Phosphoethanolamine methyltransferases in phosphocholine biosynthesis: functions and potential for antiparasite therapy

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Abstract

S-adenosyl-l-methionine (SAM)-dependent methyltransferases represent a diverse group of enzymes that catalyze the transfer of a methyl group from a methyl donor SAM to nitrogen, oxygen, sulfur or carbon atoms of a large number of biologically active large and small molecules. These modifications play a major role in the regulation of various biological functions such as gene expression, signaling, nuclear division and metabolism. The three-step SAM-dependent methylation of phosphoethanolamine to form phosphocholine catalyzed by phosphoethanolamine N-methyltransferases (PMTs) has emerged as an important biochemical step in the synthesis of the major phospholipid, phosphatidylcholine, in some eukaryotes. PMTs have been identified in nematodes, plants, African clawed frogs, zebrafish, the Florida lancelet, Proteobacteria and human malaria parasites. Data accumulated thus far suggest an important role for these enzymes in growth and development. This review summarizes published studies on the biochemical and genetic characterization of these enzymes, and discusses their evolution and their suitability as targets for the development of therapies against parasitic infections, as well as in bioengineering for the development of nutritional and stress-resistant plants.

SAM-dependent MTases play essential roles in a plethora of important biological functions such as cellular metabolism, nuclear division, transcription, signal transduction and detoxification, to name only a few. Defects in some of these enzymes have also been linked to various human diseases such as Alzheimer’s disease, attention deficit disorder and pre-eclampsia (Panula et al., 1998; Kanasaki et al., 2008). The DNA C5-cytosine MTase M, HhaI, was the first SAM-dependent MTase to be characterized structurally (Cheng et al., 1993), and today, more than 100 structures of SAM-dependent MTases have been determined (Schubert et al., 2003). Although these enzymes share little sequence identity (< 5%), they incorporate a highly conserved structural fold (Fauman et al., 1999; Martin & McMillan, 2002; Schubert et al., 2003) comprised of a seven-stranded β-sheet, with strand seven antiparallel to the other six strands and inserted into the sheet between strands five and six. This seven-stranded β-sheet is sandwiched between two layers of α-helices to form an α/β/α structure. The SAM-binding site in SAM-dependent MTases is located in the N-terminal part of the β-sheet, whereas the substrate-binding site is located in the C-terminal part of the β-sheet. The structure and...
topology of the substrate-binding site vary considerably. In many cases, additional structural domains are required to confer substrate specificity and mediate methyl group transfer from SAM to the substrate. This review focuses on one class of SAM-dependent MTases, the phosphoethanolamine N-methyltransferase (PMT) family, catalyzing the three-step methylation of phosphoethanolamine (P-Etn) to form phosphocholine (P-Cho), which serves as a substrate for the synthesis of the major eukaryotic membrane phospholipid, phosphatidylcholine (PtdCho).

PMT enzymes

Since the pioneering work of Eugene Kennedy (Kennedy & Lehninger, 1949; Kennedy & Weiss, 1956) and others in mammalian tissues and the yeast species Saccharomyces cerevisiae and Schizosaccharomyces pombe, it became widely accepted that in most nucleated cells, PtdCho is made either from choline via the CDP-choline pathway (the nucleotide pathway also known as the Kennedy pathway) or from phosphatidylethanolamine (PtdEtn) (the phospholipid methylation pathway) via three successive methylation reactions catalyzed by one or two phospholipid methyltransferases (PLMTs). The CDP-choline pathway, which is highly conserved among eukaryotes, begins with the phosphorylation of choline by a choline kinase to form P-Cho. Phosphocholine is next converted to CDP-choline by a CTP:phosphocholine cytidylyltransferase (CCT) and subsequently into PtdCho by a CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT). The phospholipid methylation pathway catalyzed by PLMT enzymes is conserved among most, but not all eukaryotes, and is absent in

Fig. 1. PtdCho biosynthesis pathways in Plasmodium falciparum (red arrows), plants (green arrows) and Caenorhabditis elegans (black arrows). Ser, serine; SD, serine decarboxylase; Etn, ethanolamine; EK, ethanolamine kinase; P-Etn, phosphoethanolamine; CDP-Etn, CDP-ethanolamine; PtdEtn, phosphatidylethanolamine; PMT, phosphoethanolamine N-methyltransferase; P-MME, phosphomonomethylethanolamine; P-DME, phosphodimethylethanolamine; GlyBet, glycine betaine; Cho, choline; CK, choline kinase; P-cho, phosphocholine; CCT, CTP:phosphocholine cytidylyltransferase; CDP-Cho, CDP-choline; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; PtdCho, phosphatidylcholine; PLD, phospholipase D; CDP-MME, CDP-monomethylethanolamine; CDP-DME, CDP-dimethylethanolamine; PtdMME, phophatidylmonomethylethanolamine; PtdDME, phosphatidyl(dimethylethanolamine, PLMT, phospholipid methyltransferase.
Plasmodium species and Caenorhabditis elegans (Fig. 1). In mammals, PLMT activity is catalyzed by an 18-kDa integral membrane protein expressed primarily in the liver (Vance & Ridgway, 1988). Mice lacking PLMT activity require dietary choline for survival, manifest abnormal choline metabolism in the liver and develop hepatic steatosis (Walkey et al., 1998). Yeast cells, on the other hand, have two PLMT enzymes, Pem1p (Cho2p) and Pem2p (Opi3p), that mediate the first and the last two transmethylation reactions on PtdEtn to form phosphatidylmonomethylethanolamine (PtdMME), phosphatidyldimethylethanolamine (PtdDME) and PtdCho, respectively (Kodaki & Yamashita, 1987; Summers et al., 1988; McGraw & Henry, 1989). Mutants lacking both genes require choline for survival. Recent studies in plants identified PLMT enzymes in Arabidopsis thaliana and soybean, homologous to the rat PLMT and the yeast Pem2p (Keogh et al., 2009). These enzymes use PtdMME and PtdDME, but not PtdEtn as substrates for the synthesis of PtdCho (Keogh et al., 2009). Thus far, no PLMT enzymes catalyzing PtdEtn methylation have been identified in plants, suggesting that a methylation pathway for the synthesis of PtdCho from PtdEtn is absent in these organisms. Keogh et al. (2009) suggested that an alternative pathway, which uses phosphomonomethylethanolamine (P-MME) and phosphodimethylethanolamine (P-DME) as substrates for the synthesis of PtdMME and PtdDME, could serve as a route for the synthesis of PtdCho.

Evidence for the presence of an alternative pathway for the synthesis of PtdCho and choline in plants originated from a series of elegant labeling studies aimed at elucidating the mechanism of synthesis and accumulation of betaine. Hitz et al. (1981) identified a novel methylation reaction in barley leaves, whereby P-Etn is methylated to form P-Cho, which then serves as a precursor for the synthesis of PtdCho. Subsequent studies provided evidence for the presence of such an enzymatic reaction in other plants including sugar-beet, Lemna and carrots (Hanson & Rhodes, 1983; Mudd & Datko, 1986; Datko & Mudd, 1988a, b), and the methylation of P-Etn to P-MME in soybean (Datko & Mudd, 1988a, b). Weretilnyk et al. (1995) used protein extraction and subcellular fractionation analyses in spinach to show the presence of at least two distinct N-methyltransferases catalyzing the N-methylation of P-Etn to P-Cho. Smith et al. (2000) later reported the purification of PMT from spinach leaves and showed that the preparation catalyzed the methylation of P-Etn, P-MME and P-DME. Using an S. pombe cho2 mutant lacking the Pem1p ortholog, the first PMT cDNA from spinach was cloned (Bolognese & McGraw, 2000; Nuccio et al., 2000). Similarly, using the S. cerevisiae opi3 mutant, which lacks Pem1p, the PMT cDNA was cloned from an Arabidopsis cDNA expression library (Bolognese & McGraw, 2000). The cloning of these PMT genes set the stage for a detailed characterization of their encoded enzymes. Since then, PMT genes have been identified and characterized in other plants such as wheat (Charron et al., 2002), oilseed rape (Ye et al., 2005) and corn (Wu et al., 2007). In a series of labeling studies in Plasmodium knowlesi using serine and ethanolamine (Etn) as precursors, Elabbbadi et al. (1997) showed that an alternative pathway for PtdCho biosynthesis from serine and Etn exists in malaria parasites. While this work identified serine decarboxylation as a novel step in phospholipid metabolism in these parasites, it suggested that Etn formed via this reaction is incorporated into PtdEtn, which is subsequently methylated into PtdCho by putative malarial PLMT enzymes. Metabolic analyses in the human malaria parasite Plasmodium falciparum by Pessi et al. (2004) showed that serine decarboxylation also occurs in this pathogen, resulting in the formation of Etn and subsequently P-Etn. This study further demonstrated that in this organism, P-Etn can serve as a precursor for the synthesis of P-Cho via a three-step methylation catalyzed by a SAM-dependent phosphoethanolamine methyltransferase, PIPMT. However, no evidence for PtdEtn transmethylation could be detected in this parasite. Thus, at least in the case of P. falciparum, an alternative pathway similar to that identified in plants is used by the parasite for the synthesis of PtdCho. This pathway, which was named the serine decarboxylase phosphoethanolamine methyltransferase (SDPM) pathway, uses serine either transported from human serum or resulting from the degradation of host hemoglobin as a starting precursor (Fig. 1) (Pessi et al., 2004, 2005; Ben Mamoun et al., 2010). Serine is first decarboxylated by a serine decarboxylase to form Etn. The gene encoding this activity has not yet been identified in Plasmodium species and no homologs of plant serine decarboxylases could be found in the malaria genome databases. Etn is next phosphorylated by an ethanolamine kinase to form P-Etn. Then PIPMT catalyzes a three-step methylation of P-Etn to form P-Cho (Pessi et al., 2004, 2005; Reynolds et al., 2008; Bobenchik et al., 2010), which is converted into PtdCho by the last two enzymes of the CDP–choline pathway: PfCCT and PfCEPT.

**Genome distribution**

As a result of the ongoing efforts to sequence the genomes of several eukaryotes and prokaryotes and the availability of sequence databases that can be searched for the presence of putative PMT genes, PMT orthologs are now found in Proteobacteria (Burkholderia pseudomallei and Burkholderia oklahomensis), many species of plants, two species of African clawed frogs (Xenopus laevis and Xenopus tropicalis), nematodes (C. elegans and Caenorhabditis briggsae), zebrafish (Danio rerio), the Florida lancelet (Branchiostoma floridae), but not in humans. In protozoa, PMTs are found in Phytophthora infestans and some Plasmodium species. No protein sequences annotated as putative PMT were found.
in silico in other protozoa including other Apicomplexa or organisms belonging to the Kinetoplastida taxa. Within the Plasmodium genus, PMTs are identified in species that infect humans and primates (Plasmodium falciparum, Plasmodium vivax and Plasmodium knowlesi). Partial genome sequences available through the Sanger Institute sequencing projects suggest the presence of PMT genes in the primate malaria parasite Plasmodium reichenowi and the bird parasite Plasmodium gallinaceum (Dechamps et al., 2010). The malarial PMT proteins share high degrees of identity and similarity (over 62%) (Table 1). Rodent malaria parasites (Plasmodium berghei, Plasmodium yoelii, Plasmodium chabaudi), however, lack PMT genes (Dechamps et al., 2010), suggesting that major differences in the metabolism of PtdCho exist between rodent and human malaria parasites. It is noteworthy that genetic studies aimed at disrupting the function of components of the CDP-choline pathway (CEPT, CCT, ECT and CK) in P. berghei failed to generate knockout strains (Dechamps et al., 2010). Although these studies need to be further validated by complementation analyses, it is presently postulated that in rodent malaria parasites, the CDP-choline pathway could be the primary route for the synthesis of PtdCho.

### Table 1. Protein sequence conservation between Plasmodium falciparum PFMT and putative PMTs from Plasmodium vivax, Plasmodium knowlesi, Plasmodium gallinaceum and Plasmodium reichenowi.

<table>
<thead>
<tr>
<th>Species</th>
<th>% of identity</th>
<th>% of similarity</th>
<th>% gaps</th>
<th>E-value</th>
</tr>
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<td>98.1</td>
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<td>1e-148</td>
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<td>77.5</td>
<td>1.5</td>
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<tr>
<td>PvPMT</td>
<td>63.7</td>
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<td>1.5</td>
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<tr>
<td>PkPMT</td>
<td>70.8</td>
<td>83.1</td>
<td>1.9</td>
<td>2e-105</td>
</tr>
</tbody>
</table>

Fig. 2. Schematic representation of the structure of the four classes of PMT enzymes. The four motifs (I, p-I, II and III) of each PMT catalytic domain are indicated as solid boxes. * Denotes conserved residues amongst PMT sequences.

### Classification and evolution of PMT enzymes

Based on their primary structure and distribution of their predicted catalytic domains, the PMT enzymes can be divided into four classes (Fig. 2). Class I includes the...
malarial (263–266 amino acid in length) and proteobacterial (264 amino acids) enzymes, which contain a single SAM-dependent catalytic domain. Class II is comprised of bipartite enzymes containing between 450 and 580 amino acids and harboring two SAM-dependent catalytic domains. This class includes PMTs from plants, frogs, zebrafish, Florida lancelet and *P. infestans*. Classes III and IV include enzymes from *C. elegans* and *C. briggsae* that are twice the size of the malarial enzymes, but contain a single SAM-dependent catalytic domain located either at the N-terminal (class III) or at the C-terminal (class IV) end (Fig. 2). The unitary SAM-dependent catalytic domain in these PMTs contains four highly conserved signature motifs defining the SAM fold (Kagan & Clarke, 1994; Pessi et al., 2004). Thus far, more than 30 PMTs have been identified in 26 organisms. Phylogenetic analyses indicate that PMTs belonging to Apicomplexa, Nematoda or Proteobacteria phyla cluster together with the *Burkholderia* enzymes diverging early during evolution, whereas other PMTs including those from plants are more distant (Fig. 3). Within plants, a clear separation between monocotyledonous and dicotyledonous PMTs is observed as monocotyledonous evolved recently from dicotyledonous in the reign of plants. The early divergence of the *Burkholderia* PMTs and their monopartite structure, a property shared with PMT enzymes from *Plasmodium*, suggests that the malarial enzymes might have evolved from an ancestral bacterial PMT enzyme. PMT enzymes from nematodes and plants might have evolved from duplication of this ancestral enzyme, followed by either specialization in nematodes or fusion in plants. Further biochemical and genetic investigations are needed to determine whether the *Burkholderia* PpPMT orthologous genes encode bona fide PMT enzymes and assess their substrate specificities and physiological functions.

### Biochemistry of PMT enzymes

Thus far, only PMT enzymes from plants *C. elegans* and *P. falciparum* have been characterized biochemically. Biochemical analyses revealed that the 266 amino acid PfPMT catalyzes the conversion of P-Etn into P-Cho using SAM as a methyl donor, and neither Etn nor PtdEtn acts as a substrate for this enzyme, implying that P-Etn is its primary methyl acceptor. The specificity of PfPMT for P-Etn was further demonstrated by functional analysis using yeast as a surrogate system (Pessi et al., 2004). Wild-type yeast cells inherently lack PMT activity. The expression of the *Pfpmt* gene in yeast conferred PMT activity and consequently allowed *pem1*Δ*pem2*Δ yeast mutant cells (which lack the ability to convert PtdEtn to PtdCho, and hence are auxotropic for choline) to grow in the absence of choline (Pessi et al., 2005; Reynolds et al., 2008). Analysis of the phospholipid content revealed that, unlike wild-type yeast cells, PpPMT-expressing *pem1*Δ*pem2*Δ yeast cells failed to
synthesize the intermediates of the methylation of PtdEtn, PtdMME and PtdDME (Pessi et al., 2005). The growth of these complemented cells was ameliorated by the addition of choline, and required an active CDP–choline pathway, suggesting that the in vivo activity of PIPMT is directly coupled to the CDP–choline pathway (Pessi et al., 2005).

Structure–function analysis of 24 conserved residues in or near the predicted catalytic motifs of PfPMT identified three residues Asp-61, Gly-83 and Asp-128 that are critical for activity in vivo (Pessi et al., 2005; Reynolds et al., 2008).

PMT activity in C. elegans is conferred by two enzymes PMT-1 and PMT-2 with low sequence identity (12.4%) to each other (Palavalli et al., 2006; Brendza et al., 2007). PMT-1 is a 475 amino acid polypeptide and uses P-Etn as a substrate to form P-MME (Brendza et al., 2007). P-MME or the dimethylated form of P-DME are not substrates of this enzyme. PMT-2 is a 437 amino acid polypeptide and catalyzes the last two methylation reactions in P-Chol synthesis, but not the initial methylation of P-Etn (Palavalli et al., 2006). The presence of two different enzymes in C. elegans, one catalyzing the initial methylation reaction and the second catalyzing the subsequent two methylation steps, may have occurred through gene duplication and divergence (Palavalli et al., 2006). No structure–function studies have been performed on these enzymes and the basis for the underlying difference between the two enzymes in substrate specificity remains to be determined. Sequence analysis revealed that of the three residues playing an important role in PIPMT activity, Asp-61 is conserved in both PMT-1 and PMT-2, whereas Gly-83 and Asp-128 are only conserved in PMT-2. Analysis of the kinetic mechanism of PMT-1 and PMT-2 using varying concentrations of SAM and either P-Etn or P-DME revealed a random-sequential for a two-substrate to a two-product (Bi Bi) reaction by these enzymes (Palavalli et al., 2006; Brendza et al., 2007). For PMT-1, the binding of P-Etn or SAM was found to have only a modest effect on the binding of the other substrate (Brendza et al., 2007). For PMT-2, the binding of either SAM or P-DME was found to result in a threefold increase in the binding of the other substrate (Palavalli et al., 2006).

In plants, PMT enzymes have been actively studied in spinach, wheat, maize and Arabidopsis due to their critical roles in membrane biogenesis, development and stress adaptation (Summers & Weretilnyk, 1993; Charron et al., 2002; Mou et al., 2002; Cruz-Ramirez et al., 2004; Tabuchi et al., 2005; Jost et al., 2009). Studies in spinach, wheat and maize demonstrated that the N-terminal domain catalyzes the first methylation step, whereas the C-terminal catalyzes the last two methylation steps to form P-Chol (Nuccio et al., 2000; Charron et al., 2002). The mechanism of this substrate specificity has not yet been unraveled. Recent studies comparing the two wheat isoforms TaPEAMT1 and TaPEAMT2 showed that TaPEAMT2 was four times more active and displayed a higher affinity for SAM and P-Etn than TaPEAMT1 (Jost et al., 2009). For both enzymes, the favored kinetic model is that of a sequential random Bi Bi mechanism (Jost et al., 2009). Kinetic analyses, however, showed that TaPEAMT1 exhibited strong cooperativity between the two SAM-binding sites, whereas TaPEAMT2 exhibited negative cooperativity between these two sites (Jost et al., 2009). Plant PMTs have also been shown to be sensitive to inhibition by P-Chol, S-adenosylhomocysteine (SAH) and other cellular metabolites and ions. Thus, it was found that native SoPEAMT was inhibited by P-Chol, SAH, calcium and magnesium (Smith et al., 2000). ZmPEAMT-like SoPEAMT was also inhibited by P-Chol, as well as SAH, whereas choline and glycine betaine (GlyBet) had no effect on enzyme inhibition. TaPEAMT1 and TaPEAMT2 were also not affected by choline or GlyBet and only mildly inhibited by potassium and calcium, but strongly inhibited by phosphatidic acid and manganese ions (Jost et al., 2009). Interestingly, both wheat enzymes were shown to bind phosphatidic acid, phosphatidylinositol (PtdIns)(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P2, PtdIns(4,5)P2 and cardiolipin (Jost et al., 2009). However, PtdCho, PtdIns(3,5)P2 and PtdIns(3,4,5)P3 did not bind to these enzymes (Jost et al., 2009).

Structural analysis of PMT enzymes

Unlike other MTases, the structures of enzymes of the PMT family remain unknown. Unraveling these structures will undoubtedly help provide a better understanding of the mechanism of catalysis and substrate specificity of these enzymes and the biochemical relevance of their monopartite and bipartite structures. The sequence alignment of PIPMT with all small-molecule SAM-dependent MTases with known function and structures revealed that these enzymes share a very low overall sequence identity (~7–16%), but high sequence conservation among residues associated with SAM binding. Among them, PIPMT is most similar to human histamine methyltransferase (HNMT) (Horton et al., 2001) as the two proteins have an overall 16% sequence identity and ~31% sequence similarity around the substrate-binding site (six out of 11 residues in the HNMT substrate-binding site are either identical or conservatively substituted in PIPMT; Table 2). Moreover, both enzymes function as monomers and contain an additional domain outside the core SAM MTase fold that plays a role in substrate or inhibitor recognition. The structure of the histamine–HNMT complex confirms that HNMT is a two-domain protein and the histamine molecule is buried at the interface of the two domains (Horton et al., 2001). Residues that are involved in histamine binding in HNMT and the corresponding 11 residues in PMTs are listed in Table 2; the plant PMTs were divided into N- and C-terminal domains (AtPEAMT-Nt and AtPEAMT-Ct) and each domain was
aligned with PfPMT separately. At first glance, there appears to be relatively low conservation of these residues across the class of PMTs (with the sole exception of the tyrosine residues corresponding to C196 of HNMT), which could be an artifact of improper alignment due to low sequence homology. However, closer examination reveals a high degree of conservation among enzymes acting on the same (known or putative) substrates. For example, the residues corresponding to Q143 of HNMT are aspartate for those enzymes acting on P-MME and P-DME, but tryptophan for those acting on P-Etn alone. This is consistent with published studies that showed loss of PMT function and activity in PfPMT mutants PfpmtD128A, PfpmtD128N and PfpmtD128E where D128 was mutated to alanine, asparagine or glutamate (Reynolds et al., 2008). These 15 residues are thus potentially involved in substrate binding and/or specificity. Efforts to resolve the structure of PMT enzymes by X-ray crystallography and nuclear magnetic resonance (NMR) are underway in several laboratories including ours. Such efforts are likely to lead to a new era in our understanding of this important class of enzymes.

### Inhibitors of PMT activity

Biochemical studies have indicated that most PMT enzymes studied thus far are inhibited by their reaction products P-Cho and SAH. Accordingly, two P-Cho analogs, hexadecylphosphocholine and hexadecyltrimethylammonium bromide, were found to inhibit the malarial PfPMT (Pessi et al., 2004) (Table 3). More recently, an enzyme-coupled spectrophotometric assay that couples the SAM-dependent transmethylation of P-Etn by PfPMT to the activity of two enzymes SAH nucleosidase and adenine deaminase has been developed (Bobenchik et al., 2010). By screening a small

<table>
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<th>Name</th>
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<th>IC50 (Parasite)</th>
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*Hexadecylphosphocholine.
†Hexadecyltrimethylammonium bromide.
‡Amodiaquine.
number of known and predicted MTase inhibitors, it was found that the antimalarial drug and HNMT inhibitor amodiaquine inhibits PIPMT activity in vitro (Bobenchik et al., 2010). Other aminoquinolines such as chloroquine, quinine and quinidine had no effect on the enzyme (Bobenchik et al., 2010) (Table 3). Using NMR analysis, it was found that amodiaquine binding results in a shift in the HSQC profile of a select number of PIPMT residues. This shift was not affected by the amodiaquine analog, chloroquine (Bobenchik et al., 2010). Alone, the inhibition of PIPMT by amodiaquine does not account for the antimalarial activity of the compound, because pfpmtΔ parasites lacking PIPMT are as sensitive to the drug as wild-type parasites. However, the inhibition of PIPMT by amodiaquine and the evidence that this drug also inhibits other eukaryotic MTases suggest that it may exert its antimalarial activity by targeting several parasite MTases. Despite the finding that amodiaquine, hexadecylphosphocholine and hexadecyltrimethylammonium bromide inhibit PIPMT activity, highly specific inhibitors of these enzymes have yet to be identified.

**Genetic analysis of PMTs**

Cell biological studies have shown that the malarial PIPMT is localized to the Golgi apparatus and its expression is highly regulated at the transcriptional and translational levels by choline (Witola et al., 2006; Witola & Ben Mamoun, 2007). The importance of PIPMT in *P. falciparum* in PtdCho biosynthesis and parasite intraerythrocytic development and survival was demonstrated by creating transgenic parasites lacking the *Pfpmt* gene. Studies aimed at characterizing the synthesis of PtdCho from choline and Etn in *pfpmtΔ* knockout parasites showed that whereas the synthesis of this phospholipid from choline via the CDP–choline pathway was intact in these parasites, the SDPM pathway from Etn was completely abolished. While these findings highlight the importance of PIPMT in PtdCho biosynthesis from Etn during *P. falciparum* in vitro development, they also suggest that this parasite lacks PtdEtn methyltransferase activity. This is consistent with the genomic data that showed the lack of *PLMT* genes in the genome sequences of different *Plasmodium* species. Interestingly, although a *pfpmtΔ* knockout could be obtained, the resulting parasites showed an altered cycle of nuclear divisions and produced mature schizonts with fewer daughter merozoites compared with wild-type parasites (Witola et al., 2008). These findings suggest that PIPMT serves an important physiological function during *P. falciparum* intraerythrocytic development. These phenotypes were observed even in the presence of choline at physiological concentrations, suggesting that both pathways are not completely redundant. With only two PtdCho biosynthesis pathways known thus far in *P. falciparum*, it was predicted that *pfpmtΔ* parasites would die in the absence of choline. Analysis of these parasites, however, showed that under these conditions, the parasites are still viable. One possible explanation is that knockout parasites survive on choline available within human red blood cells. Alternatively, the parasites might have a third mechanism for the synthesis of PtdCho that remains to be identified. Labeling of knockout parasites with 14C-ethanolamine failed to identify PtdCho derived from Etn, suggesting that if a third metabolic route exists in *P. falciparum*, it does not involve the classical methylation of PtdEtn.

The physiological functions of *C. elegans* *pmt-1* and *pmt-2* have been investigated using RNAi knockdown strategies. *pmt-1* or *pmt-2* knockdown at a first-stage larva L1 or a daf-7 dauer larva stages resulted in complete or highly penetrant parental worm sterility. At the L4 larva stage, *pmt-1* or *pmt-2* knockdown resulted in developmental arrest and death in the progeny at L1/L2 (for both knockdowns) or L3 (for *PMT-2* knockdown) larva stages (Palavalli et al., 2006; Brendza et al., 2007). The development impairment caused by the loss of *pmt-1* expression was rescued by monomethyllethanolamine, dimethylethanolamine or choline supplementation; however, that of *pmt-2* was only rescued by the addition of choline (Palavalli et al., 2006; Brendza et al., 2007). These defects are similar to those reported by Lochnit et al. (2005) following RNAi silencing of other enzymes involved in the biosynthesis of PtdCho. Together, these findings indicate an important role of PtdCho in *C. elegans* development, and imply that PMT enzymes are excellent targets for the development of compounds that could potentially inhibit the development and fertility of pathogenic nematodes.

The importance of *pmt* genes in plant development, reproduction and response to stresses was demonstrated by large-scale reverse genetics in *A. thaliana*. This species of plants contains three genes encoding PMT isoforms. Using the sense/antisense RNA expression system, Mou et al. (2002) identified a mutant line, t356, in which the *AtPEAMT1* gene was silenced. *AtPEAMT1* downregulation resulted in a drastic decrease in the choline content (~36% of the wild type) and critical developmental and survival defects. The growth of the mutants plants under continuous light at 22 °C resulted in rosette leaves that were pale green in the juvenile stages and senesced early in the late reproductive stage. These mutants produced shorter siliques and fewer seeds, and their flowers produced less pollen than the isogenic parental wild-type plants due to decreased male fertility. The male sterility phenotype was temperature sensitive, with a severe decrease in fertility observed at temperatures > 23 °C (Mou et al., 2002). The growth of the mutant was also strongly inhibited in the presence of high salt concentrations, and this salt sensitivity phenotype was reversed when plants were supplied with exogenous choline. Using the
T-DNA random insertional mutation system, Cruz-Ramirez et al. (2004) showed that disruption of the xiptol1 locus, which encodes AtPEAMT1, resulted in altered root architecture during early and late developmental stages. Analysis of the kinetics of root elongation, and root and cellular morphology in this mutant revealed a drastic decrease in the rate of primary root elongation and number of root hairs as well as major abnormalities in epidermal cell development and size compared with the wild type (Cruz-Ramirez et al., 2004). Analysis of cell membrane integrity and cell viability in this mutant revealed that loss of AtPEAMT1 results in cell death of the epidermal and cortical cells of the elongation and differentiation zones of the root (Cruz-Ramirez et al., 2004). The addition of choline or P-Chol restored the defects in root system development, epidermal cell morphology and viability. The addition of phosphatidic acid resulted in full complementation of the cell death and epidermal cell morphology phenotypes. On the other hand, phosphatidic acid supplementation had no effect on primary root growth or root hair formation (Cruz-Ramirez et al., 2004). Although both the studies by Mou and colleagues and Cruz-Ramirez and colleagues target the same PMT gene, some of the phenotypes reported are different. These differences could be due to the distinct genetic approaches used in each study (i.e. gene silencing vs. insertional mutagenesis). Nonetheless, the genetic studies in plants indicate that PMT enzymes play an important role in development, sexual reproduction and stress response, and their functions are not redundant.

Suitability of PMT enzymes as targets for bioengineering and drug development

Available data indicate that PMT enzymes play an important role in growth and development in plants, nematodes and human malaria parasites, in the production of the essential human dietary nutrient, choline, by plant cells, and in the ability of plants to resist harsh conditions such as high salinity and drought. In the past, efforts to engineer GlyBet synthesis in organisms such as tobacco, A. thaliana and other plants that lack this activity for enhanced resistance to osmotic stresses relied on the use of plant and bacterial genes encoding enzymes that oxidize choline to GlyBet (McNeil et al., 2000). Although an increase in stress tolerance was detected in these organisms, the change was only marginal and the levels of GlyBet attained were low, mostly due to low choline synthesis and the inability to transport choline into the chloroplast, where GlyBet synthesis occurs. McNeil et al. (2001) showed that the overexpression of spinach PMT enzyme in transgenic tobacco expressing transgenes encoding chloroplast enzymes for GlyBet synthesis increased the level of choline by 50-fold and this expanded choline pool led to a 30-fold increase in the synthesis of GlyBet. Engineering strategies that combine means to enhance the production of internal pools of Etn and P-Etn, overexpression of mutated PMT enzymes that are less sensitive to inhibition by P-Chol and expression of GlyBet enzymes could make it possible to produce crops with even higher choline and GlyBet levels.

While PMT enzymes could potentially be used in the bioengineering of resilient and nutritional crops, their suitability as targets for the development of antimalarial or nematocidal drugs remains debatable. In the case of the malarial PfPMT, genetic studies showed that this gene plays an important, but not essential function during the asexual stage of the parasite life cycle in human red blood cells under in vitro culture conditions. Thus, compounds that block PfPMT activity are presumably unlikely to block malaria infection in humans. Notably, the culture medium used to monitor the growth and multiplication of knockout parasites in vitro is rich in lipid precursors such as phospholipids, fatty acids and lysophosphatidylcholine, which could help overcome the loss of the SDPM pathway. Studies to determine whether PfPMT is required for development under conditions that resemble those found in human serum are thus essential in order to address its suitability as a drug. Similarly, the importance of PMT activity in nematodes has been investigated only in the model organism C. elegans. Studies in pathogenic nematodes are needed to determine whether these enzymes are critical for survival and pathogenesis and whether they are suitable for the development of nematocidal drugs.

Concluding remarks

PMT enzymes represent a novel class of MTases identified in a select number of eukaryotic organisms as well as in Proteobacteria. To date, our understanding of the biochemistry and physiological function of these enzymes has been mostly gained from studies performed in P. falciparum, C. elegans and various plant species. Data obtained thus far suggest an important role for these enzymes in membrane biogenesis, cellular development, nuclear division and other important cellular processes. However, there is more to learn about these enzymes and the molecular mechanisms by which they control various physiological functions. Structural studies are also needed in order to help better understand their mechanism of catalysis, substrate specificity and mode of inhibition. These studies could help further the design of better compounds to inhibit this class of enzymes or guide bioengineering studies aimed at modulating their activity in vivo.

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