Molecular Dissection of the S-Adenosylmethionine-binding Site of Phosphatidylethanolamine N-Methyltransferase*

Received for publication, June 16, 2003, and in revised form, July 2, 2003
Published, JBC Papers in Press, July 3, 2003, DOI 10.1074/jbc.M306308200

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Phosphatidylethanolamine N-methyltransferase (PEMT) is a quaternotopic membrane protein that catalyzes the conversion of phosphatidylethanolamine to phosphatidylcholine through three sequential methylation reactions. Analysis of mice lacking a functional PEMT gene revealed a severe reduction in plasma homocysteine levels. Homocysteine is generated by the hydrolysis of S-adenosylhomocysteine, which is also a product of the PEMT reaction. To gain insight into the PEMT transmethylation reaction and the mechanism by which PEMT regulates homocysteine levels, we sought to define residues that are required for binding of the methyl group donor, S-adenosylmethionine (AdoMet). Bioinformatic analysis of the predicted amino acid sequence of human PEMT identified two putative AdoMet-binding motifs (KCGXG100 and G180EE181). Site-directed mutagenesis experiments demonstrated the requirement for the conserved motifs in PEMT specific activity. Analysis of the AdoMet binding ability of mutant recombinant PEMT derivatives established that residues Gly100 and Glu180 are essential for binding of the AdoMet moiety. A significantly elevated $K_d$ with respect to AdoMet is observed following conservative mutagenesis of residues Gly69 (400 pmol) and Glu181 (666.7 pmol), relative to the unmodified enzyme (303.1 pmol), suggesting that these residues also participate in AdoMet binding. A model positions two separate AdoMet-binding motifs of PEMT in close proximity at the external leaflet of the endoplasmic reticulum membrane.

PEMT catalyzes the sequential transfer of three methyl groups from AdoMet to phosphatidylethanolamine to generate the essential phospholipid, phosphatidylcholine (PC). Concomitant production of one S-adenosylhomocysteine (AdoHcy) molecule occurs with each methylation step. The liver is the primary site of PEMT activity, and the PEMT-controlled pathway accounts for ~30% of hepatic phosphatidylethanolamine biosynthesis (1–4). The enzymes of the CDP-choline pathway, which are active in all nucleated cells, catalyze the remaining 70% of PC biosynthesis in the liver (2–5). In addition to being a key modulator of PC biosynthesis, the liver is the site of ~85% of all methylation reactions (6). AdoMet, the primary methyl group donor, is utilized by at least 29 mammalian methyltransferases, including DNA, RNA, protein, lipid, and small molecule methyltransferases (7, 8). Each AdoMet-dependent transmethylation reaction generates AdoHcy, which in turn is hydrolyzed to yield adenosine and the non-protein amino acid, homocysteine (Hcy) (9). Because mild hyperhomocysteinemia is an independent risk factor for cardiovascular and atherosclerotic disease, circulating plasma homocysteine levels are of significant clinical interest (10). Previous studies on PEMT focused on the PC biosynthetic function of the enzyme and, in particular, whether PEMT-derived PC was targeted to a specific hepatic fate such as very low density lipoprotein particles or bile (11–14). Recently, however, phenotypic analysis of mice homozygous for a disrupted PEMT allele revealed a novel role for PEMT in the regulation of plasma Hcy levels (15). Although the liver is the site of numerous AdoMet-dependent methylation reactions, each of which contributes to the Hcy pool, genetic ablation of the PEMT gene alone resulted in a 50% decrease in circulating Hcy levels (15). Furthermore, hepatoma cells transfected with a cDNA encoding PEMT secreted more Hcy than mock transfected cells (15). Combined, these results suggest a key yet previously unknown role for PEMT in the regulation of hepatic one-carbon metabolism.

To gain further insight into the PEMT transmethylation reaction and the mechanism by which this enzyme modulates plasma Hcy levels, we sought to identify residues that are required to bind AdoMet/AdoHcy. A plethora of enzymes bind AdoMet and/or AdoHcy, including the AdoMet-dependent methyltransferases, AdoMet synthetase, AdoMet decarboxylase, and AdoHcy hydrolase (16). Comparative amino acid sequence analysis previously identified several conserved motifs that bind the AdoMet/AdoHcy moieties, but a small number of methyltransferases including the eukaryotic PEMT family of enzymes do not contain any of these motifs (16–18).

Using bioinformatic analysis, two putative AdoMet/AdoHcy-binding motifs were identified that are conserved among the eukaryotic PEMT proteins. Here, we describe the biochemical evaluation of the motifs in the human PEMT enzyme. Understanding the nature of the interaction between AdoMet/AdoHcy and PEMT will promote resolution of the mechanism by which the enzyme modulates plasma Hcy levels and facili-

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§ Supported by a Studentship from the Alberta Heritage Foundation for Medical Research.

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EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, restriction endonucleases, and Platinum Pfx DNA polymerase were from Invitrogen. Oligonucleotides for mutagenesis were synthesized in the DNA core facility at the Department of Biochemistry of the University of Alberta. FuGENE transfection reagent was from Roche Applied Science. S-Adenosyl-L-[methyl-3H]methionine (15 Ci/ mmol) was obtained from Amersham Biosciences. S-Adenosyl-L-methionine was from Sigma. HAWP 02500 filters and Multiscreen 96-well filtration/assay plates (MHSBN4540) for AdoMet binding assays were from Millipore. Goat anti-rabbit secondary antibodies were purchased from Pierce. All other reagents were of the highest standard commercially available.

Bioinformatic Analysis—The PROWL ProteinInfo sequence analysis program (prowl.rockefeller.edu) was used to screen for putative AdoMet-binding motifs in the predicted human PEMT primary structure (accession number, NP_009100). The ALIGN program (based on the ClustalW algorithm) at the San Diego Supercomputer Biology Workbench (workbench.sdsu.edu) was utilized to analyze the conservation of motifs between orthologous eukaryotic PEMT enzymes (19).

Recombinant Plasmid Construction—Mutant PEMT derivatives for analysis of putative AdoMet-binding residues/motifs were generated by the “splice by overlap extension” PCR mutagenesis method, using the wild type hPEMT-pCI plasmid as a template for all reactions (20, 21). This plasmid consists of the human PEMT open reading frame cloned to 3’ into the XhoI and XbaI sites, respectively, of the pCI mammalian expression vector polylinker (Promega) (21). Transcription is under the control of a cytomegalovirus promoter. Full-length mutant PCR products were blunt end-ligated to Smal-cut pBluescript II (KS) (Stratagene) and recloned into the pCI expression vector using XhoI and XbaI restriction sites. All of the constructs were sequenced to confirm fidelity of amplification and orientation of the insert at the Molecular Biology Services Unit of the University of Alberta. Plasmids encoding conservative GXG mutant motifs were generated as follows. To mutagenize the glycine residue at the first position in the GXG tripeptide motif to an alanine residue and generate the plasmid, hP-G98A, PCR A was performed with oligonucleotide 1 (5’TCTAGAGATGACCGGCTGCT- GGGCTAC-3’), and oligonucleotide 2 (5’/H11032CAGAGGGCGGCGCAGC- GCTG-3’), PCR B was performed with the mutant oligonucleotide 3 (5’AGGCTGAGCGCTCGCTGACCCGCTGCTG-3’). To mutagenize both glycine residues in the GXG tripeptide motif to alanine residues and generate the plasmid, hP-G100A, oligonucleotide 3 was 5’AGGCTGGCGCTCGCTGACCCGACGGTGCTG-3’. To mutagenize both glycine residues in the GXG tripeptide motif to alanine residues and generate the plasmid, hP-GAGA, 5’AGGCTGGCGCTCGCTGACCCGCTGCTG-3’. To mutagenize both glycine residues in the GXG tripeptide motif to alanine residues and generate the plasmid, hP-G100A, oligonucleotide 3 was 5’AGGCTGGCGCTCGCTGACCCGACGGTGCTG-3’. To mutagenize both glycine residues in the GXG tripeptide motif to alanine residues and generate the plasmid, hP-GAGA, 5’AGGCTGGCGCTCGCTGACCCGCTGCTG-3’. To mutagenize both glycine residues in the GXG tripeptide motif to alanine residues and generate the plasmid, hP-G100A, oligonucleotide 3 was 5’AGGCTGGCGCTCGCTGACCCGACGGTGCTG-3’.

FIG. 1. Interrelationships between the PEMT-catalyzed biosynthesis of PC and Hcy metabolism. PEMT catalyzes the synthesis of PC via three AdoMet-dependent transmethylation reactions. AdoHcy is produced with each methylation step (1). AdoHcy hydrolyase catalyzes the hydrolysis of AdoHcy to yield Hcy and adenosine (Ado) (9). Hcy may be directed to the irreversible trans-sulfuration pathway to generate Cys, reincorporated to the Met cycle for regeneration of AdoMet or exported from the cell (36).
Fig. 2. A putative AdoMet binding motif (GXG) is conserved in PEMT orthologs. A, alignment of conserved AdoMet/nucleotide-binding motifs. Rows (i), three conserved AdoMet-binding motifs found in the majority of non-nucleic acid AdoMet-dependent methyltransferases. Row (ii), motif I of the conserved DNA methyltransferase AdoMet-binding site. Row (iii), motif I of the AdoMet synthetase AdoMet-binding site. Row (iv), ATP/nucleotide-binding motif of protein kinases and other nucleotide-binding proteins, such as the NAD-binding proteins. The shaded box indicates conservation of the GXG motif in disparate motifs. B, linear schematic map of the human PEMT enzyme indicating the position of the conserved GXG motif. C, amino acid sequence alignment of eukaryotic PEMT orthologs. The shaded box indicates conservation of the GXG motif in PEMT enzymes from different species. GenBank™ accession numbers for each protein are included.

RESULTS

Identification of a Putative AdoMet-binding Motif—PEMT catalyzes a series of AdoMet-dependent methylation reactions to produce PC and the byproduct, AdoHcy (Fig. 1). Three AdoMet-binding motifs (I, II, and III) are conserved in the majority of non-DNA AdoMet-dependent methyltransferases (Fig. 2A, row (ii)), but a small number of methyltransferases including the enzymes of the eukaryotic PEMT family do not contain the three motifs (16). However, using bioinformatic analysis, we identified a partial motif I consensus sequence that is conserved among the eukaryotic PEMT orthologs (Fig. 2, B and C) (25). Furthermore, this partial motif (GXG, where X is any amino acid) is similar to a DNA methyltransferase AdoMet-binding motif, one of the AdoMet-binding motifs of AdoMet synthetase, as well as a nucleotide-binding motif found in protein kinases and other nucleotide-binding proteins such as the NAD-binding proteins (Fig. 2A, rows (ii), (iii), and (iv)) (17, 26–28).

Conservative Mutagenesis of the GXG Motif—To probe the role of the conserved glycine residues in PEMT-mediated binding of the AdoMet moiety, each glycine residue of the GXG motif was individually mutated to an alanine residue (encoded by the mutant PEMT plasmids, hP-G98A and hP-G100A). To evaluate the effect of conservative mutagenesis of each glycine residue on PEMT activity, Cos-7 cells were transfected with plasmids encoding wild type PEMT or the mutant PEMT derivatives or mock transfected with empty vector, and cell homogenate protein was assayed for PEMT activity. Mutagenesis of Gly98 to an alanine residue decreased PEMT activity by ~25%, whereas similar mutagenesis of Gly100 completely abolished enzymatic activity in the recombinant PEMT derivatives (Fig. 3A). Immunoblots with an anti-PEMT peptide antibody demonstrated similar levels of abundance of the recombinant proteins (Fig. 3B). Enzymatic assays of homogenates performed in the presence of various concentrations of substrate revealed that conservative mutagenesis of Gly98 results in a slightly elevated Kₘ with respect to the methyl donor, AdoMet, but not with respect to the methyl acceptor, PMME (Fig. 3, C and D). Thus, mutagenesis of Gly98 would appear to have perturbed a facet of the transmethylation reaction pertaining to the AdoMet moiety. The hP-G100A mutant was inactive at all concentrations of PMME and AdoMet evaluated (data not shown).

Conservative Mutagenesis of 100G Abolishes AdoMet Binding Activity—To determine whether the changes in PEMT activity were specifically due to decreased AdoMet binding activity, we transfected Cos-7 cells with the wild type or mutant PEMT plasmids or mock transfected with empty vector. The microsomes were prepared for AdoMet binding assays. The microsomal fraction was utilized for AdoMet binding assays because we have previously demonstrated that PEMT activity is enriched in the microsomal subcompartment (22).

Microsomes from cells expressing the mutant hp-G98A protein displayed a reduction of ~20% in AdoMet binding activity compared with the unmodified enzyme (Fig. 4A). In contrast, conservative mutagenesis of Gly100 completely abolished the AdoMet binding activity of the mutant enzyme. To determine
whether the altered enzymatic activity of the mutant recombinant PEMT proteins was a consequence of direct changes in AdoMet binding activity, binding assays were performed with various concentrations of AdoMet. Mutagenesis of Gly98 to an alanine results in a PEMT derivative with an elevated $K_D$ with respect to AdoMet, relative to values for the unmodified enzyme (Fig. 4B). Thus, a specific role for the GXG motif in binding of the AdoMet moiety is supported. Increasing concentrations of AdoMet did not result in detectable AdoMet binding activity for the Gly100 mutant (data not shown), suggesting that the integrity of this residue is essential to the AdoMet binding function of PEMT.

**Nonconservative and Combinatorial Mutations of the GXG Motif**—To further investigate the role of the glycine residues in

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**Fig. 3.** *Conservative mutagenesis of the GXG motif alters PEMT specific activity.* Cos-7 cells were transiently transfected with plasmids (1 μg) containing wild type PEMT or epitope-tagged PEMT derivatives or mock transfected with empty pCI vector. A, cell homogenates (50 μg of protein) were assayed for PEMT activity. The results are expressed as the means ± S.E. of three separate experiments, each performed in duplicate, relative to the values obtained for similar assays on cells transfected with wild type PEMT. The asterisk signifies $p < 0.05$. B, immunoblot with anti-PEMT antibody using 25 μg of transfactant homogenate protein. C, cell homogenates (50 μg of protein) were assayed for PEMT activity in the presence of a constant AdoMet concentration (200 μM) and various PMME concentrations (100–1000 μM). Three separate experiments were conducted with each assay performed in duplicate. The results were subjected to nonlinear regression using the Curvefit software (Graphpad.com). The $V_{max}$ and $K_m$ values were calculated using the Michaelis-Menten equation. The units for kinetic parameters are as follows: $V_{max}$, pmol/min/mg protein; $K_m$, μM. D, cell homogenates (50 μg of protein) were assayed for PEMT activity in the presence of a constant PMME concentration (500 μM) and various AdoMet concentrations (100–1000 μM). The results were calculated as described for C.
the putative AdoMet-binding motif, we performed nonconservative mutagenesis of each glycine residue to glutamate or aspartate residues to yield the mutant plasmids hP-G98E and hP-G100D, respectively. Additionally, combinatorial mutant plasmids were generated in which both glycine residues were mutated to alanine residues (hP-GAGA) or to glutamate and aspartate residues (hP-GEGD). To assess the PEMT activity of the nonconservative and combinatorial mutants, Cos-7 cells were transiently transfected with wild type or mutant plasmids or mock transfected with empty vector. The cellular homogenates were assayed for PEMT activity.

The cells expressing the nonconservative mutant PEMT derivatives (hP-G98E and hP-G100D) were completely inactive as compared with mock transfected cells (Fig. 5A). In the case of hP-G98E, this was in contrast to the conservative G98A mutation, which only caused a decrease of 20% in PEMT activity (Fig. 3A). Cells expressing the combinatorial mutant PEMT derivatives were similarly devoid of PEMT activity (Fig. 5A). Immunoblots with an anti-PEMT peptide antibody confirmed the expression of each PEMT derivative (Fig. 5B). Assays performed in the presence of various concentrations of PMME or AdoMet did not demonstrate enzymatic activity in any of the nonconservative or combinatorial GXG mutants (Fig. 5, C and D).

**Nonconservative Mutagenesis of Gly98 Reduces AdoMet Binding Activity**—In the next series of experiments, we resolved the issue of whether the reduction in PEMT activity of the nonconservative hP-G98E mutant was due to a specific decrease in the AdoMet binding activity of the recombinant mutant protein. Cos-7 cells were transfected, and microsomal AdoMet binding assays were performed as described.

Although the cells expressing the mutant hP-G98E protein did not display any significant PEMT activity (Fig. 5A), the mutant protein still retained ~55% of AdoMet binding activity as compared with the unmodified enzyme (Fig. 6A). Binding assays performed in the presence of various concentrations of AdoMet revealed that the mutant hP-G98E protein displays an increased $K_D$ with respect to AdoMet, relative to values for the unmodified enzyme (Fig. 6B). Combined, these results suggest that although the mutant protein is partially capable of binding AdoMet, the bound AdoMet moiety is not available for PEMT-catalyzed transmethylation (i.e. the mutant PEMT enzyme is unable to transfer the methyl group from the bound AdoMet moiety). This may arise because AdoMet is bound in such a conformation that the methyl group is not accessible to the transmethylation machinery. Furthermore, because mutagenesis of Gly98 to the small amino acid, alanine, had only minimal effects on both PEMT activity and binding activity (Figs. 3A and 4A), but mutation to the larger and charged glutamate residue caused a significant decrease in binding activity (Fig. 6, A and B), Gly98 may serve to structurally position Gly100 or other residues for optimal binding activity rather than actually binding to AdoMet itself.

As expected, the hP-G100D mutant protein and the combinatorial mutant PEMT derivative, hP-GEGD did not bind AdoMet, whereas the mutant PEMT protein, hP-GAGA, retained only fractional activity (Fig. 6A). Assays performed in the presence of various concentrations of AdoMet did not enhance binding (data not shown).

**Identification of a Second Putative AdoMet-binding Motif**—A number of AdoMet-dependent methyltransferases do not contain the classical AdoMet-binding motifs (16, 17, 26). Analysis
of enzymes that lack the three classical AdoMet-binding motifs (including the yeast PEM2-encoded PEMT ortholog) identified a novel tripartite motif (Fig. 7A) (18), referred to in abbreviated form as the “RHPXY-hyd-EE” motif. This motif consists of two regions of homology (Region A and Region B) separated by a hydrophobic region of ∼30 residues (18). To determine whether the tripartite motif is conserved among the higher eukaryotic PEMT enzymes, we performed feature analysis of the aligned amino acid sequences. Although the greater part of Regions A and B do not exist in human PEMT, two specific portions of the motifs, which flank the hydrophobic region, are conserved, i.e. PXY-hydrophobic region-EE, where X represents any amino acid (Fig. 7).

Recently, we demonstrated that PEMT is a quatrotopic ER membrane protein with a topographical orientation that localizes both termini external to the ER subcompartment (22).
Such a topographical orientation would localize the PXY motif to the ER lumen and the di-glutamate motif to the external surface of the ER membrane. Because AdoMet is most concentrated in the cytosolic compartment, we hypothesize that the di-glutamate motif is more likely to function in an AdoMet binding capacity in human PEMT (29).

Mutagenesis of the Di-glutamate Motif Decreases PEMT Activity—To examine the role of the conserved di-glutamate motif in binding to AdoMet, we mutated each glutamate residue to an aspartate residue to generate the mutant PEMT plasmids hP-E180D and hP-E181D. To evaluate the conservative glutamate mutants, we assayed PEMT activity in homogenates from cells that express the recombinant PEMT derivatives. Mutagenesis of Glu180 to an aspartate residue completely abolished PEMT activity, whereas similar mutagenesis of Glu181 resulted in a 70% decrease in PEMT activity (Fig. 8A). Immunoblots with an anti-PEMT peptide antibody demonstrated similar levels of abundance of the recombinant proteins (Fig. 8B). Provision of various concentrations of PMME or AdoMet did not suffice to restore the enzymatic activity to wild type levels (Fig. 8, C and D). However, assays performed in the presence of various concentrations of AdoMet revealed an elevated $K_m$ with respect to AdoMet in the case of the hP-E181D mutant (Fig. 8D). Thus, a role for the di-glutamate motif in an AdoMet-specific aspect of the transmethylation reaction is suggested.

Mutagenesis of the Di-glutamate Motif Diminishes AdoMet Binding Activity—To elucidate whether the changes in PEMT activity could be attributed to a reduction in AdoMet binding activity, we performed microsomal AdoMet binding assays on transfected cells. Mutation of Glu180 to an aspartate residue abolished the AdoMet binding capacity of the recombinant mutant enzyme (Fig. 9A). Similar mutagenesis of Glu181 yielded a protein that exhibited a 55% decrement in AdoMet binding activity (Fig. 9A). Binding assays of the hP-E181D PEMT derivative did not detect binding activity at any AdoMet concentration evaluated (data not shown). Thus, the integrity of residue Glu180 is essential to the AdoMet binding activity of PEMT. Mutagenesis of Glu181 results in a PEMT derivative with significantly reduced AdoMet binding ability ($K_D$, 666.7 pmol) relative to the wild type enzyme ($K_D$, 303.1 pmol) (Fig. 9B). Because the changes in binding activity and enzymatic activity of the hP-E181D mutant are proportionally similar (Figs. 8A and 9A), and the replacement of Glu181 with an aspartate residue yields a protein with severely reduced AdoMet binding ability, it appears that the altered PEMT activity of the di-glutamate mutants was a consequence of specific changes in the AdoMet binding activity. Thus, a functional role for the di-glutamate motif in the AdoMet binding activity of the human PEMT enzyme is supported.

DISCUSSION

AdoMet is the most commonly used substrate after ATP (7). The AdoMet moiety is required for the synthesis of several essential biomolecules including PC, creatine, and epinephrine, as well as for the methylation of DNA and RNA (30). A measure of the importance of AdoMet is perhaps best illus-
trated in that the only known genome that lacks the gene encoding AdoMet synthetase is that of the obligate intracellular parasite *Chlamydia trachomatis* (31). Of clinical relevance, the AdoMet molecule is intrinsically linked with the non-protein amino acid, Hcy, elevated plasma concentrations of which are considered an independent risk factor for cardiovascular disease (10).

To gain insight into the PEMT transmethylation reaction and, thereby, the mechanism by which genetic ablation of PEMT can cause decreased circulating plasma homocysteine levels, we examined the role of specific PEMT amino acid residues in the binding of AdoMet/AdoHcy. We have now identified two distinct motifs that are required for PEMT to bind the methyl donor.

Three AdoMet-binding motifs are conserved in the majority of the AdoMet-dependent methyltransferases, but these motifs are absent from several enzymes including those of the eukaryotic PEMT family and the isoprenylcysteine carboxyl methyltransferase (ICMT) family of enzymes (16, 18). The ICMT enzymes constitute part of a post-translational modification process, in which proteins that terminate in a CAA motif, such as Ras, undergo isoprenylation, C-terminal proteolytic cleavage, and carboxyl methylation (10). Although absent from all eukaryotic PEMT enzymes, the three classical AdoMet-binding motifs are present in the prokaryotic *Rhodobacter sphaeroides* PEMT ortholog, encoded by the *pmtA* gene (16, 32). However, this enzyme is a soluble cytosolic protein that has little homology with the higher eukaryotic, membrane-bound PEMT orthologs (32, 33).

A partial consensus AdoMet-binding motif (98GXG100) is conserved in the eukaryotic PEMT enzymes, and site-directed mutagenesis of each glycine residue demonstrated their importance in the AdoMet binding activity of PEMT. Whereas an intact Gly100 residue is essential for binding of the AdoMet moiety, Gly98 may serve in a structural capacity, because conservative mutagenesis of the residue was quite well tolerated. Moreover, significant binding activity was retained even when Gly98 was substituted with a glutamate residue. However, because the $K_D$ with respect to AdoMet was elevated in the case of each Gly98 mutant, a role for this residue in binding of AdoMet is suggested. Our data do not allow us to exclude the possibility that the glutamate residue is also mediating the binding of AdoMet, especially because we have demonstrated the role of other PEMT glutamate residues in AdoMet binding. However, because an intact Gly100 residue is essential for AdoMet binding function, and the replacement of Gly98 with an alanine residue has a lesser impact on AdoMet binding, insertion of a glutamate residue combined with an intact Gly100 might be expected to result in enhanced AdoMet binding. Because this was not the case, we favor a structural role for Gly98 in the AdoMet binding activity of the human PEMT enzyme.

To further define the AdoMet binding site of human PEMT, we examined a novel, recently proposed, AdoMet-binding motif (RHPX-EE) (18). This nonclassical motif was derived following alignment-based analyses of the ICMT family of enzymes, homologs thereof, the yeast PEM2 enzyme, and several sterol reductases (18). By *in silico* analysis, we determined that the consensus motif is also partially conserved in the rat, mouse, and human PEMT orthologs.

Topographical analysis of Ste14p, a member of the ICMT family of methyltransferases, indicated that the enzyme is oriented in the ER membrane such that the two flanking regions of the motif are colocalized on the external face of the membrane (18). Such an orientation might be possible because...
the intervening hydrophobic domain is predicted to "double back" within the membrane plane through the use of a helical hairpin (18). The C-terminal transmembrane domain of the yeast PEM2 enzyme is proposed to adopt a similar structural conformation (18).

In contrast, the corresponding transmembrane domain in the human PEMT enzyme is shorter than the minimum length (31 amino acids) necessary for helical hairpin formation, and a topographical model based on four transmembrane domains has been elucidated (22). Such a topographical structure would position the two flanking segments of the motif on opposite sides of the ER membrane. Hence, in this model, the N-terminal portion of the motif (PXY) is lumenerally oriented and therefore diametrically opposed to the AdoMet-rich cytosol (22, 29).

FIG. 8. Site-directed mutagenesis of the di-glutamate motif decreases the enzymatic activity of PEMT. Cos-7 cells were transiently transfected with plasmids (3 μg) containing wild type PEMT or epitope-tagged PEMT derivatives or mock transfected with empty pCI vector. A, cell homogenates (50 μg of protein) were assayed for PEMT activity. The results are expressed as the means ± S.E. of three separate experiments, each performed in duplicate, relative to the values obtained for similar assays on cells transfected with wild type PEMT. B, immunoblot with anti-PEMT antibody using 25 μg of transfectant homogenate protein. C, cell homogenates (50 μg of protein) were assayed for PEMT activity in the presence of a constant AdoMet concentration (200 μM) and various PMME concentrations (100–1000 μM). The results are expressed as the means of three separate experiments, each performed in duplicate. The data points for the E180D derivative are clustered along the abscissa because this mutant did not display activity at any concentration of PMME evaluated. D, cell homogenates (50 μg of protein) were assayed for PEMT activity in the presence of a constant PMME concentration (500 μM) and various AdoMet concentrations (100–1000 μM). Three separate experiments were conducted with each assay performed in duplicate. The results were subjected to nonlinear regression using the Curvefit software (Graphpad.com). The $V_{max}$ and $K_m$ values were calculated using the Michaelis-Menten equation. Units for kinetic parameters are as follows: $V_{max}$, pmol/min/mg protein; $K_m$, μM.
Although we have not eliminated the possibility that the lumenally oriented PXXY motif might still have a role in the AdoMet binding activity of PEMT, our working hypothesis is that the cytosolic-localized di-glutamate motif is the functional AdoMet-binding portion of the RHPXY-hyd-EE motif in the human PEMT enzyme.

Mutagenesis of the di-glutamate motif coupled with AdoMet binding assays verified the importance of the glutamate pair, with Glu180 in particular being essential to the AdoMet binding activity of PEMT. However, Glu181 also has a role in AdoMet binding, because mutagenesis of the residue results in a significantly elevated $K_D$ with respect to AdoMet. Nucleotide-binding proteins such as p21, contain an invariant acidic residue and a GXGXXG motif that are separated by 20 amino acids (34). Hydrogen bonding between the acidic residue of such proteins and the 2'-hydroxy group of the adenosine ribose has been demonstrated (34). Hydrogen bonding between the acidic residue of glutamate residues and the 2'-hydroxy group of the adenosine ribose has been demonstrated (34). Thus, either or both glutamate residues in the conserved di-glutamate motif of PEMT may bind the AdoMet moiety through hydrogen bonding. Mutagenesis of each glutamate residue to an aspartate residue reduced AdoMet binding activity. Although aspartate residues may also form hydrogen bonds, the shorter length of the aspartate side chain may impair the AdoMet binding capacity of the residues in this region of the human PEMT enzyme.

From the topographical orientation of the PEMT enzyme and the predicted length of the transmembrane domains, we envision a model that would position the conserved GXG and di-glutamate motifs toward the cytosolic face of the third and fourth transmembrane helices, respectively (Fig. 10). This would provide optimal access to the AdoMet pool in the cytosol (29). Moreover, because the third and fourth transmembrane helices are adjacent domains, the conserved GXG and di-glutamate motifs may be juxtaposed (Fig. 10). Intriguingly, S-adenosylhomocysteine hydrolase, which hydrolyzes AdoHcy to Hcy and adenosine, is also localized in the cytosol (35). Thus, the combined topographical organization of these disparate elements may provide the basis of a mechanism that links the AdoMet-dependent PEMT reaction to circulating Hcy levels.
In summary, we identified two closely oriented amino acids motifs in the human PEMT enzyme that are essential for binding of AdoMet. The resolved AdoMet-binding site of PEMT is unique in that it conforms to neither the classical nor non-classical binding motifs but rather is a novel combination of both classes. Because PEMT has been shown to modulate circulating plasma Hcy levels, the enzyme represents a novel target for therapeutic intervention in patients with hyperhomocysteinemia. Resolution of key motifs that bind AdoMet will greatly accelerate the design of such therapeutic agents.

Acknowledgments—We thank Susanne Lingrell for excellent technical assistance. We are grateful to Dr. Richard Lehner for invaluable discussions.

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