A Conserved Seven Amino Acid Stretch Important for Murine Mitochondrial Glycerol-3-phosphate Acyltransferase Activity

SIGNIFICANCE OF ARGININE 318 IN CATALYSIS*

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Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the initial and committed step in glycerolipid biosynthesis. We previously cloned the cDNA sequence to murine mitochondrial GPAT (Yet, S-F., Lee, S., Hahm, Y. T., and Sul, H.S. (1993) Biochemistry 32, 9486–9491). We expressed the protein in insect cells which was targeted to mitochondria, purified, and reconstituted mitochondrial GPAT activity using phospholipids (Yet, S-F., Moon, Y., and Sul, H. S. (1995) Biochemistry 34, 7303–7310). Deletion of the seven amino acids from mitochondrial GPAT, 312IFLEGTR318, which is highly conserved among acyltransferases in glycerolipid biosynthesis, drastically reduced mitochondrial GPAT activity. Treatment of mitochondrial GPAT with arginine-modifying agents, phenylglyoxal and cyclohexanedione, inactivated the enzyme. Two highly conserved arginine residues, Arg-318, in the seven amino stretch, and Arg-278, were identified. Substitution of Arg-318 with either alanine, histidine, or lysine reduced the mitochondrial GPAT activity by over 90%. On the other hand, although substitution of Arg-278 with alanine and histidine decreased mitochondrial GPAT activity by 90%, replacement with lysine reduced activity by only 25%. A substitution of the nonconserved Arg-279 with either alanine, histidine, or lysine did not alter mitochondrial GPAT activity. Moreover, R278K mitochondrial GPAT still showed sensitivity to arginine-modifying agents, as in the case of wild-type mitochondrial GPAT. These results suggest that Arg-318 may be critical for mitochondrial GPAT activity, whereas Arg-278 can be replaced by a basic amino acid. Examination of the other conserved residues in the seven amino acid stretch revealed that Phe-313 and Glu-315 are also important, but conservative substitutions can partially maintain activity; substitution with alanine reduced activity by 83 and 72%, respectively, whereas substituting Phe-313 with tyrosine and Glu-315 with glutamine had even less effect. In addition, there was no change in fatty acyl-CoA selectivity. Kinetic analysis of the R318K and R318A mitochondrial GPAT showed an 89 and 95%, respectively, decrease in catalytic efficiency but no major change in substrate binding as indicated by the $K_m$ values for palmitoyl-CoA and glycerol 3-phosphate. These studies indicate importance of the conserved seven amino acid stretch for mitochondrial GPAT activity and the significance of Arg-318 for catalysis.

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The first committed step in glycerophospholipid biosynthesis is the acylation of glycerol 3-phosphate in the sn-1 position with a fatty acyl-CoA to form 1-acylglycerol-3-phosphate (lysophosphaticid acid) catalyzed by glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15). Lysophosphaticid acid is further acylated in the sn-2 position by 1-acylglycerol-3-phosphate acyltransferase (AGPAT) to form 1,2-diacylglycerol-3-phosphate, which is used for synthesis of all phospholipids and triacylglycerol (reviewed in Refs. 1–3). GPAT is believed to be a rate-limiting step in phospholipid biosynthesis. There are two isoforms of mammalian GPAT; one resides in the endoplasmic reticulum (ER) membrane and the other in the outer mitochondrial membrane. The two isoforms can be distinguished by their differential sensitivity to the sulfhydryl group modifying reagent, N-ethylmaleimide (NEM): mitochondrial GPAT is resistant to NEM, whereas GPAT in the ER is inactivated by NEM (4, 5). In most tissues the mitochondrial enzyme comprises about 10% of GPAT activity, whereas in liver it comprises 50% of the total activity. Mitochondrial GPAT is responsive to nutritional and hormonal regulation, whereas the ER isoform is not; mitochondrial GPAT activity is very low in diabetic or fasted animals and is increased upon administration of insulin to diabetic animals or by refeeding fasted animals a high carbohydrate, fat-free diet (6, 7). The ER isoform uses saturated and unsaturated fatty acyl-CoAs equally well, whereas the mitochondrial isoform has a preference for saturated fatty acyl-CoAs as a substrate (8, 9). This preference is believed to be responsible for the observed predominance of saturated fatty acids in the sn-1 position, in contrast to unsaturated fatty acids in the sn-2 position, in naturally occurring phospholipids (10, 11).

Despite the importance of mammalian GPAT in phospholipid and triacylglycerol biosynthesis, little is known about the structural features that determine substrate binding or catalytic activity. Due to its membrane association, mitochondrial GPAT has been difficult to purify and reconstitute (12). It is unlikely that the x-ray crystallography data needed to identify the structural information required for substrate binding and catalysis will be obtained in the near future. Our isolation of a cDNA clone to murine mitochondrial GPAT, which shares 30% identity and 72% similarity over a 300 amino acid region with the previously cloned Escherichia coli GPAT (13), has enabled us to begin characterizing the structural features required for its function. The protein has been overexpressed in baculovirus-infected insect cells. The enzyme was shown to be targeted to mitochondria, and when purified and reconstituted with phospholipids this mitochondrial GPAT preferred saturated

‡ The abbreviations used are: GPAT, glycerol-3-phosphate acyltransferase; mGPAT, AGPAT, 1-acylglycerol-3-phosphate acyltransferase; NEM, N-ethylmaleimide; ER, endoplasmic reticulum; kb, kilobase pair; MOPS, 4-morpholinepropanesulfonic acid.
fatty acyl-CoAs as a substrate (14). We showed that murine mitochondrial GPAT containing a deletion of 78 of the most conserved amino acids, residues 250–327, had no catalytic activity (14). These studies suggest that the region comprising the deletion is probably required for GPAT activity.

Here we have used amino acid sequence comparisons among known GPATs and AGPATs to predict regions of mitochondrial GPAT that may be important for its activity. We report that a deletion of a seven amino acid stretch in the region most conserved among acyltransferases, \textit{IFLEGTR} (18), and cells were harvested 3 days after transfection.

**RESULTS**

**Construction of Plasmids and Mutant Mitochondrial GPATs—**

pcDNA-mitochondrial GPAT was constructed by ligating the 2.8-kb EcoRV-FspI fragment from p3513 (15) containing the GPAT coding sequence to EcoRV-digested pcDNA/amp (Invitrogen). pAlter-mitochondrial GPAT was constructed by ligating the 2.8-kb EcoRV-NcoI fragment from pcDNA-mitochondrial GPAT containing the mitochondrial GPAT coding sequence into SmaI-NcoI-digested pAlter MAX (Promega). Polymerase chain reaction-generated mutants (R318A, R318H, R278K, and F313A) were constructed by amplifying the mitochondrial GPAT coding sequence between nucleotides 1674 and 1911 using primer P1 (5'-CAACATCAAGGCGCCGTACA-3') and the specific primer from Table I, which contains a SmaI site. The polymerase chain reaction product was purified from an agatong-glass homogenizer at moderate speed and centrifuged at 8000 rpm for 15 min to separate the two enzymes, such as fatty acyl-CoA binding or the anti-rabbit secondary antibody as described previously (14). The protein concentration was determined by the Bradford method using bovine serum albumin as the standard (19).

**Identification of a Conserved Seven Amino Acid Stretch Important for Mitochondrial GPAT Activity—**

Murine mitochondrial GPAT shares 30% identity and overall 72% similarity with \textit{E. coli} GPAT in a 300 amino acid region. We previously showed that deletion of 78 of the most conserved amino acids in this 300 amino acid region, residues 250–327, abolished mitochondrial GPAT activity, suggesting that this region was important for GPAT catalysis or substrate binding (14). In Fig. 1, we compared known GPAT and various AGPAT sequences and found a 134 amino acid conserved region within the 300 amino acids that we previously found to be conserved between GPATs. The regions that are conserved between the GPATs and AGPATs are likely to be involved in functions that are shared between the two enzymes, such as fatty acyl-CoA binding or the esterification reaction. Within this region, amino acids 312-318
Amino acids that are identical between known GPATs and various AGPATs. The align-
ments were done using ClustalW version 1.4. Amino acids are identified by *.
The following sequences, with their GenBank accession numbers, were used: murine mitochondrial GPAT (mitochondrial GPAT, accession number M77003); E. coli GPAT (eGPAT, accession number P00482); human AGPAT (hAGPAT, accession number U56417); E. coli AGPAT (eAGPAT, accession number M63491); yeast AGPAT (yAGPAT, accession number L13282); L. douglasii AGPAT (iAGPAT, accession number X83269). The seven amino acids deleted are overlined. The mutated amino acids are identified by *.

The arginine-modifying agents butanedione and phenylglyoxal inactivated E. coli GPAT. CoA, however, protected mitochondrial GPAT from inactivation by arginine-modifying agents, indicating that arginine may be involved in fatty acyl-CoA binding. We tested whether the arginine-modifying agents, phenylglyoxyl and cyclohexanedione, had similar effects on mitochondrial GPAT. As shown in Fig. 3, both phenylglyoxyl and cyclohexanedione inactivated mitochondrial GPAT in a dose-dependent manner. This result indicates that, similar to E. coli GPAT, one or more arginine residues are crucial for mitochondrial GPAT activity.

**Identification of Arg-318 as the Arginine Residue Important for Mitochondrial GPAT Activity**—Within 312IFLEGTR318 is an arginine, Arg-318, that is conserved among all the sequences shown in Fig. 1. We postulated that arginine, with its guanidino group, is well suited to interact with the phosphate groups of glycerol 3-phosphate or of the CoA moiety of fatty acyl-CoA. In fact, Green and Bell (20) previously observed that the arginine-modifying agents butanedione and phenylglyoxal inhibited mitochondrial GPAT activity from murine liver. As shown in Fig. 3, both phenylglyoxyl and cyclohexanedione inactivated mitochondrial GPAT in a dose-dependent manner. This result indicates that, similar to E. coli GPAT, one or more arginine residues are crucial for mitochondrial GPAT activity.
important for murine mitochondrial GPAT function. In addition to Arg-318 in the seven amino acid stretch, Arg-278 is also conserved among the sequences shown in Fig. 1. These are the only two conserved arginines in mitochondrial GPAT. To test whether one or both of these arginines are important for mitochondrial GPAT activity, we used site-directed mutagenesis to substitute alanine for each arginine, individually (R278A and R318A). Since alanine has a small nonpolar side chain as compared with arginine with a large basic side chain, we hypothesized that this substitution was likely to affect mitochondrial GPAT activity if either of the arginines were important for GPAT activity. Cells transfected with R278A or R318A GPAT expressed similar amounts of mitochondrial GPAT mRNA as cells transfected with the wild-type GPAT, and mitochondria from cells expressing R278A or R318A GPAT had a similar level of mitochondrial GPAT protein as mitochondria from cells expressing wild-type GPAT (Fig. 4, panels A and B). Because R278A was constructed in the pAlter Max vector and R318A was constructed in pCDNA-1/amp vector, RNA, protein, and mitochondrial GPAT activity of R278A were compared with those of wild-type mitochondrial GPAT expressed from pAlter-mGPAT and RNA, protein, and mitochondrial GPAT activity of R318A were compared with those of wild-type mitochondrial GPAT expressed from pcDNA-mGPAT. The mitochondrial GPAT mRNA transcribed from pcDNA-mGPAT is larger than that transcribed from pAlter-mGPAT because the pCDNA-1/amp vector contains about 400 nucleotides longer untranscribed flanking sequence than the pAlter Max vector. Fig. 4, panel C, shows the mGPAT activity of mitochondria isolated from cells expressing wild-type, R278A, or R318A mGPAT. Mitochondria isolated from cells expressing R278A and R318A mGPAT had only 8 and 0.8%, respectively, of the GPAT activity observed from cells expressing wild-type mGPAT. We also substituted both of these arginines with either histidine or lysine (R278H, R278K, R318H, and R318K) to test whether a positive charge at Arg-278 and Arg-318 was sufficient for GPAT activity or if arginine itself was required. Arginine to histidine substitutions for both Arg-278 and Arg-318 reduced mitochondrial GPAT activity to approximately 10 and 3%, respectively, of the wild-type activity (Table II). Substitution of Arg-278 with lysine reduced mitochondrial GPAT activity by about 25%, whereas substitution of Arg-318 with lysine reduced the enzyme’s activity by almost 90%. These data indicate that Arg-318 is important for substrate binding or catalysis of mitochondrial GPAT, because even a conservative substitution of lysine for Arg-318 drastically reduced enzyme activity. On the other hand, a positive charge at position 278 was sufficient for activity since an arginine to lysine substitution did not substantially reduce mitochondrial GPAT activity. As shown in the sequence alignment in Fig. 1, only 2 of 10 arginines (Arg-278 and Arg-318) within the 134 amino acid region are conserved among the acyltransferases. We also mutated Arg-279, which is not conserved among the sequences shown in Fig. 1, to alanine, histidine, or lysine (R279A, R279H, and R279K). The results in Table II show that, in contrast to Arg-278 and Arg-318, mutation of Arg-279 did not reduce mitochondrial GPAT activity. R279A was sufficient for enzyme activity since an arginine to lysine substitution did not substantially reduce mitochondrial GPAT activity.
and cyclohexanedione.

Mitochondrial GPAT by phenylglyoxal with increasing concentrations of phenylglyoxal (□) or ethanol alone (○) as described in Fig. 3. Right panel, mitochondria were treated for 30 min with increasing concentrations of cyclohexanedione.

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Fig. 5. Inactivation of R278K mitochondrial GPAT by phenylglyoxal and cyclohexanedione. Left panel, 25 μg of mitochondria isolated from CMT cells expressing R278K mitochondrial GPAT were treated for 30 min at 25 °C with increasing concentrations of phenylglyoxal (□) or ethanol alone (○) as described in Fig. 3. Right panel, mitochondria were treated for 30 min with increasing concentrations of cyclohexanedione.

Fig. 6. Trypsin digestion of wild-type and R318K mitochondrial GPAT mutants. Mitochondria were isolated from CMT cells expressing either wild-type (lanes 1–4) or R318K (lanes 5–8) mitochondrial GPAT. Fifteen μg of mitochondrial protein were subjected to digestion at 25 °C with 10 μg/ml trypsin for the indicated times. Mitochondrial GPAT digestion products were analyzed by Western blot. Lanes 1 and 5, 0 min; lanes 2 and 6, 10 min; lanes 3 and 7, 20 min; lanes 4 and 8, 60 min.

Fig. 5 shows a comparison of the proteolytic digestion pattern of wild-type and R318K GPAT. The time course and appearance of digestion products from wild-type and R318K GPAT were similar, indicating that the accessibility of trypsin cleavage sites, and thus the overall protein structure, were probably not different in the wild-type and the mutated proteins. These data indicate that the inactivation of mitochondrial GPAT by the R318K mutation was most likely not caused by altered folding of the protein but was due to effects of the specific mutation on GPAT activity.


drial GPAT activity compared with wild-type GPAT. Based on these results, we conclude that Arg-318 is essential for mitochondrial GPAT activity, since amino acid substitutions of Arg-318, including a conservative arginine to lysine substitution, abolished enzyme activity, whereas a lysine substitution for Arg-278 did not significantly affect activity. The Arg-278 to alanine substitution did not affect enzyme activity, demonstrating that a nonconserved arginine residue is not critical for mitochondrial GPAT activity.

To identify further the arginine residue responsible for inactivation of mitochondrial GPAT by arginine-modifying agents, we treated mitochondria isolated from CMT cells expressing R278K GPAT with phenylglyoxal and cyclohexanedione. As shown in Fig. 5, both phenylglyoxal and cyclohexanedione inactivated R278K GPAT, as in the case of wild-type GPAT. These results suggest that modification of an arginine other than Arg-278, probably Arg-318, by phenylglyoxal and cyclohexanedione inactivates mitochondrial GPAT. Due to the difficulty of studying extremely low activity of Arg-318 GPAT, we did not determine the effects of phenylglyoxal and cyclohexanedione on mutants of Arg-318. Nevertheless, the results of our mutagenesis of conserved arginine residues indicate that Arg-318 is the most important for mitochondrial GPAT activity, and mutation of Arg-278 did not change the susceptibility to arginine-modifying agents. To determine whether the overall protein conformation of R318K GPAT was altered by the mutation, we analyzed the R318K mutant by protease digestion. Fig. 6 shows a comparison of the proteolytic digestion pattern of wild-type and R318K GPAT. The time course and appearance of digestion products from wild-type and R318K GPAT were similar, indicating that the accessibility of trypsin cleavage sites, and thus the overall protein structure, were probably not different in the wild-type and the mutated proteins. These data indicate that the inactivation of mitochondrial GPAT by the R318K mutation was most likely not caused by altered folding of the protein but was due to effects of the specific mutation on GPAT activity.

Significance of Other Conserved Residues in the Seven Amino Acid Stretch—We have determined by the deletion of residues 312IFLEGTR318 that these seven conserved amino acids are essential for mitochondrial GPAT activity. Further mutagenesis demonstrated that Arg-318 is critical to enzyme function. Next, we mutated other conserved amino acids adjacent to Arg-318 in the seven amino acid stretch to determine whether they also contributed to mitochondrial GPAT catalytic activity. The phenylalanine at position 313 was substituted with either alanine (F313A) or tyrosine (F313Y), and the glutamic acid at position 315 was substituted with either alanine (E315A) or glutamine (E315Q). Substitution of Phe-313 with alanine reduced mitochondrial GPAT activity by approximately 83%, whereas substitution with tyrosine, another aromatic amino acid, did not significantly reduce activity (Table I). Substitution of Glu-315 with alanine reduced mitochondrial GPAT activity by 72%, whereas the more conservative glutamine substitution decreased mitochondrial GPAT function by 53%. These results indicate that Phe-313 and Glu-315 are important for mitochondrial GPAT activity because substitutions of either with alanine significantly reduced mitochondrial GPAT activity. However, in the case of Phe-313, an aromatic residue is probably sufficient because tyrosine could substitute for phenylalanine with no significant loss of activity. In the case of Glu-315, the polar character and/or chain length of glutamine may also have enabled E315Q mitochondrial GPAT to function, albeit at a reduced efficiency.

Fig. 7 summarizes the effects of each substitution of the conserved amino acids in mitochondrial GPAT. As discussed above, substitution of Arg-278 or Arg-318 with alanine or histidine and of Arg-318 with lysine had the most dramatic effects on mitochondrial GPAT function, reducing activity by at least 90%. Although Arg-278 may play a role, Arg-318 is the most important arginine residue for mitochondrial GPAT activity. A substitution of Phe-313 with alanine and Glu-315 with glutamine also significantly reduced enzyme activity. These results indicate that Phe-313 and Glu-315, which are adjacent to Arg-318, are also important, but substitutions with similar amino acids can restore enzyme activity.

Comparison of Kinetic Characteristics of R318K and R318A with Wild-type Mitochondrial GPAT—Presently we do not know whether Arg-318 plays a critical role in substrate binding or catalysis. Bell and co-workers (20) showed that CoA and palmitoyl-CoA protected E. coli GPAT from inactivation by arginine-modifying agents and speculated that arginines are at or near the active site of the enzyme. In contrast, we observed that CoA did not protect murine mitochondrial GPAT from inactivation by arginine-modifying agents (data not shown). These results suggest that Arg-318 might not be involved in fatty acyl-CoA binding. Therefore, we attempted to examine the potential role of this conserved region in substrate binding by comparing kinetic characteristics. Due to the very low enzymatic activity of the Arg-318 mutants, we further purified...
the mitochondria by Percoll gradient, eliminating microsomal GPAT contamination. We employed varying concentrations of palmitoyl-CoA and 1 mM glycerol 3-phosphate. As we previously reported, the inhibitory effect of fatty acyl-CoA at higher concentrations makes it difficult to determine the apparent affinity for fatty acyl-CoAs. In calculating the kinetic data, only the palmitoyl-CoA concentrations that do not inhibit GPAT activity were used. The V\textsubscript{max} for R318K and R318A were 3.6 and 1.8% (0.8 and 0.4 nmol/min/mg, respectively) that of wild-type mitochondrial GPAT (22.2 nmol/min/mg) (Table III). The K\textsubscript{m} values for palmitoyl-CoA of R318K and R318A (4.6 and 3.2 µM, respectively) were not increased relative to the K\textsubscript{m} value of wild-type mitochondrial GPAT (11.3 µM). The catalytic efficiency, as estimated by V\textsubscript{max}/K\textsubscript{m}, of R318K and R318A were decreased by 89 and 95%, respectively, relative to the wild type. These results suggest that the R318K and R318A mutations did not significantly affect the affinity of the enzyme for palmitoyl-CoA, but catalytic efficiency was reduced. In addition, the K\textsubscript{m} value for glycerol 3-phosphate of R318A was not significantly changed from the wild-type enzyme (data not shown).

We previously reported that murine mitochondrial GPAT was most active with saturated fatty acyl-CoAs of chain length 8–16. Mitochondrial GPAT was less than 30% as active when unsaturated acyl-CoAs were used as compared with palmitoyl-CoA (14). We employed E315Q GPAT, which has approximately 50% of the wild-type mitochondrial GPAT activity, to test whether the preference for saturated fatty acyl-CoA of mitochondrial GPAT was maintained. Fig. 8 shows the activity of wild-type (left panel) and E315Q mitochondrial GPAT (right panel), respectively, at increasing concentrations of the indicated fatty acyl-CoAs. Similar to the wild-type enzyme, E315Q mitochondrial GPAT was active with lauroyl-CoA (C12:0) and palmitoyl-CoA (C16:0), was 28% as active with oleoyl-CoA (C18:1), and 43% as active with linoleoyl-CoA (C18:2) as with palmitoyl-CoA when the maximal activity with each fatty acyl-CoA was compared. Overall, E315Q mitochondrial GPAT was about 50% as active as wild-type mitochondrial GPAT with each fatty acyl-CoA. We could not directly compare the apparent K\textsubscript{m} values of the mutant and wild-type enzymes for the acyl-CoAs because various acyl-CoAs were inhibitory at differing concentrations (14). However, we detected no significant differences in the ability of E315Q mitochondrial GPAT to use the various acyl-CoAs as a substrate. As with the Arg-318 mutations, there was no major change in substrate binding, as indicated by the K\textsubscript{m} value for glycerol 3-phosphate of E315Q and wild-type GPAT, 0.48 and 0.27 mM, respectively. We previously reported the K\textsubscript{m} value for mitochondrial GPAT overexpressed in Sf9 insect cells to be 0.67 mM, which is an order of magnitude higher than that of the E. coli enzyme. Regardless, the E315Q mutation does not significantly affect the preference for saturated fatty acyl-CoA as a substrate.

**DISCUSSION**

As the first committed, and possibly a rate-limiting, step in phospholipid and triacylglycerol biosynthesis, GPAT is a pivotal enzyme in lipid metabolism. Mitochondrial GPAT exhibits a preference for saturated fatty acyl-CoAs (8, 9) and is regulated by nutritional and hormonal manipulations (6, 7). Therefore, mitochondrial GPAT may be critical for establishing the observed predominance of saturated over unsaturated fatty acids in the sn-1 position of cellular phospholipids and for responding to physiological states that require alterations in the metabolism of glycerophospholipids. Despite the importance of mitochondrial GPAT, very little is known about the primary structural features that mediate substrate binding and catalysis. Understanding the mechanism of mitochondrial GPAT function has been hindered by the difficulty of purifying this membrane-bound enzyme. Our cloning of the mitochondrial GPAT cDNA and determination of the amino acid sequence enabled us to compare the sequences of known acyltransferases involved in phospholipid biosynthesis to predict regions of mitochondrial GPAT that would likely be important for function. We identified a 134-amino acid region that is conserved among known acyltransferases. We showed here that a seven amino acid deletion within this conserved region, IFLEGTR, drastically reduced mitochondrial GPAT cat-
alytic activity. We used site-directed mutagenesis to substitute highly conserved amino acids within this region. Our data revealed that Arg-318 is important for mitochondrial GPAT activity since even a conservative substitution from arginine to lysine inactivated the enzyme. On the other hand, a positively charged amino acid can replace the other conserved arginine at position 278 (Table II). Other conserved amino acid residues that are close to Arg-318 also contribute to mitochondrial GPAT function because mutations of those amino acids also resulted in substantial loss of catalytic activity, but conservative replacement could maintain partial activity.

In the absence of x-ray crystallographic or other structure data for the glycerolipid acyltransferases, we can only speculate on the role of the essential Arg-318. A possible role for arginines in mitochondrial GPAT is to mediate binding of the substrates glycerol 3-phosphate or palmitoyl-CoA by interacting with the phosphate groups, as has been proposed for other enzymes (21). Green and Bell (20) demonstrated that arginine-modifying agents inactivated E. coli GPAT and CoA or palmitoyl-CoA partly protected GPAT from inactivation, and they proposed that important arginine residues were at or near the active site. Similar to the result with E. coli GPAT, phenylglyoxal and cyclohexanone also inactivated murine mitochondrial GPAT. However, in contrast to the case with E. coli GPAT, CoA did not protect mitochondrial GPAT from inactivation. Arg-278, Arg-318, or both, which are conserved in acyltransferases involved in glycerolipid biosynthesis, could be the targets for inactivation by arginine-modifying agents. Since R278K mitochondrial GPAT was also inactivated by phenylglyoxal and cyclohexanone (Fig. 5), it is likely that modification of Arg-318 is responsible for mitochondrial GPAT inactivation. The fact that CoA did not protect mitochondrial GPAT from inactivation might indicate that Arg-318 is not involved in CoA binding. Our observation that a substitution of Arg-318 with either lysine or alanine did not alter affinity for palmitoyl-CoA suggests its potential role in catalysis. In this regard, we did not observe a significant change in $K_m$ for glycerol 3-phosphate either.

Heath and Rock (22) recently reported that the conserved histidine 306 and aspartic acid 311 of E. coli GPAT, which correspond to His-230 and Asp-235 within the 134-amino acid conserved region of murine mitochondrial GPAT, are important for the catalytic activity of E. coli GPAT because a substitution of alanine for His-306 or glutamic acid for Asp-311 significantly reduces the GPAT activity. These amino acids are part of a HX$_2$D consensus sequence that is found in glycerolipid acyltransferases from a variety of organisms. Because the $K_m$ value of D311E for glycerol 3-phosphate is not significantly different from that of wild-type E. coli GPAT, they hypothesized that the HX$_2$D consensus sequence was not involved in substrate binding and that, by analogy to chloramphenicol acetyltransferase, the histidine might function as a general base to deprotonate the hydroxyl moiety of the acyl acceptor.

Since this sequence is also conserved in murine mitochondrial GPAT, these residues may play a similar role in the mammalian mitochondrial enzyme. Regardless, we have demonstrated that amino acids $^{312}$FLEG$^{318}$, Arg-318 in particular, play a significant role in catalysis of mitochondrial GPAT. Further studies are necessary to identify the residues involved in catalysis and to define the regions responsible for determining fatty acyl-CoA substrate specificity.

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