membranes were probed with random-primed [^{32}P]dCTP-labeled CPTIα (*11*) or CPTIβ probes. A 596 bp fragment of the human β-actin probe (E00829) was obtained from CaCo-2 cell line RNA by using the primers actin-F (5′-ACCAACTGGGACGACATGGAC) and actin-R (5′-TCGTGGATGCCACAGGACTCC). Transcript size was determined using 0.28−6.58 kb of RNA marker (Promega).

cDNA Extension by SMART RACE (Clontech). This technique was used according to the manufacturer's instructions to amplify the 3′ end of pig CPTI-β using the forward primer PMCPTI-F701 [5′-CAAGTCATGGTGGGCGACTAACTATGTG (+676 to +701)] and poly(A) mRNA from pig muscle (2 μg) as template. The PCR product (1743 bp) was cloned in the vector pGEM-T (Promega) and sequenced. The generated plasmid, p3PMCPTIβGEMT, contains the pig CPTIβ cDNA from position +676 to +2419.

Pig CPTIβ cDNA Cloning. PMCPTI-F1 (5′-CTGACTAAACCCCAGGATG, a "guess-mer" forward primer corresponding to coordinates −15 to +3) and PMCPTI-R1 (5′-GTATTCCTCGTCATCCAG, a reverse primer corresponding to coordinates +604 to +586) were used to amplify a 619 bp fragment of the pig CPTIβ 5′cDNA. The RT-PCR product sequence was used to design the forward primer PMCPTI-F6 needed in the cloning strategy. Then the 5′ end of the cDNA was amplified by RT-PCR using the primers PMCPTI-F6 [5′-*CAAATT**GAATTC***ATGGCGGAAGCGCACCAG (+1/+18)] and PMCPTI-R7 [5′-GCTGTCTGTGAGGTGGTG (+1000/+979)]. The primer PMCPTI-F6 introduces an *Eco*RI restriction site (italicized and bolded in the primer sequence) just before the ATG start codon. This PCR product was digested with *Eco*RI and *Bam*HI (which cuts the pig MCPTI cDNA at position +950) and cloned into BSSK+, forming p5PMCPTI/BSSK+.

The RACE 3′ product was excised from p3PMCPTIGEMT by digestion with *Sac*I and *Aat*II (present in the pGEM-T polylinker), and the ends were filled in with Klenow DNA polymerase and cloned into *Eco*RV-cut BSSK+. The insert was released by digestion with *Bam*HI and ligated into *Bam*HI-cut 5PMCPTI/BSSK+, resulting in the plasmid pPMCPTI/BSSK+. This plasmid contains the pig CPTIβ cDNA sequence from position +1 to +2419. This plasmid was further manipulated to remove most of the 3′ UTR sequence of pig CPTIβ cDNA. pPMCPTI/BSSK+ was used as a template in a PCR reaction with Pigfw [5′-CGTGAAAGCAGACCTGCAAG (+1872 to +1892)] and Pigrv [*ACTG**GAATTC***GCCCTTAGCTGTCAGCTTTGGGAAC (+2323 to +2298)] to generate a 925 bp product, in which an *Eco*RI site (bolded in the Pigrv primer sequence) was introduced downstream of the stop codon (underlined in the Pigrv sequence). The PCR product was subcloned in the vector p-GEM-T and sequenced. The plasmid was digested with *Not*I and *Bgl*II, generating a 444 bp product, and subcloned in *Not*I−*Bgl*II-cut PMCPTI/BSSK, yielding pPMCPTISTOP/BSSK$^+$, which contains the ORF of the pig CPTIβ (from +1 to +2323) flanked by two *Eco*RI sites.

Construction of Plasmids for CPT Expression in Pichia pastoris. The ORF of pig CPTIβ cDNA was cloned into the unique *Eco*RI site, located 3′ of the glyceraldehyde-3-phosphate dehydrogenase gene promoter (*GAPp*), in the pHW010 plasmid (*8*, *17*), to produce the expression plasmid pPMCPTI-β/pHW010. This construct was linearized in the GAP gene promoter by digestion with *Avr*II and integrated into the *GAPp* locus of *P. pastoris* GS115 by electroporation (*18*). 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 (*8*, *19*). Previously reported expression plasmids [pig CPTIα (*11*), human CPTIβ (*4*), and rat CPTIα (*8*)] were processed in a similar way to obtain a mitochondrial preparation expressing the respective recombinant enzyme.

CPT Assay. CPT activity was assayed by the forward exchange method using L-[^{3}H]carnitine as previously described (*8*). In a total volume of 0.5 mL, the standard enzyme assay mixture contained 0.2 mM L-[^{3}H]carnitine (~5000 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. Reactions were initiated by addition of isolated intact yeast mitochondria. The reaction was linear up to 4 min, and all incubations were done at 30 °C for 3 min. Reactions were stopped by addition of 6%

perchloric acid and were then centrifuged at 2000 rpm for 7 min. The resulting pellet was suspended in water, and the product [^{3}H]palmitoylcarnitine was extracted with butanol at low pH. After centrifugation at 2000 rpm for 2 min, an aliquot of the butanol phase was transferred to a vial for radioactive counting.

K_m for Carnitine and Palmitoyl-CoA. The K_m for carnitine was obtained by assaying mitochondria in the presence of increasing carnitine concentrations: 50−1500 μM for pig CPTIβ, 50−2000 μM for human CPTIβ, and 25−1000 μM for CPTIα isotype assays. Determination of the K_m for palmitoyl-CoA was performed at fixed carnitine concentration (0.2 and 1 mM for CPTIα and CPTIβ isotypes, respectively) by varying the palmitoyl-CoA concentration from 0 to 500 μM. The fatty acid-free albumin concentration was increased proportionally to the palmitoyl-CoA concentration to maintain a fixed molar ratio of palmitoyl-CoA: albumin of 6.1:1 as previously described (*18*).

IC_{50} for Malonyl-CoA. The IC_{50} value was determined by assaying mitochondria in the presence of increasing concentrations of malonyl-CoA (from 0 to 50 μM for rat CPTIα or pig CPTIβ and from 0 to 15 μM for human CPTIβ or pig CPTIα). The activity (in percent) was plotted against the malonyl-CoA concentration, with 100% representing the CPT activity in the absence of malonyl-CoA. Data were fitted to exponential decay curves (linear scale) or to competition curves (logarithmic scale) for IC_{50} calculation.

Western Blot Analysis. Proteins were separated by SDS−PAGE in a 7.5% gel and transferred onto nitrocellulose membranes as previously described (*8*). A 1:2000 dilution of the anti-pig CPTIα antibody previously obtained in our laboratory (*11*) was used. This antibody also recognizes other CPT proteins (*11*, *12*). Proteins were detected using the ECL chemiluminescence system (Amersham Pharmacia Biotech). Western blot analysis in urea gels was performed by adding 9 M urea in the SDS−PAGE gel.

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

RESULTS

Pig CPTIβ Cloning. The cDNA for pig M-CPTI was obtained by PCR-derived methods (RT-PCR and SMART RACE). 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 select those in which the sequence was coincident with the PCR products.

This approach allowed determination of the sequence of full-length pig CPTIβ (GenBank accession number AY642381). Database search showed this sequence to have 99.8% identity with a GenBank reported clone (AY181062) characterized as pig M-CPTI. The pig CPTIβ cDNA encodes a protein of 772 amino acids with a predicted molecular mass of 88.2 kDa. The predicted amino acid sequence shares extensive sequence identity with human (88%), rat (85%), and mouse (86%) CPTIβ, while the degree of identity with human (61%), rat (62%), pig (61%), and mouse (60%) CPTIα is much lower. The hydropathy plot of pig CPTIβ (TMpred program) predicted that pig CPTIβ retains a folding

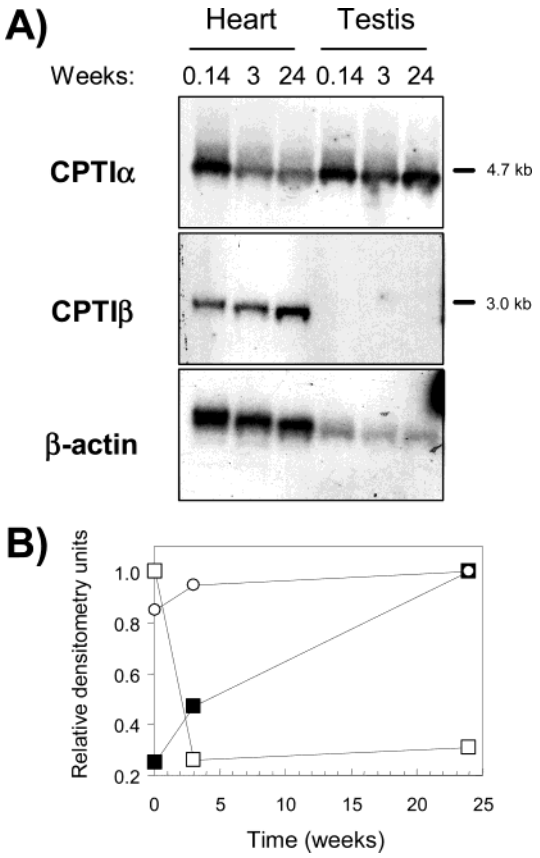

FIGURE 1: Liver and muscle CPTI expression pattern in pig heart and testis during development. (A) Northern blot showing tissue-specific expression of pig liver (CPTIα) and muscle (CPTIβ). Total RNA samples obtained from 0.14-, 3-, or 24 week-old pigs were analyzed for CPTIα and CPTIβ mRNA abundance. Equal loading of the sample in each lane was confirmed by use of the human β-actin probe. (B) mRNA levels were determined by densitometer scanning of the blot and normalized against β-actin (the highest mRNA level of each gene was normalized to a value of 1.0). Key: open squares, CPTIα expression in heart; closed squares, CPTIβ expression in heart; open circles, CPTIβ expression in testis.

pattern within the outer mitochondrial membrane similar to that previously described for CPTI proteins (data not shown).

Pig CPTIβ Expression Pattern. We previously showed that, in 3-week-old piglets, CPTIβ is expressed in muscle and heart but not in kidney, duodenum, and testis (*11*). Figure 1 shows the expression pattern of CPTIα and CPTIβ in heart and testis during development. In heart, the expression of both isotypes follows a pattern observed with other mammals (*20*), since CPTIα expression was high at birth and decreased during development, while the CPTIβ isotype followed the inverse pattern. This has been interpreted in terms of differences in affinity for the substrate by the enzymes, thus correlating with the low carnitine levels at birth in this tissue. However, this interpretation is not applicable for the pig model since CPTIβ has a low K_m for carnitine (197 μM), similar to that of the rat (100 μM) and pig (126 μM) CPTIα (see below). Figure 1 also shows that, irrespective of the age of the animal, the liver isotype but not the muscle isotype is expressed in pig testis.

Recombinant Pig CPTIβ Expression. Pig CPTIβ was expressed using the yeast *P. pastoris*, since other CPT proteins have been successfully expressed in this system (*4*, *8*, *9*, *11*). Figure 2 shows a western blot analysis of different recombinant CPTI enzymes using a pig CPTIα antibody.

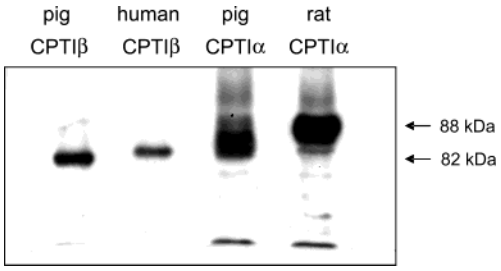

FIGURE 2: Mobility of CPTI proteins. Immunoblot showing the expression and migration patterns of yeast-expressed CPTI proteins. Mitochondria isolated from the yeast strain expressing the CPTI constructs were separated on a 7.5% SDS−PAGE and blotted onto a nitrocellulose membrane. Approximately 10 μg of protein was applied in each lane, and the immunoblot was developed using pig L-CPTI-specific antibodies.

Pig CPTIβ migrates as an 82-kDa protein while its predicted molecular mass is 88.2 kDa. The same phenomenon is observed for human CPTIβ and pig CPTIα, while rat CPTIα migrates as expected from its predicted molecular weight. Since human CPTIβ and pig CPTIα are both highly sensitive to malonyl-CoA inhibition (*4*, *11*), we previously correlated the unexpected higher mobility of pig CPTIα with the high sensitivity to malonyl-CoA inhibition (*11*). However, this correlation is not observed for the CPTIβ, because the enzyme with the higher mobility shows a lower sensitivity to malonyl-CoA inhibition (see below). Since in vitro transcribed and translated proteins retain the same anomalous fast migration pattern, this anomalous migration pattern cannot be due to posttranslational modifications (*11*). However, proteins run on gel containing 9 M urea migrate in the same fashion (data not shown), which strongly suggests that the anomalous migration pattern is not due to incomplete protein denaturation.

Pig CPTIβ Kinetic Characteristics. No CPT activity was found in the control yeast strain with the empty vector. Isolated mitochondria from the pig CPTIβ expression strain had a high level of malonyl-CoA-sensitive CPT activity [2.17 $\pm$ 0.4 nmol min^{-1} (mg of protein)$^{-1}$]. Yeast-expressed pig CPTIβ exhibited normal saturation kinetics with respect to its two substrates, palmitoyl-CoA and carnitine, as shown in Figure 3A,B. The K_m for palmitoyl-CoA was 82.58 $\pm$ 15.28 μM. The K_m for carnitine was 197.58 $\pm$ 42.46 μM. Thus, pig CPTIβ has unique kinetic characteristics, since it has the very lowest affinity for palmitoyl-CoA of any characterized CPTI and the highest affinity for carnitine of all reported β isotypes (*4*, *9*, *21*). With regard to malonyl-CoA inhibition, recombinant pig CPTIβ exhibited a low sensitivity to malonyl-CoA, with an IC_{50} of 906.33 $\pm$ 135.02 nM (Figure 3C). Thus, the kinetic characteristics of the pig CPTIβ with respect to carnitine and malonyl-CoA are very similar to those of the rat CPTIα isotype (see Figure 4A,B).

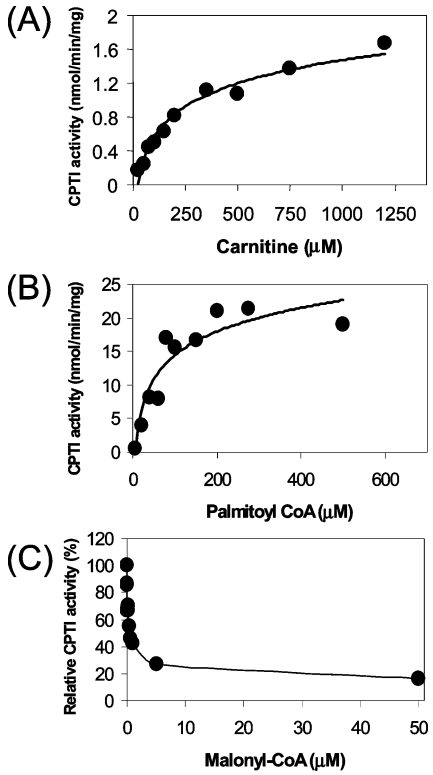

FIGURE 3: Kinetic analysis of pig M-CPTI. Mitochondria obtained from the yeast strain expressing pig M-CPTI were assayed for CPTI activity in the presence of increasing concentrations of carnitine (A), palmitoyl-CoA (B), and malonyl-CoA (C). Assays were done with 100 μg of mitochondrial protein as described in the Experimental Procedures section. Results are an average of three separate experiments with at least two independent mitochondrial preparations.

DISCUSSION

In pig, a very low rate of long-chain fatty acid oxidation has been reported in liver (*22*), while an effective β-oxidation process has been observed in muscle (*15*). The main control point of the fatty acid oxidation flux has been postulated to be the CPTI enzyme and its inhibition by malonyl-CoA, a physiological substrate produced mainly but not exclusively in lipogenic tissues (*1*). Altered malonyl-CoA sensitivity, presented by the different pig CPTI isotypes, agrees with this model of long-chain fatty acid oxidation regulation by malonyl-CoA. Thus, in pig liver, CPTIα is inhibited at low malonyl-CoA levels (*11*), and consequently, the fatty acid oxidation rate is slowed. On the contrary, pig muscle CPTIβ is less sensitive to malonyl-CoA inhibition, and consequently, the fatty acid oxidation rate is high (see Figure 4A). Therefore, species-specific variations in malonyl-CoA sensitivity of CPTI enzymes are in agreement with the rate of fatty acid oxidation observed in the whole animal.

When expressed in *P. pastoris*, recombinant CPTI shows kinetic characteristics similar to those observed in different tissues from different species (*3*−*7*, *11*, *12*). Figure 4A shows that the pig CPTIβ reported here has a low sensitivity to malonyl-CoA inhibition (IC_{50} of 906 nM) when compared with the human CPTIβ (IC_{50} of 69 nM). Figure 4A also shows that this decreased sensitivity to malonyl-CoA inhibition is characteristic of the liver isotypes of the enzyme (i.e., rat CPTIα has an IC_{50} of 1900 nM). The high IC_{50} obtained here agrees with previous data reported for pig muscle mitochondria and could explain the effective β-oxidation process observed in muscle (*15*). However, the substrate K_m obtained here are unique. Thus, pig CPTIβ has a lower affinity for palmitoyl-CoA (K_m of 82.58 μM) than recombinant human (K_m of 43 μM) or rat (K_m of 57 μM) CPTIβ isotypes (*4*, *21*), while the carnitine K_m of pig CPTIβ (197 μM) that was obtained is lower than previously reported (*14*) and closer to the K_m of the α isotypes (ranging between 100 and 126 μM) rather than the β isotype (ranging between 666 and 800 μM; see Figure 4B).

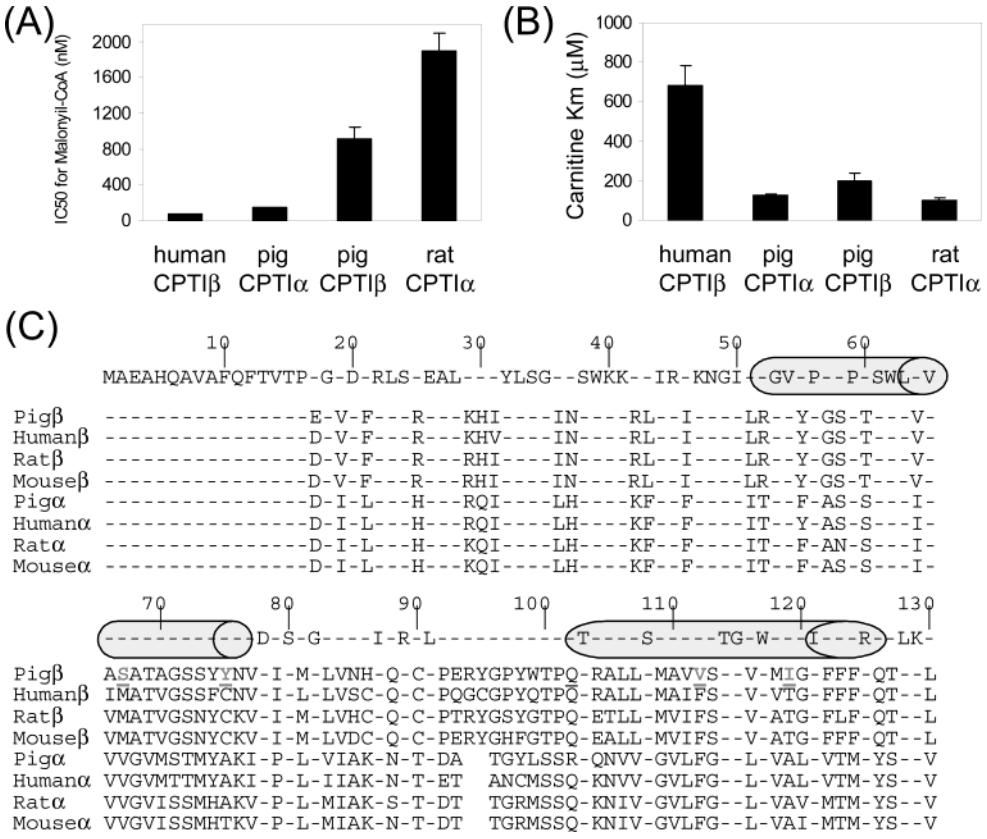

FIGURE 4: Kinetic characteristics and N-terminal alignment of different CPTI isotypes. (A) IC_{50} for malonyl-CoA inhibition of the different isotypes of CPTI. (B) K_m for carnitine for the same isotypes of CPTI. (C) Alignment of the N-terminal and the transmembrane domain sequences of the enzymes studied. In the top of the alignment are depicted the residues shared among all CPTI isotypes; nonidentical residues are only depicted in the respective sequences. Underlined are the four characteristic residues of pig CPTIβ (Ser^{67}, Tyr^{75}, Val^{111}, and Ile^{119}). The two transmembrane domains, TM1 and TM2, are also indicated.

Diet-induced changes in carnitine K_m have been reported in pig liver CPTI, but the CPTIβ isotype expressed in muscle is not sensitive to these types of changes (*14*). Nevertheless, the low carnitine K_m observed with the recombinant enzymes agrees with recent data in which carnitine supplementation does not accelerate the kinetics of triacylglycerol utilization by colostrum-deprived newborn pigs (*23*). In addition, this result suggests that the recombinant pig CPTIβ isotype is a muscle isotype with kinetics characteristics of the rat liver isotype, i.e., low K_m for carnitine and high IC_{50} (low malonyl-CoA sensitivity). The residues responsible for the unique α isotype kinetic characteristics of the pig CPTIβ probably reside in the N-terminus of the enzyme.

N-Terminal deletion experiments (*18*, *24*−*26*), site-directed mutagenesis (*27*−*29*), and analysis of natural or recombinant chimeras (*12*, *21*) have been important in revealing the role of the N-terminus in the kinetic behavior of the CPTI enzymes. Thus, it is generally agreed that the N-terminus of the CPTI enzymes plays an important role in determining the kinetic characteristics of the enzymes such as the IC_{50} for malonyl-CoA inhibition or the K_m for the substrate. Interestingly, the N-terminus of the enzymes is highly conserved between different CPTI isotypes that show different kinetic parameters, which indicates that the interaction between the cytosolic N- and C-terminal regions of the enzyme is an important determinant of the overall kinetics of both CPTIα and CPTIβ isotypes (*12*, *27*).

In accordance with its role in malonyl-CoA sensitivity, the N-terminus of the CPTIα enzyme can be divided into two different domains that constitute the positive (residues 1−18) and negative (residues 19−30) determinants of malonyl-CoA sensitivity of the enzyme (*24*, *26*). Site-directed mutagenesis studies of the second domain suggest that Ser^{24} and Gln^{30} may be responsible for the effect on malonyl-CoA sensitivity (lower IC_{50}) (*29*). All CPTIβ isotypes have Ser^{24} but have His^{30} instead of Gln^{30} (see Figure 4C). In addition, this second domain (negative determinant) appears to be absent in the CPTIβ isotype (*26*), which can be correlated with the high malonyl-CoA sensitivity observed with this isotype until now (*4*). Therefore, it could be hypothesized that the absence of the negative malonyl-CoA determinant in CPTIβ gives rise to an enzyme more sensitive to malonyl-CoA inhibition. Interestingly, our studies with the pig CPTIβ demonstrate that the enzyme has low sensitivity to malonyl-CoA inhibition when compared with the rat or human CPTIβ enzymes but still has higher sequence homology to CPTIβ (His^{30}) than CPTIα (Gln^{30}) isotypes (Figure 4A,C). Studies to determine the presence of a putative negative determinant in the N-terminal of pig CPTIβ are currently being carried out in our laboratory.

The sequence alignment shown in Figure 4C may also shed light into our understanding of the kinetic parameters such as the K_m for carnitine of CPTI. Previous studies suggest that the interaction between the two putative transmembrane domains of CPTI enzymes (TM1 and TM2) is important in determining the K_m for carnitine (*18*, *21*). The behavior of pig CPTIβ, with a low K_m for carnitine, narrows down the putative amino acids involved in this kinetic parameter to a

few residues, since its transmembrane domains are more similar to CPTIβ (with high K_m for carnitine) than CPTIα (with low K_m for carnitine) isotypes.

In summary, the cloning and kinetic characterization of pig CPTIβ reported here show that it has kinetic properties similar to those of rat CPTIα (*8*) rather than the CPTIβ enzyme from human heart (*4*). Therefore, the pig CPTIβ sequence may be useful in our understanding of the kinetic behavior of different CPTI isotypes.

ACKNOWLEDGMENT

We thank Dr. Feike R. Van Der Leij (Department of Pediatrics, University of Groningen, The Netherlands) for kindly supplying the ODC1 and ODC2 primers.

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BI0488597