Structure and Reaction Mechanism of Phosphoethanolamine Methyltransferase from the Malaria Parasite Plasmodium falciparum

AN ANTIPARASITIC DRUG TARGET*§•

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Background: In the malaria parasite, Plasmodium falciparum, a phosphoethanolamine methyltransferase (PfPMT) is critical for membrane biogenesis. Results: Structures and mutagenesis of PfPMT suggest Tyr-19 and His-132 as a catalytic dyad. Conclusion: The reaction sequence of PfPMT likely involves structural changes with the Tyr-19-His-132 forming an active site latch. Significance: This is the first structure of an enzyme essential for the survival of the malaria parasite.

In the malarial parasite Plasmodium falciparum, a multifunctional phosphoethanolamine methyltransferase (PfPMT) catalyzes the methylation of phosphoethanolamine (pEA) to phosphocholine for membrane biogenesis. This pathway is also found in plant and nematodes, but PMT from these organisms use multiple methyltransferase domains for the S-adenosylmethionine (AdoMet) reactions. Because PfPMT is essential for normal growth and survival of Plasmodium and is not found in humans, it is an antiparasitic target. Here we describe the 1.55 Å resolution crystal structure of PfPMT in complex with AdoMet by single-wavelength anomalous dispersion phasing. In addition, 1.19–1.52 Å resolution structures of PfPMT with pEA (substrate), phosphocholine (product), sinefungin (inhibitor), and both pEA and S-adenosylhomocysteine bound were determined. These structures suggest that domain rearrangements occur upon ligand binding and provide insight on active site architecture defining the AdoMet and phosphobase binding sites. Functional characterization of 27 site-directed mutants identifies critical active site residues and suggests that Tyr-19 and His-132 form a catalytic dyad. Kinetic analysis, isothermal titration calorimetry, and protein crystallography of the Y19F and H132A mutants suggest a reaction mechanism for the PfPMT. Not only are Tyr-19 and His-132 required for phosphobase methylation, but they also form a “catalytic” latch that locks ligands in the active site and orders the site for catalysis. This study provides the first insight on this antiparasitic target enzyme essential for survival of the malaria parasite; however, further studies of the multidomain PMT from plants and nematodes are needed to understand the evolutionary division of metabolic function in the phosphobase pathway of these organisms.

Malaria is a major worldwide health threat as this disease, caused by different species of Plasmodium parasites, results in over 1 million deaths and 300 million clinical cases each year (1). In particular, infections by Plasmodium falciparum are the most severe with the highest rates of mortality and morbidity. The global prevalence of malaria, the fact that ∼40% of mankind lives in endemic areas, and the emergence of drug-resistant strains drive the need to identify new biochemical targets for the development of therapeutics targeting this protozoan parasite (2).

The rapid growth and replication of Plasmodium in human erythrocytes requires a significant source of phospholipids to support membrane biogenesis, and phospholipid biosynthesis has been explored as a target for antimalarial compounds (3, 4). In mammals, phosphatidylcholine is the major phospholipid in cellular membranes and is synthesized either by the de novo pathway which converts dietary choline to phosphocholine (pCho)2 and then to the phospholipid via CDP intermediates, or by methylation of phosphatidylethanolamine to phosphatidylcholine through the Bremer-Greenberg pathway (5). In contrast, plants methylate phosphoethanolamine (pEA) into pCho (Fig. 1A), which then enters the Kennedy pathway, through the action of S-adenosylmethionine (AdoMet)-dependent phosphoethanolamine methyltransfer-

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The atomic coordinates and structure factors (codes 3UJ6, 3UJ7, 3UJ8, 3UJA, 3UJ9, 3UJB, 3UC, and 3UD) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: pCho, phosphocholine; pDME, phospho-dimethylethanolamine; pEA, phospho-ethanolamine; pMME, phosphomonomethylethanolamine; AdoCys, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; Se-Met, selenomethionine; ITC, isothermal titration calorimetry; PMT, phosphoethanolamine methyltransferase(s); r.m.s.d., root mean square deviation.
The PMT family of enzymes catalyzes the sequential methylation of phosphoethanolamine (pEA) to form phosphocholine (pCho). This process is essential for the synthesis of phospholipids, which are major components of cellular membranes. Three types of PMTs are known: plant PMT, Plasmodium PMT, and nematode PMT. Each type has a different domain organization and catalyzes a specific set of reactions.

**Structure of PfPMT**

![Diagram of PfPMT](https://example.com/diagram)

**FIGURE 1.** Phosphobase methylation and domain arrangement in the PMT family. **A**, the PMT pathway showing the sequential methylation of pEA to pCho. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine. **B**, organization of the methyltransferase (MT) domains in the plant, *Plasmodium*, and nematode PMT is shown. MT-1 domains catalyze the conversion of pEA to pMME, and MT-2 domains catalyze the reactions from pMME to pCho. 100 aa, 100 amino acids.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Mutagenesis**—For bacterial expression, the PfPMT cDNA (7) was synthesized (GenScript) with *Escherichia coli* codon optimization and introduction of Ndel and BamHI sites at the 5′- and 3′-ends of the gene, respectively. The Ndel/BamHI fragment from the synthetic gene was excised and subcloned into pET-28a (Novagen). Expression in *E. coli* BL21(DE3) and purification by nickel-affinity and size-exclusion chromatographies were as described for the *C. elegans* PMT (11, 12). Selenomethionine (Se-Met)-substituted protein was produced by inhibition of the *E. coli* methionine biosynthesis pathway with the AdoMet vector and bacterial strain used for native protein expression (18). Incorporation of Se-Met was confirmed by mass spectrometry to compare intact molecular masses of native and derivatized protein. Purification of Se-Met-PfPMT was as for native protein. Site-directed mutants were generated using the QuikChange PCR method (Stratagene) with expression and purification as above.
Structure of PfPMT

Enzyme Kinetics and Isothermal Titration Calorimetry—Activity assays were performed using the standard PMT radiochemical assay at fixed concentrations of AdoMet (0.5 mM) and pEA (2 mM) (11, 12). For determination of kinetic parameters, reactions were performed either with fixed AdoMet (0.5 mM) and varied phosphobase (0.010–2 mM) or with fixed phosphobase (2 mM) and varied AdoMet (5–500 μM). All data were fit to the Michaelis-Menten equation in SigmaPlot. Calorimetric analysis of AdoCys and pCho binding to PfPMT was performed, and data were analyzed as described previously for the nematode PMT (19).

Protein Crystallography—Crystals of Se-Met-PfPMT in complex with AdoMet were grown by the vapor diffusion method in hanging drops of a 1:1 mixture of protein (13.5 mg ml⁻¹) and crystallization buffer (20% PEG8000, 0.1 M sodium cacodylate, pH 6.5, 0.2 M sodium acetate, 20 mM tris(2-carboxyethyl)phosphine, and 5 mM AdoMet). Crystals of native and mutant PfPMT in complex with various ligands (5 mM for each ligand except 0.5 mM AdoCys) were obtained in similar conditions (20–30% PEG8000, 0.1 M sodium cacodylate, pH 6.5, 0.2 M sodium acetate, 5 mM β-mercaptoethanol).

The PfPMT structure was solved by single-wavelength anomalous dispersion phasing. Diffraction data collected at beamline 19ID of the Argonne National Laboratory Advanced Photon Source were indexed, integrated, and scaled using HKL3000 (20). SHELX (21) was used to determine initial Se-Met positions and parameters. Anomalous signal from three Se-Met. The resulting model used for molecular replacement into the PfPMT monomer was overexpressed in E. coli as an N-terminal hexahistidine-tagged protein and purified by Ni²⁺-affinity and size-exclusion chromatographies. As observed for the C. elegans PMT (11, 12), PfPMT was monomeric upon gel filtration. Kinetic analysis of PfPMT revealed less than 2-fold differences in kcat/Km for pEA, pMME, and pDME (Table 2), which contrasts with the 6–20-fold higher efficiency of CePMT1 versus CePMT2 (11, 12). These results confirm that PfPMT efficiently catalyzes all reactions in the phosphobase pathway.

Overall Structure of PfPMT—The x-ray crystal structure of PfPMT in complex with AdoMet and PO₄²⁻ was determined by single-wavelength anomalous dispersion phasing using the anomalous signal from three Se-Met. The resulting model used to solve, build, and refine a native 1.55 Å resolution structure (Table 1 and Fig. 2A). The PfPMT monomer consists of an

Table 1. Summary of PfPMT crystallographic statistics

<table>
<thead>
<tr>
<th>Crystal</th>
<th>Space group</th>
<th>a (Å)</th>
<th>b (Å)</th>
<th>c (Å)</th>
<th>α (°)</th>
<th>β (°)</th>
<th>γ (°)</th>
<th>Resolution (Å)</th>
<th>Rsym (%)</th>
<th>Rcryst/RFree (%)</th>
<th>No. of water molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se-Met-PfPMT</td>
<td>CCP4</td>
<td>89.8</td>
<td>89.8</td>
<td>89.8</td>
<td>90.0</td>
<td>90.0</td>
<td>90.0</td>
<td>36.5-1.97 (2.00-1.97)</td>
<td>6.3 (10.7)</td>
<td>14.9/18.5</td>
<td>325</td>
</tr>
<tr>
<td>PfPMT + pEA</td>
<td>CCP4</td>
<td>89.8</td>
<td>89.8</td>
<td>89.8</td>
<td>90.0</td>
<td>90.0</td>
<td>90.0</td>
<td>36.5-1.97 (2.00-1.97)</td>
<td>6.7 (38.2)</td>
<td>16.7/18.5</td>
<td>875</td>
</tr>
<tr>
<td>PfPMT + pMME</td>
<td>CCP4</td>
<td>89.8</td>
<td>89.8</td>
<td>89.8</td>
<td>90.0</td>
<td>90.0</td>
<td>90.0</td>
<td>36.5-1.97 (2.00-1.97)</td>
<td>4.7 (40.9)</td>
<td>16.5/18.3</td>
<td>516</td>
</tr>
<tr>
<td>PfPMT + pDME</td>
<td>CCP4</td>
<td>89.8</td>
<td>89.8</td>
<td>89.8</td>
<td>90.0</td>
<td>90.0</td>
<td>90.0</td>
<td>36.5-1.97 (2.00-1.97)</td>
<td>2.4 (39.1)</td>
<td>14.4/18.1</td>
<td>11</td>
</tr>
</tbody>
</table>

Steady-state Kinetic Analysis—Previously, only pEA was used as a substrate for PfPMT (7). To examine phosphobase specificity, PfPMT was overexpressed in E. coli as an N-terminal hexahistidine-tagged protein and purified by Ni²⁺-affinity and size-exclusion chromatographies. As observed for the C. elegans PMT (11, 12), PfPMT was monomeric upon gel filtration. Kinetic analysis of PfPMT revealed less than 2-fold differences in kcat/Km for pEA, pMME, and pDME (Table 2), which contrasts with the 6–20-fold higher efficiency of CePMT1 versus CePMT2 (11, 12). These results confirm that PfPMT efficiently catalyzes all reactions in the phosphobase pathway.
α-helical “lid” domain (α1, α2, α7, α9-α10) and a canonical AdoMet binding domain defined by a central β-sheet (β1-β7) flanked by two α-helical regions (α3/α4/α5 and α6/α8). The overall fold of PfPMT was most similar to those of cyclopropane-fatty-acyl-phospholipid synthase (2.4 Å r.m.s.d.; 17% identity) and methoxymycolic acid synthase (2.5 Å r.m.s.d.; 16% identity), both N-methyltransferases from *Mycobacterium* (28, 29). In addition, other methyltransferases, including glycine N-methyltransferase (2.9 Å r.m.s.d.; 13% identity), histamine N-methyltransferase (3.3 Å r.m.s.d.; 14% identity), phenylethanolamine N-methyltransferase (3.0 Å r.m.s.d.; 13% identity), and guanidinoacetate N-methyltransferase (3.3 Å r.m.s.d.; 12% identity), were also identified by a DALI search as structurally similar (28, 29). In addition, other methyltransferases, including glycine N-methyltransferase (3.0 Å r.m.s.d.; 13% identity), *Mycobacterium* N-methyltransferases from *Mycobacterium* (3.3 Å r.m.s.d.; 12% identity), and guanidinoacetate N-methyltransferase (3.3 Å r.m.s.d.; 12% identity), were also identified by a DALI search as structurally similar (28, 29). In addition, other methyltransferases, including glycine N-methyltransferase (3.0 Å r.m.s.d.; 13% identity), *Mycobacterium* N-methyltransferases from *Mycobacterium* (3.3 Å r.m.s.d.; 12% identity), and guanidinoacetate N-methyltransferase (3.3 Å r.m.s.d.; 12% identity), were also identified by a DALI search as structurally similar (28, 29).

### TABLE 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$</th>
<th>$K_m$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEA</td>
<td>109 ± 2</td>
<td>54.2 ± 3.8</td>
<td>33,518</td>
</tr>
<tr>
<td>pMME</td>
<td>218 ± 24</td>
<td>181 ± 45</td>
<td>20,074</td>
</tr>
<tr>
<td>pDME</td>
<td>185 ± 14</td>
<td>66.8 ± 12</td>
<td>46,158</td>
</tr>
<tr>
<td>AdoMet (pEA)</td>
<td>23.5 ± 0.9</td>
<td>29.9 ± 3.8</td>
<td>13,099</td>
</tr>
<tr>
<td>AdoMet (pDME)</td>
<td>29.2 ± 1.2</td>
<td>33.1 ± 4.2</td>
<td>14,703</td>
</tr>
</tbody>
</table>

### FIGURE 2. PfPMT structure.**A.** A ribbon diagram of the PfPMT-AdoMet-Po$_4^{2-}$ complex with α-helices and β-strands colored gold and blue, respectively. Positions of AdoMet (green) and Po$_4^{2-}$ (rose) are indicated by the stick molecules. **B,** representative electron density. The 2F$_{o}$-F$_{c}$, omit map (1.5 σ) for pCho in the 1.19 Å resolution PfPMT-pCho complex is shown. **C,** view of the AdoMet binding site and the position of the α1 lid helix. PfPMT is shown as a surface rendering with the exception of Arg-179 and residues 8–22 (resi 8–22) of α1. Surfaces for Asp-61, Asp-85, and Asp-110 are highlighted in red.
Waters forming hydrogen bonds with the ligands are shown as spheres.

Arg-179 differs in position depending on whether PO₄

Likewise, the conformations of residues and ligands in the phosphobase site, as observed for the methyl group of AdoMet.

Sinfungin is oriented toward Tyr-19 (2.84 Å), and not the active site when PO₄

From the active site when PO₄

These data indicate that the phosphobase site is highly organized with multiple specific interactions for efficient catalysis, most likely through binding interactions with substrate.

Structural and Functional Analysis of the Catalytic Dyad—Mutagenesis of either Tyr-19 or His-132 in the putative catalytic dyad severely affects PFPMT activity. The His-132, Tyr-19, His-132Q, and Y19A mutants displayed no detectable activity, and with yields comparable with wild-type PFPMT for activity assays. Using standard assay conditions, the methylation reaction was not detected for the Y19A, D85A, D85N, H132A, H132Q, H132N, Y160A, Y160F, R179A, K247A, and K247M mutants, but varying levels of activity were observed for other mutants (Fig. 5). Deviations in relative activity can result from either changes in turnover rates and/or elevated $K_m$ values.

Within the AdoMet site, Asp-85 and Ile-86 provide critical interactions with the substrate as mutations of either residue (D85N/D85A or I86F/I86A) abrogated activity, likely through a loss of AdoMet binding. Mutation of Ser-37, Asp-61, and Asp-110 resulted in mutants with ~35–85% of wild-type activity (Fig. 5). The higher activity in the D61N (68%) and D110N (82%) mutants suggests that substitution of the side-chain carboxylate for an amide retains hydrogen-bonding patterns with AdoMet, whereas the D61A (35%) and D110A (35%) mutants disrupt these interactions.

Mutant analysis of residues in the phosphobase site indicate that the electrostatic interactions provided by Arg-179 and Lys-247 are required for activity as the K247A and K247M mutants were inactive and the R179A and R179K mutants displayed 110 resulted in mutants with 35–85% of wild-type activity (Fig. 5). In addition, each of the tyrosine residues in the site (Tyr-27, Tyr-160, Tyr-175, and Tyr-181) were also important for PFPMT activity as removal of the hydroxyl or the phenol moieties severely disrupted methylation of pEA, yielding mutants that were inactive (Y160F/Y160A) or with 1–10% of wild-type activity (Fig. 5). Similarly, the Q18N mutant also reduced activity to 22% (Fig. 5). These data indicate that the phosphobase site is highly organized with multiple specific interactions for efficient catalysis, most likely through binding interactions with substrate.

the turnover rate 60-fold and increased the
showed that removal of the side-chain hydroxyl group reduced
of which is 2.7 Å from the hydroxyl group of Tyr-19. In the
His-132 (2.7 Å), which shifts slightly in position when com-
position of 30 injections of 10 μl AdoCys (650 μM) into a solution containing PMT protein (51.5 μM) at 20 °C. The bottom panel shows the integrated
of the Y19F active site (Fig. 7A), mutation of Tyr-19 to a phenylalanine provides space for a water molecule to enter the site and interact with the Nη of
His-132 (2.7 Å), which shifts slightly in position when com-
pared with wild type. This water approximates the position of
the hydroxyl group of Tyr-19 and maintains the overall struc-
tural and functional analysis of the Tyr-19 and His-132 PfPMT
the Y19F mutant retained 24% activity (Fig. 5). Because of the
potential role of these residues in the reaction mechanism of
PfPMT, a more detailed analysis of the Y19F and H132A
mutants was performed, which included kinetic analysis of the
Y19F mutant, isothermal titration calorimetry (ITC) of AdoCys
and pCho binding to wild-type, Y19F, and H132A PfPMT, and
crystallographic analysis of Y19F and H132A mutants, both in
complex with pCho.

Determination of the steady-state kinetic parameters for the
Y19F mutant (kcat = 1.85 ± 0.3 min⁻¹; kcat/pEA = 644 ± 40 μM)
showed that removal of the side-chain hydroxy group reduced the
turnover rate 60-fold and increased the kcat/pEA 12-fold when compared with wild-type enzyme. Evaluation of AdoCys
and pCho binding to the wild-type, Y19F, and H132A PfPMT
was performed using ITC (Fig. 6 and Table 3) (24). This analysis
shows that these proteins bind each ligand with comparable Kd values; however, the enthalpic and entropic contributions to
ligand binding change in the mutants (Table 3). Crystallo-
graphic analysis of the Y19F and H132A mutants (Fig. 7) reveal
that the overall active site structures are similar to that of the
PfPMT-AdoCys-pEA complex with differences localized where

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**TABLE 3**

**ITC analysis of AdoCys and pCho binding**

Titrations were performed at 20 °C and data fit to a single-site binding model as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Protein-Ligand</th>
<th>Kd</th>
<th>ΔG</th>
<th>ΔH</th>
<th>ΔAΔS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfPMT-AdoCys</td>
<td>7.9 ± 0.8</td>
<td>-6.8 ± 0.7</td>
<td>-11.0 ± 0.3</td>
<td>4.2</td>
</tr>
<tr>
<td>Y19F-AdoCys</td>
<td>12.0 ± 1.8</td>
<td>-6.6 ± 1.0</td>
<td>-5.3 ± 0.2</td>
<td>-1.3</td>
</tr>
<tr>
<td>H132A-AdoCys</td>
<td>12.2 ± 2.4</td>
<td>-6.6 ± 1.3</td>
<td>-1.6 ± 0.1</td>
<td>-5.0</td>
</tr>
<tr>
<td>PfPMT-pCho</td>
<td>23.4 ± 1.6</td>
<td>-6.2 ± 0.4</td>
<td>-22.3 ± 0.7</td>
<td>16.1</td>
</tr>
<tr>
<td>Y19F-pCho</td>
<td>20.2 ± 6.8</td>
<td>-6.3 ± 2.1</td>
<td>-1.9 ± 0.2</td>
<td>-4.4</td>
</tr>
<tr>
<td>H132A-pCho</td>
<td>26.0 ± 11.3</td>
<td>-6.2 ± 2.7</td>
<td>-1.6 ± 0.2</td>
<td>-4.5</td>
</tr>
</tbody>
</table>

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FIGURE 6. **ITC analysis of ligand binding.** A, titration of PfPMT with AdoCys. The top panel shows data plotted as heat signal (μcal sec⁻¹) versus time (min). The experiment consisted of 30 injections of 10 μl AdoCys (650 μM) into a solution containing PMT protein (51.5 μM) at 20 °C. The bottom panel shows the integrated heat response per injection from the upper panel plotted as normalized heat per mol of injectant. SAH, S-adenosylhomocysteine. The solid line represents the fit to the data. B, titration of PfPMT (65.1 μM) with pCho (850 μM). The top panel shows data plotted as heat signal (μcal sec⁻¹) versus time (min). The bottom panel shows the integrated heat response per injection from the upper panel plotted as normalized heat per mol of injectant. SAH, S-adenosylhomocysteine. The solid line represents the fit to the data. Top and bottom panels are as in A.
mutants suggests a likely reaction mechanism for the PMT family of enzymes (Fig. 8).

**DISCUSSION**

The phosphobase methylation pathway from pEA to pCho (Fig. 1A) provides *Plasmodium* with a novel and essential metabolic route for membrane biogenesis that is not found in mammals (7–10). In addition, this pathway is also required for growth and development in *C. elegans* (11–14, 19). Given the biological function of the PMT in *Plasmodium* and nematodes and that these enzymes are not found in mammals, the PMT are potential targets for the development of antiparasitic therapeutics (10, 13, 14, 17). The PfPMT structure (Fig. 2) provides the first three-dimensional view of this enzyme from any species, yields new insight on catalysis and ligand binding in this group of methyltransferases, and may be useful for the development of inhibitors targeting this enzyme from *P. falciparum*, the major causative agent of malaria.

Comparisons of PfPMT with other N-methyltransferases show structural conservation of the canonical AdoMet binding fold in PfPMT but also highlight the diversification of the lid domain, most likely for recognition of chemically distinct substrates (supplemental Fig. S1). Although the organization of methyltransferase domains in the *Plasmodium*, nematode, and plant PMT differs (Fig. 1B), sequence alignment of PfPMT with the C-terminal methyltransferase domains of *C. elegans* PMT2 (11), *Haemonchus contortus* (sheep barber pole worm) PMT2 (19), and *Arabidopsis thaliana* PMT (34) shows that residues forming the sequence motifs (I, post-I, II, and III) defining the AdoMet binding region in the C terminus are conserved and located in the β-strands at the core of this scaffold (supplemental Fig. S3). Low sequence identity between PfPMT and the N-terminal methyltransferase domains from *C. elegans* PMT1, *H. contortus* PMT1, and *A. thaliana* PMT preclude accurate sequence alignments. Previously, mutagenesis of PfPMT targeting residues in the conserved motifs yielded inactive proteins (15), which likely resulted from disruption of protein folding and not binding and/or catalysis. Within the AdoMet binding site, Ser-37, Gly-63, Asp-61, Asp-85, and Asp-110 are conserved in PfPMT, CePMT2, HcPMT2, and AtPMT, but other residues (Asp-10, Ile-36, Thr-44, Ile-86, and Ile-111) that are generally at the periphery of the AdoMet binding site vary. Functional analysis of PfPMT mutants in the AdoMet binding site showed that Asp-85 and Ile-86 are important for activity with other positions of varied importance (Fig. 5).

All of the residues forming the phosphobase site are invariant between PfPMT, CePMT2, HcPMT2, and the second domain of AtPMT (Figs. 3 and 4 and supplemental Fig. S3). Site-directed mutagenesis of amino acids in the phosphobase site also highlights the functional importance of interactions with the substrate phosphate group for activity (Fig. 5). Interestingly, conservation of residues in the phosphobase binding site of the PMT raises the question of what determines substrate specificity in these enzymes as PfPMT accepts pEA, pMME, and pDME as substrates (Table 2), but the nematode PMT2 and the C-terminal domain of the plant PMT do not use pEA as a substrate (6, 11, 19). Structural comparison of PfPMT with pEA-, pCho-, and AdoCys-pEA-bound forms shows that the active site can readily accommodate pEA and its methylated products (Fig. 4B). The molecular basis for substrate specificity in the different types of PMT is currently unclear; however, it is possible that the multidomain organization of the plant and nematode PMT alters the active site structure to favor pMME and pDME versus pEA as substrates. Further structural studies of the PMT from nematodes and plants are needed to understand the evolutionary division of metabolic function in the phosphobase pathway of these organisms.

Structural and functional analysis of PfPMT, together with previous work on the nematode and plant PMT, suggests a plausible reaction sequence for phosphobase methylation. The nematode and plant PMT use a random bi bi kinetic mechanism in which binding of either AdoMet or phosphobase occurs first followed by binding of the other ligand (11, 12, 16). Crystal structures of PfPMT in complex with pEA, pCho, AdoMet, and AdoCys-pEA are consistent with this kinetic mechanism (Figs. 2–4). However, these structures suggest that conformational changes between apoenzyme- and ligand-bound forms likely occur as both phosphobase and AdoMet binding sites are largely solvent-inaccessible (Fig. 2C). In particular, movement of the N-terminal region of PfPMT, including α1, upon ligand binding appears important for locking of AdoMet/AdoCys in the active site. Similar movement of the N-terminal regions in other N-methyltransferases upon ligand binding has been observed (30–33). Previously, thermodynamic analysis of AdoCys binding to the two PMT from *H. contortus* suggested that ligand binding is accompanied by changes in protein structure (19). Moreover, localized conformational changes in the PfPMT active site are also likely as the conformations of Tyr-27, Phe-31, and Arg-179 differ between PO₄⁻⁻, pEA⁻⁻, and pCho-bound forms (Fig. 4). Thus, structural changes likely order the active site for catalysis.

Within the PfPMT active site, Tyr-19 and His-132 are positioned between the AdoMet and phosphobase sites to function as a catalytic dyad in the SN₂ transfer of a methyl group from AdoMet to the amine on pEA, pMME, or pDME (Figs. 3 and 8). These two amino acids are also invariant in the nematode and plant PMT (supplemental Fig. S3). In the PfPMT-AdoCys-pEA complex, the hydroxyl group of Tyr-19 is 3.5 Å away from the S₆ of AdoCys, 2.5 Å from the amine of pEA, and 2.8 Å from the N₆ of His-132. In the proposed reaction mechanism (Fig. 8), His-132 functions as a general base to abstract a proton from the hydroxyl group of Tyr-19 to activate the residue. The negatively charged oxygen interacts with a hydrogen on the substrate.
amino acids to distort the electron configuration from \(sp^2\) to \(sp^3\), which orients the lone pair electrons toward the methyl group of AdoMet to facilitate methylation of the phosphorybse.

In this mechanism, His-132 is the critical driver for catalysis. The loss of activity in the H132A, H132Q, and H132N mutants is consistent with the proposed mechanism. Binding analysis of the H132A mutant by ITC demonstrates that this substitution does not significantly change the \(K_d\) values for AdoCys and pCho when compared with wild-type (Table 3), which indicates that the loss of activity does not result from an inability to bind ligands. In addition, the 1.19 Å resolution structure of H132A shows that the active site structure is essentially identical to that of wild-type with the exception of two water molecules that fit in the mutant site left by removal of the side-chain imidazole in the mutant (Fig. 7B). This structure also indicates that the presence and positioning of Tyr-19 alone are insufficient to drive the methylation reaction.

Although the lack of activity in the Y19A mutant agrees with the reaction mechanism, the reduced activity in the Y19F mutant appears inconsistent. Removal of the hydroxyl group from Tyr-19 reduces activity; however, the Y19F mutant binds AdoCys and pCho with affinities similar to wild type (Table 3). Structural analysis of the Y19F mutant reveals the presence of a water molecule that approximates the position of the Tyr-19 hydroxyl group and forms a hydrogen bond with His-132. Activation of this water molecule by His-132 in the Y19F mutant likely substitutes for the wild-type hydroxyl group, albeit with compromised efficiency.

ITC analysis of AdoCys and pCho binding to the wild-type, Y19F, and H132A PfPMT also suggests a possible role for the Tyr-19-His-132 interaction as a “catalytic latch.” As noted above, the structures of PfPMT show that the active site is occluded by \(\alpha_1\), which includes Tyr-19. We suggest that movement of the N-terminal helix upon AdoMet/AdoCys binding not only locks the substrate in the active site, but also positions the tyrosine for interaction with His-132 to form the catalytic dyad. ITC analysis of AdoCys and pCho binding to the Y19F and H132A mutants indicates that these mutations do not change binding affinity; however, the enthalpic and entropic contributions decrease and increase, respectively, when compared with wild-type enzyme (Table 3). The greater entropy contribution in the mutants suggests that alteration of either residue in the dyad increases flexibility that becomes more ordered upon ligand binding.

The overall reaction catalyzed by the PMT resembles that of other N-methyltransferases, including the well studied glycine and guanidinoacetate methyltransferases; however, how different methyltransferases chemically perform the transfer reaction differs (30–33, 35–39). The structure of the glycine methyltransferase in complex with AdoMet and acetate led to the proposal that an active site glutamate facilitated the reaction as a general acid (35). Later structures of this enzyme suggested that an arginine drives catalysis primarily by proximity and orientation (30), but computational modeling of the reaction energetics suggests that this is unlikely (36, 37). In guanidinoacetate methyltransferase, a critical aspartate residue is positioned to abstract a proton from the substrate amine (33, 38, 39). Although all these enzymes bind AdoMet using an evolutionarily conserved scaffold, the residues responsible for recognizing substrates, positioning the amine for methylation, and facilitating catalysis differ.

The structural and mechanistic differences between the PMT and other methyltransferases suggest that the development of specific inhibitors is possible. Although the identification of compounds targeting the Plasmodium and nematode PMT is in the early stages, a handful of known compounds, such as sinefugin (an AdoMet analog), miltefosine (a choline analog), and amodiaquine (a 4-aminoquinoline), are inhibitors (7, 17, 19). Previous work on the inhibition of PfPMT by amodiaquine suggested a possible binding site near Gly-32 and Gly-68 for this compound based on NMR titration studies (17). In the PfPMT crystal structure, Gly-32 is in a loop after \(\alpha_2\) and Gly-68 in \(\alpha_4\), which is also positioned near \(\alpha_2\). Given the possible structural changes in PfPMT upon ligand binding, the shifts observed in the NMR spectra in the presence and absence of amodiaquine may result not from direct interaction with the molecule but from conformation changes in the N-terminal region upon binding. Further structural studies with inhibitors are required to elucidate how these molecules interact with PfPMT.

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Structure and Reaction Mechanism of Phosphoethanolamine Methyltransferase from the Malaria Parasite Plasmodium falciparum: AN ANTIPARASITIC DRUG TARGET

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