Mitochondrial Glycerol Phosphate Acyltransferase Contains Two Transmembrane Domains with the Active Site in the N-terminal Domain Facing the Cytosol*

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The topography of mitochondrial glycerol-3-phosphate acyltransferase (GPAT) was determined using rat liver mitochondria and mutantized recombinant rat GPAT (828 aa (amino acids)) expressed in CHO cells. Hydrophobicity analysis of GPAT predicts two transmembrane domains (TMDs), residues 472–493 and 576–592. Residues 224–323 correspond to the active site of the enzyme, which is believed to lie on the cytosolic face of the outer mitochondrial membrane. Protease treatment of rat liver mitochondria revealed that GPAT has a membrane-protected segment of 14 kDa that could correspond to the mass of the two predicted TMDs plus a loop between aa 494 and 575. Recombinant GPAT constructs containing tagged epitopes were transiently expressed in Chinese hamster ovary cells and immunolocalized. Both the C and N termini epitope tags could be detected after selective permeabilization of only the plasma membrane, indicating that both termini face the cytosol. A 6–8-fold increase in GPAT-specific activity in the transfected cells confirmed correct protein folding and orientation. When the C terminus and loop-tagged GPAT construct was immunostained, the epitope at the C terminus could be detected when the plasma membrane was permeabilized, but loop-epitope accessibility required disruption of the outer mitochondrial membrane. Similar results were observed when GPAT was truncated before the second TMD, again consistent with an orientation in which the loop faces the mitochondrial intermembrane space. Although protease digestion of the HIA-tagged loop resulted in preservation of a 14-kDa fragment, consistent with a membrane-protected loop domain, neither the truncated nor loop-tagged enzymes conferred GPAT activity when overexpressed, suggesting that the loop plays a critical structural or regulatory role for GPAT function. Based on these data, we propose a GPAT topography model with two transmembrane domains in which both the N (aa 1–471) and C (aa 593–end) termini face the cytosol and a single loop (aa 494–575) faces the intermembrane space.

Glycerol phosphate acyltransferase (GPAT) (EC 2.3.1.15)

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† The abbreviations used are: GPAT, mitochondrial glycerol 3-phosphate acyltransferase; HA, hemagglutinin; OMM, outer mitochondrial membrane; TMD, transmembrane domain; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; aa, amino acids; GFP, green fluorescent protein; CHAPS, 3-(3-cholamidopropyl)dimethylammonium-1-propanesulfonic acid; LPAAT, lysophosphatidic acid acyltransferase.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal anti-GPAT antibody raised against the gel-purified protein expressed in bacteria was commercially produced by Immunodynamics, La Jolla, CA. Monoclonal antibodies against the epitopes used were from Sigma (anti-FLAG), Invitrogen (Carlsbad, CA, anti-Myc), and Covance (Princeton, NJ; anti-HA). Mitochondrial marker Mito-Tracker CMX-Ros was from Molecular Probes (Eugene, Oregon).

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OR. Tissue culture and transfection media and reagents were from Life Technologies-Invitrogen (Princeton, NJ). Proteases and protease inhibitors were purchased from Sigma and Life Technologies.

**Animals and Isolation of Liver Mitochondria**—Female (150–200 g) Sprague-Dawley rats were housed on a 12-h/12-h light/dark cycle with free access to Purina rat chow. They were fed ad libitum with Purina rat chow. They were then fasted for 48 h and refeed for 24 h with a high sucrose (69.5%), low fat (0.5%) diet (Dyets Inc.) to up-regulate mitochondrial GPAT expression (10). Animals were killed by CO₂ narcosis. After dissection, livers were immediately placed on ice and homogenized in Buffer A (0.25 mM sucrose, 25 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 mM dithiothreitol). Mitochondria were isolated by differential centrifugation at 20,000 × g for 20 min. The cellular particulate pellet was resuspended in Buffer A, and stored in aliquots at −80 °C. Protein concentrations were determined by the BCA method (Pierce) using bovine serum albumin as the standard.

**Limited Proteolysis of Mitochondria**—Mitochondria were analyzed for protease-protected fragments after preincubation with buffer (Control) or with 1% Triton X-100 to disrupt the OMM. The integrity of the OMM was determined by measuring the activity of the intermembrane space enzyme, adenylate kinase (12). To determine the accessibility of GPAT domains to protease, 350 μg of mitochondrial protein was then incubated in a final volume of 0.1 ml with selected proteases. For protease K, 20 μg of protease was added with 50 mM Tris-HCl, pH 7.4, 0.25 mM succrose, and 2 mM CaCl₂, for 30 min on ice. The reaction was stopped by adding 2 mM phenylmethylsulfonyl fluoride, 0.1% SDS, and 2 μg/ml aprotinin, and then centrifuged at 20,000 × g for 5 min. The supernatant was then centrifuged at 105,000 × g for 1 h and the supernatant was used for immunodetection of GPAT fragments. For Glu-C endoproteinase (V8 protease), the mitochondria (350 μg of protein) were incubated with 0.062 V8 protease units in 50 mM ammonium acetate buffer, pH 4.0, for 90 min at 22 °C. The reaction was stopped by adding 0.1 mM 2,4-diisochlorocoumarin and incubating for 5 min on ice. For chymotrypsin, mitochondria (350 μg of protein) were incubated with 20 μg of the protease in 0.125 mM succrose, 0.5 mM CaCl₂, and 25 mM Tris-HCl, pH 8, at 22 °C for 30 min. The reaction was stopped by adding 2 mM phenylmethylsulfonyl fluoride. For trypsin, mitochondria (200 μg) were incubated with 25 μg/ml protease in 0.125 mM succrose and 10 mM Tris-HCl, pH 8, at 22 °C for 20 min. The reaction was stopped with the addition of 2 mg/ml soybean trypsin inhibitor.

**Construction of Epitope-tagged GPAT**—Four GPAT constructs were made (Fig. 3). The cDNA encoding the complete open reading frame of rat liver mitochondrial GPAT (4) was first subcloned in pcDNA3.1 (Invitrogen) digested with BamHI-XhoI (pcDNA3.1-GPAT). A FLAG epitope at the N terminus was added by a two-step polymerase chain reaction procedure in which the 30 nucleotides encoding the epitope, an XhoI restriction site, and a stop codon were added at the 3′ end of the open reading frame (13). The polymerase chain reaction product was then inserted in the BamHI-XhoI sites of the multi cloning site of pcDNA3.1. This construct pcDNA3.1-GPAT-FLAG was referred to as GFLAG.

To insert hemagglutinin (HA) epitopes, mutagenesis was performed using the Gene Editor system (Promega). For the mutagenic reactions, GPAT-FLAG (BamHI-XhoI) was subcloned into pGEM-11Zf (pcDNA3.1-GPAT). A FLAG epitope at the C terminus was added by a two-step polymerase chain reaction procedure in which the 30 nucleotides encoding the epitope, an XhoI restriction site, and a stop codon were added at the 3′ end of the open reading frame. For chemiluminescent detection, immunoreactive bands were visualized with a fluorescent microscope equipped with green and blue filters.

**Immunoblotting**—The mitochrondrial protease digestion products were separated on a 4–20% gradient (8% for the cellular particulate of CHO cells expressing recombinant GPAT) polyacrylamide gel containing 100 mM Tris-HCl, pH 8.9, 0.1% SDS, and transferred to a polyvinylidene difluoride membrane (Bio-Rad). For chemiluminescent detection, immunoreactive bands were visualized by incubating the membrane with horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG and PicoWest reagents (Pierce).

**GPAT assays**—GPAT was assayed in rat liver mitochondria (20–80 μg of protein) and in total particulate preparations from CHO cells expressing the recombinant GPAT constructs (40–80 μg of protein). The assay was performed at 23 °C with 300 μM [³H]glycerol 3-phosphate and 112.5 μM palmitoyl-CoA in the presence or absence of 1 mM N-ethylmaleimide to inhibit the microsomal isoform (16). Microsomal GPAT was estimated by subtracting the N-ethylmaleimide-resistant activity (mitochondrial GPAT) from the total. All assays measured initial rates (5). [³H]Glycerol-3-phosphate was synthesized enzymatically (17). GPAT activity was also assayed with immobilized palmitoyl-CoA on agarose beads (180 μg/mL Sigma) using rat liver mitochondria or CHO cell total particulate preparations under isosmotie conditions (in tact) or hyposmotic conditions (10 mM Tris-HCl, pH 7.4), which disrupted the OMM. Disruption of the OMM was monitored by loss of adenylyl kinase activity.

**RESULTS**

**GPAT Is Predicted to Have at Least Two Transmembrane Domains**—The GPAT amino acid sequence deduced from the cDNA complete open reading frame was examined using algorithms that predict the possible location of TMDs based on the hydrophobicity of the residues. Four different programs available on the Internet (TMPrEd (18), TMHMM (19), DAS (20), and SOSUI) strongly predicted the presence of two TMDs between amino acids 472–493 and 575–595 (Fig. 1A). These two TMDs were the only ones predicted by TMHMM and SOSUI. These TMDs also identify two putative non-membrane segments: an N-terminal region (aa 1–471), a loop region (aa 494–575), and a C-terminal region (aa 593–828).
Two additional membrane-associated segments are predicted to lie at the hydrophobicity characteristics of the amino acid residues. Two TMDs are predicted for the segments 472–493 and 576–592 (black blocks). Two additional membrane-associated segments are predicted to lie at residues 184–203 (a) and 235–254 (b) (gray blocks). The putative membrane-associated segment b is located within the domain containing the acyltransferase homology blocks (dark block), which comprises the active site of the enzyme. B, epitope-tagged GPAT cDNA constructs used to determine the protein topography. The antigenic epitope FLAG (diamonds) was added in the C terminus of the GPAT (GFLAG), HA epitopes (triangles) were inserted within the loop (HA496) or near the N terminus (HA33) before the first predicted transmembrane domain, or a Myc epitope (oval) was added to the truncated protein as shown (Tr576Myc).

of GPAT (5, 6, 8), this predicted TMD is highly improbable because it would cause portions of the active site to lie on opposite faces of the OMM. The TMD predicted for aa 184–203 is the only possible one between the N terminus and the active site domain. Specific experiments were carried out to determine the existence of this TMD and the two prime TMD predictions.

The Outer Mitochondrial Membrane Protects Some GPAT Segments from Protease Hydrolysis—When rat liver mitochondria were treated with different specific and nonspecific proteases and the reaction products were blotted with a polyclonal antibody, several immunoreactive bands were detected (Fig. 2, lane 2). The digestion products were analyzed by 4–20% gradient polyacrylamide gel electrophoresis under denaturing conditions, and proteins were detected with anti-GPAT polyclonal antibody. The percent activity of the intermembrane space enzyme adenylate kinase was measured as a control for OMM integrity. Control activity was 9.1 μmol ATP/min for the conditions used in lane 2. The arrows indicate the proteolytic fragments: A, 16 and 23 kDa; B, 13.5 and 26 kDa; C, 23 and 31 kDa. The asterisks indicate native GPAT (92 kDa).

Epitope-tagged GPAT Can Be Expressed in CHO Cells—To determine the orientation of GPAT, several constructs were designed to contain specific epitopes in the domains delimited by the two TMDs or truncations of GPAT proximal to the second TMD (Fig. 1B). CHO K1 cells were transiently transfected with these constructs, and the expression of GPAT was monitored by Western blot both with the specific antibodies for the epitopes (anti-FLAG, anti-HA, and anti-Myc) and with assays for GPAT activity (Fig. 3). Although the four proteins corresponding to the different constructs were expressed with the correct predicted molecular weights, only the GPAT transfectants with epitopes added at the C terminus (GFLAG) or near the N terminus (HA33) were active. Compared with vector-transfected control cells membranes, GFLAG and HA33 cells had 6–8-fold increases in mitochondrial GPAT specific activity. Cells transfected with GPAT constructs containing the HA epitope in the loop (HA496) or with the truncated construct (Tr576Myc), however, expressed GPAT activity similar to the vector-transfected control. This lack of activity was surprising because neither of the loop constructs was near the active site domain, and in both, the active site domain remained on the opposite, cytosolic side of the OMM (see below).

Because previous studies indicated that the active site of GPAT faces the cytosol (21–23), we tested GPAT activity in CHO cell total particulate preparations by using immobilized palmitoyl-CoA as a substrate to corroborate the correct insertion of the recombinant proteins (Table I). GPAT was able to access agarose-bound palmitoyl-CoA under isosmotic conditions when the OMM was intact, showing that the active site of the enzyme faces the cytosol and can interact with agarose beads that cannot cross the membrane. Furthermore, disruption of the OMM under hypotonic conditions did not increase...
GPAT activity, indicating a lack of latency of GPAT activity. The integrity of the OMM under each condition was confirmed by assaying the activity of the intermembrane marker, adenylate kinase. Full adenylate kinase activity was present in both total cellular particulate and intact CHO cells was 100% of control activity, showing a lack of protease entry into the intermembrane space. In contrast, cells or total cellular particulates permeabilized with 1% Triton X-100 lost 75% of the adenylate kinase activity, indicating disruption of the OMM that allowed protease K to have access to the enzyme.

Fluorescent images for the FLAG, HA, and Myc epitope antibodies (green) or the mitochondrial marker (red) were analyzed either after the plasma membrane was permeabilized with digitonin or after the intracellular membranes were permeabilized with Triton X-100 (Fig. 4). Specificity of the antibody signal was tested by probing control CHO cells transfected with the empty plasmid with both the primary antibody (anti-HA, anti-FLAG, or anti-Myc) and the fluorescein isothiocyanate-labeled secondary antibody. CHO cells expressing the epitope-tagged GPAT constructs were also probed with only the secondary antibody. In both cases, the fluorescent signal re-
mained at background values (results not shown). The CHO cells expressing recombinant epitope-tagged GPAT exhibited an antibody-staining pattern within discrete structures in the cytoplasm, similar to the mitochondrial localization signal.

The results show that the C terminus of GPAT faces the cytosol, because in the full-length GPAT construct tagged in the C terminus (GFLAG), this epitope was recognized in digitonit-treated cells when only the plasma membrane was permeabilized (Fig. 4). As expected, no change in staining was observed when these cells were permeabilized with Triton X-100. Similarly, the full-length GPAT containing an HA epitope near the N terminus (HA33) could be probed with anti-HA antibody after permeabilization with either digitonin or Triton X-100, showing that in this construct the N terminus is exposed to the cytosol. Both GFLAG and HA33 transfectants expressed high GPAT activity (8- and 6-fold more than control cells) (Fig. 3) and had their active sites exposed on the cytosolic surface, as evidenced by activity with agarose-linked palmitoyl-CoA substrate (Table I). These data indicate that the N and C termini and the active site of GPAT all face the cytosolic face of the OMM.

Two constructs were made to test the presence of the putative loop between residues 472–493 and 576–592. CHO cells transfected with HA496 were identified with the HA antibody only after permeabilization with Triton X-100 but not after digitonin. This difference indicates that the HA epitope was not accessible to the antibody unless the OMM was disrupted. As was observed in GFLAG and HA33 transfectants, the C-terminal FLAG epitope present in the HA496 transfectants remained accessible to the FLAG antibody in digitonin-permeabilized cells, indicating that the C terminus of this construct was in its usual position on the cytoplasmic face of the OMM. The correct orientation of the GPAT-HA496 construct was further verified in total membrane fractions obtained from CHO cells that expressed HA496. When these membranes were exposed to protease K, anti-HA antibody detected a membrane-protected fragment whose molecular mass (~14 kDa) matches the calculated molecular mass of the loop plus the two TMDs (Fig. 5). This fragment did not react with the anti-FLAG antibody, consistent with protease degradation of the FLAG epitope and C terminus on the cytosolic face of the OMM. Adenylate kinase activity corroborated the integrity of the OMM.

Similarly with the second loop construct, Tr576Myc, which is truncated just before the second TMD, cells became stained with the Myc antibody only after intracellular membranes had been disrupted with Triton X-100; the Myc epitope was not accessible to the antibody in cells permeabilized with digitonin, indicating that the shortened C terminus was located in the intermembrane space. Taken as a whole, these results indicate that GPAT C- and N-terminal domains are located in the cytosolic face of the OMM and that the loop lies in the intermembrane space.

**DISCUSSION**

The mitochondrial isofrom of GPAT is an intrinsic membrane protein (1) and requires detergents for isolation and purification (26, 27). Studies of intrinsic membrane proteins in internal mammalian membranes have most commonly investigated proteins of the endoplasmic reticulum where the methyloxyn B activator were added in a soluble form or immobilized on agarose beads (23), which cannot pass through the OMM (30). We corroborated the finding that the active site lies on the cytoplasmic surface when we found that agarose-linked palmitoyl-CoA was a substrate for GPAT in intact mitochondria and it difficult to use conventional labeling or protein modification reagents to determine the sidedness of a residue.

With protease digestion of native intact mitochondria, we found that at least one GPAT segment is protected from hydrolysis by V8 protease or chymotrypsin and that the size of this fragment matches the theoretical molecular mass of the loop plus the two TMDs. Additional higher molecular weight fragments, however, were observed when intact mitochondria were exposed to protease K. Some membrane proteins have a tight tertiary structure lying outside the membrane-spanning regions (32), and this folded structure may prevent proteases from cleaving at all their potential recognition sequences. Furthermore, some proteins contain domains that have a compact conformation with helices parallel to the membrane plane, and these may not be accessible to proteases (33). It is likely that GPAT has one or more protease-protected regions in extramembrane domains, especially at its active site, because its acyl-CoA substrate and its lysophosphatic acid product are both amphipathic molecules and may need to interact with the mitochondrial membrane itself or with another hydrophobic or amphipathic structure.

Mutagenesis studies performed on the highly homologous *Escherichia coli* GPAT (5, 6) and mouse GPAT (7) have clearly demonstrated that the active site of GPAT is located in a highly conserved region corresponding to the amino acids 224–323. Designation of this area as the active site is also supported by studies of mutations in the human acyl-CoA:diacylglycerol acyltransferase, in which the substitution of His or Cys for Arg-211 in the homologous active site region inactivates the enzyme completely (34). It was previously concluded that the GPAT active site faces the cytosol because GPAT activity was similar when either the palmitoyl-CoA substrate or a poly-
in the GFLAG and HA33 constructs and that disruption of the OMM did not reveal latent GPAT activity measured with the immobilized substrate. Based on these data, we conclude that the GPAT active site region from aa 224–323 faces the cytosol.

TMD prediction algorithms varied in their predictions. All predicted two TMDs that followed the active site domain, but some also predicted a TMD that lay between aa 184 and 203 (Fig. 1A) and just before the active site domain (aa 224–323). If the N terminus were cytosolic, this TMD would place the active site domain in the intermembrane space. To delineate the topography of GPAT and to determine accurately where the cytosolic and intermembrane domains lay, we transfected epitope-tagged GPAT constructs into CHO cells and localized the epitopes by immunofluorescence. Epitope tagging has been used successfully to elucidate the topography of proteins in the plasma membrane (35) and endoplasmic reticulum (15, 24, 25, 36). We designed the mutated constructs so that the epitopes would be located near the N terminus, at the C terminus, and in the putative loop. Although little is known about the targeting of OMM proteins, the first amino acids may provide a targeting signal (22), so we inserted the HA epitope 33 residues after the N terminus. No predicted TMDs are present between the N terminus and aa 33. In each instance, this C terminus HA33 epitope was visualized after digitonin permeabilization of the plasma membrane, indicating that the C terminus is exposed to the cytosol. Antibodies did not interact with the epitopes located in either the beginning or the end of the hydrophilic domain of the loop (HA496 and Tr576Myc) in the cells unless the OMM was permeabilized, whereas epitopes located at the N and C termini could be probed without disrupting the OMM. Furthermore, immunofluorescence studies and protease treatment of the native enzyme and the HA496 construct showed that GPAT contains one domain that is not accessible from the cytosolic side and has a molecular mass consistent with the loop plus two TMDs (aa 472–593). Thus, the N terminus and the active site domain face the cytosolic surface without an additional intervening TMD. The C terminus faces the cytosol in the three full-length constructs and transfected these into Cos-7 cells. One 120-kDa construct contained a GFP sequence inserted at aa 115, and the second expressed GFP at the C terminus. Trypsin did not cleave the fusion protein in intact mitochondria but digested the GFP domains after the membrane had been solubilized with CHAPS. Like an earlier report by these investigators (21), we also observed that trypsin does not digest GPAT efficiently (Fig. 2D), indicating that this protease is uninformative for any investigation of GPAT topography (37). Susceptibility to a protease only in the presence of a detergent is not informative because the detergent may expose residues that are not ordinarily accessible (38), or with recombinant proteins, the lack of digestion may occur because the protein is mis-oriented in the membrane. Furthermore, these investigators did not show that the transfected GFP-GPAT fusion constructs express GPAT activity or that the enzyme could use palmitoyl-CoA provided to the cytosolic face of intact mitochondria, as they had previously reported (21, 39) and as would be expected if GPAT were correctly oriented. Thus, we conclude that the topography inferred by the previous report was in error.

Protein topography deduced from our results (Fig. 6) is opposite that previously proposed (9), which postulated a model of GPAT with N and C termini facing the intermembrane space and a loop that faces the cytosol. In the previous report, peptide antibodies were raised against an internal N-terminal domain distal to the active site (aa 420–435), against a loop sequence (aa 543–559), and against a sequence near the C terminus (aa 726–740) (see Fig. 1). These antibodies were tested on native GPAT in rat liver mitochondria. Only the antibody raised against the loop abolished GPAT activity. None of these three peptide antibodies was directed against the GPAT active site region confirmed by site-directed mutagenesis to be responsible for catalysis and substrate binding (5–8). This active site region is further substantiated by a mutation in a closely related human gene (34). Thus, it is not entirely surprising that the antibodies to the N and C regions had no apparent effect on GPAT activity in intact mitochondria. Although the antibody directed to the loop region did inhibit GPAT activity, no marker was used to prove continued OMM integrity, and it is possible that GPAT inactivation occurred because of structure-function changes similar to our observations with the two loop constructs. These investigators also made two GPAT-GFP fusion constructs and transfected these into Cos-7 cells. One 120-kDa construct contained a GFP sequence inserted at aa 115, and the second expressed GFP at the C terminus. Trypsin did not cleave the fusion protein in intact mitochondria but digested the GFP domains after the membrane had been solubilized with CHAPS. Like an earlier report by these investigators (21), we also observed that trypsin does not digest GPAT efficiently (Fig. 2D), indicating that this protease is uninformative for any investigation of GPAT topography (37). Susceptibility to a protease only in the presence of a detergent is not informative because the detergent may expose residues that are not ordinarily accessible (38), or with recombinant proteins, the lack of digestion may occur because the protein is mis-oriented in the membrane. Furthermore, these investigators did not show that the transfected GFP-GPAT fusion constructs express GPAT activity or that the enzyme could use palmitoyl-CoA provided to the cytosolic face of intact mitochondria, as they had previously reported (21, 39) and as would be expected if GPAT were correctly oriented. Thus, we conclude that the topography inferred by the previous report was in error.
cause an altered relationship of the associated TMDs (42). Thus, some loop residues might interact with other molecules that stabilize GPAT structure and/or activity. Alternatively, the modified loop in our constructs could alter the spatial conformation of the cytosol-facing domains that are required for interaction of the active site region and the C-terminal domain. This interpretation is consistent with the fact that the LPAAT proteins lack a predicted TMD after their active site domain. This interpretation is consistent with the fact that the conformation of the cytosol-facing domains that are required that stabilize GPAT structure and/or activity. Alternatively, some loop residues might interact with other molecules of the GPAT loop and C-terminal domains which will require future experiments.

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