Structure and Biophysical Characterization of the S-Adenosylmethionine-dependent O-Methyltransferase PaMTH1, a Putative Enzyme Accumulating during Senescence of Podospora anserina*

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Background: PaMTH1, a putative O-methyltransferase protects Podospora anserina from oxidative stress during senescence and acts as a longevity assurance factor.

Results: Crystal structures of PaMTH1/PaMTH1-SAM/SAH co-complexes and NMR-based characterization of enzymatic methylation of its substrate were obtained.

Conclusion: PaMTH1 catalyzes methyl group transfer from the co-factor to the substrate in a cation-dependent manner.

Significance: This work facilitates the identification of endogenous polyphenolic compounds directly involved in metal-induced oxidative stress.

Low levels of reactive oxygen species (ROS) act as important signaling molecules, but in excess they can damage biomolecules. ROS regulation is therefore of key importance. Several polyphenols in general and flavonoids in particular have the potential to generate hydroxyl radicals, the most hazardous among all ROS. However, the generation of a hydroxyl radical and subsequent ROS formation can be prevented by methylation of the hydroxyl group of the flavonoids. O-Methylation is performed by O-methyltransferases, members of the S-adenosyl-L-methionine (SAM)-dependent O-methyltransferase superfamily involved in the secondary metabolism of many species across all kingdoms. In the filamentous fungus Podospora anserina, a well-established aging model, the O-methyltransferase (PaMTH1) was reported to accumulate in total and mitochondrial protein extracts during aging. In vitro functional studies revealed flavonoids and in particular myricetin as its potential substrate. The molecular architecture of PaMTH1 and the mechanism of the methyl transfer reaction remain unknown. Here, we report the crystal structures of PaMTH1 apoenzyme, PaMTH1-SAM (co-factor), and PaMTH1-S-adenosyl homocysteine (by-product) co-complexes refined to 2.0, 1.9, and 1.9 Å, respectively. PaMTH1 forms a tight dimer through swapping of the N termini. Each monomer adopts the Rossmann fold typical for many SAM-binding methyltransferases. Structural comparisons between different O-methyltransferases reveal a strikingly similar co-factor binding pocket but differences in the substrate binding pocket, indicating specific molecular determinants required for substrate selection. Furthermore, using NMR, mass spectrometry, and site-directed active site mutagenesis, we show that PaMTH1 catalyzes the transfer of the methyl group from SAM to one hydroxyl group of the myricetin in a cation-dependent manner.

Organisms growing under aerobic conditions are constantly exposed to several forms of reactive oxygen species (ROS)4 gen-

The abbreviations used are: ROS, reactive oxygen species; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl homocysteine; PaMTH1, P. anserina O-methyltransferase; MT, methyltransferase; SAM-MT, SAM-dependent methyltransferase; COMT, catechol-O-methyltransferase; CCoAOMT, caffeoyl-Co-A-O-methyltransferase; LiOMT, L. interrogens O-methyltransferase; SEC, size exclusion chromatography; MALS, multiangle laser light scattering; TSP-d4, 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid; HSQC, heteronuclear spin quantum correlation; CSP, chemical shift perturbation; RMSD, root mean square deviation; TCEP, tris(2-carboxyethyl)phosphine; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol; MAD, multiple-wavelength anomalous dispersion.
tered either endogenously during metabolic processes or exogenously due to environmental exposure (1). Accumulation of ROS can potentially damage proteins, lipids, carbohydrates, and DNA, resulting in several pathological occurrences like aging and age-associated diseases, including cancer (2, 3). ROS interfere with cellular functions by either inactivating enzymes with sulfhydryl groups via oxidation, lipid peroxidation, and subsequent increase in membrane permeability; depolymerization of polysaccharides; or degradation of DNA (4, 5). Although low concentrations of ROS (nitric oxide (NO), superoxide anion (O2•−), and hydrogen peroxide (H2O2)) facilitate important signaling and biological functions, high concentrations of H2O2, hydroxyl radicals (OH•), and O2•− can be damaging to biologically significant targets and constitute the basis for the free radical (oxidative stress) theory of aging (1, 6). Among all three ROS, the hydroxyl radical is the most reactive and toxic, and no detoxification system exists for it (7).

Some transition metals, in particular iron and copper, catalyze the formation of hydroxyl radicals from superoxide radicals or hydrogen peroxide (Haber-Weiss and Fenton’s reaction) (4). Apart from generating ROS via the above reaction, metals like iron and copper are also known to interact with naturally occurring antioxidants like polyphenolic compounds (flavonoids) or vitamin C, thereby transforming an antioxidant into a prooxidant (8). Flavonoids, a frequent component of the human diet, in general exhibit their beneficial antioxidant properties by inhibiting several enzymes, including oxidases, lipases, and protein kinases (9–13). However, apart from these beneficial effects, some flavonoids in vitro are known to be mutagenic, due to their prooxidant behavior (14). For example, quercetin, a plant flavonoid, in the presence of ferrous ions generates hydroxyl radicals and leads to enhanced sulfhydryl groupoxidation of the enzyme glyceraldehyde 3-phosphate dehydrogenase and its subsequent inactivation. In the absence of metal ions, quercetin becomes protective in function and prevents sulfhydryl oxidation (15, 16). Therefore, the presence/absence of metal ions modulates the biological or pharmacological behavior of flavonoids to act as an antioxidant or prooxidant (17). Several studies in model organisms have established substantial correlation between age-related accumulation of metal ions and enhanced formation of ROS (18, 19). Most of the polyphenols, including flavonoids, react with metals via their vicinal dihydroxyl system and lead to generation of ROS (20).

Several defense mechanisms (enzymes, vitamins, and metabolites) in the cells are involved in either preventing the formation of ROS or inactivating them (21). Particularly, in flavonoids, methylation of the vicinal hydroxyl groups and their subsequent conversion to methyl ethers prevent their interaction with metals, thereby abolishing their prooxidant activity (22). Methylation of biological compounds is also an integral part of various cellular processes, such as protein trafficking, signal transduction, biosynthesis, metabolism, and gene expression (23). Methyltransferases (MTs), via an SN2-like nucleophilic substitution reaction mechanism, catalyze the transfer of a methyl group from a donor to an acceptor molecule, resulting in the formation of methylated products and a by-product. Most often, methylation is catalyzed by S-adenosyl-l-methio-
Here, we present crystal structures of the PaMTH1 apoenzyme, a PaMTH1-SAM (co-factor) co-complex, and a PaMTH1-SAH (by-product) co-complex refined to 2.0, 1.9, and 1.9 Å, respectively. The enzyme is a highly stable dimer both in solution and in the crystal due to swapping of the N termini. Structural analysis of the apo- and holoenzyme reveals an overall architecture similar to SAM-MTs but shows significant differences in the substrate binding region. The α2-α3 loop, involved in substrate binding, adopts a “closed” conformation in the presence of SAM/SAH, as observed in other SAM/SAH-bound structures, and an “open” conformation in the apoenzyme. In addition, using sequence and structural homology, residues important for substrate binding were predicted and probed using site-directed mutagenesis. Based on NMR chemical shift perturbations, we show that SAM and the substrate (myricetin) bind to PaMTH1 and catalyze the methyl group transfer from SAM to one hydroxyl group of myricetin in a cation-dependent manner. Furthermore, by using mass spectrometry, we confirmed the conversion of myricetin to monomethylated myricetin.

Experimental Procedures

Protein Expression and Purification—The PaMth1 gene was cloned into the expression vector pETTEV-16b and transformed in Escherichia coli BL21 (DE3) cells (Invitrogen) for overexpression of the fusion protein with an N-terminal His7 tag. Transformed E. coli cells were grown at 37 °C in autoinduction medium ZYM-5052 (30) containing 100 µg/ml ampicillin to an A600 of ~0.6, and then the temperature was reduced to 20 °C to support soluble protein expression. After 48–60 h of cell growth (due to the long induction time, hydrolisis of the antibiotic can occur, so ampicillin was replenished every 24 h), bacteria were harvested by centrifugation (20 min, 4000 rpm) and resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10 mM β-mercaptoethanol) with the addition of complete protease inhibitor (1 tablet/100 ml of EDTA-free; Roche Applied Science). Selenomethionine-derivated protein was expressed according to standard EMBL protocols (see the EMBL Web site). The cells were lysed using microfluidizer (15,000 p.s.i., 2 cycles), and the cell debris was separated by centrifugation (30 min, 16,000 rpm). The supernatant was filtered and passed through a 5-ml nickel-nitrirotiacetic acid column (GE Healthcare), washed with 10 bed volumes of wash buffer (same as lysis buffer), and the His-tagged protein was eluted using an isocratic gradient (0–100%) of wash buffer and elution buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 500 mM imidazole, 15 mM β-mercaptoethanol) for 8 column volumes. The eluted fractions were analyzed by SDS-PAGE, and the ones containing PaMTH1 were pooled, incubated with tobacco etch virus protase to remove the N-terminal His tag (1 mg of tobacco etch virus protase (in-house produced) per 10 mg of PaMTH1) and dialyzed for 24 h at 4 °C against 25 mM Tris, pH 8.0, 100 mM NaCl, 15 mM β-mercaptoethanol, and 5% glycerol. The cleaved protein was reloaded onto a nickel-nitrirotiacetic acid column for removal of the tag and the tobacco etch virus protase. Further purification of protein was achieved by gel filtration chromatography on a Superdex-200 column (GE Healthcare) equilibrated with 25 mM HEPES, pH 7.5, 100 mM NaCl, and 1 mM DTT. Fractions containing the target protein were pooled and concentrated up to ~5 mg/ml and stored at −80 °C or immediately used for experiments. 15N-Labeled protein was expressed in M9 minimal medium containing 15NH4Cl (1 g/liter) as the sole nitrogen source.

Isothermal Titration Calorimetry—All titration experiments were performed at 25 °C using a VP-ITC microcalorimeter (MicroCal Inc.). All titrations were performed using buffer containing 25 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM TCEP. SAM at a concentration of 940 µM was titrated into 36 µM PaMTH1 protein solution in 26 steps; SAH (930 µM) was titrated into 34 µM PaMTH1 protein in 21 steps. In the presence of 10 mM MgCl2, the concentrations of compounds and protein were reduced in order to avoid regularly present aggregation of the protein/complex (300 µM SAM into 19 µM PaMTH1 in 26 steps; 220 µM SAH into 22 µM PaMTH1 in 31 steps).
The protein concentrations were calculated from the UV absorption at 280 nm with a Nanodrop spectrophotometer (Thermo Fisher Scientific) using the extinction coefficient obtained from the amino acid sequence (51,910 liters/cm mol). Concentrations of compounds were obtained similarly using the known extinction coefficient for the dilution heat of the compounds.

**Protein Crystallization and Structure Determination**—The purified native and selenomethionine PaMTH1 protein was concentrated up to 5 mg/ml in a buffer containing 20 mM Tris (pH 8.0), 200 mM NaCl, 10 mM DTT and crystallized at 277 K by hanging drop vapor diffusion against a reservoir containing 100 mM BisTris (pH 7.0), 200 mM NaCl, 20% (w/v) PEG 3350, and 20 mM spermine tetrahydrochloride. Crystals were transferred to mother liquor with 30% PEG 400 for flash freezing. For co-crystallization experiments, the following conditions were used: PaMTH1-SAM (6 mg/ml PaMTH1 + 1.1 mM MgCl₂ + 0.22 mM SAM in 0.2 mM lithium sulfate, 100 mM BisTris, pH 6.5, 25% (w/v) PEG 3350; 4 °C; sitting drop; reservoir, 300 mM). The initial model was obtained from the amino acid sequence (51,910 liters/(cm² mol)) for both SAM and SAH. The raw ITC data were analyzed with the ITC-Origin version 7.0 software with a “one-site” binding model after correction for the dilution heat of the compound.

**NMR Spectroscopy**—NMR experiments were performed at T = 298 K on Bruker 800-, and 600-MHz spectrometers equipped with room temperature TXI-HCN probes. NMR samples were prepared with 10% D₂O to lock the spectrometers, and 3-(trimethylsilyl)-2,2,3,3'-tetradeteropropionic acid (TSP-d₄; 1 mM) was used as an internal standard for spectral referencing. The processing and analysis of NMR spectra were done in Topspin version 2.1 (Bruker Biospin).

All ligands, including co-factor (SAM), by-product (SAH), and putative substrates (apigenin, kaempferol, pyrocatechol, and myricetin) were purchased from Sigma-Aldrich and used without further purifications. The solubility of ligands in H₂O and buffer 25 mM Tris, pH 7.5, 100 mM NaCl, 5 mM TCEP were determined by a one-dimensional ¹H NMR experiment (Bruker, 600 MHz) using peak integrals of ¹H peaks of the ligands, which were normalized against the calibrated peak of 1 mM TSP at 0 ppm. The assignments of one-dimensional ¹H peaks corresponding to SAM (BMRB ID: bmse000059), SAH (BMRB ID: bmse000289), and pyrocatechol (BMRB ID: bmse000385) were obtained from the Biological Magnetic Resonance Bank (BMRB) metabolomics database, and the assignment for myricetin (HMDB ID: HMD802755) was obtained from the Human Metabolomics Data Base (HMDB).

PaMTH1-ligand interactions were studied by recording two-dimensional ¹H,15N HSQC (42) (Bruker, 800 MHz) of PaMTH1 (500 μM ¹⁵N-labeled PaMTH1 in 25 mM Tris, pH 6.5, 100 mM NaCl, 5 mM TCEP) in the presence or absence of the ligands. The ligands were added to the protein in 2-fold excess (2:1).

NMR titration experiments were performed by adding increasing amounts of ligand (0.125, 0.25, 0.375, 0.5, 1, and 2 mM SAM, 0.0625, 0.125, 0.25, 0.5, and 1 mM SAH) to 480 μM ¹⁵N-PaMTH1 at pH 6.5 and acquiring a series of two-dimensional ¹H,15N HSQC spectra at 298 K. Chemical shift perturbations (CSPs) from NMR titrations were quantified using the following equation (43).

\[ \Delta = \sqrt{\left(\delta H\right)^2 + 0.17 \times \left(\delta N\right)^2} \]  

(Eq. 1)

The affinity for each titration was determined by a simultaneous fit of the titration parameters to the observed CSPs. The affinity of the titrated ligand and the chemical shifts in the complex structure were used as fitting parameters. A single step binding mechanism in the fast or intermediate exchange regime was presumed. The differential equations were solved numerically to determine the equilibrium concentrations, presuming a given affinity. No statistical evidence for more complicated binding mechanisms could be found.

The enzymatic reaction of PaMTH1 (wild type and mutants) was monitored by recording a series of one-dimensional ¹H NMR experiments (Bruker 600 MHz) of the reaction mixture containing 100 μM PaMTH1, 1 mM SAM (unlabeled), and 1 mM myricetin (1:10:10) in deuterated Tris buffer containing MgCl₂ (25 mM d-Tris, pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 5 mM TCEP). The methoxyl peak of the product was identified by measuring one-dimensional ¹H NMR spectra of the reaction mixture after removal of the protein (denaturation by heat at
TABLE 1
Crystalllographic data and refinement statistics for PaMTH1

| Structure | PaMTH1 apo | PaMTH1 SAM | PaMTH1 SAH |
|-----------|------------|------------|------------|
| Protein Data Bank code | 4QVK | 4YMG | 4YMH |
| Space group | P 2 1 2 1 | P 2 1 2 1 | P 2 1 2 1 |
| Resolution (Å) | 26.27-1.97 (2.02-1.97) | 41.36-1.90 (1.95-1.90) | 48.77-1.88 (1.92-1.88) |
| Unit cell dimensions a, b, c (Å) | 73.38 79.72 84.39 | 75.04 78.89 72.72 | 84.23 239.30 50.56 |
| α, β, γ (degrees) | 90.00 90.00 90.00 | 90.00 90.00 90.00 | 90.00 90.00 90.00 |
| Unique reflections | 35,407 | 39,322 | 84,455 |
| Completeness (%) | 99.3 (99.7) | 99.9 (98.7) | 99.5 (97.3) |
| Average redundancy | 5.1 | 6.6 | 6.6 |
| Rmerge (%) | 6.7 (93.9) | 13.7 (95.1) | 11.7 (89.3) |
| Wilson B-factor (Å²) | 17.0 (1.8) | 12.75 (2.08) | 13.00 (2.30) |
| Mosaicity (degrees) | 0.155 | 0.232 | 0.117 |
| Rwork (%) | 17.4 (26.8) | 15.9 (24.9) | 16.4 (24.1) |
| Rfree (%) | 20.6 (30.4) | 20.2 (26.2) | 20.4 (30.6) |
| RMSD bonds (Å) | 0.012 | 0.008 | 0.008 |
| RMSD angles (degrees) | 1.264 | 0.999 | 1.062 |
| RMSD dihedral angles (degrees) | 13.693 | 14.839 | 14.424 |
| RMSD chirality (degrees) | 0.058 | 0.041 | 0.043 |
| Ramachandran favored (%) | 97.4 | 97.4 | 98.1 |
| Ramachandran allowed (%) | 2.6 | 2.6 | 1.9 |
| Ramachandran outliers (%) | 0 | 0 | 0 |

*Values in parentheses are for the highest resolution shells.

Results

Overall Structure of PaMTH1—Recombinant PaMTH1 was overexpressed in E. coli and purified both in native and selenomethionine-substituted forms. The crystal structure refined to 2.0 Å was solved using experimental Se-MAD (Table 1). The protein crystallizes as a homodimer (Fig. 1A). On SEC, PaMTH1, a 27-kDa protein, eluted as a single peak and with an elution volume corresponding to a 44-kDa protein. To perform in-line SEC-MALS, the measured molecular weight confirms that PaMTH1 exists as a dimer in solution (Fig. 1A, inset).

Most of the structurally characterized plant O-MTs are dimers (47), including the caffeoyl-CoA O-methyltransferase (CCoAOMT), which displays significant sequence homology to PaMTH1 (Fig. 2). Previous structures of O-MTs have shown that the SAM-binding site is located in the vicinity of the dimer interface. In addition, many MTs contain an additional N-terminal extension or dimerization domain also important for substrate binding and specificity (24, 25). PaMTH1 lacks such an additional dimerization domain, and the interaction between the monomers in the dimer appears to be unique. The dimerization interface buries 6270 Å² of surface area and accounts for 32% of the total available surface area of the dimer, consistent with previously reported small molecule O-MTs. Importantly, the first 10 N-terminal loop residues are swapped between the two monomers (Fig. 1, A and B). Additionally, the interface includes interactions involving helices α1, α3, and α9 and β3-strands β6 and β7. The N-terminal loop directly inserts into the catalytic pocket of the neighboring molecule and fixes the positions of helix α3 and the α8-α9 loop in the substrate binding region via a hydrogen bond network, suggesting a critical role in substrate binding (Fig. 1B). The hydroxyl group of Ser4 (chain A) in the loop hydrogen bonds with the hydroxyl group of Ser50 (chain B) of helix α3, and the carbonyl oxygen of the Pro7 (chain A) hydrogen bonds with the Nε1 hydrogen of Trp188 (chain B) in the α8-α9 loop (Fig. 1B). An analysis of the PaMTH1 dimer using PISA (48) shows that the dimer interface is predominantly composed of 34 hydrogen bonds, 17 salt bridges, and several non-bonded interactions. The regions involved in hydrogen bonding include N-terminal loop (residues Gly3, Ser4, Ile5, Pro7, and Phe8), α1 (Asp14, Arg15, Ser17, and Tyr173), α1-α2 loop (Ser24 and His25), α3 (Ser50 and Arg62–Lys65), α8-α9 loop (Ile181, Asp184, Cys185, and Trp188), α9 (Asp205), β6 (Val219 and Leu221), and β7 (Arg232 and Asp235). CCoAOMT (Medicago sativa) (Protein Data Bank entry 1SUI) is one of the closest structural homologues of PaMTH1 and also crystallizes as a dimer. The dimerization interface buries 25% of the total available surface area of the dimer. In the context of catalysis, dimerization of CCoAOMT is not critical for its activity (49). However, similar to other plant O-MTs, dimerization of PaMTH1 might be required either for catalysis or to impart substrate specificity, because its N-terminal loop contribtues to the active site of the dyad-related monomer. Interestingly, in the dimeric structures of two bacterial O-MTs (LiOMT from Leptospira interrogens (50) and SynOMT from cyanobacterium Synechocystis sp. (51)), the N-terminal loop (first 10–13 residues) plays an important role in substrate binding and specificity.
role either in dimerization or catalysis. The N-terminal loop in LiOMT is domain-swapped and involved in dimerization, although the functional significance is not known. However, in the case of SynOMT, although the loop is not involved in dimerization, it inserts into the monomer catalytic pocket and plays a critical role in catalysis.
Each monomer subunit of PaMTH1 consists of nine α-helices and seven β-strands, and folds into a globular tertiary structure consisting of a core α/β Rossmann fold (Fig. 1 C), typical for many SAM-binding MTs. The seven-stranded β-sheet core is sandwiched between two helical regions. As in all Rossmann fold SAM-dependent MTs, the β-sheet adopts a strand topology 3,2,1,4,5,7,6, with all strands oriented parallel to each other except for 7, which is antiparallel (Fig. 1 D).

**PaMTH1 Binding to Co-factor (SAM), By-product (SAH), and Substrate—**We used NMR spectroscopy to investigate the PaMTH1-SAM/SAH interaction in solution. 1H,15N HSQC experiments can detect ligand binding to a target through CSPs and serve as a qualitative tool for identifying binders. In addition to the CSPs, the complete disappearance of signals can also occur upon complexation, which is also indicative of binding. An overlay of 1H,15N HSQC spectra of PaMTH1 in the presence and absence of SAM/SAH shows that the addition of the ligand induces both CSPs and disappearance of a subset of NMR signals, indicating specific binding (Fig. 3, A and B). Previous studies have shown that both SAM and SAH occupy the same binding pocket in most methyltransferases. A comparison between SAM- and SAH-induced NMR-spectral changes reveals that there is a significant overlap, suggesting an identical or at least overlapping binding pocket for SAM and SAH in PaMTH1.

Subsequently, we co-crystallized SAM/SAH with PaMTH1 and solved the three-dimensional structures of the co-complexes refined to 1.9 Å (Table 1). The structures of PaMTH1 in complex with SAM and SAH clearly reveal a conserved SAM/SAH binding motif (Fig. 1 E). Positional conservation of the amino acids involved in the co-factor/by-product binding is directly evident from the crystal structures of SAM/SAH bound to PaMTH1 as well as sequence alignments of the closely related MTs (Fig. 2). Analogous to other SAM-MTs, the binding pocket is located on the C-terminal end of the β-strands. SAM/SAH binding within the active site pocket of PaMTH1 is mediated through hydrogen bond networks and van der Waals and π-π stacking interactions (Fig. 1 E). The GXGXXG motif is highly conserved in Rossmann fold SAM-dependent MTs and is considered to be a hallmark of the SAM-binding site (25). In PaMTH1, this region (GCYSG) is well conserved except that...
The central glycine of the GXGXG motif is substituted by a tyrosine (Tyr\textsuperscript{75}). In the apoenzyme, Tyr\textsuperscript{75} points into the SAM/SAH binding pocket, and upon SAM/SAH binding, it flips and reorients to establish direct hydrogen bonding with Asp\textsuperscript{44}, thereby stabilizing the H\textsubscript{9251}-H\textsubscript{9251} loop (Fig. 1E). This reorientation of the Tyr\textsuperscript{75} and the subsequent stabilization of the H\textsubscript{9251}-H\textsubscript{9251} loop in a closed conformation might possibly function to allow substrate specificity and binding.

Flavonoids are potential substrates of PaMTH1. Previous biochemical functional assays tested several phenolic putative PaMTH1 substrates and showed that the flavonoid myricetin was the best recognized substrate (28). However, the biophysical and structural evidence for methylation by PaMTH1 remains unknown. Three putative flavonoids (apigenin, kaempferol, and myricetin) and pyrocatechol were chosen for characterization of their interaction with PaMTH1 by NMR. Solubility of a compound is one of the essential factors for interaction studies. One-dimensional NMR-based solubility analysis of the chosen compounds in the interaction buffer revealed that two compounds (apigenin and kaempferol) were insoluble under the conditions of the experiment, whereas myricetin and pyrocatechol were soluble (data not shown). \textsuperscript{1}H,\textsuperscript{15}N HSQC spectra of PaMTH1 in the presence and absence of myricetin or pyrocatechol show that the addition of the ligand induces both CSPs and disappearance of a subset of NMR signals, indicating specific binding (Fig. 3, C and D) even in the absence of co-factor.

Cation-dependent Methylation of Substrate by PaMTH1—Previously, based on the sequence and the size of PaMTH1 (27 kDa), it had been suggested that the protein belongs to the small cation-dependent class I and not to the cation-independent class II with a molecular mass of about 40 kDa (28). Sequence comparison shows that the residues Asp\textsuperscript{144}, Lys\textsuperscript{147}, Asp\textsuperscript{172}, and Asn\textsuperscript{173}, which are important for divalent cation binding, are
highly conserved in PaMTH1. Cation binding is important for substrate binding and orientation for the transmethylation reaction. We investigated the cation dependence of the myricetin O-methylation catalyzed by PaMTH1 using NMR spectroscopy. The protons of the methyl group bound to the oxygen of an aromatic ring (methoxy) resonate around at ~4 ppm, whereas protons of aliphatic methyl groups typically are found at ~1 ppm. SAM acts as a methyl group donor required for methylation of the substrate. In a proton one-dimensional NMR spectrum of SAM, the thiomethyl group appears as a singlet peak at 2.98 ppm (Fig. 3A, inset). We then took advantage of the typical chemical shifts of methyl/methoxy groups and based our analysis on the fact that the catalytic transfer of a methyl group from SAM to the aromatic hydroxyl oxygen of the substrate will result in a decrease in the intensity of the thiomethyl signal of SAM and the appearance of the methoxy methyl signal of the product at distinct chemical shifts.

To determine the metal requirements of the myricetin methylation reaction, we investigated the effect of the presence and absence of Mg2+ and Ca2+ ion on the catalytic activity of PaMTH1. The one-dimensional 1H NMR spectrum of a reaction mixture of SAM, substrate (myricetin), and PaMTH1 shows that in the absence of cations, the intensity of the methyl group signal of SAM remains constant even after 8 h, indicating no catalysis (Fig. 4A). In the presence of Mg2+, we observe a fast decrease in the intensity of the methyl group signal, indicating catalysis of the methyl transfer reaction (Fig. 4B). In the presence of Ca2+, the enzymatic activity is significantly slowed down (Fig. 4C). This strongly suggests that PaMTH1 is a divalent cation (Mg2+)-dependent methyltransferase. The influence of the Mg2+ ion on the catalytic activity of PaMTH1 may be directly associated with the enhanced ability of the enzyme to form a more stable complex with myricetin (see also below). Although pyrocatechol binds to PaMTH1 (Fig. 3D), even in the absence of Mg2+, the enzyme does not catalyze its methylation even in the presence of Mg2+ (Fig. 4D), suggesting that flavonoids are the preferred substrates.

During enzymatic methyl group transfer to myricetin, SAM donates the methyl group and is converted to SAH. Comparison of the aromatic region of the one-dimensional 1H NMR spectrum reveals a decrease in the signal intensity of the adenine protons of SAM and a simultaneous increase in the signal intensity for SAH adenine protons, confirming the formation of SAH (Fig. 5A). It should also be possible to directly detect the formation of methoxymyricetin by inspecting the spectral region at ~4 ppm typical for the methoxy signal. However, due to significant overlap with protein signals in this region, a methoxy signal could not be resolved. Upon removal of the protein by heat denaturation, however, a clear signal for the methoxy protons could be resolved at 3.93 ppm (Fig. 5B, inset), confirming the formation of methoxymyricetin. Further, using 13C,15N-labeled SAM, we were able to detect and confirm the formation of methoxymyricetin by measuring multiplicity-edited two-dimensional 1H,13C HSQC spectra in which the methyl group signals are inverted in comparison with CH and CH3 signals. Only one clear cross-peak (H = 3.93 and 13C = 58.9 ppm) for the methoxy group was detected, indicating the specific methylation of a single hydroxyl group of myricetin (Fig. 5B). Further, mass spectrometry analysis of the reaction mixture confirmed that myricetin is monomethylated (Fig. 5C). However, several attempts to isolate methoxymyricetin by HPLC and characterize the position of methylation were unsuccessful, presumably due to its low stability.

Feedback Inhibition of PaMTH1 by SAH—SAH, the by-product of methionine transmethylation, acts as feedback inhibitor of many SAM-dependent MTs and plays a role in the control of the overall methyl transfer rates (52). The time-dependent one-dimensional 1H NMR spectra recorded for a reaction mixture of SAM, substrate (myricetin), and PaMTH1-SAH (PaMTH1 saturated with SAH, 1:20 ratio) shows a significant decrease in the reaction rate as monitored by the decrease in intensity of the thiomethyl group NMR signal of SAM. This result demonstrates that SAH saturation of PaMTH1 (Fig. 6A) significantly reduced the catalytic efficiency compared with free PaMTH1 (Fig. 5B). Further, the 1H,15N HSQC spectrum of PaMTH1 bound to SAH shows no changes upon the addition of SAM, indicating that SAH has a higher binding affinity to PaMTH1 compared with SAM (Fig. 6B). We then used isothermal titration calorimetry to determine the binding affinities of SAM and SAH to PaMTH1, respectively. In line with the NMR results, ITC data show that SAH binds (KD = 3.2 μM) to PaMTH1.
>30-fold tighter than SAM ($K_D = 113 \mu M$). Additionally, the presence of Mg$^{2+}$ enhances the binding affinity ~10-fold for both SAM ($K_D = 21 \mu M$) and SAH ($K_D = 0.35 \mu M$). The $K_D$ values obtained from $^1H, ^15N$ HSQC-based NMR titration experiments are in good agreement with the ITC measurements (Fig. 7 and Table 2).

**Structural Comparisons with Other O-MTs**—The unavailability of structural information for the ternary complex of PaMTH1 bound to co-factor (SAM) and the substrate hinders a complete description of the binding pocket involved in catalysis. Instead, we used sequence homology and the Dali server to compare PaMTH1 with the structures of other O-MTs with known function.

PaMTH1 presents significant homology to various MTs. A DALI (53) search for three-dimensional structure similarity resulted in 40 structures with $Z$ scores greater than 15. The first five structures with the highest $Z$ scores are from bacterial O-MTs with $Z$ scores ranging from 26.2 to 25.7, followed by CCoAOMT (25.6) from the plant M. sativa and human COMT with 24.3 (Table 3). It is interesting to note that most of the
The closest bacterial structural homologues are involved in antibiotic synthesis pathways, whereas the plant CCoAOMT and human-COMT are involved in lignin biosynthesis and the metabolism of catecholamine neurotransmitters, respectively. A superposition of each of the closest homologue structures with a monomer of PaMTH1 revealed an overlap with root mean square deviations (RMSDs) ranging from 1.3 to 1.8 Å for Cα atoms (RMSDs generated using PyMOL (54)). ClustalW-based multiple-sequence alignment of the amino acid sequence shows that PaMTH1 contains eight motifs (motifs I–VIII) with a high degree of identity to the corresponding stretches of plant CCoAOMT, bacterial LiOMT, rat COMT, and human COMT (Fig. 2). The individual motifs in a tertiary fold together form the defining feature of the bipartite active site to accommodate SAM and in addition Mg$^{2+}$ that can bind catechol substrates. Motifs I–V are mainly involved in SAM binding. Asp$^{144}$, Lys$^{147}$, Asp$^{172}$, and Asn$^{173}$ are important for divalent cation binding, which in turn is required for substrate binding and hydroxyl orientation before the transmethylation reaction of some O-MTs. All four residues are highly conserved in PaMTH1 and structurally adopt a conformation similar to that of CCoAOMT and human COMT, suggesting that PaMTH1 might share a similar metal-dependent catalytic mechanism (Fig. 1E). Mutation of these residues (K147A and double mutants D144A/K147A and D172A/N173A) either abrogates or slows down the transmethylation reaction, suggesting a crucial role in catalysis (Fig. 8).

In the substrate binding region, conformational differences in the α2–α3, β5–α9, and β6–β7 loops play an important role in mediating divalent cation and substrate binding at least in rat and mouse COMT (55). Further, conformational changes in the α2–α3 loop upon ligand binding reposition the hydrophobic side chain of a conserved methionine (within the loop) onto the aromatic ring of the catechol and shield the Mg$^{2+}$ binding site, displaying a critical role during catalysis, at least in COMT. In PaMTH1, both apo- and holoenzyme superpose well with each other with an RMSD of 0.42 Å calculated over all Cα atoms excluding the α2–α3 loop region, which shows an RMSD of 1.7 Å (PaMTH1-SAM) and 2.3 Å (PaMTH1-SAH), respectively. The conformation of the α2–α3 loop is not dominated by crystal contacts. Additionally, the side chain of the Met$^{48}$ in SAM/SAH-bound PaMTH1 is swung 5.4 Å into the active site (“closed”), whereas it is solvent-exposed in the apoenzyme (“open”) (Fig. 1F). The two conformational states observed for Met$^{48}$ indicate that the α2–α3 loop in PaMTH1 could play a similar role as observed in rat and mouse COMT and could mediate substrate binding or specificity.

Discussion

Small molecule natural product MTs play an important role in modulating diverse biological processes, such as cell signaling and biosynthesis. Most of the natural product MTs belong to two (Class I and Class III) of 15 currently known protein fold superfamilies of SAM-binding proteins (25, 56). Depending on the substrate atom accepting the methyl group, O-directed MTs (O-MTs) are the most abundant natural product MTs found across all kingdoms of life. Many of these O-MTs belong to the Class I or Rossmann-like fold family. Subtle modifications of this conserved fold are responsible for the wide variety in substrate selectivity and specificity. The explicit selectivity is achieved through the movement of the loops decorating the conserved core together with the side chain variation in the substrate binding region, leading to reconfiguration of the active site surface. Additionally, structural elaborations in the N-terminal region modulate the dimerization interface, thereby introducing diversity in the active site topology. In certain bacteria and plants, the O-MT subfamily has undergone significant functional and genetic expansion to accommodate a wide variety of substrates. Certain plant MTs, such as caffeic acid O-MT and CCoAOMT, show promiscuity in substrate selection, whereas most other plant O-MTs act on unique substrates. However, in humans, only two O-MTs (catechol O-MT and N-acetyl-serotonin O-MT) are found, which are involved in neurotransmitter metabolism and melatonin biosynthesis, respectively.
FIGURE 7. Binding of SAM/SAH to PaMTH1. A, isothermal titration calorimetry. The top panel of each graph shows the raw heat changes with each injection as a function of time. The bottom panel shows the recalculated enthalpies per injection versus the molar ratio of ligand (squares). The data were fitted (lines) to a single-site binding model using the MicroCal Origin software package. The $K_D$ value of each interaction is indicated. Left panels, PaMTH1 + SAM without Mg$^{2+}$ (top) and PaMTH1 + SAM with 10 mM Mg$^{2+}$ (bottom). Right panels, PaMTH1 + SAH without Mg$^{2+}$ (top) and PaMTH1 + SAH with 10 mM Mg$^{2+}$ (bottom). B, two-dimensional NMR-based titration. Binding curve fits for titrations using SAM (left) and SAH (right), in which combined chemical shift perturbations are plotted against increasing ligand (SAM and SAH) concentration. Representative peak spectra for one of the binding curves (B) are shown above (B). All spectra were acquired at 800 MHz ($T = 298$ K) in the following buffer: 25 mM Tris, pH 6.5, 100 mM NaCl, 5 mM TCEP, 10% D$_2$O, and 1 mM TSP-d$_4$. 

Structure and Biophysical Characterization of PaMTH1
PaMTH1 shows significant sequence identity to other known O-MTs (bacterial LiOMT, alfalfa CCoAOMT, rat COMT, and human COMT). The highest degree of sequence identity is found for the stretches involved in the co-factor SAM-binding amino acids and shows subtle differences in the substrate binding regions. Further, a Dali search for structural homology identified 10 structures with a Z-score greater than 24. All 10 structures belonged to cation-dependent O-MTs and can be superimposed to PaMTH1 with an RMSD of less than 3 Å. Sequence and structural comparison demonstrates that a high degree of structural similarity is observed for the SAM-binding region in many species across all kingdoms of life, despite differences in the substrate specificity. Although the overall fold of the protein is conserved, the divergence between bacterial, plant, and animal MTs results in altered oligomerization states, different substrate recognition modes, or even altered catalytic mechanism of methyl transfer. PaMTH1 exists as a dimer both in solution and crystals. An SEC analysis shows that in the presence of putative substrate (myricetin) and co-factor (SAM), the oligomeric state of PaMTH1 is not altered that in the presence of putative substrate (myricetin) and co-factor (SAM), the oligomeric state of PaMTH1 is not altered as a dimer both in solution and crystals. An SEC analysis shows that in the presence of putative substrate (myricetin) and co-factor (SAM), the oligomeric state of PaMTH1 is not altered as a dimer both in solution and crystals. An SEC analysis shows that in the presence of putative substrate (myricetin) and co-factor (SAM), the oligomeric state of PaMTH1 is not altered as a dimer both in solution and crystals. 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Structure and Biophysical Characterization of PaMTH1

PaMTH1 secondary structure

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PaMTH1_FUNGI  LOMT_BACTERIA(28%)  LOMT_PROTEIN(2%)  LOMT_HUMAN(20%)  LOMT_YEAST(20%)  COMT_RAT(22%)  COMT_HUMAN(20%)  CCoAOMT (dark gray), and CCoAOMT (light gray) with the capping loop from PaMTH1 shown in dark blue. Top, sequence alignment of the amino acid sequences of PaMTH1, LiOMT, CCoAOMT, COMT_RAT, and COMT_HUMAN with the amino acids forming the capping loop highlighted with a blue box.
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forming a unique dimerization interface. The side-chain Ne of Trp<sup>188</sup> (chain B) in the capping loop hydrogen-bonds with the carbonyl oxygen of the Pro<sup>7</sup> (chain A) on the N terminus of the dyad-related molecule and stabilizes the interaction interface. Both mutation of key N-terminal residues (S4A/P7A double mutant) and the N-terminal deletion mutant (Δ1–10 PaMTH1) considerably slow down the catalysis, suggesting a definitive role in substrate binding (Fig. 8). These observations suggest that the capping loop and the residues therein might play a critical role in substrate selection and binding. Insertions to the common core Rossmann fold in the structures of MTs seem to be an evolutionary way of imparting unique features necessary for substrate selectivity (57, 58). Furthermore, the conformational flexibility within the residues of the α2-α3 loop and in particular a well conserved methionine whose side chain sequesters aromatic moieties of the substrates at least in other MTs (55) could also play a similar role in PaMTH1.

The natural in vivo substrate of PaMTH1 is unknown. However, previous studies suggested that PaMTH1 specifically methylates flavonoids and not 1,3,4-dihydroxyphenylalanine, a substrate of mammalian COMT (28). In this study, we have given direct biophysical evidence and show by NMR and mass spectrometry that PaMTH1 binds to the co-factor (SAM) and methylates the flavonoid myricetin in a cation-dependent manner. Taking into account the three-dimensional structure of PaMTH1, we suggest a catalytic mechanism; the side chains of conserved residues Asp<sup>144</sup>, Asp<sup>172</sup>, and Asn<sup>173</sup> and the hydroxyl groups of the flavonoid coordinate the Mg<sup>2+</sup>. Mg<sup>2+</sup> probably acts as weak Lewis acid, and during catalysis, the conserved Lys<sup>147</sup> can act as a Brønsted base. Further, the positive charge of Mg<sup>2+</sup> may also result in a decrease of the pK<sub>a</sub> for the Lys<sup>147</sup> side chain, followed by positioning of the side chain amino group of Lys<sup>147</sup> against the hydroxyl group to be methylated resulting in an abstration of a proton and generation of a reactive phenolic oxyanion near the reactive methyl group of SAM. Such a metal-dependent catalytic mechanism of methyl transfer persists in several related MTs (24, 55).

Understanding the aging process and improving the life span of organisms has become a specific aim of many research activities, resulting in significant fundamental advances (59). Aging and several age-related diseases, including cancer, are fuelled by damage to the macromolecules in cells and tissues (60, 61). Studies involving genetically tractable model organisms like the ascomycete <i>Podospora anserina</i>, yeast, <i>Caenorhabditis elegans</i>, <i>Drosophila melanogaster</i>, and mice have shown that specific genes are involved in the regulation of aging (62). PaMTH1 protects <i>P. anserina</i> from oxidative stress during senescence and acts as a longevity assurance factor. Identification of its natural substrates and thus elucidation of the underlying principle mechanisms contributing to life span increase still remains a challenge. To this end, the structures (apo- and holoenzyme) of PaMTH1 and biophysical characterization of its interaction with putative substrates will facilitate future studies involving the identification of endogenous polyphenolic compounds directly involved in metal-induced oxidative stress.

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