The Extreme C Terminus of Rat Liver Carnitine Palmitoyltransferase I Is Not Involved in Malonyl-CoA Sensitivity but in Initial Protein Folding*

Yong Pan‡, Isabelle Cohen§, Fanny Guillerault, Bruno Feve¶, Jean Girard, and Carina Prip-Buus§

From the Endocrinology Department, Cochin Institut, INSERM U567, CNRS Unité Mixte de Recherche 8104, Université René Descartes, 24 Rue du Faubourg Saint-Jacques, 75014 Paris, France, and the ¶Unité Propre de Recherche de l’Enseignement Supérieur Associé au CNRS 7079, 15 Rue de l’Ecole de Médecine, 75006 Paris, France

Received for publication, August 7, 2002, and in revised form, September 23, 2002

The carnitine palmitoyltransferase I (CPTI, EC 2.3.1.21) catalyzes the rate-limiting step of mitochondrial long-chain fatty acid oxidation in all mammalian tissues that converts long-chain acyl-CoA to acylcarnitine (1). A unique feature of CPTI is its potent inhibition by malonyl-CoA, the first committed intermediate of fatty acid biosynthesis (2). This provides a mechanism for physiological regulation of β-oxidation in liver and other tissues and for cellular fuel sensing based on the availability of fatty acids and glucose (1, 3, 4). In recent years the concept has emerged that lipid disorders, such as inefficient fatty acid oxidation, may contribute in the etiologies of insulin resistance, non-insulin-dependent diabetes mellitus, coronary artery disease, and other heart diseases (5–8). Acute metabolic complications of uncontrolled insulin-dependent diabetes mellitus, life-threatening diabetic ketoacidosis, mainly stem from excessive fatty acid oxidation (9). An ideal design of a drug to control the CPT system in such metabolic disorders has not come into being (10), largely because the molecular mechanisms underlying CPTI inhibition by malonyl-CoA have not thoroughly been understood.

In mammalian tissues, there are two CPTI isoforms, the liver type (L-CPTI) and the muscle type (M-CPTI), encoded by distinct genes (1). The M-CPTI is 30–100-fold more sensitive to malonyl-CoA inhibition than L-CPTI. L-CPTI is a polytopic outer mitochondrial membrane (OMM) protein harboring two hydrophobic transmembrane segments (TM1 and -2) (11). Its membrane topology is such that both the N-terminal domain (residues 1–47) and the C-terminal catalytic domain (residues 123–773) are located on the cytosolic face of mitochondria (12). The N-terminal domain (1–147 residues) of L-CPTI is not only responsible for mitochondrial targeting and import into the OMM but is also involved in maintenance of a folded active and malonyl-CoA-sensitive conformation of the enzyme (13). Recently, TM2 was shown to be essential for the correct folding of the catalytic domain of L-CPTI, suggesting that some N/C intramolecular interactions may be directly involved in the establishment and/or maintenance of the native functional conformation of L-CPTI (14).

The malonyl-CoA binding site as well as the molecular basis for malonyl-CoA inhibition remain largely unknown despite clues that have begun to emerge from functional mutagenesis studies. Deletion of the highly conserved N-terminal residues of L-CPTI or mutation of Glu3 and His5 led to a decreased malonyl-CoA sensitivity and impairment of malonyl-CoA binding (15–17). However, whether Glu3 participates directly to malonyl-CoA binding or allows the extreme cytosolic N terminal to interact with the catalytic C-terminal domain in order to maintain a conformational malonyl-CoA binding site is still unclear. We have previously shown that the N-terminal domain of L-CPTI cannot confer malonyl-CoA sensitivity to the malonyl-CoA-insensitive CPTII (13), which allowed two hypotheses to be formulated. The first one was that, if malonyl-CoA binds only the N terminus of L-CPTI, the CPTII component of the CPTI (1–147)-CPTII chimera must have a tertiary conformation that is unable to interact with this domain. The
second hypothesis was that the C-terminal region of L-CPTI is critical for malonyl-CoA binding and that the necessary site(s) is not present in CPTII. Since this observation, different studies have indicated that (i), the different malonyl-CoA sensitivities of the CPTI isoforms result primarily from differences in their C termini (16, 18, 19) and (ii), the nature of the cytosolic N/C interaction of the L-CPTI is involved in the degree of malonyl-CoA sensitivity (20, 21). The existence of such a physical N/C interaction was recently supported by the identification of a highly tryptophan-resistant 60-kDa folded core within the catalytic C-terminal domain that is hidden in the native protein by its cytosolic N-terminal residues (14). However, whether the extreme N terminus (and particularly Gnu3) participates directly to malonyl-CoA binding or allows the extreme cytosolic N terminus to interact with the catalytic C-terminal domain in order to maintain a conformational malonyl-CoA binding site is still unclear.

In the present study, we examined whether the N-terminal domain of L-CPTI exhibits a high affinity for malonyl-CoA binding independently of the presence of the C-terminal domain of L-CPTI, and we investigated for the first time whether the extreme C terminus of L-CPTI, which exhibits notable C-terminal residues, respectively. The fidelity of all PCR reactions, the constructs encode an L-CPTI protein deleted of the last 7 and 31 C-terminal residues, respectively. The fidelity of all PCR reactions, the constructs were obtained as described previously (13, 22). Briefly, pOM29-CPTII were obtained as described previously (13, 22). The PCR product was cut by SacI restriction site of L-CPTI cDNA and the PCR primer (5′-TATGTGGTGTCCAAGTAT-3′) located upstream the unique SacI primer (5′-CCCGCGGTTAAGAATTGATGGTGAG-3′) coding sequence of the rat L-CPTI by using the 5′ stretch of the 3′ primer but to those used for the construction of CPTI mutants. All DNA manipulations were performed by the method of Ref. 25 with bovine serum albumin as a standard. All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and Zymolase 20T was from Amersham Biosciences.

Construction of Expression Plasmids—The S. cerevisiae strains expressing rat L-CPTI, pOM29-CPTII, pOM29-CPTII Δ148, and CPTI (1–147)-CPTII were obtained as described previously (13, 22). Briefly, pOM29-CPTII and -CPTII Δ148 correspond to the fusion of pOM29 (a specific OMM signal anchor sequence), to the mature form of CPTII, or to L-CPTI lacking the first 148 N-terminal residues, respectively. CPTI (1–147)-CPTII is the fusion of the mature form of CPTII to the N-terminal domain of L-CPTI. Escherichia coli DH5α strain was used to propagate various plasmids. The yeast expression vector pYDPAS1-10 containing the full-length rat L-CPTI cDNA (pYEDP1/8–10-L-CPTI (22)) was used to generate the deletion mutants. All DNA manipulations (restriction and ligation) were performed according to the instructions provided by the manufacturer’s protocols for the respective enzymes. The C-terminal deletion rat L-CPTI mutants were constructed as follows.

CPTI Δ772–773—PCR was performed to copy a 336-nucleotide stretch of the 3′ coding sequence of the rat L-CPTI by using the 5′-primer (5′-TATGTGGTGTCCAAGTAT-3′) located upstream the unique SacI restriction site of L-CPTI cDNA and the 3′-primer (5′-CCCGCGGTTAAGAATTGATGGTGAG-3′) introducing a stop codon and a SacI site immediately downstream the codon encoding Ser771. The PCR product was cut by SacI and SacII and ligated into pYEDP1/8–10-L-CPTI cut by the same enzymes. This construct encodes an L-CPTI lacking the last two C-terminal amino acids.

CPTI Δ767–773 and CPTI Δ743–773—These constructs were obtained in a similar manner to CPTI Δ772–773 by using the same 5′-primer but different 3′-primers as follows: CPTI Δ767–773, 5′-TCCGCGGTTAGCAGAAACAGGTGAT-3′; CPTI Δ743–773, 5′-TCCGCGGTTAGCAGAAACAGGTGAT-3′; CPTI Δ767–773, 5′-TCCGCGGTTAGCAGAAACAGGTGAT-3′. All subsequent procedures were identical to those used for the construction of CPTI Δ772–773. These constructs encode an L-CPTI protein deleted of the last 7 and 31 C-terminal residues, respectively. The fidelity of all PCR reactions, the DNA subcloning mutants, and the equality of DNA subcloning were confirmed by DNA sequence analysis.

Yeast Culture, Isolation of Yeast Mitochondria, and Subcellular Fractionation—Each DNA was placed under the control of the inducible GAL10 promoter, and the constructs were used to transform S. cerevisiae (haploid strain W303: MATa, his3, leu2, trp1, ura3, ade2–1, can1–100, trp1–1, leu2–3,112, ura3–1). Methods for yeast transformation and cultivation, subcellular fractionation, and isolation of yeast mitochondria were as described previously (22).

CPT Assay—CPT activity was assayed at 30 °C as palmitoyl-L-[methyl-3H]carnitine formed from L-[methyl-3H]carnitine (200 μM; 10 Ci/mol) and palmitoyl-CoA (80 μM) in the presence of 1% bovine serum albumin (w/v) as described previously (22). Malonyl-CoA concentration varied over 0.01 to 150 μM for estimation of the IC50 value. The apparent Kd for carnitine was measured at 600 μM palmitoyl-CoA with 5–800 μM carnitine and the apparent Kd for palmitoyl-CoA at 200 μM carnitine with 10–900 μM palmitoyl-CoA in the presence of a fixed molar ratio of palmitoyl-CoA/albumin (6.1:1) (22).

Malonyl-CoA Binding Assay—[14C]Malonyl-CoA binding was determined by a modified centrifugation assay as described previously (23). Isolated mitochondria (400 μg of protein) from wild-type and mutants were resuspended in 0.4 ml of ice-cold medium containing 250 mM sucrose, 60 mM KCl, 10 mM Hepes (pH 7.4), 1 mM EGTA, 1% bovine serum albumin (w/v), 0.01–5 μM [14C]malonyl-CoA (56 mCi/mmol) (Amersham Biosciences) in the absence or presence of 200 μM unlabelled malonyl-CoA. Incubations were started by addition of the mitochondria and were continued for 20 min at 0 °C with gentle mixing at 4-min intervals. Bound and free malonyl-CoA were then separated by centrifugation at 15,000 × g for 10 min at 4 °C. The supernatants were discarded, and 0.25 ml of 1 M KOH was immediately added to the pellets. Subcellular fractionation of the mitochondria was facilitated by heating at 50 °C for 30 min, after which time the contents of the tube, together with 0.8 ml of water, were transferred to counting vials and assayed for radioactivity after addition of 10 ml of Aquasol. The final malonyl-CoA binding values for the wild-type and mutants were corrected for background malonyl-CoA binding by the yeast control strain that purified with the vector but without the yeast insert. All binding experiments were analyzed with the non-linear least squares curve-fitting procedure of the EBBDA/LIGAND program (Biosoft Elsevier, Cambridge, UK). After subtraction of nonspecific malonyl-CoA binding to the CPTI-free yeast control strain, data were always best fitted according to a one-site model. When needed, the validity of a one-site model was confirmed using an F-test.

Assessment of the Folding State of CPTI Mutants—Proteolytic analysis of the full-length L-CPTI and mutants was performed as described previously (22) with the following modifications. Eighty μg of mitochondria isolated from yeast strains expressing the different proteins were resuspended in SH buffer (0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4) at a concentration of 0.5 mg of protein/ml in the absence or in the presence of 30 μg/ml trypsin. Samples were kept on ice for 30 min, and then soybean trypsin inhibitor (STI) was added in a 30-fold excess. After 10 min, mitochondria were resolubilized, washed once with SH buffer containing 1 mM EDTA and 1 mg/ml STI, and then lysed directly in Laemmli buffer. Samples were analyzed by SDS-PAGE and immunoblotting.

Western Blot Analysis—Aliquots of proteins were subjected to SDS-PAGE (24) in an 8% gel. The detection of proteins after blotting onto nitrocellulose was performed as described previously (22) using the ECL detection system (Fierce) according to the supplier’s instructions. Miscellaneous and Chemicals—Protein concentration was determined by the method of Ref. 25 with bovine serum albumin as a standard. All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. PCR reagents and T4 DNA polymerase were obtained from Invitrogen. Yeast culture media products were from Difco, and Zymolase 20T was from ICN Biomedicals, France. Others chemicals were purchased from Sigma.

Statistics—Results are expressed as means ± S.E. Statistical analysis was performed using the Mann-Whitney U test.

RESULTS

Malonyl-CoA Sensitivity and [14C]Malonyl-CoA Binding of Chimeric CPTs—The first purpose of this study was to determine whether the N-terminal domain (the first 147 N-terminal amino acids) of L-CPTI was able to bind malonyl-CoA with a high affinity, independently of the presence of the C-terminal domain. For this purpose, we further characterized some chimeric CPTs previously generated and expressed in S. cerevisiae (13), a system devoid of endogenous CPT enzyme (22, 26). CPTI (1–147)-CPTII (fusion of the mature form of CPTII to the N-terminal domain of L-CPTI) offered the possibility of directly

Functional Analysis of the C Terminus of Rat Liver CPTI

47185
addressing the former question. pOM29-CPTII and pOM29-CPTIΔ148 were used as negative control proteins, pOM29 being a specific OMM signal anchor sequence (27). All of these chimeric proteins were expressed in yeast at similar steady-state levels and were located at the OMM, their C terminus exposed to the cytosol (13). As previously reported (13), both pOM29-CPTII and CPTI (1–147)-CPTII were catalytically active, whereas pOM29-CPTIΔ148 retained only 8% of that observed with L-CPTI (Table I). In contrast to L-CPTI, which exhibited an IC₅₀ for malonyl-CoA of 0.6 μM (Table I and Fig. 1), pOM29-CPTII, pOM29-CPTIΔ148, and CPTI (1–147)-CPTII were largely insensitive to malonyl-CoA whatever the malonyl-CoA concentration tested. Malonyl-CoA binding to mitochondria from yeast strains expressing L-CPTI and chimeric CPT was clearly saturable (Fig. 2 A). As shown by the Scatchard plots in Fig. 2B, saturation binding experiments were resolved into a single high affinity site (L-CPTI) or a single low affinity site (chimeric CPT). Replacement of the N-terminal domain of L-CPTI by pOM29 completely abolished the high affinity malonyl-CoA binding (Fig. 2B), increasing the Kᵥ value by 20-fold whereas the Bᵥ values decreased only slightly (Table I). In agreement with the fact that CPTII does not possess a malonyl-CoA binding domain, pOM29-CPTII exhibited a Kᵥ value 30-fold higher than that of L-CPTI (Table I). Anchorage of CPTII at the OMM by the N-terminal domain of L-CPTI instead of pOM29 did not result in the appearance of a high affinity

### Table I

| Strain                  | Activity | IC₅₀ | Kᵥ  | Bᵥ  |
|-------------------------|----------|------|-----|-----|
| L-CPTI                  | 2.65 ± 0.28 | 0.56 ± 0.10 | 11 ± 2 | 20 ± 3 |
| pOM29-CPTII             | 2.92 ± 0.14 | N/A              | 312 ± 71 | 20 ± 4 |
| pOM29-CPTIΔ148          | 0.19 ± 0.06 | N/A              | 264 ± 74 | 15 ± 3 |
| CPTI (1–147)-CPTII      | 2.07 ± 0.25 | N/A              | 281 ± 7 | 12 ± 2 |

Fig. 1. Effect of increasing concentrations of malonyl-CoA on the activities of yeast-expressed wild-type L-CPTI and chimeric CPTs. Results are expressed relative to activity in the absence of malonyl-CoA (100%) and are the means ± S.E. of three to six separate experiments. (○), wild-type L-CPTI; (●), pOM29-CPTIΔ148; (□), pOM29-CPTII; (■), CPTI (1–147)-CPTII.

![Fig. 1](http://www.jbc.org/)

Fig. 2.Binding of [2-¹⁴C]malonyl-CoA to mitochondria isolated from the yeast strains expressing the wild-type L-CPTI and the chimeric CPTs. 400 μg of protein were used for the binding assay. Malonyl-CoA binding values for the wild-type and chimeric CPTs were corrected for malonyl-CoA binding to the mitochondria from the yeast strain with the vector but no insert. A, specific [2-¹⁴C]malonyl-CoA binding. B, Scatchard plots for binding of [2-¹⁴C]malonyl-CoA. (○), wild-type L-CPTI; (●), pOM29-CPTIΔ148; (□), pOM29-CPTII; (■), CPTI (1–147)-CPTII. Results are means ± S.E. of three to five separate experiments.

![Fig. 2](http://www.jbc.org/)

Fig. 3. Outline of the C-terminal deletion CPTI constructs. Aligned amino acid sequences of the C-terminal end of known CPTI isoforms in mammalian species with conserved (shared area) and non-conserved (boxed area) amino acids highlighted. The position of each of the deletion mutants is shown by an arrow. Sources of the sequences were GenBank for mouse M-CPTI cDNA (AB0108226) and the Swiss Protein data base for rat (F92198), human (P50416), and mouse (P97742) L-CPTI, and rat (Q63704) and human (Q92523) M-CPTI.
malonyl-CoA binding (Fig. 2B and Table I). This clearly shows that the insensitivity of CPTI (1–147)-CPTII to malonyl-CoA resulted from the absence of high affinity malonyl-CoA binding.

Generation of C-terminal Deletion Mutants of Rat L-CPTI and Expression in S. cerevisiae—Amino acid sequence alignment of the last 33 C-terminal residues of CPTI isoforms from different mammalian species is shown in Fig. 3. In contrast to the N terminus, the C terminus exhibits a low degree of sequence conservation because no more than 13 of the last 33 residues are identical. The major difference between the L- and M-CPTI isoforms is the presence of two lysine residues at the C-terminal end of all known mammalian L-type isoforms. Secondary structure prediction methods predict that residues 747–768 of the rat L-CPTI form an amphipathic a-helix, as for the corresponding residues in the other CPTI isoforms. Therefore, three deletion mutants of rat L-CPTI were constructed in which the last 2 (2 lysines), 7 (downstream the a-helix), or 31 (encompassing the a-helix) C-terminal residues were deleted (Fig. 3). All these constructs, as well as the full-length L-CPTI, were expressed in S. cerevisiae.

Western blot analysis of mitochondria isolated from yeast cells expressing the different proteins using a polyclonal anti-(Fig. 4). Deletion of the last 2 or 7 C-terminal residues did not affect malonyl-CoA sensitivity, whatever the concentration of the inhibitor tested (Fig. 5A), and the IC50 values for malonyl-CoA of the wild-type L-CPTI, Δ772–773, and Δ767–773 were similar (Table II). All active CPTI mutants exhibited normal saturation kinetics when the carnitine concentration varied relative to a fixed concentration of palmitoyl-CoA (Fig. 5B) or when palmitoyl-CoA concentration varied when the molar ratio of palmitoyl-CoA:albumin was fixed at 6.1.1 (Fig. 5C). The calculated Km for carnitine and palmitoyl-CoA of Δ772–773 and Δ767–773 were similar to those of the wild-type L-CPTI (Table II).

Assessment of the Folding State of the C-terminal Deletion Mutants—To understand the abolition of CPT activity upon deletion of the last 31 C-terminal residues, we examined the possibility that the conformational state of L-CPTI could have been affected. We have previously shown that native or yeast-expressed L-CPTI exhibited a highly folded conformation resistant to trypsin proteolysis (13, 14, 22). When intact mitochondria isolated from the different expressing yeast strains were incubated in the presence of trypsin, both Δ772–773 and Δ767–773 remained largely resistant to the protease treatment, indicating that their conformation was not dramatically affected by the deletion (Fig. 6). By contrast, Δ743–773 was totally digested by trypsin (Fig. 6) without apparent detectable proteolytic fragments (results not shown). Thus, in the absence of the last 31 C-terminal residues, the C-terminal domain of L-CPTI did not harbor the highly folded core characteristic of the native enzyme. Therefore, we concluded that, in the case of Δ743–773, disappearance of functional activity directly resulted from an unfolded state of the protein.

### DISCUSSION

N-terminal Domain of L-CPTI and Malonyl-CoA Sensitivity—The N-terminal domain of rat L-CPTI is not only responsible for protein targeting to the outer mitochondrial membrane but is also essential for malonyl-CoA sensitivity (13–15, 17, 20). Inhibition of L-CPTI activity by malonyl-CoA involves two sites of malonyl-CoA interaction. The first one is the low affinity site, near the catalytic acyl-CoA-binding domain (28, 29) as recently modeled by Ref. 30, whereas the second site, the high affinity site, is separated from the active site and does not compete with acyl-CoA (31–34). Although previous studies have shown that the N-terminal domain influences the degree of malonyl-CoA sensitivity, it has not been ascertained whether the N-terminal residues of L-CPTI contribute directly to a malonyl-CoA binding site. Using heterologous expression in S. cerevisiae, we have previously shown that fusion of the N-terminal domain of L-CPTI (1 to 147 amino acids) to the mature form of the malonyl-CoA-insensitive CPTII allowed a specific OMM targeting of the resulting protein leaving the CPTII moiety on the cytosolic face of the mitochondria, as for the catalytic domain of L-CPTI (12). This chimeric CPTI (1–147)-CPTII protein was functionally active but totally malonyl-CoA-insensitive (13). In the present study, we show that chimeric
CPTI activity was assayed at 80 μM palmitoyl-CoA and 200 μM carnitine in the absence or presence of increasing concentrations of malonyl-CoA (Δ), at 600 μM palmitoyl-CoA with increasing concentrations of carnitine (B), or at 200 μM carnitine with increasing concentrations of palmitoyl-CoA in the presence of a fixed 6.1:1 molar ratio of palmitoyl-CoA:albumin (C) using intact yeast mitochondria (50 μg of protein). Results are means ± S.E. of three to six separate experiments.

Mitochondria were isolated from the yeast strains deletion mutants. 

Kinetic analysis of wild-type L-CPTI and C-terminal deletion mutants. Mitochondria were isolated from the yeast strains expressing the wild-type L-CPTI (C), Δ772–773 (∆), and Δ767–773 (▲). CPTI activity was assayed at 80 μM palmitoyl-CoA and 200 μM carnitine in the absence or presence of increasing concentrations of malonyl-CoA (A), at 600 μM palmitoyl-CoA with increasing concentrations of carnitine (B), or at 200 μM carnitine with increasing concentrations of palmitoyl-CoA in the presence of a fixed 6.1:1 molar ratio of palmitoyl-CoA:albumin (C) using intact yeast mitochondria (50 μg of protein). Results are means ± S.E. of three to six separate experiments. A, values are expressed as percentage of control activity measured in the absence of malonyl-CoA. B and C, values are expressed as percentage of Vmax obtained for the same preparation. The Vmax values for carnitine (mean ± S.E.) for L-CPTI, Δ772–773, and Δ767–773 were 40.29 ± 2.73, 41.72 ± 5.67, and 51.47 ± 7.19 nmol of palmitoylcarnitine formed/min/mg of protein, respectively. The Vmax values for palmitoyl-CoA (mean ± S.E.) for L-CPTI, Δ772–773, and Δ767–773 were 30.32 ± 6.87, 31.18 ± 0.62, and 30.21 ± 6.74 nmol of palmitoylcarnitine formed/min/mg of protein, respectively.

CPTI (1–147)-CPTII does not exhibit a high affinity for malonyl-CoA binding when compared with pOM29-CPTII. These results indicate that (i), the N-terminal domain of rat L-CPTI has not the ability by itself (i.e. in the absence of the C-terminal domain) to bind malonyl-CoA and (ii), the high affinity binding site for malonyl-CoA likely involves either residues located both in the N- and C-terminal domains or only residues within the C-terminal domain. In the first hypothesis, physical N/C interactions are necessarily involved in order to constitute the malonyl-CoA binding site. Any modification within the N terminus would then be expected to alter profoundly the high affinity malonyl-CoA binding. Initial deletion mutation analysis of the conserved first 18 N-terminal residues of rat L-CPTI was in agreement with this statement (15, 17), whereas further studies indicated that the mechanism of malonyl-CoA inhibition is more complicated because the cytosolic N terminus contained both positive and negative determinants of malonyl-CoA sensitivity (20). Moreover, functional characterization of several L-CPTI chimeras has predicted that malonyl-CoA binding sites were located in the cytosolic C-terminal domain (16, 19). If this is true, the cytosolic N terminus should (i), stabilize the high affinity malonyl-CoA binding site through its interaction with the catalytic C-terminal domain and (ii), modulate the degree of malonyl-CoA sensitivity through conformational changes that alter these N/C intramolecular interactions, as previously suggested (19, 20).

C Terminus of L-CPTI and Malonyl-CoA Sensitivity—Whatever the location of the high affinity malonyl-CoA binding site within L-CPTI, it might be a conformational site that is highly sensitive to interaction(s) with the N terminus. Which part(s) of the C-terminal domain of L-CPTI do interact with the N terminus? Because the negative charge of Glu7 has been shown to be essential for mediating the inhibitory effects of malonyl-CoA (17, 21), we asked whether the positive charges of the 2
Lys residues present at the COOH terminus of all known L-CPTI species (1) could play a role in these N/C intramolecular interactions and/or explain the discrepancies in the degree of malonyl-CoA sensitivity between the L- and M-CPTI isoforms. The kinetic properties of the L-CPTI mutants lacking the last 2 or 7 C-terminal amino acids were indistinguishable from those of the wild-type. We concluded that the two highly conserved Lys residues in the C terminus of all known L-CPTI species are essential for neither functional activity nor malonyl-CoA sensitivity and hence are not involved in a physical interaction with the N terminus of the enzyme. To further investigate the role of the C terminus of L-CPTI, we constructed two other deletion mutants, Δ743–773 and Δ719–773. Among the seven independent yeast clones transfected with pYeDP1/8-Δ719–773, no protein expression was detected. This was in contrast with the three other deletion mutants (Δ772–773, Δ767–773, and Δ743–773) for which we observed correct protein expression in all tested yeast clones. This observation suggested that the last 55 C-terminal amino acids are critical for protein stability. Moreover, Δ743–773 mutant was correctly imported into the OMM but was not folded, as shown by its high sensitivity to trypsin proteolysis and, hence, was functionally inactive. The effect of this deletion was unexpected from our previous observations (14). Indeed, we have shown that once imported into the OMM, L-CPTI adopts its native functional conformation that is characterized by a highly folded state resistant to trypsin proteolysis (Fig. 7, a and b). However, when the loop connecting TM1 and -2 is first cleaved by the protease, such as during the swelling procedure (Fig. 7c), possible trypsin cleavage sites occur C-terminal to Arg598 or Arg599 and Lys631 or Lys634, and the highly folded trypsin resistant core (likely to be in part the catalytic core) is still detected after solubilization by Triton X-100 in the presence of trypsin (Fig. 7d). This indicates that the last 139–178 C-terminal amino acids of L-CPTI can be cleaved from the native protein without impairing the folding of the remaining C-terminal domain. By contrast, if deletion of the last C-terminal amino acids occurs prior to mitochondrial protein import, such as in Δ743–773, then the resulting imported protein is unable to reach its correct folded conformation (Fig. 7e). This observation indicates that the last 51 C-terminal amino acids, which are predicted to form an amphipathic α-helix, are critical for initial protein folding of the catalytic C-terminal domain of L-CPTI. Once folded, the tertiary structure of this domain is thereafter stabilized by other intramolecular interactions independently of the last 31 C-terminal residues. The functional relevance of these results is of great importance and must be kept in mind when performing functional analysis of L-CPTI mutants. Indeed, not enough caution has been taken in differentiating between whether a specific mutation within the C-terminal domain directly affects catalytic activity without drastically altering protein folding (functional determinant) or whether it induces protein unfolding (structural determinant) that secondarily decreases functional activity. During their course of folding, proteins undergo different types of structural rearrangements, ranging from local to large-scale conformational changes that lead to a series of sequential transition folding states. In this protein folding pathway, cooperative interactions of specific residues may be critical in establishing a bonding network that transiently stabilizes the intermediate conformations (35, 36). For instance, point mutations can increase local flexibility or affect kinetic folding without altering the average native conformation of the protein (37–39). Such a direct effect of mutations on the degree and/or rate of protein folding independently of function could be proposed for the L-CPTI-H277A and L-CPTI-H277A/H483A mutants described recently in Ref. 30. These mutants expressed in yeast showed different sensitivity to malonyl-CoA inhibition, depending on the time after galactose induction, the kinetics of inhibition by malonyl-CoA being affected only at the time of 1 h of induction but not after 20 h. Such time-dependent behavior of the mutants could be due to a slower kinetic of folding and not necessarily be linked to the level of protein expression and/or organization within mitochondrial membrane. In conclusion, the present study indicates that the last 31 C-terminal residues of rat L-CPTI are not involved in malonyl-CoA sensitivity but constitute a “secondary structural specificity determinant” that may prevent neighboring residues from adopting an alternate protein fold by acting as a folding initiation site.

REFERENCES
1. McGarry, J. D., and Brown, N. F. (1997) Eur. J. Biochem. 244, 1–14
2. McGarry, J. D., Leatherman, G. F., and Foster, D. W. (1978) J. Biol. Chem. 253, 4128–4136
3. Pretkni, M., and Corkey, B. E. (1996) Diabetes 45, 273–283
4. Zammit, V. A. (1999) Biochem. J. 343, 505–515
5. Buderam, N. B., Saha, A. K., Varvas, D., and Witters, L. A. (1999) Am. J. Physiol. 276, E1-E18
6. Bergman, R. N., and Ader, M. (2000) TEM 11, 351–356
7. Unger, R. H., and Orci, L. (2001) FASEB J. 15, 312–321
8. McGarry, J. D. (2002) Diabetes 51, 7–18
9. McGarry, J. D. (1994) J. Cell. Biochem. 55S, 29–38
10. Anderson, R. C. (1998) Curr. Pharm. Des. 4, 1–16
11. Eser, V., Britton, C. H., Weis, B. C., Foster, D. W., and McGarry, J. D. (1993) J. Biol. Chem. 268, 5817–5822
12. Fraser, F., Corstorphine, C. G., and Zammit, V. A. (1997) Biochem. J. 333, 711–718
13. Cohen, I., Kohl, C., McGarry, J. D., Girard, J., and Prip-Buus, C. (1998) J. Biol. Chem. 273, 29986–29994
14. Cohen, I., Guillerault, F., Girard, J., and Prip-Buus, C. (2001) J. Biol. Chem. 276, 5430–5431
15. Shi, J., Zhu, H., Arvidson, D. N., Cregg, J. M., and Woldegiorgis, G. (1998) Biochemistry 37, 11033–11038
16. Swanson, S. T., Foster, D. W., McGarry, J. D., and Brown, N. F. (1998) Biochem. J. 335, 513–519
17. Shi, J., Zhu, H., Arvidson, D. N., and Woldegiorgis, G. (1999) J. Biol. Chem. 274, 9421–9426
18. Shi, J., Zhu, H., Arvidson, D. N., and Woldegiorgis, G. (2000) Biochemistry 39, 712–717
19. Jackson, V. N., Cameron, J. M., Fraser, F., Zammit, V. A., and Price, N. T. (2000) J. Biol. Chem. 275, 19560–19566
20. Jackson, V. N., Zammit, V. A., and Price, N. T. (2000) J. Biol. Chem. 275, 37026–37031
21. Jackson, V. N., Price, N. T., and Zammit, V. A. (2001) Biochemistry 40, 14629–14634
22. Prip-Buus, C., Cohen, I., Kohl, C., Eser, V., McGarry, J. D., and Girard, J. (1998) FEBS Lett. 429, 173–178
23. Mills, S. E., Foster, D. W., and McGarry, J. D. (1983) Biochem. J. 214, 83–91
24. Laemmli, U. K. (1970) Nature 227, 685–688
25. Lowry, O. H., Rosebrough, N. J., Lewis, F. A., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
26. Brown, N. F., Eser, V., Foster, D. W., and McGarry, J. D. (1994) J. Biol. Chem. 269, 26438–26442
27. Li, J.-M., and Shore, G. C. (1992) Science 256, 1815–1817
28. Dai, J., Zhu, H., Shi, J., and Woldegiorgis, G. (2000) J. Biol. Chem. 275, 20209–20214
29. Morillas, M., Gomez-Puertas, P., Roca, R., Serra, D., Asins, G., Valencia, A., and Hegardt, F. G. (2001) J. Biol. Chem. 276, 45001–45008
30. Morillas, M., Gomez-Puertas, P., Rius, B., Clotet, J., Arino, J., Valencia, A., Hegardt, F. G., Serra, D., and Asins, G. (2002) J. Biol. Chem. 277, 11473–11480
31. Bird, M. I., and Saggerson, E. D. (1984) Biochem. J. 222, 639–647
32. Zammit, V. A., Corstorphine, C. G., and Gray, S. R. (1984) Biochem. J. 222, 335–342
33. Cook, G. A., Mynatt, R. L., and Kashfi, K. (1994) J. Biol. Chem. 269, 8803–8807
34. Kashfi, K., Mynatt, R. L., and Cook, G. A. (1994) Biochim. Biophys. Acta 1212, 245–252
35. Horvitz, A., and Fersht, A. R. (1992) J. Mol. Biol. 224, 733–740
36. Kurokawa, T., Kurokawa, R., Nakamura, K., Perez-Cerda, C., Ibellidou, A., Barrionuevo, C. R., Castello, F. G., Kohno, Y., Ugarte, M., and Kondo, N. (2002) Mol. Genet. Metab. 75, 235–243
The Extreme C Terminus of Rat Liver Carnitine Palmitoyltransferase I Is Not Involved in Malonyl-CoA Sensitivity but in Initial Protein Folding
Yong Pan, Isabelle Cohen, Fanny Guillerault, Bruno Fève, Jean Girard and Carina Prip-Buus

J. Biol. Chem. 2002, 277:47184-47189.
doi: 10.1074/jbc.M208055200 originally published online September 25, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208055200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 22 of which can be accessed free at http://www.jbc.org/content/277/49/47184.full.html#ref-list-1