Self-association of Transmembrane Domain 2 (TM2), but Not TM1, in Carnitine Palmitoyltransferase 1A

ROLE OF GXXXG(A) MOTIFS

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Carnitine palmitoyltransferase 1 (CPT1) controls the rate of entry of long-chain fatty acids into the mitochondrial matrix for β-oxidation and has been reported to exist as an oligomer. We have investigated the in vivo oligomerization of full-length rat CPT1A (rCPT1A) along with those of the N-terminal truncation/deletion mutants Δ(1–82), Δ(1–18), and Δ(19–30) expressed in yeast mitochondria. The data indicate that in liver mitochondria in vivo CPT1A exists as a hexamer but that during preparation and storage of mitochondria the order of oligomerization is rapidly reduced to the trimer, such that a mixture of hexamer and trimer is observed in isolated mitochondria in vitro. Mutants bearing deletions of different segments of the N terminus (including the more N-terminal of the two transmembrane domains) have the same pattern of oligomerization when expressed in yeast mitochondria. The self-association of the individual CPT1A transmembrane (TM) domains (TM1, TM2) was also studied using the TOXCAT assay (which measures TM self-association in the Escherichia coli inner membrane). There was minimal self-association of the sequence corresponding to TM1 but significant self-association of TM2 in TOXCAT. Chemical cross-linking and analytical ultracentrifugation of a TM2-derived synthetic peptide showed oligomerization with a similar trimer/hexamer equilibrium to that observed for native rCPT1A in isolated mitochondria. Therefore, there was a correlation between the oligomerization behavior of TM2 peptide and that of the full-length protein. In silico molecular modeling of rCPT1A TM2 highlighted the favorable orientation of GXXXG and GXXXA motifs in the formation of the TM2 hexamer.

Carnitine palmitoyltransferase 1 (CPT1)2 catalyzes the reaction that exerts the strongest control over the rate of mitochondrial long-chain fatty acid β-oxidation (1). Changes in mitochondrial function, particularly their ability to oxidize fatty acids, accompany the development of obesity and pre-diabetes (2). The activity of CPT1 also determines the availability of one of its substrates, long-chain acyl-CoA, in the cytosol such that when the rate of fatty acid oxidation is low, there is increased availability of acyl-CoA esters which are highly active intermediates involved in complex lipid synthesis (3), activation of inflammatory pathways (4), and activation of ion channels (particularly the KATP Channel (5)), actions that have the potential to result in important cellular effects (6). Therefore, regulation of CPT1 activity is important not only in determining the rate of fatty acid oxidation but in the maintenance of a multiplicity of cell functions (7).

CPT1A is one of three isoforms of CPT1 that are products of distinct genes that share considerable sequence similarity (~65%) (8). It is a polytopic membrane protein of the mitochondrial outer membrane (9, 10) having two transmembrane domains (TM1, TM2) that link the large C-terminal (catalytic, 602-residue) and N-terminal (regulatory, 47-residue) segments of the molecule (9). The N- and C-terminal regions are exposed on the cytosolic face of the membrane; the functional consequences of this topology have been described previously (11). In CPT1A, interactions between the N- and C-terminal domains of the protein are central to the regulation of the activity of the enzyme and particularly the modulation of its sensitivity to the physiological inhibitor, malonyl-CoA (11). The close interaction between the N- and C-terminal segments has been demonstrated via intramolecular cross-linking studies (12) and supported by recent in silico molecular modeling of human CPT1A (13). The TM domains play an important role in mediating and modulating N-C interactions (12) as does the loop linking the two TMs (14, 15).

More recently, it has been reported that rat CPT1A exists as an oligomer and that the N-terminal region of the protein containing TM1 and TM2 (residues 1–147 in the rat enzyme, rCPT1A) is involved in driving the oligomerization of CPT1A (16). Thus, it was shown that full-length rCPT1A (whether in rat liver mitochondria or when expressed heterologously in the yeast Pichia pastoris) forms oligomeric complexes when analyzed using gel filtration chromatography or Blue Native (BN)-PAGE. The fundamental oligomeric unit was suggested to be the trimer, which associates into a dimer of trimers to yield hexamers (16). The protein-protein interactions that stabilize these complexes were narrowed down to a 51-amino acid stretch of CPT1A (residues 97–147) that includes TM2 and a...
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non-cleavable matrix-targeting signal sequence. When expressed as a fusion protein with dihydrofolate reductase, this region induced the formation of hexamers. However, any complementary role of the oligomerization behavior of rat CPT1A TM1 could not be investigated (16). These observations point toward an important role for the TM domains in the oligomeric assembly of rCPT1A. This role can be conceptualized using the “two-stage model” of membrane protein folding (17) which describes the side-by-side interactions of TM α-helices in the plane of the bilayer as strong determinants of correct folding and assembly of membrane proteins.

In the present study we have investigated the role of the TM domains in the oligomerization of full-length rCPT1A. Self-association of each individual TM domain was studied directly in natural membranes (Escherichia coli) using the TOXCAT assay (18). The resulting data indicate that whereas TM2 has a strong propensity for homo-oligomerization, TM1 does not. A synthetic peptide corresponding to the TM2 sequence of rat CPT1A was used to obtain quantitative biophysical evidence of TM2-peptide oligomerization, including the stoichiometry of assembly in detergent micelles. The results have enabled us to construct molecular models that highlight potential points of contact between TM2 helices and suggest that oligomer formation is favored by the relative positioning of tightly packing GXXXG and GXXXA motifs within the predicted TM2 helix.

EXPERIMENTAL PROCEDURES

Preparation of Rat Liver Mitochondria—Mitochondria were prepared from livers of fed male Wistar rats. The liver was rapidly excised from the rat and placed in 20 ml of ice-cold homogenization medium containing 300 mM sucrose, 5 mM Tris-HCl, 1 mM EDTA (pH 7.4). All procedures were carried out at 4 °C. The liver was minced using scissors and washed 2–3 times in ice-cold medium. The minced liver was homogenized using a Porter-Elvenheim Teflon glass homogenizer. The homogenates were centrifuged at 4000 g for 10 min to give a mitochondrial fraction. For rapid preparation of the mitochondria, the second centrifugation step was omitted.

Preparation of P. pastoris Mitochondria—Yeast cultures were grown for 24 h at 30 °C with shaking at 200 rpm. All initial steps were carried out at room temperature. Cells were collected by centrifugation at 4000 g for 5 min, and the resultant pellet was weighed. The appropriate amounts of Tris-SO₄ (2 ml g⁻¹ yeast) and dithiothreitol (3.084 mg g⁻¹ yeast) were added. The mixture was incubated at 30 °C for 10–15 min with shaking at 150 rpm. The cells were then sedimented by centrifuging at 9000 g for 10 min to give a mitochondrial fraction. For rapid preparation of the mitochondria, the second centrifugation step was omitted.

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Blue Native PAGE of CPT1A—Samples were prepared and subjected to Blue Native PAGE using the Novex Bis-Tris gel system (Invitrogen). Briefly 100 μg of mitochondrial fraction were collected by centrifugation for 12 min at 12,000 g in a pre-cooled centrifuge at 4 °C and resuspended in 100 μl of Native sample buffer (Invitrogen) containing 0.5% digitonin. After solubilization for 15 min on ice, the extracts were centrifuged for 30 min at 50,000 g, and the supernatants were recovered and supplemented with 0.5 μl of G-250 additive (Invitrogen) for each 10 μl of extract before electrophoresis on 4–16% gradient Novex Bis-Tris gels (Invitrogen). The calibration standard kit used was comprised of eight proteins with a molecular mass covering the range between 20 and 1200 kDa.

Western Blot Analysis—Separated proteins were blotted onto polyvinylidene difluoride membranes using Tris-glycine transfer buffer. After transfer, the membrane was washed for 15 min in 8% acetic acid and allowed to air-dry. The membrane was then washed in methanol, and proteins were detected as described previously using the ECL detection system (Pierce). The antibodies used were antipeptide antibodies raised against the Arg⁴²⁸-Lys⁴⁴⁷ sequence within the C-segment of rat CPT1A (19).

TOXCAT Assay and Construction of Chimera—The self-association of rCPT1A transmembrane domains in a natural membrane was studied using the TOXCAT assay, the details of which have been described previously (18). Briefly, TOXCAT employs a chimeric protein in which the α-helical TM domain of interest is inserted between the N-terminal DNA binding domain of ToxR, a dimerization-dependent transcriptional activator, and maltose-binding protein, a monomeric periplasmic anchor protein. The fusion protein is constitutively expressed in E. coli together with a chloramphenicol acetyltransferase (CAT) reporter gene under the control of a ToxR-responsive ctx promoter. Oligomerization of the TM domains within the bacterial inner membrane results in oligomerization of the ToxR domain, transcriptional activation of the ctx promoter, and CAT expression. The amount of CAT expressed in this system is proportional to the strength of oligomerization of the TM domains. The expression vectors (pccKAN, pccGpA-wt, and pccGpA-G83I) and E. coli strain NT326 were kindly provided by Prof. Donald M. Engelman. TOXCAT chimera were constructed according to a reported protocol (18) and expressed in E. coli. Before performing the assay, membrane insertion of all constructs was confirmed using sodium hydroxide washes (20), and correct orientation of the TOXCAT constructs in the membrane was confirmed through protease sen...
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Sensitivity in a spheroplast assay (18). Similar expression levels for all constructs were confirmed via Western analysis against the maltose-binding protein domain before performing CAT assays. The CAT assays were performed using the FAST CAT kit (Molecular Probes and Invitrogen).

Peptide Synthesis and Purification—A peptide corresponding to the second TM domain (TM2) of CPT1A was synthesized at the Keck Facility, Yale University using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. The sequence of the TM2 peptide was COCH3-KKNIVSVGLFTGMTLWAVIMT-MRK-CONH2, containing residues Lys102-Arg123 as well as non-native lysine residues (to aid solubility) and end-caps on the C and N termini (Mr = 2689 Da). The peptide was purified by reversed-phase high performance liquid chromatography using a linear acetonitrile gradient from 30 to 100% containing 0.1% trifluoroacetic acid on a Phenomenex C4 semipreparative column. The purity of pooled peptide fractions was confirmed by electrospray ionization time-of-flight mass spectroscopy (ESI-TOF-MS microTOF, Bruker) before subsequent lyophilization. Peptides were stored as dry powders until use.

Circular Dichroism (CD)—CD spectra were measured using a Jasco J715 spectropolarimeter (Jasco UK, Great Dunmow, UK) and 1.0-mm path-length quartz cuvettes (Starlab, Optiglass Ltd, Hainault, UK). All spectra were recorded from 190 to 260 nm (data below 200 nm are not shown due to high noise of light scattering) using a 2.0-nm spectral bandwidth, 0.2-nm step resolution, 100-nm min⁻¹ scanning speed, and 1-s response time. The TM2 peptide was prepared in 50 mM sodium phosphate buffer (pH 7) containing 10 mM SDS or 46 mM dodecylphosphocholine (DPC) (Avanti Polar Lipids, Alabaster AL) and 100 mM NaCl to a final peptide concentration of 40 μM. CD spectra were collected at room temperature.

Peptide Cross-linking in Detergents—Cross-linking reactions were carried out for 20 μM solutions of the TM2 peptide dissolved in DPC micelles at peptide:micelle ratios ranging from 1 to 20 (corresponding to DPC concentrations ranging from 2.7 to 24 μM). All samples were prepared in 20 mM sodium phosphate buffer and 150 mM NaCl (pH 8). Bis[sulfosuccinimidyl]suberate (BS3, Pierce) was used to cross-link the peptide in solution via primary amine groups according to the manufacturer’s protocol. The cross-linking reaction was terminated after 30 min by the addition of 1 M Tris-HCl (pH 8). Uncross-linked and SDS cross-linked samples were prepared as controls. All samples were analyzed by gel electrophoresis, and peptides were visualized on gels by staining either with Coomassie Blue or with silver nitrate.

Analytical Ultracentrifugation—Sedimentation velocity measurements were carried out on a Beckman XL-1/A analytical ultracentrifuge (Beckman Coulter, Fullerton CA) housed in the Department of Biological Sciences, University of Birmingham, Birmingham, UK. TM2 peptide samples were prepared in 50 mM Tris-HCl (pH 7.5), containing 15 mM DPC (Avanti Polar Lipids), 100 mM NaCl, and 52.5% D₂O (Cambridge Isotope Laboratories, Andover MA) to match the buoyant density of the detergent. When the solvent matches the buoyant density of the detergent micelles, the only contribution to the buoyant molecular weight is from the peptide, as described (21).

Data were collected using absorbance optics set to 280 nm at two peptide concentrations (142 and 62 μM) using a double-sector centerpiece at a speed of 40,000 rpm and a temperature of 25 °C. 400 scans were recorded for each sample, with 50 s between each scan. The moving boundary was monitored by repetitive radial scanning at a constant step size of 0.003 cm at 280 nm using a UV absorption optical system. Fitting of the resulting profiles was achieved using SEDFIT (22) to generate a continuous sedimentation coefficient distribution, which was subsequently converted to a molecular mass distribution using a peptide monomeric molecular mass of 2689 Da, a buffer density of 1.05971 g ml⁻¹, a buffer viscosity of 1.0267 centipoise, and a partial specific volume of 0.7792 ml g⁻¹ (calculated using SEDNTERP).

Computational Searches Using CHI—Structural calculations were performed using the CNS searching of helix interactions (CHI) package, the details of which have been described previously (23–25), on an 8-node dual 2.66-GHz Xenon processor Linux cluster (Streamline Computing, Warwick). CHI was used to create models of rCPT1A TM2 homotrimers and homohexamers. Using CHI, either three (for trimer) or six (for hexamer) canonical α-helices containing residues Lys102-Arg123 of TM2 were built. The starting geometries incorporated both right-handed (−25°) and left-handed (25°) crossing angles and an axis-to-axis distance between the helices of 10.4 Å. In a search of approximately symmetrical interactions, the helices were simultaneously rotated about their central axis in 45° increments from 0° to 360°. After each rotation, molecular dynamics simulations were performed using simulated annealing of atomic coordinates. Four different molecular dynamics simulations were performed for each starting geometry, and energy minimization of structures was carried out both before and after molecular dynamics simulations. Groups of structures with a backbone root-mean-square deviation of ≤1 Å were placed in clusters of 10 or more members followed by a calculation of an average structure for each cluster and energy minimization.

RESULTS

Oligomerization of Full-length and Truncated rCPT1A

The oligomeric states of native rCPT1A in both intact rat liver mitochondria or expressed in mitochondria of the yeast P. pastoris were studied in addition to those of several yeast-expressed N-terminal truncation/deletion mutants (Δ1–82, Δ1–18, Δ19–30) to investigate which regions of the protein contribute to oligomer formation. A schematic of the various regions of rCPT1A is shown in Fig. 1a. The digitonin-solubilized mitochondrial extracts were analyzed using BN-PAGE followed by Western blotting and immunodetection with anti-peptide antibody raised against the C-terminal segment of the protein (19) (Fig. 1b). In both rat liver and yeast extracts, Western blotting revealed bands at ~264 and 528 kDa corresponding to rCPT1A trimers and hexamers, respectively. In preliminary experiments, extracts prepared in digitonin or 1% Triton-X-100 gave the same results (data not shown). Subsequent experiments were performed using only digitonin extraction. The results are at variance with previously published BN-PAGE
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As the BN-PAGE results given in Fig. 1b differ from previous results in that both trimers and hexamers of rCPT1A were present in digitonin extracts of mitochondria isolated from rat liver and *P. pastoris*, we addressed the question of whether the trimer/hexamer ratio was altered during preparation of the mitochondria, as this would be indicative of whether the two oligomeric forms exist in equilibrium or whether one is preferentially present *in vivo*. Therefore, crude mitochondrial fractions were prepared as rapidly as possible from rat liver followed by storage at either 0 or 37 °C before extraction with digitonin, BN-PAGE analyses of the proteins, and Western blotting. The data shown in Fig. 1c indicate that when mitochondria were kept at 0 °C for less than 30 min, both trimers and hexamers were observed. However, at longer incubation times (t ≥ 30 min; T = 0 °C) the proportion of CPT1A present as the hexamer was rapidly diminished, whereas that of the trimer was increased. When mitochondria were stored at 37 °C, the hexamer was not observed at all (even after the shortest incubation times), and only the trimer was detected. These data suggest that rCPT1A may exist mainly as hexamers in the rat liver mitochondrial outer membrane *in vivo* and that changes in the mitochondrial membrane that occur during isolation and storage result in rapid destabilization of the higher order oligomeric state.

Self-association of the Individual TM Domains rCPT1A

Although the present data and those published previously (16) strongly suggest that TM2 plays a key role in oligomer formation of rCPT1A, no direct measurements of self-association for either TM of rCPT1A have been reported. To address this, we have examined the homo-oligomerization of rCPT1A TM1 and TM2 using the TOXCAT assay, a ToxR-based transcriptional assay linked to CAT expression. TOXCAT is a method used to quantify the ability of individual TM domains to self-associate in a natural (*E. coli*) membrane bilayer in which the degree of association can be measured in terms of the CAT activity generated as a result of the homo-oligomerization of TM domains (18) (see “Experimental Procedures”). The sequences corresponding to the respective TM domains of rCPT1A that were investigated using TOXCAT are shown in Fig. 2a. The selection of these sequences was based on an analysis of the full-length sequence of rCPT1A using the bioinformatics program TMHMM (27, 28) to predict the location of TM domains. TOXCAT data were also obtained for a positive control (the TM domain of glycophorin A (GpA), which is known to dimerize strongly (29)), and a negative control, GpA-G83I, a dimerization-impaired mutant. The data are shown in Fig. 2b. Comparison of the rCPT1A TM1 signal in the TOXCAT assay to that of the controls indicated that TM1 has very little propensity to self-associate. Indeed, TM1 yielded homo-oligomerization-dependent CAT activities that were lower than those produced by the negative control (GpA-G83I). Conversely, rCPT1A TM2 showed a higher propensity to form homo-oligomers, yielding CAT activities approximately half those observed for the TM domain of GpA.

It is important to note that the TM-spanning regions for TM1- and TM2-containing TOXCAT constructs are very different in length; the TM1 insert contained 24 amino acids,
whereas the TM2 insert contained 16 amino acids. It has been shown previously that in a similar assay the length of the TM can affect ToxR-based transcriptional activity (30). To test whether differences in the lengths of TM1 and TM2 lead to an artificial difference in CAT activity, additional TOXCAT constructs were prepared. The TM1 sequence was shortened to 21, 20, 18, and 16 residues and tested in the assay (data not shown). TM1 inserts shorter than 21 residues failed to insert into the *E. coli* membrane, and the construct containing a 21-residue TM1 (Ile50–Ile70) yielded a signal ~50% smaller than that shown in Fig. 2b, thus confirming that TM1 has little or no ability to self-associate.

Therefore, these data support the inferences from the present BN-PAGE data on rCPT1A(1–82) and those obtained previously (16) that TM2 is the only one of the TM domains of CPT1A that is required for homo-oligomerization of the protein. In view of these observations, only the oligomerization of TM2 was studied further.

**Secondary Structure of TM2 Peptide**

A synthetic peptide corresponding to TM2 (residues Lys102–Arg123, Fig. 3a) was studied to determine whether the oligomeric states attained in *vitro* correspond to those observed for full-length rCPT1A in rat liver mitochondria and in heterologously expressed rCPT1A. In synthesizing the peptide, lysine residues were added to the sequence at both its N and C termini to aid solubility and avoid nonspecific aggregation (see “Results”). CD spectra of the TM2 peptide in SDS (solid line) and DPC (broken line) detergent micelles. SDS-PAGE analysis of the TM2 peptide dissolved in DPC micelles, visualized using Coomassie-G250. B S3-mediated cross-linking of TM2 peptide dissolved in DPC micelles. Cross-linking reactions were carried out in DPC detergent micelles at various micelle:peptide concentration ratios as indicated below each lane. Molecular weight markers are shown in the far left-hand lane. Protein bands were visualized by staining with silver nitrate. Oligomeric states (e.g. dimer indicated by n = 2) are indicated at the far right of the gels. A negative control reaction in which cross-linking was carried out for the peptide dissolved in SDS buffer is shown in the last lane.

**Oligomeric State of rCPT1A TM2 Peptide**

Chemical Cross-linking—Although providing a strong indication that the sequence of TM2 can support self-association of this domain, the TOXCAT assay cannot report on the order of
the oligomeric state achieved. Therefore, to characterize the oligomeric state(s) of the rCPT1A TM2 domain, in vitro approaches were used. The synthetic peptide corresponding to TM2 (see above) was electrophoresed under denaturing conditions (SDS-PAGE) before and after treatment with a chemical cross-linker. As shown in Fig. 3c, in the absence of cross-linker the peptide migrated as the monomeric species in SDS detergent. To investigate its oligomeric state in a less denaturing detergent, the peptide was dissolved in DPC detergent micelles and then treated with the water-soluble chemical cross-linker BS3, which reacts specifically with the terminal NH2 of the peptide as well as the NH2 groups on lysine side chains provided that the reactive groups are within 11.4 Å of one another (34). Cross-linking reactions were carried out at increasing DPC micelle:peptide (M:P) concentration ratios to investigate the effect of detergent concentrations on oligomeric state. Cross-linked species were analyzed using SDS-PAGE and visualized by staining with silver nitrate (Fig. 3d). At the lowest detergent micelle:protein ratio (M:P = 1), bands corresponding to the hexamer (16.1 kDa) and dodecamer (32.2 kDa) were observed. No bands corresponding to lower-order oligomeric states (e.g. trimer) or monomer were evident. As the M:P ratio was increased to a value of 2, a monomer band could be observed (2.69 kDa) as well as weak bands corresponding to dimer (or potentially monomer plus bound BS3, 5.38 kDa) and trimer (8.07 kDa). Further increases in the M:P ratio resulted in increasing amounts of monomeric, dimeric, and trimeric species accompanied by decreasing populations of hexamers and dodecamers. The results shown in Fig. 3d demonstrate that the oligomeric states of the TM2 peptide differ with increasing detergent concentrations. It is well established that increasing detergent concentration can destabilize TM helix oligomers (35). The rapid loss of the dodecamer of TM2 with increasing M:P ratios suggests that this is the least stable species. The hexamer is evidently more stable, as its presence can be detected at all M:P ratios studied, although the concentration of hexamer is greatly reduced at M:P > 5. The oligomeric species that are most stable at the highest M:P ratios are the dimer and trimer. These two species (together with the monomer) steadily increased in concentration at high M:P ratios. As a negative control, cross-linking was carried out with the peptide dissolved in SDS; as expected, only a band corresponding to the monomer was observed (Fig. 3d).

**Analytical Ultracentrifugation**—The oligomeric state of the TM2 peptide was also studied in the absence of cross-linker using analytical ultracentrifugation. Sedimentation velocity experiments were employed in this study as they provide information about the shape and size of peptides in solution. Because of the high rate of back diffusion expected from low molecular weight peptides, high speeds are sometimes required to obtain reliable sedimentation velocity data. In the present studies, a speed of 40,000 rpm was sufficient to generate data with the required mass resolution for the analysis of the oligomeric state of the TM2 peptide. Two peptide concentrations were analyzed, 62 μM (M:P ∼ 4) and 142 μM (M:P ∼ 1) in buffered DPC solution. The fitting residuals for both concentrations are shown in Figs. 4, a and b (sedimentation profiles not shown), corresponding to fits with root-mean-square deviation values of 4.9 × 10⁻³ and 6.5 × 10⁻³, respectively. The sedimentation coefficient profile for the higher of the two concentrations (Fig. 4c) contained two species centered at S = 0.747 and S = 1.15, corresponding to a molecular mass for the TM2 peptide of 16.1 and 30.9 kDa. When compared with the calculated molecular weights for the TM2 hexamer (16.1 kDa) and dodecamer (32.2 kDa), the experimental values agreed to within 4% of the theoretical values. This result agrees with the cross-linking results shown in Fig. 3d, where at M:P = 1, only hexamers and dodecamers were present. At the lower concentration of peptide (M:P ratio ∼ 4), the resulting sedimentation coefficient profile contained a single species centered at S = 0.787 (Fig. 4c), corresponding to a molecular mass for the TM2 peptide of 16.3 kDa, i.e. within 2% of the theoretical molecular
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FIGURE 5. Molecular models of CPT1A TM2 trimers and hexamers. Molecular models of CPT1A TM2 trimers and hexamers were created using the program CHI (see “Experimental Procedures”). a, side view of a right-handed TM2 trimer in which individual helices are represented as ribbons. In this model the trimer is stabilized by packing of the GXXXG motifs (Gly107, Gly111) shown in yellow) against side chains from an adjacent helix as well as interhelical hydrogen bonding of Thr112 (shown in red, bond representation) at the center of the bundle. b, model of a left-handed trimer in which the GXXAX motif (Gly113, Ala117) shown in dark blue packs against the adjacent helix, whereas the GXXXG motif is predicted to be on the outside of the bundle. c, top-down view of the left-handed TM2 hexamer. d, side view of the left-handed TM2 hexamer, showing the simultaneous packing of both the GXXXG (yellow) and GXXAX (dark blue) motifs against adjacent TM helices. The hexamer is the only observed oligomeric state for which it is predicted that both of these oligomer-favoring TM domain motifs are used to stabilize the quaternary structure of the peptide.

FIGURE 6. Conservation of GXXXG and GXXAX motifs in the TM2 sequences of CPT1A from several mammalian species and their proposed role in hexamer formation. a, alignment of the sequences of the predicted TM2 domains for CPT1A proteins sequenced to date. The TM domain is very highly conserved, as are the highlighted GXXXG and GXXAX motifs. b, orientation of the GXXXG and GXXAX motifs relative to each other on a helical wheel (left panel) and in a canonical α-helix (built using CHI; see “Experimental Procedures”) containing residues Lys102–Arg123 of TM2. Both models predict that the relative angle between the two motifs corresponds closely to the apex angles of a hexamer (120°). Also highlighted is the apex angle in a trimer (60°), which cannot accommodate both motifs.

weight of the hexamer (16.1 kDa). These data are in accordance with the results obtained after cross-linking at M:P ratios of 3 and 5 (Fig. 3d). Within the range of these M:P ratios, the concentration of cross-linked dodecamer decreased significantly, whereas the hexamer remained detectable.

Fitting of the data described above produced a frictional ratio of 1.88, suggesting a slightly elongated ellipsoidal shape for the hexamer as would be expected for a bundle of TM α-helices. Therefore, the sedimentation velocity data suggest that the TM2 peptide in DPC detergent solution exists as an equilibrium mixture of hexamers and dodecamers at the concentrations studied. The dodecamer species, which was also detected in the cross-linking experiments, is present only at low micelle:peptide concentration ratios.

Structural Models of TM2 Trimmers and Hexamers

The results presented here establish that TM2 can associate to form hexamers and trimers, directly mirroring the behavior of the full-length rCPT1A both in freshly prepared rat liver mitochondria and in mitochondria isolated from yeast in which the protein (and its mutants) were heterologously expressed. To gain insight into the structural features of TM2 that may have a role in stabilizing both the trimeric and hexameric forms of the peptide, computational models were produced using the program CHI (see “Experimental Procedures”). CHI searches were performed on the basis of either three or six parallel α-helices containing the predicted sequence of the TM2 peptide. The trimer models suggest that the TM domain can form chemically plausible right- and left-handed coiled coils. However, in the all of the trimer models obtained (Fig. 5, a and b), the interaction interface contains either a GXXXG motif (Gly107, Gly111) or a GXXAX motif (Gly113, Ala117), respectively. Both of these motifs are highly conserved in TM2 domains of the CPT1A isoform across all mammalian species (Fig. 6a) and are known to stabilize TM helix-helix interactions in several other membrane proteins (see “Discussion”) (36–38). The in silico models of the trimer of rCPT1A TM2 also suggest that this oligomer is capable of being stabilized by the interhelical hydrogen bonding of Thr112, as observed in other interacting TM domains (39) (in the present analyses, hydrogen bonds were assigned whenever a hydrogen bond donor and acceptor were present within 3 Å of one another).

The modeling of the TM2 hexamer (Fig. 5c) suggests that TM2 can form a plausible left-handed helical bundle (no right-handed solutions were found) in which both the GXXXG and the GXXAX motifs pack against neighboring helices (Fig. 5d). In this way the stabilizing effects of both motifs can be exploited simultaneously through interactions with adjacent helices. This is possible only in a hexameric arrangement, which has apex angles of 120°, the predicted angle between the two motifs in the α-helical model of TM2 (Fig. 6b). Such simultaneous packing of the two motifs is not possible in the trimer, as the angle between the two motifs is such that they can never both pack at once (apex angles = 60°), resulting in exclusion of one motif from interfacial interactions with the other two helices (Fig. 6b).

DISCUSSION

Oligomer Formation in Natural Membranes—The involvement of the TM domains of CPT1A in the regulation of its activity and its sensitivity to malonyl-CoA has been strongly inferred for some time through numerous findings on the modulation of the interaction between the N- and C-terminal seg-
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ments (for review, see Ref 40). This interaction between the N- and C-terminal segments of the molecule has now been modeled using in silico techniques based on homology with other transferases for which x-ray crystal structures have been determined (13). Moreover, we have shown that such a role for TM1–TM2 interactions occurs very much in the context of protein–lipid interactions, making CPT1A sensitive to changes in lipid composition that accompany changes in diet and pathophysiological state (e.g. diabetes, fasting) (15). Previous studies have also raised the possibility of an additional role for the TM domains, namely that of driving the oligomerization of CPT1A (16). In those studies, analysis of the native protein either in rat liver mitochondria or that expressed heterologously in yeast yielded either trimeric or hexameric oligomers in a manner that suggested that the fundamental oligomeric state is the trimer and that the hexamer results from the dimerization of the trimer (16). In general, trimers were observed after gel filtration, whereas hexamers were observed after Blue Native gel electrophoresis (16). The CPT1A protein N-terminally truncated at residue 82 (i.e. lacking TM1 but retaining most of the loop region and TM2) was similarly found to yield only trimers. Although these data indicated that TM2 was sufficient for oligomerization to occur, they did not exclude the possibility that TM1 plays an additional/complementary role in oligomerization and may be interpreted as indicating that TM1 may determine the order of oligomerization. Therefore, in our studies, in addition to characterization of the oligomeric characteristics of the native rCPT1A and truncation/deletion mutants, we have quantified the oligomerization propensities of both TM1 and TM2 in isolation using the TOXCAT assay and have explored in more detail the biophysical properties of TM2 (which is the only one to give a substantial signal in TOXCAT) with respect to its oligomerization properties.

Importantly, our experiments on CPT1A in rat liver mitochondria and the native protein expressed heterologously in yeast mitochondria showed that both trimers and hexamers can be detected using the same technique (Blue-Native gel electrophoresis). Similar observations were made for all the truncation and deletion mutants tested when expressed in yeast (Fig. 1b). In particular, contrary to previous observations (16), we found that the (Δ1–82) CPT1A truncation mutant also exists in a mixture of the two oligomeric states (Fig. 1b). These observations suggested that the two oligomeric forms may exist in equilibrium in vivo. However, our time-course experiments on rapidly prepared rat liver mitochondria suggest that in vivo CPT1A is likely to occur as a hexamer which de-oligomerizes rapidly during preparation of subcellular fractions and especially with storage of the mitochondria, particularly at 37 °C. The observations obtained on rapidly fractionated rat liver tissue indicate that the trimer (although stabilized by non-covalent interactions that we have modeled; see below) may not be a physiologically relevant entity but may arise as an artifact during the necessarily lengthy preparation of purified mitochondrial fractions by differential centrifugation (this study) or on self-forming gradients (16).

It has also been observed that there was no difference in the order of oligomerization of CPT1A in rat liver mitochondria isolated from animals in different physiological states (fasting, diabetic) known to result in a wide range of sensitivities of the enzyme to malonyl-CoA inhibition (16). Therefore, in this study we have tested this observation directly by studying the oligomerization state of mutants that contained deletion mutations within the N-terminal 47 amino acid residues that have previously been shown (see e.g. Ref. 26) to display a range of several orders of magnitude in their malonyl-CoA sensitivities. The data in Fig. 1b show that we always observed similar proportions of trimer and hexamer irrespective of which N-terminal mutant was studied. This strengthens the evidence that malonyl-CoA sensitivity is independent of the quaternary structure of CPT1A and is instead determined by intramolecular (N–C) interactions (40) to which TM1–TM2 (hetero-oligomeric) interactions are likely to be central.

Self-association of TM2, but Not TM1, Reflects the Oligomerization of the Native Protein—The above interpretations are supported by the biophysical experiments we performed on the peptide corresponding to TM2 in vitro. The experimental demonstration using TOXCAT that only TM2 has homo-oligomerization potential enabled us to focus on the biophysical properties of a peptide corresponding to this TM. When the TM2 peptide was dissolved in DPC micelles and cross-linked with BS3, oligomers corresponding to the hexamer were observed, suggesting that the TM2 sequence has inherent primary and secondary structural properties that favor hexamerization. The fact that the hexamer also appears to be a favored oligomeric state in vivo suggests that these interactions are also important in the context of the full-length protein when this is stabilized within a lipid bilayer. De-oligomerization to the trimeric form occurs rapidly during preparation and storage of mitochondria (when disruption of contacts with other intracellular systems, loss of contact site density (20) and changes in outer membrane lipid composition because of phospholipase activity are known to occur). It is likely that de-oligomerization to the trimeric form of the native rCPT1A protein is also favored during digitonin- or Triton X-100 solubilization of mitochondria, in preparation for BN-PAGE or gel filtration (16). In this context, it is of interest to note that when the size of the molecular entity C-terminal of TM2 was much smaller than that of native CPT1A (e.g. the truncated CPT1A-dihydrofolate reductase chimera, CPT1A-(97–147) dihydrofolate reductase), only the respective hexamer was observed (16). This suggests that whereas TM2-driven oligomerization results in the hexamer as a default quaternary structure for the native protein, the large C-terminal domain of the native protein imposes intermolecular constraints within that oligomeric structure that are overcome only within the mitochondrial outer membrane in vivo and which result in the relatively rapid deoligomerization to the trimer.

Such close correspondence between the oligomerization behavior of rCPT1A in vivo and those of TM2 in vitro provide further evidence that TM2 plays a central role in the oligomerization of the native protein, as suggested by the strong propensity of this TM to oligomerize in E. coli inner membrane (in the TOXCAT assay).

Molecular Modeling of the Role of the GXXXG(A) Motifs in TM2 Oligomerization—As discussed above, the hexamer of rCPT1A is likely to be the most important oligomeric form of
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rCPT1A in vivo, although the inability to prepare mitochondria sufficiently rapidly from intact rat liver does not allow a definitive exclusion of the co-existence of the trimer. Certainly, the hexameric form of TM2 is the predominant species when the corresponding peptide is studied in DPC-micelle solution; other oligomeric states are observed only at very low or very high M:P ratios. The molecular models shown in Fig. 5 illustrate predicted conformations that may be adopted by the TM2 trimers and hexamers. Importantly, Fig. 5d highlights the possible role of the GXXXG and GXXXA motifs present within TM2 (and conserved in all CPT1A proteins sequenced to date) in stabilizing the hexameric structure. In both of these motifs, Gly or other small residues (e.g. Ala) are found four residues apart (one turn of the helix) and, therefore, appear on the same helical face. The presence of a “patch” of these small residues allows the helices to pack tightly together, and this close-packaging stabilizes helix-helix interactions (36, 41, 42).

The angular separation of the GXXXG and GXXXA motifs within the predicted helical structure of TM2 (Fig. 6b) is approximately equal to the apex angles of a hexagon (120°). Therefore, in the hexamer both motifs could theoretically pack against adjacent TM2 helices, hence favoring this oligomeric form. However, in the native protein, intermolecular steric hindrance may destabilize this tertiary structure such that during preparation of mitochondria the trimer is formed (Fig. 1c). This is also evidenced by data showing that when a smaller molecular entity replaced the bulky C-terminal segment of native rCPT1A with the structure of dihydrofolate reductase, only hexamers were observed (16). Although further work is required to confirm the mechanism of association, GXXXG and GXXXA motifs are well known to contribute to TM interactions.

The trimer, although appearing not to be the physiologically relevant quaternary structure of rCPT1A, is predicted to be stabilized by the possible close packing of at least one of the GXXXG or GXXXA motifs and by interhelical hydrogen bonding, as shown in Figs. 5, a and b. The relative disposition of the GXXXG or GXXXA motifs within the predicted α-helical structure of TM2 is incompatible with favorable packing of both of these motifs simultaneously in the formation of homo-trimers, which would require an angle of ~60° (Fig. 6b).

Functional Significance of CPT1A Oligomerization—Previous data (16) as well as data from the present study raise the question as to the function of CPT1A hexamer formation. It has been suggested that oligomerization (to form trimers or a dimer of trimers) may be a mechanism for the formation of a channel in the mitochondrial outer membrane through which the acylcarnitine product of the reaction catalyzed by CPT1 may gain access to the intermembrane space (16). The molecular models in Fig. 5 suggest that the hexamer (but not the trimer) is capable of forming a pore of significant dimensions; however, validation of its possible function as a channel for acylcarnitines awaits further study. In this context, two considerations are pertinent. First, we have previously shown by performing functional target size analysis on CPT1A in whole rat liver mitochondrial and purified outer membranes that the functional size of rCPT1A (i.e. the size of the molecular entity required for catalytic activity) is the same as that of the monomeric molecular mass (~88 kDa) of the protein (43). Therefore, in the rat liver mitochondrial outer membrane each monomer within the hexameric form would function as an independent entity in terms of its catalytic function. Second, if as suggested (16) the putative channel formed by the oligomerization of CPT1A were necessitated by the inability of porin (VDAC1) to allow acylcarnitine esters access into the intermembrane space (a prospect raised previously (44)), it would need to be suggested that either this effect of porin is unidirectional or that transport of acylcarnitines out of mitochondria (which is considerable) also occurs necessarily through the putative pore/channel created by CPT1A oligomerization. These are now testable hypotheses, and the present studies suggesting that stabilization of TM2 hexamerization by GXXXG and GXXXA interactions may be central to rCPT1A oligomerization will enable further studies on CPT1A mutants that are unable to oligomerize. These should be useful in obtaining information regarding the function of CPT1A oligomerization with respect to long-chain fatty acyl moiety entry into intact mitochondria.

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