The Mitochondrial Intermembrane Loop Region of Rat Carnitine Palmitoyltransferase 1A Is a Major Determinant of Its Malonyl-CoA Sensitivity*

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Carnitine palmitoyltransferase (CPT) 1A adopts a polytopic conformation within the mitochondrial outer membrane, having both the N- and C-terminal segments on the cytosolic aspect of the membrane and a loop region connecting the two transmembrane (TM) segments protruding into the inter membrane space. In this study we demonstrate that the loop exerts major effects on the sensitivity of the enzyme to its inhibitor, malonyl-CoA. Insertion of a 16-residue spacer between the C-terminal part of the loop sequence (i.e. between residues 100 and 101) and TM2 (which is predicted to start at residue 102) increased the sensitivity to malonyl-CoA inhibition of the resultant mutant protein by more than 10-fold. By contrast, the same insertion made between TM1 and the loop had no effects on the kinetic properties of the enzyme, indicating that effects on the catalytic C-terminal segment were specifically induced by loop-TM2 interactions. Enhanced sensitivity was also observed in all mutants in which the native TM2-loop pairing was disrupted either by making chimeras in which the loops and TM2 segments of CPT 1A and CPT 1B were exchanged or by deleting successive 9-residue segments from the loop sequence. The data suggest that the sequence spanning the loop-TM2 boundary determines the disposition of this TM in the membrane so as to alter the conformation of the C-terminal segment and thus affect its interaction with malonyl-CoA.

Carnitine palmitoyltransferase (CPT)1 is a mitochondrial outer membrane protein that catalyzes the conversion of long chain acyl-CoA esters to acylcarnitines. This is the step that commits fatty acids to β-oxidation within mitochondria and plays an important role in determining the availability in the extramitochondrial compartment of long chain acyl-CoA esters, which are potent effectors of multiple aspects of cell function, such as gene transcription, ion channel regulation, and secretory processes (1). CPT 1 is a polytopic integral membrane protein, with two segments (N- and C-terminal) that are exposed on the cytosolic aspect of the mitochondrial outer membrane and two transmembrane segments (TM1 and TM2) linked by a loop region that protrudes into the intermembrane space of the mitochondria (2). The membrane topology of the protein is highly relevant to the action of its physiological inhibitor, malonyl-CoA. The sensitivity to malonyl-CoA inhibition of the isoform originally identified in the liver (L-CPT 1 or CPT 1A) is modulated by physiological state. Thus, CPT 1A in mitochondria isolated from the liver of rats in pathophysiological conditions characterized by high glucagon/insulin molar ratios is much less sensitive to malonyl-CoA inhibition (3–6). Dietary lipid composition is also able to modulate the kinetic characteristics of the enzyme (7). These changes in sensitivity are thought to result from a response of the enzyme to the molecular order of the lipids of the mitochondrial outer membrane (8, 9) and can be mimicked in vitro by conditions that alter membrane fluidity (10). Therefore, it has been suggested (9) that the membrane-integral nature of the protein enables its tertiary structure (and kinetic properties) to respond to changes in membrane composition induced by different pathophysiological states. Previous studies have concentrated on the role of potential changes in the interaction between the catalytic C-segment (to which malonyl-CoA also binds) and the regulatory N-segment, both of which are exposed on the cytosolic aspect of the membrane. Recent intramolecular cross-linking experiments have borne out this hypothesis by showing that a cross-linker with a spacer arm 15.7 Å long is able to form a covalent link between a lysine and a cysteine residue on the N- and C-terminal segments, respectively, of rat CPT 1A (11). The previous descriptions of several critical residues within the N-terminal segment that are either strong positive or negative determinants of malonyl-CoA sensitivity (12–14) also suggest that the precise interaction, or docking, of the N-segment with the C-segment determines this important kinetic parameter of CPT 1A.

However, a potential role for the loop linking the two parts of the molecule in determining malonyl-CoA sensitivity of CPT 1A has hitherto not been considered. Studies on other polytopic proteins have shown that loops can greatly influence the angle at which TM segments lie within and emerge from membranes so as to influence the conformation of integral membrane proteins (15–17). Loops between transmembrane helices constrain the location of TM segments and may dictate specific orientations that favor particular tertiary structures, e.g. by promoting or resisting folding events that bring TM helices together (17). Loops of polytopic proteins range in size from a few amino acid

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2The abbreviations used are: CPT, carnitine palmitoyltransferase; TM, transmembrane; C-segment, C-terminal segment; N-segment, N-terminal segment; TEV, tobacco etch virus.
Structure-Function Relationships of CPT 1A

(A) 76 – 102
Wild Type
KVDP5LMIKASRALTDCGKSSQ-

Insert at TM2
KVDP5LMIKAI5TLGTDCGKSSQ-

Insert at TM1
KV5P5LMIKASRALTDCGKSSQ-

Random insert at TM2
KVDP5LMIKASRALTDCGKSSQ-

(B) 76 – 102
Wild Type
KVDP5LMIKASRALTDCGKSSQ-

Δ (77–84)
KV5P5LMIKASRALTDCGKSSQ-

Δ (85–93)
KVDP5LMIKASRALTDCGKSSQ-

Δ (94–101)
KV5P5LMIKASRALTDCGKSSQ-

(C) 73 – 135
Wild Type
MHI5P5LMIKASRALTDCGKSSQ-

b-TM2
MHI5P5LMIKASRALTDCGKSSQ-

b-loop
MHI5P5LMIKASRALTDCGKSSQ-

FIGURE 1. Sequence alignment of mutations made within the loop or TM2 regions of rat CPT 1A. A, the sequences of the loop regions for the wild type CPT 1A and the same sequence with three different insertion mutations (underlined) placed adjacent to either TM1 or TM2. B, sequences of the loop region of the wild type CPT 1A and of the three deletion mutants of the loop sequence. This was subdivided into three approximately equal sections, and each section was individually deleted, as indicated by the dashed lines. C, sequences of chimeric constructs in which either the loop (b loop) or TM2 sequence (b TM2) of CPT 1B were substituted, as shown underlined, for the corresponding sequences of native CPT 1A.

residues to very large domains; in the case of CPT 1A, the loop between TM1 and TM2 is predicted to be 27 residues long. Initial experiments in our laboratory, which were aimed at introducing a TEV protease site within the loop of CPT 1A, indicated that such an insertion greatly affected the malonyl-CoA sensitivity of the enzyme. Therefore, we have conducted an investigation into the role that loop structure may play in determining the kinetic properties of CPT 1A. The data indicate that the sequence spanning the boundary between TM2 and the region of the loop adjacent to it is particularly important in determining the malonyl-CoA sensitivity of the catalytic C-terminal segment of CPT 1A.

MATERIALS AND METHODS

The materials were obtained as in Refs. 13, 14, and 18. The methods for transformation, culturing of Pichia pastoris and preparation of yeast extracts for PCR 1 assay were also as described previously (13, 14, 18). All of the clones were selected with 0.1 mg/ml zeocin. Protein assays, SDS-PAGE, and Western blotting were performed as in Ref. 19; the primary antibody used was anti-peptide C raised in sheep against a synthetic peptide corresponding to residues 428–441 of rat CPT 1A, as described in Ref. 2.

Construction of Mutants of Rat CPT 1A—Fig. 1 gives the sequences of the mutations studied. Construction of wild type, E3A, S24A/Q30A, and Δ (1–82) rat CPT 1A pGAPZ expression plasmids have been described previously (18). The point mutant P79I and the deletion mutant Δ (77–84) were prepared by replacing the 3′ SphI-AflII fragment of CPT 1A with cleaved PCR-generated fragments. For the mutant P79I, primers 5′-GCATGCAGATGCGAC-3′ (forward) and 5′-AGTCGAC-3′ (reverse) were used with the wild type construct as template. The forward primer 5′-CTCTGATGCTAACCTGAGAC-3′ was used in combination with 5′-CTGCGGCGCATTGGTTCTGCA-3′ to generate Δ(77–84). The Δ(94–101) mutation was prepared similarly using primers 5′-TCTGTCTGGAAGATGCGAC-3′ (forward) and 5′-TCTGTGCTGGAAGATGCGAC-3′ (reverse) and restriction enzymes Csp45I and BspEI.

The chimeric construct in which the loop of rat CPT 1B was substituted for that of CPT 1A (b-Loop) and the deletion mutant Δ (85–93) was prepared by replacing the 3′ SphI-BspEI fragment of CPT 1A with cleaved PCR-generated fragments. For the mutant B-Loop mutants 5′-TTATGCATGCTAAAAGAATGCGAC-3′ (forward) and 5′-AGATCGCGGACACAACTTTTTGTC-3′ were prepared similarly using primers 5′-AGATCGCGGACACAACTTTTTGTC-3′ (forward) and 5′-AGATCGCGGACACAACTTTTTGTC-3′ (reverse) were annealed and extended by heating to 70 °C for 5 min and allowed to cool to room temperature. Three units of T4 DNA polymerase were added with dNTPs (1 mM) and bovine serum albumin (1 mM). The reaction was allowed to proceed for 1–2 h. The resultant fragment was cleaved with BstEII and HindIII for TEV adjacent to TM1. The randomized sequence of the TEV site was designed using randomizing software. Primers 5′-CTATGCACTGTTTGCTGATGCTGCA-3′ and 5′-AATTGCTAACCAATTTGGCG-3′ were used with the M-CPT 1 wild type template. For the mutant Δ (85–93) primers 5′-GCATGCCAAGATGCGAC-3′ (forward) and 5′-TGACCGGACACAACTTTTTGTC-3′ (reverse) were annealed and extended as above. The cleaved product was cloned into a pGEM-T vector. Primers 5′-TTATGCATGCTAAAAGAATGCGAC-3′ and 5′-AATTGCTAACCAATTTGGCG-3′ were annealed and extended as above. The cleaved product replaced the BstEII-HindIII fragment of CPT 1A. For preparation of other insertion mutants, initial PCR products were generated to create extra restriction enzyme sites: BstEII and HindIII for the TEV site insert adjacent to TM2 and BamHI for TEV adjacent to TM1. The respective primers were: 5′-AATGCTAAGAGAACTTTTTGTC-3′ (forward) and 5′-CTATGCACTGTTTGCTGATGCTGCA-3′ (reverse) and 5′-TCATTTTGCAAGATGCGAC-3′ (forward) and 5′-GCAGCAGCTTAAAGGCAATGCGAC-3′ were annealed and extended as above. The cleaved product replaced the BstEII-HindIII fragment of CPT 1A.
Structure-Function Relationships of CPT 1A

FIGURE 2. Detection of heterologously expressed rat CPT 1A mutants in mitochondria-enriched fractions of P. pastoris. The mutant proteins were expressed in P. pastoris, and mitochondria-enriched fractions were prepared. For each preparation, 50 μg of fraction protein was subjected to SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membrane. CPT 1A C-segment was detected with an anti-peptide antibody raised against residues 428–441 of rat CPT 1A.

Corresponding fragment of CPT 1A. All of the constructs were verified by DNA sequencing.

Assay of CPT Activity of Proteins Expressed in P. pastoris—CPT activity was measured in cell-free mitochondria-enriched fractions obtained from P. pastoris extracts as described previously (18) using variable concentrations of [3H]carnitine and palmitoyl-CoA as substrates and of malonyl-CoA. The rate of formation of [3H]palmitoylcarnitine was quantified by extraction of the acidified reaction mixture with butanol. Briefly, for the determination of the Km for carnitine, palmitoyl-CoA was fixed at 135 μM, and the carnitine concentrations varied up to 1 mM. For determination of the IC50 for malonyl-CoA, the concentrations of palmitoyl-CoA and carnitine were fixed at 35 and 500 μM, respectively. Preliminary experiments were performed to ensure linearity with the amounts of yeast protein assayed and the duration of the assay.

Data Analysis—Statistical analyses and velocity versus substrate or inhibitor concentration curves were fitted using Sigma-Plot software (Adept Scientific).

RESULTS

Expression Levels of Native and Mutant Proteins—To ascertain that the native and mutant proteins were all expressed at the predicted sizes and within the same order of magnitude of expression, we detected the C-segment (which was present in all the mutants) with an antipeptide antibody raised against residues 428–441 of rat CPT 1A. The Western blots in Fig. 2 show that proteins of the predicted sizes were expressed and that their level of expression (per unit of yeast mitochondria-enriched fraction) was always of the same magnitude. The activities of the expressed proteins measured at high substrate concentrations were also all of the same magnitude (Table 1). The apparent Km values for carnitine and palmitoyl-CoA are also given in Table 1.

Effects on CPT 1A Kinetics of Amino Acid Sequence Insertions into the Loop—CPT 1A has several potential tryptic sites within the loop segment that only become accessible to exogenously added protease when the mitochondrial outer membrane is disrupted. Thus, the native protein is resistant to trypsin in intact mitochondria because of its tightly folded state but loses its stability to trypsin once the mitochondrial outer membrane is ruptured (2, 11). Therefore, action of trypsin on this loop-located site(s) appears to result in partial unfolding of the C-terminal segment of the protein, because it causes multiple (otherwise) tryptic sites within the C-segment to become highly sensitive to the protease (2). In an attempt to generate a form of the enzyme that had a specific protease-sensitive site in the loop region so that the loop could be cleaved without risk of further proteolysis of the rest of the protein, we engineered the insertion of the TEV protease-sensitive site (ASENLYFQGLDAAAAV) within the C-segment of CPT 1A. All of the constructs were verified by DNA sequencing.

The mutant proteins were expressed in P. pastoris, and enzyme activity was measured in mitochondria-enriched cell-free extracts. The values are the means for three independent preparations. The activities listed in the second column were measured at high substrate concentrations (500 μM carnitine and 135 μM palmitoyl-CoA). The apparent Km values were determined by varying the concentration of one substrate while keeping the other constant at these maximal concentrations. Activities are expressed as nmol palmitoylcarnitine formed/min/mg yeast protein. The apparent Km values were calculated using Sigma-Plot software. Three separate preparations were assayed for each mutant. Insert refers to the TEV-protease site sequence (ASENLYFQGLDAAAAV). B-loop and B-TM2 refer to chimeras in which the loop and TM2 sequences of CPT 1B were substituted for the corresponding sequences of CPT 1A within the full-length sequence of the latter.

Table 1

| Mutation       | Activity | Carnitine Km μM | Palmitoyl-CoA Km μM |
|----------------|----------|-----------------|---------------------|
| Wild type      | 5.9 ± 1.3| 184.2 ± 17.0    | 46.5 ± 10.8         |
| Insert adjacent to TM2 | 6.5 ± 1.2| 170.8 ± 17.6    | 64.1 ± 6.3          |
| Randomized insert | 12.3 ± 0.8| 142.6 ± 25.0*   | 80.3 ± 6.4*         |
| Insert adjacent to TM1 | 6.3 ± 0.8| 187.8 ± 20.4    | 41.5 ± 7.0          |
| E3A + insert   | 4.9 ± 1.2| 153.7 ± 12.9    | 58.3 ± 8.8          |
| Δ(1–82) + insert | 4.8 ± 0.7| 143.8 ± 16.8    | 59.7 ± 8.9          |
| S24A/3Q30A + insert | 2.9 ± 0.4| 181.9 ± 6.3    | 96.4 ± 26.0         |
| CPT 1A (B-Loop) | 8.1 ± 0.8| 156.2 ± 14.3    | 46.5 ± 10.8         |
| CPT 1A (B-TM2) | 3.7 ± 0.2| 227.6 ± 13.1    | 102.6 ± 12.1*       |
| Δ(77–84)      | 2.2 ± 0.8| 235.1 ± 25.9    | 47.6 ± 5.8          |
| Δ(85–93)      | 4.5 ± 0.5| 259.6 ± 39.9    | 64.2 ± 10.5         |
| Δ(94–101)     | 8.3 ± 2.2| 202.1 ± 35.4    | 49.3 ± 35.4         |
| Pro79 → ile   | 7.0 ±1.2 | 213.7 ± 31.2    | 68.6 ± 12.6         |

* Indicates a value that was significantly different (p > 0.05) from that of the wild type.
Structure-Function Relationships of CPT 1A

Interaction of the Effects on Malonyl-CoA Sensitivity of Disruption of Loop-TM2 Interaction and Point Mutations in the N-terminal Segment—Positive and negative determinants of malonyl-CoA sensitivity have been identified within the N-terminal cytosolic segment of CPT 1A (12–14). Specifically, Glu³ has a strong positive effect on malonyl-CoA sensitivity (12), whereas Ser²⁴ and Gln³⁰ act synergistically to reduce malonyl-CoA sensitivity (13, 14). Therefore, we investigated whether the increase in sensitivity mediated by the sequence insertion adjacent to TM2 is additive to, or dependent on, the action of either of these critical residues within the N-terminal segment. The insertion did not overcome the requirement of the enzyme for Glu³ to express high affinity malonyl-CoA inhibition in the context of the full-length protein (IC₅₀ for malonyl-CoA, 92.5 ± 4.7 μM (cf. E3A mutant) (13)). When the insertion was engineered into the S24A/Q30A mutant, which is itself much more highly malonyl-CoA-sensitive than the native enzyme (13), the sensitivity of the triple mutant was of the same order as that induced by the S24A/Q30A mutations alone (IC₅₀ for malonyl-CoA, 0.36 ± 0.04 μM). These observations indicated that both the insertion between the loop and TM2 and the N-terminal point mutations result in maximal malonyl-CoA interaction with the catalytic C-segment, because the two effects were not additive.

To test this, we made a mutant that lacked the sequence N-terminal to the loop. This deletion mutant, Δ(1–82), lacks TM1 and the N-terminal segment. Previous studies have established that this mutant is active and substantially malonyl-CoA-sensitive (14). When the extra 16-residue spacer was introduced adjacent to its sole TM (TM2), i.e. between residues Phe¹⁰⁰ and Tyr¹⁰¹, it too showed an increase in malonyl-CoA sensitivity (IC₅₀ = 10.5 ± 3.7 μM versus 76.8 μM for the Δ(1–82) mutant (14)). This confirmed that Loop-TM2 interaction (continuity) is a major determinant of the properties of the C-segment even when its interaction with the N-segment is no longer possible and suggests that the disposition of TM2 within the membrane is affected, independently of the presence of TM1.

No changes in the respective affinities for carnitine or palmitoyl-CoA were evident for any of the mutations (Table 1), making it unlikely that the mechanism of inhibition was altered and suggesting specificity of the effects to malonyl-CoA interaction with the catalytic segment.

Effects of Site-specific Deletions within the Loop—We addressed the question as to whether changes in the conformation of the loop brought about by deletion-mediated shortening of this sequence would also affect the TM2-mediated changes in conformation of the C-segment sufficiently to affect malonyl-CoA sensitivity. For this purpose, we subdivided the loop sequence into three sections and deleted each one in turn. The data in Fig. 4 show that deletion of each section resulted in the sensitization of the resulting protein to malonyl-CoA inhibition compared with the wild type protein, but that the effect was highest (50–100-fold) when the more C-terminal sections were deleted.

Effect on CPT 1A Malonyl-CoA Sensitivity of Exchanging the Loop or TM2 Sequences of CPT 1B for Those of CPT 1A—In view of the position-specific effects revealed by the above observations, we hypothesized that it is the pairing of the sequence of

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**FIGURE 3. Kinetic properties CPT 1A mutants with insertions in the loop region adjacent to either TM1 or TM2.** The sequence ASENLYFGLDAAAAY (TEV protease-sensitive site) was inserted adjacent to either TM2 or TM1. The randomized was AYASGLAEAODFANLY was inserted only adjacent to TM2. The mutant proteins were expressed in *P. pastoris*, and mitochondria-enriched yeast membrane fractions were prepared and assayed under the conditions described under “Materials and Methods.” The velocity-substrate saturation curves are shown for carnitine (at 135 μM palmitoyl-CoA, A), and palmitoyl-CoA (at 500 μM carnitine, B). The dependence of the inhibition of the enzyme activities by palmitoyl-CoA is shown in C, in which the palmitoyl-CoA and carnitine concentrations were fixed at 35 and 500 μM, respectively. The palmitoyl-CoA IC₅₀ values were: wild type (○), 16.2 ± 0.4 μM; TEV protease-sensitive site insert adjacent to TM2 (●), 1.0 ± 0.1 μM; randomized insert adjacent to TM2 (△), 1.6 ± 0.2 μM; and TEV site insert 14.2 ± 3.0 μM. The values are the means (±S.E.) for determinations on three separate yeast preparations; each extract was assayed in duplicate.
the TM2-adjacent region of the loop and TM2 itself, rather than the total length of the loop, that is a determinant of malonyl-CoA sensitivity of the protein. To test this hypothesis, we engineered chimeric constructs of CPT 1A in which the loop or TM2 sequences of the CPT 1A isoform were substituted for those of CPT 1B, in the context of the otherwise intact, full-length sequence of CPT 1A (i.e. retaining the CPT 1A catalytic C-segment). The data in Fig. 5 show that, irrespective of whether either the loop or TM2 sequences were exchanged between CPT 1A and CPT 1B, the IC_{50} for malonyl-CoA was decreased by an order of magnitude. These observations confirm that it is the loop-TM2 pairing (i.e. the retention of the native sequence spanning the N-terminal TM2-membrane boundary) that is important in the maintenance of the inherent malonyl-CoA sensitivity of CPT 1A.

Investigation of the Possible Role of Residue Pro^{79} in the Loop of CPT 1A—Fasting- and diabetes-induced changes in malonyl-CoA sensitivity occur for CPT 1 in isolated liver mitochondria (see Introduction), which express CPT 1A, but not in heart, in which expression of CPT 1B predominates (20). Although, overall, the sequence of the inter-TM loop of CPT 1A is not very well conserved between species, Pro^{79} is highly conserved in CPT 1A but is replaced by Ile in CPT 1B. Because proline is a residue known to introduce rigidity in the backbone of polypeptide chains, we hypothesized that Pro^{79} might be a critical residue for the adoption of the native conformation of the loop in CPT 1A and thus may be important in determining loop-TM2 interactions. However, the P79I mutant of CPT 1A showed no significant difference from the wild type protein with respect to its kinetic characteristics (Table 1). Therefore, any effects that the Pro^{79} may have on loop secondary structure in CPT 1A do not affect the loop-TM2 interactions.

DISCUSSION

The inhibition of CPT 1A and CPT 1B by malonyl-CoA is at the center of many responses of different cell types and tissues to changing physiological conditions, ranging from the development of insulin resistance under conditions of excessive fatty acid supply to tissues (21) to the fuel-sensing of neurones within the hypothalamus (22) and the insulin-secretory responses of the pancreatic β-cells (23). CPT 1A undergoes changes in its malonyl-CoA sensitivity depending on the molecular order of the lipids constituting the mitochondrial outer membrane of which it is an integral protein (8). This behavior has been suggested (24) to be due to the polytopic nature of its membrane topology, having two transmembrane segments from which N- and C-terminal segments protrude on the cytosolic aspect of the membrane, with a 27-residue intermembrane space loop connecting the two TM segments (2). Such a topology is anticipated to enable the interaction between the N- and C-terminal segments, such that the former can dock with the latter to modulate its malonyl-CoA binding characteristics at the active site, thus altering its sensitivity to the inhibitor (24). In agreement with this model, the N-terminal segment has both strongly positive and negative determinants of malonyl-CoA sensitivity within the predicted N-terminal first and second helices of the protein, respectively (24). That close interaction of the N- and C-terminal segments of the protein does occur and is capable of modulation by physiologically and pharmacologically induced alterations in membrane fluidity has recently been demonstrated experimentally through cross-linking experiments (11). Moreover, other observations have shown that larger differences in the microenvironment of CPT 1A, such as those that are anticipated to occur between populations of CPT 1A mol-
ecules resident in contact sites and those in the bulk outer membrane, result in major changes in the kinetics of inhibition by malonyl-CoA with respect to acyl-CoA substrate (25).

The present data suggest that the precise pairing of the sequences on either side of the loop-TM2 boundary is likely to affect the orientation of TM2 in the membrane in a manner that affects the tertiary structure of the C-segment of the protein so as to alter its binding of malonyl-CoA. This role of TM2 appears to be determined by the identity of the loop sequence immediately adjacent to it, such that any alterations to either the C-terminal loop region or to TM2 itself results in a C-segment with at least an order of magnitude greater sensitivity to malonyl-CoA inhibition, without major changes in affinity for either carnitine or palmitoyl-CoA. Lengthening of the loop by the same number of residues, but adjacent to TM1, had no effect on malonyl-CoA sensitivity, emphasizing the importance of the specific TM2-loop interaction required for this effect. Shortening of the loop also increased the sensitivity of the resulting mutant CPT 1A to malonyl-CoA, even if the sequence immediately adjacent to TM2 was unaltered. However, the effect was much greater if the sequences nearer to TM2 were deleted. These data suggest that the secondary structure of the loop sequence is important in determining the relationship between TM2 and the C-segment of the protein and thus the properties of the latter. However, the point mutation P79I does not appear to influence loop secondary structure sufficiently to affect loop-TM2 interactions.

Substitution of either the loop or of TM2 of CPT 1B for those of CPT 1A also had the effect of increasing the sensitivity to malonyl-CoA by an order of magnitude, confirming that the TM2-loop interactions are isoform-specific. This is a significant observation because the loop and TM2 of CPT 1B are physiologically relevant sequences similar to those of CPT 1A. It is evident from these data that loop-TM2 interactions are sensed by the C-segment with a high degree of discrimination that detects the (in)correct pairing of the adjacent loop and TM2 segments in an isoform-specific manner. It is important to note that these data are in contrast to the previous observation (14) that simultaneous exchange of loop-plus-TM2 sequences between CPT 1A and CPT 1B does not affect malonyl-CoA sensitivity. The combination of these two sets of data provides further strong evidence that the intactness of the sequence spanning the N-terminal membrane boundary of TM2 (which was preserved in Ref. 14 but not in the B-Loop or B-TM2 mutants in the present study) is a crucial determinant of malonyl-CoA sensitivity of the native protein.

Changes in the interaction between the cytosolically exposed N- and C-terminal segments are predicted to be determined by the degree of interaction between the two TM segments (TM1 and TM2, respectively) as well as that of the TMs with other membrane components. Inter-TM segment interactions are determined by their respective primary and secondary (presumed helical) structures and are commonly accepted as major determinants of the interactions between the more hydrophilic, extramembranous, parts of integral membrane proteins, including inter-TM loops (17). Conversely, loops can act to facilitate or disrupt interactions between TMs with the particular sequences adjacent to the TMs being important in determining the angle at which TMs emerge from the membrane at the lipid-aqueous interface (17). Therefore, it is not surprising that, for an enzyme such as CPT 1A, the kinetic characteristics of which are very sensitive to membrane composition and fluidity, the structure of the loop connecting its two TMs should play a major role in determining its overall tertiary structure, including that of its catalytic C-segment. Indeed, our data show that TM2 can transmit information about the identity of the loop sequence flanking it on its N-terminal side, across the membrane to the C-terminal segment.

The significance of these findings is that they provide the strongest evidence to date that malonyl-CoA sensitivity of CPT 1A is determined by the disposition of the TMs and particularly of TM2 (which is adjacent to the catalytic and malonyl-CoA binding C-terminal segment of the protein) within the membrane. TM2 function is likely to be affected by changes in membrane properties such as fluidity and lateral pressure, the latter varying depending on lipid and protein composition, as well as membrane curvature (e.g. at contact sites). The present data also explain why the protein obtained when the first N-terminal 82 amino acid residues are deleted (i.e. missing the N-terminal segment and TM1) is substantially sensitive to malonyl-CoA (14). They suggest that in $\Delta$(1–82), which is freed of the negative effects of residues Ser$^{24}$ and Gln$^{30}$, the combination of a largely intact loop sequence and TM2 is sufficient to result in a functional C-segment despite the absence of Glu$^9$, which in the native protein is essential for expression of high affinity malonyl-CoA inhibition (12). The observation that the loop-TM2 interactions still exert an effect on the C-segment in this truncated protein, albeit of a different magnitude, is evident from the marked change in malonyl-CoA sensitivity of the protein when an insertion is made between it and TM2.

Interestingly, the previous observation that tryptic cleavage of the loop (potential sites exist at peptide bonds C-terminal to residues Lys$^{86}$, Arg$^{89}$, and Arg$^{96}$) results in sufficient unfolding of the C-segment so as to make its otherwise cryptic tryptic sites accessible to the protease (2) further strengthens the evidence that an intact loop region adjacent to TM2 is essential for the tight folding of the C-segment, as previously observed (2, 11, 26).

Recently, in silico models of the three-dimensional structure of the catalytic core of CPT 1A have been obtained by mathematical modeling based on the crystal structure of soluble members of the carnitine acyltransferase family with which the C-segment of CPT 1A has a high degree of primary sequence similarity (27–30). However, the in silico model for CPT 1A is necessarily limited to the region of the C-segment between residues 166–773, i.e. it does not incorporate either of the TMs or the loop region. Consequently, the very large effects of loop-TM2 interactions on the malonyl-CoA binding characteristics of the active site cannot yet be modeled in the absence of additional crystallographic data obtained on CPT 1A proteins minimally including the loop and TM2.

In conclusion, the malonyl-CoA sensitivity of the catalytic C-segment of CPT 1A is highly influenced by the sequence spanning the loop-TM2 membrane boundary. As a result, it is suggested that TM2 is able to transmit information about the loop structure across the membrane to the C-segment so as to alter its interaction with malonyl-CoA. Therefore, it is sug-
gested that modulation of the protein conformation around the loop-TM2 boundary in the native protein, e.g. through changes in membrane composition and/or fluidity, may affect TM2-membrane interactions and is likely to be important in the modulation of the malonyl-CoA sensitivity of CPT 1A in vivo by pathophysiological conditions that affect the physico-chemical properties of the mitochondrial outer membrane.

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