The First 28 N-Terminal Amino Acid Residues of Human Heart Muscle Carnitine Palmitoyltransferase I Are Essential for Malonyl CoA Sensitivity and High-Affinity Binding†

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ABSTRACT: Heart/skeletal muscle carnitine palmitoyltransferase I (M-CPTI) is 30−100-fold more sensitive to malonyl CoA inhibition than the liver isoform (L-CPTI). To determine the role of the N-terminal region of human heart M-CPTI on malonyl CoA sensitivity and binding, a series of deletion mutations were constructed ranging in size from 18 to 83 N-terminal residues. All of the deletions except Δ83 were active. Mitochondria from the yeast strains expressing Δ28 and Δ39 exhibited a 2.5-fold higher activity compared to the wild type, but were insensitive to malonyl CoA inhibition and had complete loss of high-affinity malonyl CoA binding. The high-affinity site (K_{D1}, B_{max1}) for binding of malonyl CoA to M-CPTI was completely abolished in the Δ28, Δ39, Δ51, and Δ72 mutants, suggesting that the decrease in malonyl CoA sensitivity observed in these mutants was due to the loss of the high-affinity binding entity of the enzyme. Δ18 showed only a 4-fold loss in malonyl CoA sensitivity but had activity and high-affinity malonyl CoA binding similar to the wild type. Replacement of the N-terminal domain of L-CPTI with the N-terminal domain of M-CPTI does not change the malonyl CoA sensitivity of the chimeric L-CPTI, suggesting that the amino acid residues responsible for the differing sensitivity to malonyl CoA are not located in this N-terminal region. These results demonstrate that the N-terminal residues critical for activity and malonyl CoA sensitivity in M-CPTI are different from those of L-CPTI.

Carnitine palmitoyltransferase I (CPTI)[1] catalyzes the conversion of long-chain fatty acyl CoAs to acyl carnitines in the presence of L-carnitine (*1, 2*). As an enzyme that catalyzes the first rate-limiting step in fatty acid oxidation, CPTI is tightly regulated by its physiological inhibitor malonyl CoA, the first intermediate in fatty acid synthesis, suggesting coordinated control of fatty acid oxidation and synthesis (*1, 2*). Understanding the regulation of CPTI by malonyl CoA is important in the design of drugs for control of excessive fatty acid oxidation in diabetes mellitus (*3*), and in myocardial ischemia where accumulation of long-chain acylcarnitines has been associated with arrhythmias (*4*).

Mammalian tissues express two isoforms of CPTI—a liver isoform (L-CPTI) and a heart/skeletal muscle isoform (M-CPTI)—that are 62% identical in amino acid sequence (*5*−*11*). Although adult heart expresses both isoforms of CPTI, the predominant form is M-CPTI (*5*−*7*). The IC_{50} for malonyl CoA inhibition of heart mitochondrial M-CPTI is ∼30−100-fold lower than that of L-CPTI, but both tissues have similar malonyl CoA concentration (*2, 8*). It is estimated that about 60−80% of the energy requirement of the heart is derived from fatty acid oxidation (*12*). The important question of how fatty acid oxidation can proceed in heart in the presence of high tissue levels of malonyl CoA appears to be resolved in part by recent reports of the transcriptional regulation of M-CPTI gene expression by long-chain fatty acids via the peroxisome proliferator-activated receptor α (PPARα) (*13*−*15*). Long-chain fatty acids activate PPARα, which then heterodimerizes with the 9-*cis*-retinoic acid receptor, binds to the fatty acid response element on the promoter region of the M-CPTI gene, and in turn activates M-CPTI gene transcription (*14*). In heart, the flux of high levels of long-chain fatty acyl CoAs through the CPT system directly competes for the malonyl CoA binding site on M-CPTI to overcome inhibition by malonyl CoA. In addition, high levels of long-chain fatty acyl CoAs stimulate the AMP-activated protein kinase, inhibit acetyl CoA carboxylase, and turn off malonyl CoA synthesis, thus decreasing M-CPTI inhibition (*16*).

We have expressed both human heart M-CPTI, L-CPTI, and CPTII in the yeast *Pichia pastoris*, an organism devoid of endogenous CPT activity (*8, 17, 18*). Recently, we showed that deletion of the conserved first 18 N-terminal amino acid residues of rat L-CPTI abolishes malonyl CoA inhibition and high-affinity binding (*19*). We further demonstrated that substitution of glutamate-3 in the N-terminal region of L-CPTI abolishes malonyl CoA inhibition and binding, while a mutant L-CPTI with a change of histidine-5 to alanine

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[1] Abbreviations: CPT, carnitine palmitoyltransferase; L-CPTI, liver isoform of CPTI; M-CPTI, heart/skeletal muscle isoform of CPTI; PPARα, peroxisome proliferator-activated receptor α; Δ, deletion.

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causes partial loss in malonyl CoA inhibition (*20*). These results demonstrate that glutamate-3 and histidine-5 are necessary for malonyl CoA inhibition and high-affinity binding, but not for catalysis (*20*). In this communication, we report that, unlike the rat L-CPTI, deletion of the first 28 but not 18 N-terminal residues of M-CPTI abolishes malonyl CoA inhibition and high-affinity binding.

EXPERIMENTAL PROCEDURES

Construction of Plasmids for the N-Terminal Mutants of Human Heart M-CPTI. Deletion mutants of M-CPTI were constructed by PCR using pGAP-M-CPTI plasmid DNA as a template, as previously demonstrated (*8*). For example, to construct HMΔ18, a 668 bp fragment was obtained after using primer H18D, 5′-AAGACAATTGATGGTCGACTTCCGGCTCAGT, as the forward PCR primer, and HR1700, 5′-CCACCAGTCACTCACATA, as the reverse primer, followed by restriction digestion of the PCR product with the restriction enzymes *Mun*I and *Eco*RI. Note that for each forward primer the introduced *Mun*I site is shown underlined and is followed by the new start codon. The resulting fragment was then ligated with *Eco*RI-linearized pHWO10, and the product, pHZ18N, with the proper orientation was confirmed by restriction analysis with *Eco*RI. To complete the HMΔ18 mutant CPT gene, the 2.0 kb *Eco*RI fragment from pHZ01 was then ligated to the *Eco*RI site of pHZ18N as described (*8*). Deletion mutants HMΔ28, HMΔ39, HMΔ51, HMΔ72, and HMΔ83 were constructed as above but with the following forward primers: H28D, 5′-AAGACAATTGATGAAACACGTCTACCTGTC; H39D, 5′-AAGACAATTGATGAAGAAACGCCTGATCC; H51D, 5′-AAGACAATTGATGAGGGGCGTGTACCCTGGCA; H72D, 5′-AAGACAATTGATGTCCTTCTGCAACGTGGA; and H83D, 5′-AAGACAATTGATGGTCAGTTGCATCCAGAGAT.

Construction of Plasmids Carrying Chimeric CPTI Genes. A plasmid carrying the N-terminal portion of human M-CPTI was prepared by PCR using pGAP-M-CPTI as a template with the forward primer HHRI, 5′-GAGGAATTCATATGGCGGAAGCTCACCAG, which introduced an *Eco*RI site (underlined) 2 bp upstream of the start codon and the reverse primer HR1700; the product was cut with *Eco*RI, and the 670 bp fragment was ligated to *Eco*RI-cut pUC119 to produce pJS200. To construct a CPTI chimera in which 41 N-terminal amino acids of human M-CPTI are fused to the C-terminal portion of rat L-CPTI, we took advantage of the *Xmn*I present in this position for rat L-CPTI and introduced a translationally silent matching site into human M-CPTI. pJS200 was used as a template for PCR with forward primer HHRI and reverse primer CHX41, 5′-CTTGAAGCTTATGAACCTTTTCTTCCAGGAGTTG, which introduced the *Xmn*I site (underlined). The 130 bp PCR fragment was blunt-end-ligated to *Srf*I-cut pCR-Script (Stratagene) to produce pJS109. pJS109 was linearized with *Xmn*I and ligated to *Xmn*I-cut pM1R (*17*) to produce pJS301. The final step in the construction was to subclone the chimeric CPTI to the *P. pastoris* expression vector pHW010. pJS301 was digested with *Eco*RI to release the full-length chimeric CPTI which was then ligated into *Eco*RI-cut pHW010 to produce pCHH41RL.

A CPTI chimera in which the 79 N-terminal amino acids of human M-CPTI are fused to the C-terminal portion of rat L-CPTI was constructed as follows: The initial construct introduced a translationally silent *Hin*dIII restriction site into the N-terminal coding region of RLCPTI. A 320 bp *Hin*dIII−*Kpn*I fragment was produced by restriction enzyme digestion of the PCR product prepared using pYGW9 as a template with the forward primer RLC79, 5′-GACCCAAGCTTGGGCATGATCGCAAA, and the vector-specific reverse primer RL655, 5′-CAGGAAACAGCTATGAC, and subcloned into *Hin*dIII−*Kpn*I-cut pYGW9 to produce pJS102. A fragment encoding the N-terminal portion of HMCPTI was prepared by PCR using the plasmid pJS200 as a template, the forward primer RIHH, and the reverse primer HHC79, 5′-GCCCCAAGCTTATGTCCACGTTGCAGA, which introduced a translationally silent *Hin*dIII restriction site (underlined) into the N-terminal coding region of HMCPTI. The 250 bp PCR product was blunt-end-ligated into the pCR-Script vector to produce pJS106. Next, the heart/liver CPTI chimeras were ligated together. pJS106 was digested with *Hin*dIII, and the resulting fragment was ligated into the *Hin*dIII site of pJS102 to produce pJS302. Note that pJS302 carries the 79 N-terminal amino acids of HMCPTI fused to RLCPTI at the introduced *Hin*dIII restriction site. PJS302 was digested with *Eco*RI to release the full-length chimeric CPTI which was then ligated into the *Eco*RI-cut pHW010 to produce pCHH79RL.

A CPTI chimera, in which 130 N-terminal amino acids of human M-CPTI are fused to the C-terminal portion of rat L-CPTI, was constructed in a manner similar to pCHH79RL. A 170 bp *Hin*dIII−*Kpn*I fragment was produced by restriction enzyme digestion of the PCR product prepared using pYGW9 as a template with the forward primer RLC130, 5′-GCTGAAGCTTCTGCTCTCCTACCACGGCTGGAT, and the reverse primer RL655 and ligated with *Hin*dIII−*Kpn*I-cut pYGW9 to produce pJS115. A fragment encoding the N-terminal portion of HMCPTI was prepared by PCR using as a template the plasmid pJS200, the forward primer RIHH, and the reverse primer HCH130, 5′-CTTAAGCTTCAGGGTTTGGCGGAAGAA, which introduces a translationally silent *Hin*dIII site (underlined). The 390 bp PCR product was blunt-end-ligated into *Sur*fI-cut pCR-Script to produce pJS113. pJS113 was digested with *Hin*dIII, and the resulting fragment was ligated into the *Hin*dIII site of pJS115 to produce pJS303. pJS303 was digested with *Eco*RI to release the full-length CPTI chimera which was then ligated into the *Eco*RI-cut pHW010 to produce pCHH130RL.

A CPTI chimera in which the 197 N-terminal amino acids of human M-CPTI are fused to the C-terminal portion of rat L-CPTI was constructed by cutting pJS200 with *Kpn*I and *Hin*dIII and ligating the 563 bp fragment to *Kpn*I- and *Hin*dIII-cut pYGW9 to form pJS316. pJS316 was then cut with *Eco*RI and the fragment ligated to pHW010 to form CHH197RL. The DNA sequences of all mutants were confirmed by sequencing.

Integration of Mutant Human Heart M-CPTI DNA into the P. pastoris Genome. Each plasmid was linearized by digestion with the restriction enzyme *Bsp*EI (*8*). The linear DNA was introduced into *P. pastoris* by electrotransformation. Integrants were recovered as histidine prototrophic transformants after selection on YND plates and grown on YND medium containing glucose. Mitochondria were isolated from the wild-type and mutant M-CPTIs and L-CPTIs, as described previously (*8, 17*).

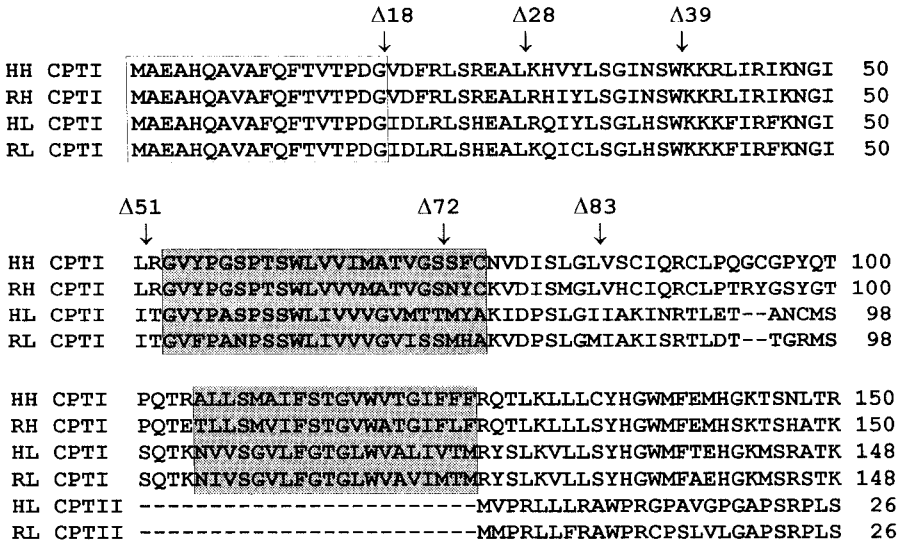

FIGURE 1: Amino acid sequence of the first 150 N-terminal residues of human and rat liver CPTs. The shaded areas represent the positions of the two predicted membrane-spanning domains of all known CPTIs. The position of each of the deletion mutants is shown by an arrow. Sources of the sequences from the data bank were from refs *8*−*11* as indicated in the text. HH, RH = human, rat heart; HL, RL = human, rat liver.

CPTI Assay. CPTI and L-CPTI activities were assayed in isolated mitochondria from the yeast strains expressing the wild-type and mutant CPTIs by the forward exchange method using L-[^{3}H]carnitine, as described previously (*8, 17, 21*). The K_m for palmitoyl CoA was determined by varying the palmitoyl CoA concentration in the presence of a fixed albumin concentration (1%) or a fixed molar ratio (6.1:1) of palmitoyl CoA to albumin (*22, 23*).

^{14}C-Malonyl CoA Binding Assay. ^{14}C-Malonyl CoA binding in isolated mitochondria from the yeast strains expressing the wild-type and mutant CPTIs was determined by a modified centrifugation assay as described previously (*19, 20, 24*). The CPT activity and IC_{50} values are given as a mean ± SD for at least three independent assays with different preparations of mitochondria. The K_D values are averages of at least two independent experiments.

Western Blot Analysis. Proteins were separated by SDS−PAGE in a 7.5% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with either the M-CPTI- or the L-CPTI-specific polyclonal antibodies as described previously (*8, 17, 18*).

Sources of materials and other procedures were as described in our previous publication (*20*).

RESULTS

Generation of Deletion Mutants and Chimeras and Expression in P. pastoris. Construction of plasmids carrying the N-terminal deletions of human heart M-CPTI and chimeras was performed as described under Experimental Procedures. The deletions and chimeras were confirmed by DNA sequencing. The deletions ranged from the smallest, 18, to the largest, 83, amino acid residues as shown in Figure 1. *P. pastoris* was chosen as the expression system for M-CPTI, the deletion mutants and the chimeras, because it does not have endogenous CPT activity (*8, 17*−*19*). The *P. pastoris* expression plasmids expressed M-CPTI and L-CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter (*17, 27*).

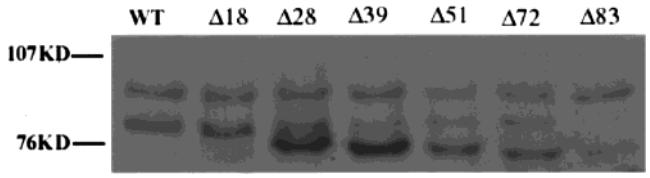

FIGURE 2: Immunoblot showing expression of wild-type and deletion mutant human heart M-CPTIs in the yeast *P. pastoris*. Mitochondria (50 μg of protein) from the wild-type yeast strain and the strains expressing each of the deletion and point mutants were separated on a 7.5% SDS−PAGE and blotted onto a nitrocellulose membrane. The immunoblot was developed as described under Experimental Procedures. WT = wild-type M-CPTI; Δ = deletions 18, 28, 39, 51, 72, and 83, respectively.

Western blot analysis of wild-type M-CPTI (80 kDa) and the mutants, using a C-terminal polyclonal antibody directed against a maltose binding protein−M-CPTI fusion protein (*8*), is shown in (Figure 2). For the wild type and all the deletion mutants, proteins of the predicted sizes were synthesized and were expressed at similar steady-state levels.

Effect of Deletions on L-CPTI Activity and Malonyl CoA Inhibition. All of the deletion mutants except Δ83 retained significant CPT activity. For deletion mutants Δ18, Δ51, and Δ72, the CPT activity level was 84%, 56%, and 20%, respectively, of that observed with the wild-type yeast strain expressing M-CPTI (Table 1). In contrast, Δ28 and Δ39 showed over 2.5-fold higher CPT activity compared to the wild-type strain expressing M-CPTI. Δ83 had no CPT activity. The IC_{50} for malonyl CoA inhibition of the wild-type strain expressing M-CPTI was 70 nM (*8*), while the IC_{50} for the minimal deletion mutant Δ18 was 300 nM, representing only a 4-fold decrease in malonyl CoA sensitivity compared to the 190-fold decrease in sensitivity observed with the corresponding L-CPTI Δ18 (*19*). Deleting 28, 39, 51, and 72 amino acid residues from the N-terminus of M-CPTI increased the IC_{50} for malonyl CoA inhibition in each of the mutants from 70 nM in the wild-type strain to 3.5−7.5 μM in the deletion mutants, thus decreasing the malonyl CoA sensitivity by 50−100-fold (Table 1). Δ28

Table 1: CPT Activity, Malonyl CoA Sensitivity, and Binding in Yeast Strains Expressing Wild-Type M-CPTI and N-Terminal Deletion Mutants[a]

strain	activity [nmol/ (mg·min)]	IC_{50} (μM)	K_{D1} (nM)	K_{D2} (nM)	B_{max1} (pmol/mg)	B_{max2} (pmol/mg)
wild-type	2.5 ± 0.4	0.07 ± 0.01	5.7	35.0	13.0	34.8
Δ18	2.1 ± 0.2	0.3 ± 0.05	4.6	37.0	6.3	24.4
Δ28	6.7 ± 1.3	7.5 ± 0.3	–	684.9	–	8.6
Δ39	6.3 ± 0.9	7.5 ± 0.4	–	285.0	–	12.5
Δ51	1.4 ± 0.2	3.5 ± 0.2	–	115.7	–	5.3
Δ72	0.5 ± 0.2	7.0 ± 0.4	–	744.6	–	4.0
Δ83	no activity					

[a] Mitochondria were isolated from the yeast strains separately expressing M-CPTI and the deletion mutants, and were assayed for CPT activity, malonyl-CoA sensitivity, and binding as described under Experimental Procedures. IC_{50} is the concentration of malonyl CoA needed to inhibit 50% of the activity of the yeast-expressed M-CPTI, and results are mean ± SD of at least three independent experiments with different mitochondrial preparations. The K_D and B_{max} values are averages of two independent experiments with different mitochondrial preparations.

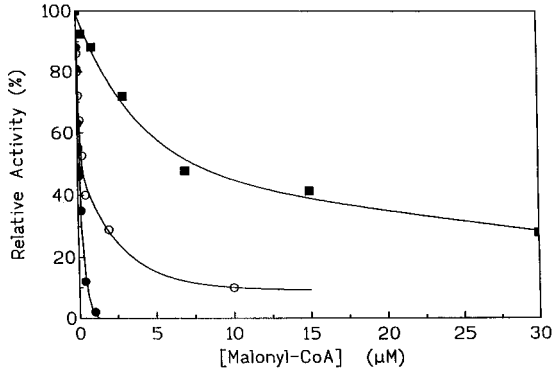

FIGURE 3: Effect of increasing concentrations of malonyl CoA on the activities of yeast-expressed wild-type and deletion mutant M-CPTIs. Approximately 150 μg of mitochondrial protein was used for the assay. ● = wild type; ○ = Δ18; ■ = Δ28.

showed decreased malonyl CoA sensitivity at all levels of the inhibitor tested compared to the wild type, as shown in Figure 3.

Kinetic Properties of Wild-Type and Mutant M-CPTs. Δ28 exhibited normal saturation kinetics when the carnitine concentration was varied relative to a second substrate, palmitoyl CoA, but showed a much higher activity compared to the wild type at all levels of carnitine tested (Figure 4A) under standard assay conditions. The calculated K_m for carnitine for Δ28 was 408 μM, which is similar to the 530 μM for the wild type, but the V_{max} [19.1 nmol min^{-1} (mg of protein)$^{-1}$] for Δ28 was 2.5-fold higher than that of the wild-type strain [7.6 nmol min^{-1} (mg of protein)$^{-1}$]. With respect to the second substrate, palmitoyl CoA, both the wild type and Δ28 showed non-Michaelis−Menten saturation kinetics at a fixed concentration of albumin (1% w/v) (Figure 4B), characteristics similar to our previous report (*8*). Δ28 exhibited significantly higher activity than the wild type at all levels of palmitoyl CoA tested. However, both Δ28 and the wild type showed normal saturation kinetics when the molar ratio of palmitoyl CoA to albumin was fixed at 6.1:1 (Figure 4C). For Δ28, the calculated K_m for palmitoyl CoA was 56.8 μM, and the V_{max} was 24.9 nmol min^{-1} (mg of protein)$^{-1}$, which is similar to the wild-type values of 93.8 μM and 25.0 nmol min^{-1} (mg of protein)$^{-1}$. Thus, deletion

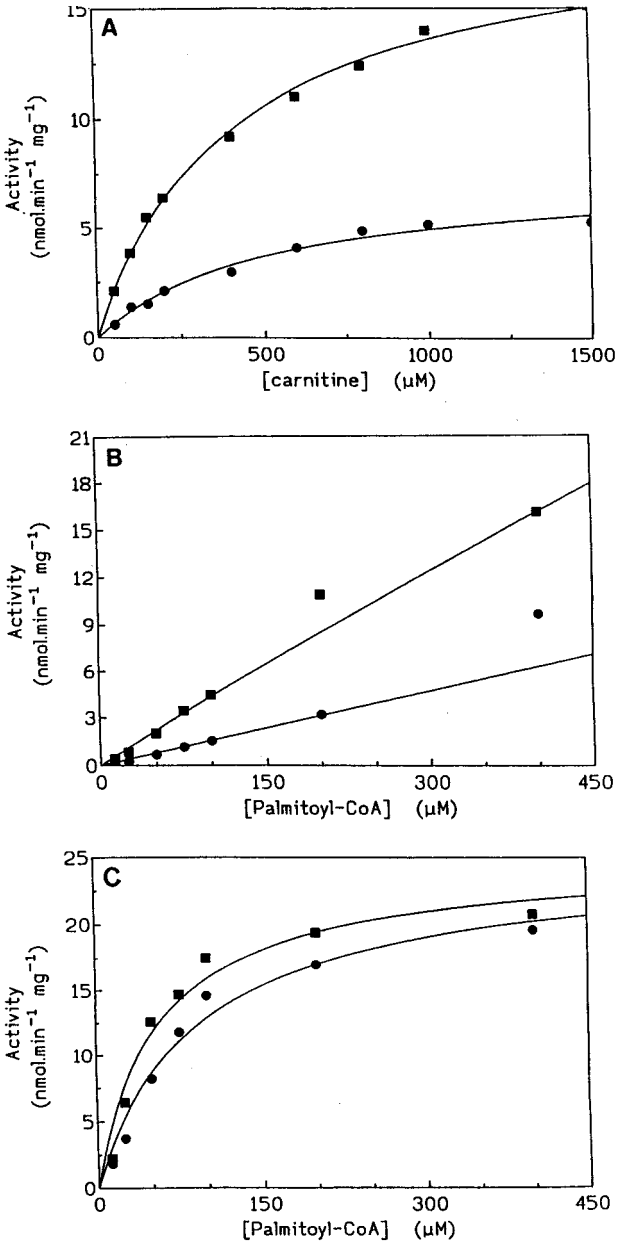

FIGURE 4: Kinetic analysis of wild-type and Δ28 mutant M-CPTI activities. Isolated mitochondria (150 μg of protein) from the yeast strains expressing the wild-type (●) and Δ28 (■) mutant M-CPTIs were assayed for CPTI activity in the presence of increasing concentrations of carnitine and palmitoyl CoA as described under Experimental Procedures. The figures show the resulting dose−response curves for M-CPTI: (A) carnitine; (B) palmitoyl CoA with fixed albumin concentration (1% w/v); (C) palmitoyl CoA with fixed molar ratio of palmitoyl CoA to albumin (6.1:1).

of the first 28 and 39 N-terminal amino acid residues abolishes malonyl CoA sensitivity and increases catalytic activity of M-CPTI, while loss of malonyl CoA inhibition in Δ51 and Δ72 was associated with decreased catalytic activity. Unlike Δ18 of L-CPTI (*19*), deletion of the first 18 N-terminal amino acids of M-CPTI had minimal effect on malonyl CoA inhibition and catalytic activity.

^{14}C-Malonyl CoA Binding in Yeast-Expressed Wild-Type and Mutant M-CPTIs. Malonyl CoA binding to the mitochondria from the yeast strain expressing Δ28 was significantly lower compared to that observed in the mitochondria from the wild-type strain and Δ18, but was saturable (Figure 5). Malonyl CoA binding clearly resolved into a high-affinity

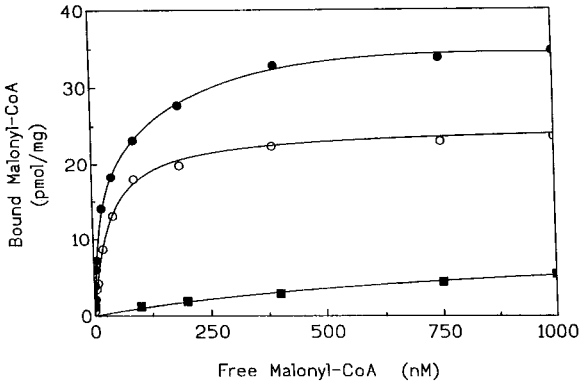

FIGURE 5: Binding of ^{14}C-malonyl CoA to mitochondria isolated from the yeast strain expressing the wild-type M-CPTI, Δ18, and Δ28. Approximately 200 μg of protein was used for the binding assay. Malonyl CoA binding values for the wild-type and deletion mutants were corrected for malonyl CoA binding to the mitochondria from the yeast strain with the vector but no insert. ● = wild type; ○ = Δ18; ■ = Δ28.

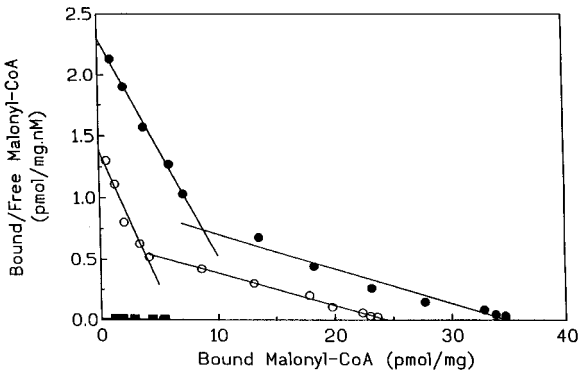

FIGURE 6: Scatchard plot for binding of ^{14}C-malonyl CoA to mitochondria from yeast strains expressing wild-type and mutant M-CPTIs. ● = wild type; ○ = Δ18; ■ = Δ28.

and a low-affinity site in the mitochondria from the wild-type M-CPTI and Δ18 as shown by the Scatchard plots in Figure 6, but only very-low-affinity binding was observed in the mitochondria from Δ28. Deletion of the first 28 N-terminal residues completely abolished high-affinity malonyl CoA binding (K_{D1}) and further decreased the low-affinity binding (K_{D2}) by 20-fold (Table 1). A complete loss in high-affinity malonyl CoA binding (K_{D1}) was also observed for Δ39, Δ51, and Δ72, which was associated with a decrease in the low-affinity binding (K_{D2}). The increase in K_{D2} for the low-affinity binding site due to the deletions correlated with a decrease in the calculated B_{max2}, suggesting that the observed loss in malonyl CoA sensitivity and binding could partially be attributed to the decreased abundance or availability of the second malonyl CoA binding entity of M-CPTI.

Activity and Malonyl CoA Sensitivity of Chimeric CPTI Enzymes. Four chimeric L-CPTI enzymes, in which the first 41, 79, 130, and 197 N-terminal amino acid residues of human heart M-CPTI replaced the corresponding portion of rat L-CPTI, were constructed and expressed in *P. pastoris*. Mitochondria from the yeast strains expressing the chimeric rat liver enzymes were monitored for changes in malonyl CoA sensitivity as a result of transplanting the N-terminal residues of the human heart enzyme to the liver enzyme. Chimeras 41, 79, and 130 showed similar CPT activity as the wild-type L-CPTI, which was 3-fold higher than that of

Table 2: CPTI Activity and Malonyl CoA Sensitivity of Wild-Type L-CPTI and Chimeric L-CPTIs[a]

strain	activity [nmol/(mg·min)]	IC_{50} (μM)
wild-type L-CPTI	7.8 ± 0.5	2.0 ± 0.2
wild-type M-CPTI	2.5 ± 0.4	0.07 ± 0.01
ML-41	6.9 ± 0.6	1.5 ± 0.3
ML-79	6.2 ± 0.4	9.0 ± 1.0
ML-130	6.6 ± 0.4	2.0 ± 0.2
ML-197	no activity	

[a] Mitochondria were isolated from the yeast strains separately expressing L-CPTI and the chimeric L-CPTIs, and were assayed for CPT activity and malonyl CoA sensitivity as described under Experimental Procedures. Results are mean ± SD of at least three independent experiments with different mitochondrial preparations. ML = muscle−liver chimera.

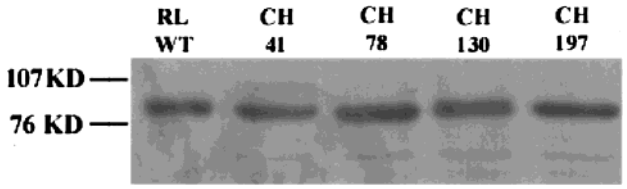

FIGURE 7: Immunoblot showing expression of wild-type L-CPTI and chimeric L-CPTs in the yeast *P. pastoris*. Mitochondria (30 μg of protein) from the wild-type L-CPTI yeast strain and the strains expressing each of the chimeric L-CPTIs were separated on a 7.5% SDS−PAGE and blotted onto a nitrocellulose membrane; the immunoblot was developed as described under Experimental Procedures using L-CPTI-specific polyclonal antibodies. RL, WT = rat liver CPTI, wild-type; CH = human M-CPTI−rat L-CPTI chimera.

the wild-type M-CPTI (Table 2). Chimera 197 had no CPT activity. The IC_{50} values for malonyl CoA inhibition of chimeras 41 and 130 were similar to the wild-type L-CPTI. Chimera 79 exhibited a 4.5-fold decrease in malonyl CoA sensitivity compared to the wild-type L-CPTI, suggesting that interaction between the two membrane-spanning α-helices of the same isoform may be important for malonyl CoA sensitivity. For the wild-type L-CPTI and chimeric enzymes, proteins of predicted sizes were synthesized and were expressed at similar steady-state levels as shown by western blot analysis using the L-CPTI-specific C-terminal antibodies (Figure 7). Thus, replacement of the first 130 N-terminal amino acid residues of L-CPTI with the corresponding M-CPTI residues did not increase the malonyl CoA sensitivity of the chimeric liver enzyme.

DISCUSSION

To determine the role of the N-terminal region of human heart M-CPTI on malonyl CoA sensitivity and binding, a series of deletion mutations were constructed ranging in size from 18 to 83 N-terminal residues. All of the deletions except Δ83 had 20−268% of the wild-type M-CPTI activity. Δ28 and Δ39 were insensitive to malonyl CoA inhibition and had complete loss of high-affinity malonyl CoA binding. However, Δ18 showed only a 4-fold loss in malonyl CoA sensitivity but had activity and high-affinity malonyl CoA binding similar to the wild type. This is in contrast to the L-CPTI Δ18 which showed complete loss in malonyl CoA sensitivity and high-affinity binding (*19*), suggesting that the same conserved first 18 residues play a different role in L-CPTI and M-CPTI. Mutant Δ72 had only 20% of the wild-type activity, and Δ83 had no activity. Our data show that

loss of residues necessary for optimal catalysis started with the Δ51 mutant. The corresponding deletion mutants for L-CPTI had ~70% of the wild-type activity, but had lost malonyl CoA sensitivity and high-affinity binding (*19*), suggesting that for M-CPTI, unlike L-CPTI, the first transmembrane domain is essential for catalytic activity. This is the first report to demonstrate isoform differences in the role of the first transmembrane domain in M-CPTI and L-CPTI activity.

The V_{max} for carnitine suggests that Δ28 has lost residues that interfere with catalysis, which may comprise part of the high-affinity malonyl CoA binding site. Binding of malonyl CoA to the high-affinity malonyl CoA binding site of wild-type M-CPTI may inhibit binding of palmitoyl CoA to the active site. Alternatively, the malonyl CoA binding site may merely sequester palmitoyl CoA, reducing its availability—a form of substrate inhibition. This model is supported by the V_{max} data for palmitoyl CoA. The V_{max} for carnitine, at fixed palmitoyl CoA and albumin concentrations, suggests palmitoyl CoA is limiting such that the effect of sequestration is observable. However, when the molar ratio of palmitoyl CoA to albumin is fixed, the effect disappears. Alternatively, since the increase in CPT activity was only observed with Δ28 and Δ39, but not Δ18 and Δ51, deletion of the first 28 and 39 residues could induce a conformational change optimal for palmitoyl CoA binding and catalysis.

Our data clearly show that there are two classes of malonyl CoA binding sites in M-CPTI, a high-affinity and a low-affinity binding site, similar to results of earlier studies with heart and skeletal muscle mitochondria (*29*−*31*). It is now well established that M-CPTI is 30−100-fold more sensitive to malonyl CoA inhibition than L-CPTI, but our binding data show no differences in the K_{D1} or K_{D2} between the heart and liver yeast-expressed wild-type CPTI isoforms, suggesting that the observed differences in malonyl CoA sensitivity may not be due to differences in the K_Ds for the two isoforms.

Replacement of the N-terminal domain of L-CPTI with the N-terminal domain of M-CPTI does not change the malonyl CoA sensitivity of the chimeric L-CPTI, suggesting that the amino acid side chains responsible for the differences in sensitivity to malonyl CoA are not located in this region. More recently, replacement of the N-terminal domain of M-CPTI with that of L-CPTI resulted in a chimeric M-CPTI with malonyl CoA sensitivity similar to wild-type M-CPTI (*32*). The decrease in malonyl CoA sensitivity observed with chimera 79 but not chimera 130 could be due to reduced interaction between the first transmembrane domain of M-CPTI and the second transmembrane domain of L-CPTI that may be necessary for malonyl CoA sensitivity of CPTI, suggesting that the membrane-spanning α-helices must be changed as a pair. Our malonyl CoA binding studies with the yeast-expressed wild-type and mutant CPTIs, and the chimera data, suggest that malonyl CoA sensitivity of L-CPTI and M-CPTI is an intrinsic property of each enzyme and that the N-terminal domain of M-CPTI cannot confer malonyl CoA sensitivity to L-CPTI or vice versa, or to CPTII (*32*, *33*). We are currently constructing substitution mutations of N-terminal amino acid residues 19−28 to identify specific residue(s) involved in malonyl CoA binding and inhibition of M-CPTI.

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