Identification of Two Transmembrane Regions and a Cytosolic Domain of Rat Mitochondrial Glycerophosphate Acyltransferase*

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The topography of rat glycerophosphate acyltransferase (GAT) in the transverse plane of the mitochondrial outer membrane (MOM) was investigated. Computer analysis of the amino acid (aa) sequence derived from rat mitochondrial GAT cDNA (GenBank® accession nos. U36771 and AF021348) predicts the presence of two possible transmembrane domains (aa 473–493 and 574–594) separated by an 80-aa stretch (aa 494–573). To determine the actual orientation of the native protein, we prepared anti-peptide antibodies to three regions: one in between (aa 543–559) and the other two (aa 420–435 and 726–740) flanking the two putative transmembrane regions. Both immunoreaction and immunoprecipitation experiments employing intact and solubilized mitochondria indicate that regions on the N- and C-terminal sides of the transmembrane regions are sequestered on the inner surface of the MOM, while the region between the transmembrane domains is present on the cytosolic face of the MOM. Additionally, two green fluorescent protein (GFP) fusion proteins consisting of full-length GAT fused to GFP at either the C terminus or inserted 115 amino acids from the N terminus were also constructed to determine the orientation of the N and C termini. COS-1 cells expressing these fusion proteins were fractionated to obtain mitochondria. Protease digestion of intact and solubilized COS-1 cell mitochondria revealed that the GFP domains of these fusion proteins are sequestered on the inner side of the MOM. The present findings indicate that GAT is a dual-spanning, transmembrane protein adopting an inverted “U” conformation in the transverse plane of the MOM, where the N and C termini are sequestered on the inner surface of the MOM, while aa 494–573 are exposed on the cytosolic surface of the MOM.

The mitochondrial outer membrane (MOM)† is the demarcatory barrier between the mitochondrial interior and cytosol. Its significance as such is especially relevant in light of the role the MOM plays in cellular glycerolipid synthesis. The three enzymes that mediate this process in the MOM are acyl-CoA synthetase (ACS), glycerophosphate acyltransferase (GAT), and monoacylglycerophosphate acyltransferase. ACS initiates the first step of the pathway via production of activated fatty acids in the form of fatty acyl-CoAs. GAT mediates the committed step of glycerolipid synthesis by converting the fatty acyl-CoAs to lysophosphatidic acid (1). The lysophosphatidic acid produced can be further acylated by monoacylglycerophosphate acyltransferase to produce phosphatic acid (1, 2), which can either move into the mitochondria for further conversion to cardiolipin (3–5) or can move to other cellular sites for conversion to other glycerolipids (6–10).

These MOM enzymes can also interact with other cellular sites of glycerolipid synthesis. Microsomal ACS, GAT, and monoacylglycerophosphate acyltransferase (6, 7, 11–13) as well as peroxisomal ACS (14) contribute to the cytosolic pool of fatty acyl-CoAs, lysophosphatidic acid, and phosphatidic acid. The participation between the mitochondrial, microsomal, and peroxisomal enzymes and the cytosolic pool is facilitated by the carrier proteins acyl-CoA-binding protein (15, 16) and fatty acid-binding protein (17), which can presumably shuttle intermediates between the surface of the three sites. This level of interplay suggests that the orientation of these enzymes in their respective membranes would be such as to maximize this interaction.

In this present work, we investigated the topography of GAT in the MOM. The biochemical characterization (6, 18–20) and transcriptional regulation (21) of mitochondrial GAT are well established; however, there is only limited characterization of the structural qualities of the enzyme. Previous studies offer indirect evidence of the orientation of the protein in the MOM. In intact mitochondria, GAT has been shown to be resistant to trypsin and chymotrypsin (18, 19, 22–24); however, activity of the enzyme can be abolished by proteinase K or subtilisin treatment, indicating that the GAT has a cytosolic domain (25). Additionally, when right-side-out trypsin-loaded MOM vesicles are generated, the activity of the enzyme is also diminished, suggesting that there are portions of GAT exposed at the inner aspect of the MOM as well (25). These results argue that, at the very least, rat mitochondrial GAT must possess one transmembrane domain.

There also are some empirical data regarding the topography of mitochondrial GAT. The cDNA of rat mitochondrial GAT (GenBank® accession nos. U36771 and AF021348) was previously isolated and mapped (26, 27). Computer analysis of the signal sequence suggested that the protein folding takes place at the cytosolic side of the MOM. The present findings confirm these previous suggestions and indicate that GAT is a dual-spanning, transmembrane protein adopting an inverted “U” conformation in the transverse plane of the MOM, where the N and C termini are sequestered on the inner surface of the MOM, while aa 494–573 are exposed on the cytosolic surface of the MOM.
The Topography of Mitochondrial GAT

31669

derived amino acid (aa) sequence revealed the presence of several hydrophobic regions that may interact with the MOM (Fig. 1). However, only two of these regions (aa 473–493 and 574–594) are characteristic of transmembrane domains. Dependent on the orientation of these putative transmembrane domains, the 80 aa between the two transmembrane domains (aa 494–573) may be exposed at the cytosolic surface of the MOM (while the N- and C-terminal regions are sequestered at the inner leaflet of the MOM) or face the inter-membrane space (N- and C-terminal regions exposed to the cytosol). The earlier of the two models was preferred as more likely since the controlled protease digestion data (25) suggest only a limited presence of GAT at the cytosolic surface of the MOM.

In the present investigation, we have employed immunological methods as well as green fluorescent protein (GFP) fusion proteins to elucidate the topography of GAT in the MOM. The results suggest aa 475–493 and 574–594 do serve as transmembrane domains, while the 80 aa between them (aa 494–573) are exposed on the cytosolic face of the MOM. Additionally, protease digestion of the GFP fusion protein constructs reveal that both the N- and C-terminal regions of rat mitochondrial GAT are sequestered on the inner side of the MOM.

EXPERIMENTAL PROCEDURES

Materials—Male Harlan Sprague-Dawley rats were purchased form Taconic Farms (Germantown, NY), sn-5,12-[H]Glycero 3-phosphate was purchased from American Radiolabeled Chemicals Inc. (specific activity: 1.64 × 107 cpm/nmol). Protein A-agarose, protein A-Sepharose, equine heart cytochrome c, trypsin, soybean trypsin inhibitor, and anti-mouse antibodies conjugated with horseradish peroxidase were purchased from Sigma. Restriction enzymes were obtained from New England Biolabs. Enhanced chemiluminescence (ECL) reagents and the pClneo mammalian expression vector were purchased from Promega Corp.

The Topography of Mitochondrial GAT

The amino acid sequence deduced from rat mitochondrial GAT cDNA was analyzed using the TopPred2 computer-based algorithm, which plots a hydrophobicity grid based on the physico-chemical characteristics of the consecutive amino acids (28). Analysis windows were set for 10 aa. Confirmation of TopPred2 results were made by comparison to predictions made by the TMpred program, a statistical algorithm based on the transmembrane sequences in the TMBase data base (29).

Antigenicity profiles for the sequence of rat mitochondrial GAT were obtained from the database of Parker et al. (30) contained in the Internet software package AnTheProt version 4.6b, available via the World Wide Web.

Preparation of Rat Liver Mitochondria—Rat liver mitochondria were isolated from 175–200-g male Harlan Sprague-Dawley rats as described previously (11). Purity of the preparation was established by performing the GAT assay in the presence and absence of 2 mM N-ethylmaleimide, a potent inhibitor of microsomal GAT (6). According to these results, microsomal cross-contamination of the mitochondrial preparation was less than 6%. Integrity of the mitochondrial membranes was confirmed by the latency of cytochrome c oxidase activity assayed in 40 mM phosphate buffer, containing 1.35 mg/ml reduced cytochrome c in the presence and absence of 0.244% Nonidet P-40.

Antibody Production and Purification—Anti-peptide antibodies to three regions of rat mitochondrial GAT were purchased from Genemed Synthesis, Inc. Briefly, the company was supplied with the amino acid sequences for three distinct regions of rat mitochondrial GAT (aa 420–435, 543–599, 726–740) that exhibited acceptable antigenic ratings by the method of Parker et al. (30). Synthetic peptides were made with an extra cysteine residue at their N terminus that was consequently used to insert keyhole limpet hemocyanin. The keyhole limpet hemocyanin-peptide conjugates were separately injected into three rabbits. Prior to the primary inoculation, pre-immune serum was obtained from the rabbits. Shortly after the secondary inoculation, the rabbits were bled and the anti-serum was isolated. Enzyme-linked immunosorbent assay titers performed by Genemed for each the three anti-serum types against their respective synthetic peptides were similar at 1:104. Total IgG fractions were purified by passage of the anti-serum through either a protein A-agarose or protein A-Sepharose column, elution in 0.1 M glycine buffer, pH 3.0, neutralized, and dialyzed overnight against phosphate-buffered saline, pH 7.4. The final concentration of the total IgG fraction was as follows: IM2GAT (specific to aa 420–435), 1.19 mg/ml; IM3GAT (specific to aa 726–740), 1.56 mg/ml; and CTVGAT (specific to aa 543–599), 0.59 mg/ml.

Immunoreaction and Immunoprecipitation of GAT—Rat liver mitochondria were suspended in 0.3 M sucrose, 1 mM EDTA, pH 7.4, at a concentration of 8–10 mg/ml. As needed, mitochondria were solubilized on ice for 30 min by the addition of CHAPS to a final concentration of 50 mM. The resulting amounts of solubilized mitochondria (200 µg) were treated with varying amount of each antibody to determine maximum GAT inhibition. The three antibodies were similar in that 10 µl of each antibody produced maximum inhibition of GAT activity in 200 µg of mitochondria. This amount was kept constant in all experiments.

In immunoreaction experiments, 10 µl of anti-peptide antibodies were added separately to 200 µg of either intact or solubilized mitochondria and incubated at 5 °C for 1 h in a revolving rack. Aseptic was added to solubilized samples to a final concentration of 7 mg/ml. Aliquots of these samples were used to assay GAT activity. Immunoprecipitation reactions required the addition of 20 µg of protein A (in the form of 4% bead coated protein A-agarose; 2 mg of protein A/ml of agarose) after incubation of intact or solubilized mitochondria with the antibodies. Immunoprecipitations were carried out at 4 °C in a revolving rack. Samples were then allowed to stand for 1 min at 5 °C to sediment the agarose beads by gravity. The supernatant was removed, and the pellet was washed and sedimented in phosphate-buffered saline (PBS) three times to remove any co-sedimented mitochondria or solubilized membranes. The pellet was then resuspended in 0.3 M sucrose, 1 mM EDTA, 7 mg/ml aselin, pH 7.4. Supernatant and pellet fractions were assayed for GAT activity. In immunoprecipitation experiments involving intact mitochondria and pre-immune serum, cytochrome c oxidase activity indicated 7% nonspecific co-sedimentation of mitochondria with the washed agarose pellets.

Extension of the N Terminal of Rat Mitochondrial GAT—The cDNA sequence of rat mitochondrial GAT was previously established by our group (GenBank accession no. U567711) (26). This sequence was ligated into the mammalian expression vector pClneo, and termed plasmid T38.2. In comparison to rat mitochondrial GAT cDNA isolated by others (GenBank accession no. AF0213448) (27) the open reading frame (ORF) of our sequence is abridged by 165 base pairs at the N-terminal coding region. A two-step procedure was designed to extend the ORF to encompass this missing region. Two complimentary primers were designed to amplify from rat mtDNA a 5′ fragment (5'-AGAAGGGAGATCAAGAGTCAGGCTGACTG) and a 3′ fragment (5'-TCTGCTGACTGACTG). The 5′ fragment was ligated into plasmid T38.2 cut with the same enzymes. The resultant plasmid, T215 contained a novel BstEII site that was designed into the insert. Two primers (5'-AGAAGGGAGATCAAGAGTCAGGCTGACTG and 5'-TCTGCTGACTGACTG) and M2 (5'-GAGAGGCTGAGGGAGTCGACCA-CGTCTCTTACGATTGATCTGAGGATGAGTCGAC-ACTGATAATTCTATCTGACCCAACTCTACCGGATCTGGCGCGGTAAGACGAGGTTCATCTGACCCAACTCTACCGGATCTGGCGCGGTAAGACGAGGTTCATCT-3') were used in PCR reactions employing plasmid T215 as the template. The −1.4-kilobase pair PCR product and plasmid T215 were cleaved with BstEII and A/III and ligated. The resultant plasmid was termed pVP1 and contains the complete ORF of rat mitochondrial GAT.

Construction of GFP Fusion Proteins—The ORF of GAT was PCR-amplified from plasmid pVP1 using two primers; 5'-CTGACTGAGGATGAGTCGACCA-CGTCTCTTACGATTGATCTGAGGATGAGTCGAC-ACTGATAATTCTATCTGACCCAACTCTACCGGATCTGGCGCGGTAAGACGAGGTTCATCTGACCCAACTCTACCGGATCTGGCGCGGTAAGACGAGGTTCATCT-3') were used in PCR reactions employing plasmid T215 as the template. The −1.4-kilobase pair PCR product and plasmid T215 were cleaved with BstEII and A/III and ligated. The resultant plasmid was termed pVP1 and contains the complete ORF of rat mitochondrial GAT.

Construction of GFP Fusion Proteins—The ORF of GAT was PCR-amplified from plasmid pVP1 using two primers; 5'-CTGACTGAGGATGAGTCGACCA-CGTCTCTTACGATTGATCTGAGGATGAGTCGAC-ACTGATAATTCTATCTGACCCAACTCTACCGGATCTGGCGCGGTAAGACGAGGTTCATCTGACCCAACTCTACCGGATCTGGCGCGGTAAGACGAGGTTCATCT-3') were used in PCR reactions employing plasmid T215 as the template. The −1.4-kilobase pair PCR product and plasmid T215 were cleaved with BstEII and A/III and ligated. The resultant plasmid was termed pVP1 and contains the complete ORF of rat mitochondrial GAT.
drial GAT fused at its C terminus to GFP via a 5-aa linker region. Primers N1 (5'-TACATCTTTTCTGACAAAGGCGCGGTGTC) and N2 (5'-CTAGTCTTCTGAGAAGGCGCGGTCGTC) were used in PCR reactions employing pVP1 as template. The 2.2-kilobase pair PCR product (containing a novel BsiWI site that was designed into primer N1) was digested with BsiWI and XbaI and ligated into plasmid pVP1 cut with BspGI and XbaI. The resultant plasmid was termed pVP15. This plasmid encodes GFP located 115 aa from the N-terminal end of the full-length mitochondrial GAT. The calculated molecular mass of this fusion protein is 121 kDa. All constructs were confirmed by nucleotide sequencing.

Expression in COS-1 Cells—COS-1 cells were grown to a density of 2 × 10^5 cells/well in six-well plates. Approximately 1 μg/well of either pVP11 or pVP15 were used to transiently transfect COS-1 cells using the Superfect transfection reagent (Qiagen). Cells were allowed to grow for 48 h after transfection. Ice-cold PBS with 1 mM CaCl_2 and 0.5 mM MgCl_2, pH 7.4, was added to the wells, and the COS-1 cells were harvested by scrapping. All subsequent steps were carried out at 4 °C or on ice. Cells were pelleted by centrifugation at 120 × g for 10 min. The supernatant was aspirated and cells resuspended in ice cold Ca^2+/Mg^2+-free buffer (PBS, pH 7.4) and centrifuged at 180 × g for 10 min. The size of the pellet was noted. Cells were resuspended and pelleted repeatedly (approximately five times) in ice-cold Ca^2+/Mg^2+-free buffer until the cell pellet became swollen to roughly twice the original size. The pellet was then resuspended in SEM (0.3M sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.4) buffer and homogenized using 4–6 strokes of a Teflon-coated homogenizer. Cell rupture was monitored by trypan blue staining before and after homogenization. KCl was added to a final concentration of 20 mM when greater than 90% of the cells were ruptured. A small sample of the whole cell homogenate was set aside. The remaining homogenate was then centrifuged at 750 × g for 10 min to pellet unbroken cells and nuclei. The supernatant was then centrifuged at 6800 × g, and the resulting mitochondrial pellet was washed three times with SEM buffer and finally resuspended in the same buffer to a concentration of 1–2 mg/ml.

Immunodetection of GFP Fusion Proteins—Approximately 40 μg of each sample was separated along with pre-stained molecular weight markers in 7.5% SDS-polyacrylamide minigels, transferred to nitrocellulose, blocked with 5% dry milk in PBS with 0.1% Tween 20, and probed with mouse monoclonal anti-GFP antibodies. GFP-antibody complexes were identified with goat anti-mouse antibody conjugated to horseradish peroxidase. ECL was used to detect horseradish peroxidase complexes were identified with goat anti-mouse antibody conjugated to horseradish peroxidase. ECL was used to detect horseradish peroxidase activity according to the manufacturer's instructions. X-ray film was exposed to developed blots for 7, 14, or 21 s to determine which time point produced a signal that was within the linear range of exposure of the film. All blots required only 7 s of exposure to x-ray film.

RESULTS

Transmembrane Domains Predicted by Computer Analysis—The nucleotide sequence of rat mitochondrial GAT cDNA has been documented previously (26, 27). The derived amino acid sequence was analyzed to determine the hydrophobic character of the protein. The Topred2 program (28) was utilized for this purpose, and the results are presented in the form of a hydrophobicity plot (Fig. 1A). In addition to the graphical output, the program also predicts possible transmembrane regions and scores each with a “putative” or “certain” rating. Analysis of rat mitochondrial GAT resulted in six candidate hydrophobic regions (Fig. 1A, boxes 1–6), wherein two of those regions, aa 473–493 and 574–594, elicited a rating of “certain” transmembrane regions (Fig. 1A, boxes 3 and 5). The predicted transmembrane character of these two regions was confirmed by using the statistical algorithm, TMpred (data not shown), which makes direct comparison of the tested sequences against the TMBase data base of transmembrane sequences (29).

The two transmembrane regions may afford GAT two possible orientations in the MOM; 1) the N- and C-terminal portions may be exposed to the cytosol, while the 80 aa between the two transmembrane domains is exposed to the inner aspect of the MOM; or 2) the N- and C-terminal portions are sequestered in the inner aspect of the MOM, while the 80 aa between the two transmembrane domains is exposed to the cytosol. The latter orientation was conscripted as our model of GAT topography (Fig. 1B) based on previous protease digestion data (25), which suggest a limited cytosolic exposure of mitochondrial GAT.

Effects of Antibodies on GAT Activity in Intact and Solubilized Rat Liver Mitochondria—In order to experimentally test this model, we prepared three anti-peptide antibodies to three specific regions: two regions flanking the putative transmembrane domains (aa 420–435 and 726–740) (Fig. 1B, circles a and b, respectively) and one region between the two transmembrane domains (aa 543–559) (Fig. 1B, circle c) of rat mitochondrial GAT. Optimization of the amount of each antibody to produce a maximal inhibition of GAT activity in a fixed amount of solubilized mitochondria (200 μg) was performed, and this amount was kept constant in all experiments (data not shown). We opted to use both intact and solubilized mitochondria to determine if membrane sequestration of portions of GAT is evident in the ability or inability of the antibodies to bind to their respective targets.

Freshly isolated rat liver mitochondria were treated separately with each of the three antibodies: IM1GAT (specific to aa 420–435), IM2GAT (specific to aa 726–740), and CYTGAT (specific to aa 543–559). Mitochondria were also treated with total IgG from pre-immune serum to serve as a control. IM1GAT and IM2GAT failed to decrease GAT activity (1% and 2%, respectively) (Fig. 2). However, CYTGAT was able to decrease activity by 50%, which indicates that the region of GAT that CYTGAT was raised against is present on the cytosolic surface of the MOM.

Since IM1GAT and IM2GAT did not produce a decrease in GAT activity in intact mitochondria, the effect of membrane solubilization on the ability of these antibodies to bind to their respective targets was determined. Mitochondria were pre-treated with 50 mM CHAPS for 30 min on ice. Following antibody incubation, GAT activity was reconstituted with asolectin to promote micelle formation. Under these conditions, all three antibodies decreased GAT activity (Fig. 3). IM1GAT and IM2GAT were able to decrease GAT activity by 33% and 25%, respectively, while CYTGAT was able to reduce activity by
Mitochondria were then solubilized prior to immunoprecipitation. IM1GAT, IM2GAT, and CYTGAT were all able to localize a large percentage of GAT activity to their pellet fractions (88%, 82%, and 93%, respectively) (Fig. 5). Considering the similarity in the percentage of total GAT activity found in their respective pellet fractions, the efficiency of IM1GAT, IM2GAT, and CYTGAT binding to the GAT protein may be considered nearly the same. This substantiates the solubilized mitochondrial immunoreaction experiments (Fig. 3) in that the greater inhibition of GAT activity effected by CYTGAT is not due to higher binding efficiency of the antibody, but rather a specific spatial or conformational effect CYTGAT binding has on GAT activity.

Trypsin Digestion of GFP Fusion Constructs—The results of the immunoreaction and immunoprecipitation experiments indicate that rat mitochondrial GAT has at least two transmembrane domains and the region between them is exposed on the cytosolic surface of the MOM. To further elucidate the topology of GAT, two GFP fusion proteins were designed to determine the orientation of the N and C termini. Plasmids encoding GFP fused either to the C terminus of GAT (pVP11) or inserted 115 aa from the N terminus (pVP15) were constructed. GFP was not fused directly to the N terminus because it may interfere with or abolish the mitochondrial targeting properties of the N terminus of GAT.2 The absence of any hydrophobic regions within the 115 aa at the N terminus would allow the submitochondrial localization of GFP to be equated with the actual N terminus of GAT. Mitochondria were isolated from COS-1 cells transiently transfected with pVP11 and pVP15. Intact and solubilized mitochondria were treated with trypsin to determine on which side of the MOM the GFP domain of the fusion proteins are present (Fig. 6). Western blots probed with anti-GFP antibodies indicate that, in intact mitochondria, the GFP domains of both C-terminal (Fig. 6, lane 3) and N-terminal (Fig. 6, lane 6) fusion proteins were resistant to trypsin digestion. However, when the mitochondria were solubilized prior to trypsin treatment, the GFP domains became susceptible (Fig. 6).
Fifty mM. Fifty mitochondria were solubilized with CHAPS to a final concentration of 4% beaded protein A-agarose was then added to the samples and rotated for 15 min. The agarose pellet and supernatant were separated as described under “Experimental Procedures.” Values are an average of two independent experiments. Percentage activity values are in relation to total GAT activity in both the pellet and supernatant fractions for each antibody, which is taken as 100%.

Fig. 5. Immunoprecipitation of GAT from solubilized rat liver mitochondria. Rat liver mitochondria were solubilized by addition of CHAPS to a final concentration of 50 mM and incubated with each of the antibodies. 4% beaded protein A-agarose was then added to the samples and rotated for 15 min. The agarose pellet and supernatant were separated as described under “Experimental Procedures.” Values are an average of two independent experiments. Percentage activity values are in relation to total GAT activity in both the pellet and supernatant fractions for each antibody, which is taken as 100%.

6, lanes 4 and 8), indicating that the N- and C-terminal regions of GAT are sequestered on the inner side of the MOM.

DISCUSSION

In the present investigation, immunological experiments have established that aa 473–493 and 574–594 serve as transmembrane domains in rat mitochondrial GAT and the region between the two (aa 494–573) is exposed on the cytosolic surface of the MOM (Figs. 2–5). Additionally, protease digestion of GFP fusion proteins demonstrates that the N and C termini of GAT are sequestered on the inner surface of the MOM (Fig. 6).

These findings corroborate the proposed “(aa 1–472)in-out(aa 494–573)-in(aa 595–)” topographical model of GAT (Fig. 1B), whose design was based on hydrophobicity analysis of the derived amino acid sequence of rat mitochondrial GAT (Fig. 1A) as well as previous biochemical experiments (25). However, this model contains only two transmembrane domains (Fig. 1A, boxes 1 and 2), although the hydrophobicity plot indicates that there are four other hydrophobic regions in GAT that may be potential transmembrane domains (Fig. 1A, boxes 1, 2, 4, and 6). These regions were excluded as transmembrane domains for various reasons. The hydrophobic regions, aa 176–196 and 235–255 (Fig. 1A, boxes 1 and 2, respectively), were not considered as possible transmembrane regions based on their hydrophobicity scores obtained from TopPred2 analysis. In comparison to the established transmembrane regions, aa 473–493 and 574–594, which received scores of 1.080 and 1.293, respectively, aa 176–196 and 235–255 only received scores of 0.728 and 0.683, respectively. To be consistent with the findings presented in this paper, both or neither of these segments would have to be transmembrane domains. If only one was a transmembrane domain, the antigenic site for IM1GAT (aa 420–435) and the N-terminal region would be on opposite sides of the membrane. In view of the relatively low hydrophobicity scores for both these segments, we conclude that neither of them is a transmembrane domain. More likely, these hydrophobic regions are probably closely associated with the inner leaflet of the MOM.

The hydrophobic region, aa 551–571 (Fig. 1A, box 4), was rejected as a possible transmembrane domain based on the results of the immunological data. In intact mitochondria, CYTGAT was able to bind to its antigenic site (aa 543–559), which overlaps this hydrophobic region. If this region were a transmembrane domain, more than half of the CYTGAT binding site would be masked by the MOM leading to the inability of the antibody to bind, which is not the case (Figs. 2 and 4). Additionally, if aa 551–571 were a transmembrane domain, the antigenic site recognized by IM2GAT would not be on the inner side of the MOM as demonstrated (Figs. 2–5), but instead would be present on the cytosolic surface of the MOM. The last hydrophobic region, aa 721–741 (Fig. 1A, box 6), was discounted as a possible transmembrane domain from the results of the protease digestion of the GFP fusion protein consisting of GFP fused to the C terminus of GAT. The GFP domain of this protein was resistant to protease digestion in intact mitochondria (Fig. 6, upper panel, lane 3) and becomes susceptible after solubilization of the mitochondria (Fig. 6, upper panel, lane 4), which implies that the C terminus of GAT is sequestered inside the mitochondria. If aa 721–741 did serve as a transmembrane region, then the GFP domain would be present on the outer surface of the mitochondria and would be susceptible to trypsin digestion.

TopPred2 hydrophobicity analysis of GAT from other species was also performed, and the predicted transmembrane domains were compared with those of rat mitochondrial GAT to determine if any topographical information of these other enzymes may be inferred (data not shown). Murine mitochondrial GAT is greater than 97% identical to rat GAT and possesses the identical transmembrane domains. GAT from the nematode worm, Caenorhabditis elegans, only exhibits one possible transmembrane region (aa 407–427), which contains 44.4% identical residues with the first transmembrane region of rat GAT (aa 473–493). Escherichia coli GAT is also predicted to have only one transmembrane domain (aa 610–630), which exhibits nearly 20% identical residues, and over 75% similar residues with the second transmembrane domain of rat GAT (aa 574–
Mycobacterium tuberculosis GAT is predicted to have two transmembrane domains (aa 294–314 and 480–500), although neither is significantly similar to those in rat GAT. Nevertheless, the presence of putative transmembrane regions in GAT of several different species indicates that membrane insertion may be a conserved characteristic among them and might even be necessary for proper function of the enzymes. In the case of rat mitochondrial GAT, for example, the transmembrane domains impart a specific topography to the enzyme, which may be crucial in the orientation of its catalytic site.

Previous studies using immobilized substrates have shown that the catalytic site of rat mitochondrial GAT is present on the cytosolic surface of the MOM (31). The identification of aa 494–573 as a cytosolic domain of GAT may implicate this region as the location of the catalytic site. Indirectly, there may be some evidence to support this hypothesis. In intact and solubilized mitochondria, immunoreaction with the CYTGAT antibody results in a drastic inhibition (50% and 75%, respectively) of GAT activity in comparison to the effects of IM1GAT and IM2GAT antibodies (Figs. 2 and 3). This may indicate that the cytosolic domain of GAT may also be essential in modulating enzyme activity. Indeed, the possibility that CYTGAT binding, in comparison to IM1GAT and IM2GAT binding, simply produces a greater conformational change in GAT resulting in the large inhibition of activity cannot be completely dismissed. Clearly, further studies are needed to directly establish that this cytosolic domain of GAT is truly the location of the catalytic site.

The cytosolic domain of GAT may also be essential in modulation of enzyme activity. Recently, AMP-activated protein kinase was shown to phosphorylate and inhibit mitochondrial GAT in vitro (32). Additionally, protein kinase C and tyrosine kinase have been shown to potently stimulate GAT activity in intact rat liver mitochondria. The size of AMP-activated protein kinase, protein kinase C, and tyrosine kinase prevents their passage across the MOM, which implies that these phosphorylation sites are on the cytosolic domain of GAT as well.

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