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S-adenosylmethionine synthases in plants: Structural characterization of type I and II isoenzymes from *Arabidopsis thaliana* and *Medicago truncatula*

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Abstract

S-adenosylmethionine synthases (MATs) are responsible for production of S-adenosylmethionine, the cofactor essential for various methylation reactions, production of polyamines and phytohormone ethylene, *etc.* Plants have two distinct MAT types (I and II). This work presents the structural analysis of MATs from *Arabidopsis thaliana* (*AtMAT1* and *AtMAT2*, both type I) and *Medicago truncatula* (*MtMAT3a*, type II), which, unlike most MATs from other domains of life, are dimers where three-domain subunits are sandwiched flat with one another. Although MAT types are very similar, their subunits are differently oriented within the dimer. Structural snapshots along the enzymatic reaction reveal the exact conformation of precatalytic methionine in the active site and show a binding niche, characteristic only for plant MATs, that may serve as a lock of the gate loop. Nevertheless, plants, in contrary to mammals, lack the MAT regulatory subunit, and the regulation of plant MAT activity is still puzzling. Our structures open a possibility of an allosteric activity regulation of type I plant MATs by linear compounds, like polyamines, which would tighten the relationship between S-adenosylmethionine and polyamine biosynthesis.

Keywords

AdoMet; Methionine adenosyltransferase; S-adenosylmethionine synthase

1. Introduction

S-adenosylmethionine (SAM) is produced from methionine and adenosine triphosphate (ATP) in a stereospecific reaction catalyzed by S-adenosylmethionine synthases (MATs, EC

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Bartosz Sekula: Conceptualization, Investigation, Methodology, Formal analysis, Validation, Writing - original draft. **Milosz Ruszkowski:** Investigation, Writing - original draft. **Zbigniew Dauter:** Formal analysis, Supervision.

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2020.02.100>.

2.5.1.6), also known as methionine adenosyltransferases. SAM is an important cofactor used as a versatile donor of methyl group in methylation reactions catalyzed by a number of SAM-dependent methyltransferases [1]. In fact, most of the methionine pool in the cell is utilized to produce SAM, which is the second most widely used cofactor after ATP [2].

Methyl group of SAM can be transferred to a variety of molecules, including hormones, neurotransmitters, lipids, proteins and nucleic acids [3]. Methylation rate regulates gene expression and signaling, it keeps cell membranes fluid, and regulates the mobility of membrane receptors [4]. SAM is also important for other crucial metabolic processes, *e.g.* transsulfuration [5] or ethylene and polyamine biosynthesis [6]. SAM, as a precursor of ethylene (a senescence inducer) and polyamines (antisenescence molecules), takes part in the regulation of senescence in plants [7]. Additional role of SAM in plants is the regulation of threonine synthase activity as its allosteric activator [8].

Multiple MAT isoenzymes (usually three or more) in plant genomes likely appeared by duplication, which occurred in a narrow timeframe [9]. Probably, it was caused by the need to introduce various regulation mechanisms or development of new SAM functions in plants, like ethylene production. Plant MATs are localized in nucleus and cytoplasm [10]. *Arabidopsis thaliana* genome encodes four MAT isoforms, *AtMAT1–4* [11], while in *Medicago truncatula* there are 5 isoforms. *AtMAT1* and *AtMAT2* are most similar in sequence and expression patterns. *AtMAT1*, *AtMAT2*, and *AtMAT4* are expressed in all plant organs, while *AtMAT3* is expressed mainly in pollen [12–14] and plays an important role at the early stages of pollen germination [11]. Plant MATs were divided into two types (I and II), distinguished by characteristic amino acid substitutions at specific positions [9,15]. The double *Arabidopsis thaliana* mutant *mat1/2* showed decreased ethylene levels [13], while the *mat4* mutant accumulated less lignin [16]. The loss of *AtMAT4* function was not compensated by other MATs. Altering MAT expression in tobacco resulted in growth of yellow-green leaves or stunned phenotypes [17]. Another aspect of SAM metabolism is the rate of DNA methylation. It was shown in studies on rice that MATs play an essential role in histone and DNA methylation to regulate gene expression related to flowering [10]. Malfunctions in MAT production led to development of pleiotropic phenotypes, including dwarfism, reduced fertility, delayed germination, as well as late flowering.

After decarboxylation to S-adenosylmethioninamine (dc-SAM), SAM exclusively supplies polyamine production with aminopropyl group. The process is catalyzed by aminopropyltransferases, a family of enzymes found in all domains of life that solely rely on dc-SAM as a coenzyme in polyamine extension. Plants have evolved several distinctive features of aminopropyltransferases which predestine them towards specific production of spermine, spermidine, or thermospermine [18]. Polyamine production and methylation rates are strictly connected with SAM/dc-SAM ratio [19]. dc-SAM may act as a competitive inhibitor of methyltransferases, thus limiting transmethylation reactions, including methylation of nucleic acids [20]. Also, changes in the cellular polyamine levels are linked to the degree of DNA methylation and, as a consequence, gene expression [21,22]. Moreover, it has been suggested that polyamines may directly influence MAT activity as well [23].

2. Results and discussion

2.1. Characterization of plant MATs

Within the scope of this manuscript, focused on structural characterization and comparison of plant MATs, we have solved three novel crystal structures of plant MATs from *Arabidopsis thaliana* (*AtMAT1* and *AtMAT2*) and *Medicago truncatula* (*MtMAT3a*). Additionally, we have determined four structures of plant MAT complexes with ligands, which capture different stages of SAM biosynthesis.

Four MAT isoforms are found in *A. thaliana*, whereas *M. truncatula* has five isoforms (Fig. 1). *AtMATs* share over 85% identical residues. *AtMAT1* and *AtMAT2* are most similar (almost 97% identity), while *AtMAT3* is slightly different, and it is 3-residue shorter than other *AtMATs*. *M. truncatula* MATs have the length between 390 and 396 amino acids and the overall sequence identity over 81%. Altogether, *AtMATs* and *MtMATs* share 78% identical positions in their sequences. It was previously proposed that plant MATs group into two MAT types, type I and II [9,15]. We have updated phylogenetic analysis of all available *Viridiplantae* MAT sequences by calculating sequence similarity network (1395 records classified to INTERPRO family IPR022636 [24]) using EFI-EST server [25]. The analysis confirmed that plant genomes encode two separate MAT types (Fig. S1) with type I being 3 times more abundant than type II. *A. thaliana* has three type I MATs (*AtMAT1*, *AtMAT2*, and *AtMAT4*), whereas *AtMAT3* is the only type II isoenzyme in this species. *M. truncatula* also has three MATs of type I (we propose to name *MtMATs* based on the closest relationship to *AtMATs* (*MtMAT1*, *MtMAT2*, and *MtMAT4*). Since *M. truncatula* has two, very similar isoforms of type II MATs (both with the length of 390 residues and over 92% sequence identity with *AtMAT3*) we named them *MtMAT3a* and *MtMAT3b* (Fig. 1).

2.2. Plant MATs are symmetrical homodimers

A subunit of plant MAT is a disk-shaped polypeptide built of three similar intertwined domains related by threefold pseudosymmetry (Fig. 2). Each domain is an α/β two-layer sandwich built of one β sheet (three- or four-stranded) covered on one side by two or three helices. Central part of the molecule is built by a long winding coil which connects the central domain with C-domain. Structurally, subunits of both plant MAT types are very similar to each other with the same architecture and distribution of the secondary structure elements (Fig. 2). The superposed isolated subunits of the type I and type II MATs present R.M.S.D. of Ca atoms no greater than 0.9 Å. All investigated plant MATs are examples of the highly conserved and very characteristic fold for the MAT superfamily [26].

Our crystallographic structures present symmetric and tightly bound MAT dimers formed by the interactions of the residues from β sheets of two monomers (Fig. 3A). In other words, the extended dimer interface is formed by two MAT subunits sandwiched flat with each other, in a way that α -helices are positioned at the outer surface of the dimer and hydrophobic β -sheets create a solvent-inaccessible interface between MAT subunits. However, dimeric assembly of type I and type II MATs is not as similar as single subunits. Although dimers of type I and II MATs are similar at a first glance, superposition of single subunits shows that their dimer mates are mutually rotated by about 13° (Fig. 3B);

the R.M.S.D. of the superposed dimers of both types is greater than 2.5 Å despite high homology of single subunits.

Plant homodimeric MATs are rather unusual members of the MAT family. MAT representatives in other domains of life, like *E. coli* MAT (cMAT) [27] or *Sulfolobus solfataricus* MAT (sMAT) [28], more often occur as homotetramers [26] built of two pairs of tightly connected MAT subunits positioned perpendicularly to each other by interacting central domains. However, the tight pairs of subunits are assembled similarly to plant MAT dimers. Other examples of dimeric MATs are found in distant archaeal MATs [26] and mammalian MATIII isoenzyme [29], which is built of two MAT α 1 (catalytic) subunits. Interestingly, mammalian MATs also assemble into homotetramers [30]. Moreover, they interact with MAT β noncatalytic regulatory subunit forming heterohexamers with the stoichiometry (α)₄(β)₂ [30]. However, human MAT α 2 equilibrates in solution between monomer, dimer, and tetramer [31]. In the case of investigated plant MATs, SEC-SAXS analysis (Fig. 4) and size exclusion chromatography elution profiles did not indicate the existence of MAT multimers other than dimers. The SAXS *ab initio* envelopes of A α MAT1 and A α MAT2 clearly correspond to the crystallographic MAT dimers (Fig. 4). Also, the molecular mass of the plant MATs, based on SAXS curves, is estimated as 81 kDa (A α MAT1) and 85 kDa (A α MAT2), agreeing with the theoretical dimer mass of the MAT constructs (87 kDa).

Three big cavities can be found in the structures of plant MATs. They are formed on the dimer interface between neighboring domains. Two of these cavities, related by the local 2-fold axis, are the active sites responsible for the conversion of ATP and Met to SAM (Fig. 3A) and they are neighboring the central domain. Architecture of the active site is highly conserved in MATs [9]. The third symmetrical cavity between N-domain and C-domain acts in MATs as a regulatory site (see below) and is placed ideally at the dimer 2-fold symmetry axis.

2.3. Active site closes when ligands are bound

Even though the investigated MATs crystallized in a way that the crystals diffracted very well (the structure of unliganded A α MAT1 was determined at nearly atomic resolution), we have struggled to capture MAT complexes with bound ligands in the active site. The crystal packing analysis of the unliganded structures showed that in M α MAT3a and A α MAT1 crystal contacts between neighboring dimers in the crystal lattice precluded the proteins from appropriate conformational changes necessary to fully structuralize the active site, which is required for the catalysis (see below). In M α MAT3a structure the catalytic loop is even involved in the crystal contacts with the symmetry-related molecule.

The active sites of MAT enzymes are occluded by flexible gate regions formed by residues 98–123 (numbering of A α MAT2 sequence). In all unliganded structures this region was not fully defined by the electron density, therefore, it is partially missing in the final models (Fig. 2). This was also the case in the structure of A α MAT1 in complex with 5'-methylthioadenosine (A α MAT1-MTA). When ligands are present in the active site, the gate region folds into two helices (α 4 and η 5) and closes the active site to stabilize substrates during the catalysis (Figs. 5A and S2A). The structure of A α MAT2-AMPCPP visualized the

substrate-bound state of MAT; inside the catalytic site both, precatalytic Met and the ATP analogue, AMPCPP, revealed the exact pre-reaction state of the enzyme (Figs. 5B and S2B).

The main residue from the gate loop that interacts with Met in the catalytic site is Gln99. It creates a hydrogen bond with the carboxyl group of Met, which also makes water-mediated H-bonds with Ser100, G245', Lys277, and G121 (Fig. S2B, an apostrophe denotes residues from the other MAT subunit of the dimer). The amino group of Met interacts through H-bonds with carboxyl groups of Glu56 and Asp246' and forms a water-mediated H-bond with β -phosphate of AMPCPP. Deep inside the active site, phosphates of AMPCPP are bound by the side chain amines of Lys273, Arg252', Lys253', Lys169', and by the main chain amide of Ala269 (Fig. 5B). They are also involved in the coordination of two Mg^{2+} and one K^+ ions (Fig. S2B). Ribose moiety of AMPCPP is positioned close to the Met binding site by the interaction with two aspartates (Asp167' and Asp246'). Asp246' is the only residue that interlocks both MAT substrates (Fig. 5B). Adenine moiety of AMPCPP is π -stacked with the phenyl ring of Phe238'; from the other side it is occluded by $\alpha 4$ from the gate loop, where Ile103 participates in the ligand stabilization (together with Ile310 from C-domain). Adenine moiety is also directly H-bonded with the side chain of Ser235' and carbonyl oxygen of Arg237'; it also creates water-mediated interactions with residues of both MAT subdomains (Fig. 5B), including Ser100 from the gate loop. The closed conformation of the gate loop was also captured with already produced SAM, in the structures of *AtMAT2*-SAM-PPNP and *AtMAT2*-SAM.

2.4. Structures of *AtMAT2* complexes support two-step SAM biosynthesis

The captured snapshots of plant MATs over the course of the catalysis show: (i) the unliganded state of the protein (structures *MtMAT3a*, *AtMAT1*, and *AtMAT2*); (ii) MAT in the partially bound state with MTA (structures *AtMAT1*-MTA); (iii) the fully bound, pre-catalytic state of the enzyme (*AtMAT2*-AMPCPP with bound Met and AMPCPP); (iv) the enzyme after SAM synthesis and triphosphate cleavage (*AtMAT2*-SAM-PPNP with bound SAM and the triphosphate analogue PPNP); (v) MAT complex with SAM after reaction (*AtMAT2*-SAM with bound SAM and two phosphates). It is worth noting that the different catalytic states of MAT were captured using two different ATP analogues, AMPCPP and AMPPNP. The omit electron density maps around the ligands in the active site of presented structures leave no doubt about the conformation and the form of bound ligands (Fig. 6A–E). They fully explain the mechanism of SAM synthesis by plant MATs presenting the following detailed snapshots: (i) formation of the MAT-ATP-Met complex (structure *AtMAT2*-AMPCPP, Fig. 6B), (ii) intermediate state of the event when SAM is already synthesized when only triphosphate is cleaved from ATP (*AtMAT2*-SAM-PPNP, Fig. 6C), and (iii) the state after reaction with captured product, which was synthesized *in vitro* (structure *AtMAT2*-SAM, Fig. 6D).

After the incorporation of ATP and Met, the gate region closes, thereby fully isolating the active site. When the substrates are appropriately positioned, two simultaneous events occur to initialize SAM synthesis which follows by S_N2 displacement mechanism as proposed earlier [32]. The cleavage of C5'-O5' bond of ATP is caused by the action of His15 (which acts as an acid in the reaction); simultaneously C5' of ATP is the target of a nucleophilic

attack by S8 of Met. This leads to the bond formation between Met and C5' of ATP to produce SAM and the release of triphosphate. The reduced pK_a of neutral His15 (with protonated N ϵ) is most likely achieved by its location at the N-terminus of a long α -helix [33]. The fact that we observed non-hydrolyzed AMPCPP and free Met in the structure of *A*MAT2 can be explained as follows: substitution of the bridging oxygen between α -P and β -P of ATP with -CH₂- group changes polarization of the triphosphate of AMPCPP; it leads to insufficient proton attraction by O5' from His15 which limits the electron shift necessary to initialize the C5'-O5' cleavage. The other factor was a relatively short soaking time with Met, which was enough for the ligand diffusion inside the crystal, but insufficient for the reaction turnover.

After being cleaved from ATP, the triphosphate remains in the active site to be hydrolyzed at γ -position by the triphosphatase activity of MAT. This is the second step of MAT turnover. The unusual coordination of two Mg²⁺ cations plus the interactions with Arg252', Lys253', and Lys273, and presence of the K⁺ ion help with accommodation of the characteristic bent conformation of the triphosphate. Additionally, all cationic components attract the negative charge of the triphosphate so that the activated water molecule (initially H-bonded with the carbonyl oxygens of Arg252' and Gly267) could attack the γ -phosphate atom, which, after the charge transfer, leads to release of the γ -P group. After the γ -P release, diphosphate and Pi most likely rotate due to steric disturbances. They push SAM out from the active site and initialize gate region opening. In plant MATs they would present similar conformation to sMAT structure with bound SAM and diphosphate (PDB ID: 4L7I) [28].

The general reaction mechanism is widely accepted for the MAT family, however the initial position of Met before the hydrolysis was uncertain. Altogether, three MAT structures were interpreted with free Met: Rat MAT α 1 (PDB ID: 1O9T) [29], human MAT α 2 (PDB ID: 5A1I) [34], and eMAT (PDB ID: 1P7L) [32]. Structures of both mammalian MATs suggest a highly doubtful substantial conformational changes of the Met sidechain during the reaction from the neighborhood of the bound ions to C5' of ATP. Human MAT α 2 structure (solved at nearly atomic resolution) presents unusual interpretation of the atomic occupancies of ligands which are modeled with a clear disagreement with the electron density. The resolution of Rat MAT is significantly worse and very high temperature factors of Met and ATP in the model (two times higher than the structure average) indicate a high degree of disorder in the active site. Unfortunately, there are no structure factors available for the structure. The third case, where Met position was suggested, is eMAT (PDB ID: 1P7L) structure determined at 2.5 Å. The authors interpreted positive electron density peaks as a kind of "mixed" state where ATP was partially hydrolyzed with also partially synthesized SAM.

In our study, the use of AMPCPP instead of ATP allowed to capture the "clear" precatalytic state, where Met is in position ready to attack C5' of ATP, but before ATP hydrolysis (Fig. 6B). Therefore, the position of Met S8 before and after SAM synthesis is roughly preserved (Fig. S3). Conformational changes concern ribose moiety after the triphosphate cleavage. Met position in *A*MAT2-AMPCPP structure is very similar to that proposed in the structure of eMAT, which suggest that it likely represents the initial conformation for other MAT enzymes as well, including eukaryotic MATs.

2.5. Plant MATs have a binding niche on the surface of the closed gate loop

The gate region of *A*MAT2 stays closed not only when the substrates are bound, it is also closed when SAM is already produced. Most likely, the energy release from triphosphate hydrolysis is the motor for gate opening. However, we clearly observe closed gate with SAM and two Pi molecules bound in the active site (Fig. 6D) instead of pyrophosphate and Pi. This structure was obtained by cocrystallization of *A*MAT2 with ATP and Met in order to perform *in vitro* SAM synthesis. The fact that we observe two single Pi molecules suggests that SAM was synthesized before crystallization. Activity of MAT enzymes is known to be regulated by product (SAM) inhibition [35,36]. Our structure suggests that SAM is a competitive inhibitor of plant MATs, as well.

Interestingly, when the gate loop is closed, a positively charged region is created in a form of a small niche (Fig. S4A) on the surface of *A*MAT2. We have observed 3-morpholinopropane-1-sulfonic acid (MOPS) molecule (one of the buffer components) bound inside this niche (Fig. 5A) in all structures with structuralized gate region. MOPS molecule penetrates this site with its sulfate moiety which interacts with Lys39 and Lys355. In other MAT structures deposited in the PDB (with ordered gate loop) there is no trace of such niche nor the positive charge accumulation outside the gate region (Fig. S4B–E). The comparison of the vicinity of the niche in *A*MAT2 and human MAT α 2 highlights two Pro residues (conserved in plants) in position 115 and 275 of *A*MAT2 (instead of Glu and Tyr in human MAT α 2). These two residues with relatively small sidechains significantly change this area; they open the space to Lys39, Lys355 and His108, which are responsible for the positive potential of the niche and, most likely, its binding properties.

The function of this niche in plant MATs could be similar to the SAM binding site in *Ureaplasma urealiticum* MAT [37] where SAM is bound outside the catalytic site (at the interface of MAT subunits) and blocks the active-site loop. In fact, plant MATs show a much higher conservation of the gate loop than MATs from other domains of life. In *A*MATs and *M*MATs only 5 out of 26 positions in the gate loop are variable (Fig. 1), however most of the substitutions preserve amino acid character. The only striking difference is Cys114, which is present only in *A*MAT1 instead of Arg or Lys in the same position in other compared MATs. It was shown that Cys114 may be S-nitrosylated [38], which bluntly inhibits *A*MAT1, while other *A*MATs stayed insensitive to nitrosylation. Now, when the structure of *A*MAT2 is known, it becomes clearer how S-nitrosylated Cys114 inhibits the enzyme. Modified Cys114 would interact with the surrounding residues in the similar way as the sulfate moiety of bound MOPS, locking the gate loop in a closed conformation. The physiological “key” to this lock in other plant MATs remains unknown. Architecture of this site and a close relationship of SAM synthesis to epigenetics and ethylene production bring to mind 1-aminocyclopropane-1-carboxylate, the byproduct of SAM breakdown in ethylene pathway, as a potential ligand. This hypothesis needs of course future experimental verification. However, the structures open a possibility for the design of selective activity modulators (specific only for plant MATs), which could be used for future herbicide design. This seems even more attractive when we consider the unique features of this niche that distinguish plant MATs from other kingdoms of life.

2.6. MAT regulatory site

Another big cavity in the MAT structure, which also exhibits binding properties, is placed between N-domain and C-domain (Fig. 3A). It is responsible for binding of the regulatory subunit MAT β [30] in human (PDB ID: 4NDN) and other mammalian MATs. It was shown that the association of α and β subunits changes kinetic properties of MAT enzymes [39]. It is also the binding site of tricyclic benzodiazepine analog (PF-9366, PDB ID: 5UGH) which acts as an allosteric inhibitor of human MAT α 2 [31]. In our structures, *A*MAT2-SAM-PPNP and *A*MAT2-SAM, which present the bound state of the enzyme with SAM bound inside the catalytic venue, we observed that the regulatory site incorporated a long 8-mer of PEG, which occupies the analogical site as MAT β and PF-9366 in human MAT α 2 (Fig. 7A). In our structures, PEG adopts a horseshoe-like conformation within a symmetrical and rather hydrophobic cavity, where it is H-bonded with carbonyl oxygen of Gly261 and Gly261' (Fig. 7B).

The regulatory site is accessible for ligands in various MATs, regardless of their oligomeric assembly. By comparing plant MATs and human MAT α 2 (Fig. 7B) two striking differences arise, Asp321 and Phe319 of *A*MAT2 are in human MAT α 2 replaced with Phe333 and Ser331, respectively. Looking at the regulatory site of human MAT α 2, three Phe residues (Phe333, Phe20, Phe18, sequence positions of human MAT α 2) seem to be important for the stable interaction with ligands [31]. In *A*MAT2, only two of them are the same; the residue corresponding to Phe333 is replaced with Asp321 in *A*MAT2. Such a significant substitution at the entrance of the cavity from a bulky hydrophobic residue to a negatively charged acidic residue, substantially changes the entrance to this site (Fig. S5), which likely impacts the binding properties as well. The other mentioned difference (substitution of Phe319 in *A*MAT2 to Ser331 in human MAT α 2) limits the boundaries of the cavity in *A*MAT2, which in practice would create a steric clash in the interactions with ligands such as PF-9366. Plant genomes, contrastingly to mammals, do not encode regulatory β subunits [26], therefore a binding counterpart of the regulatory cavity in plants remains unknown.

The presence of 8-mer of PEG in *A*MAT2, well defined in the electron density maps (Fig. 7A), is not a usual case in protein structures, which might suggest a relatively strong interaction between PEG and the protein. In our recent study on aminopropyltransferases [40], we have also observed a bound short PEG fragment inside the polyamine groove of spermidine synthase active site. Hydroxyl group of PEG in the polyamine groove of spermidine synthase was bound in the same position as the amine group of polyamine substrate. Therefore, it seems plausible that the carbonyl oxygen of Gly261 in *A*MAT2 may serve as an acceptor of the hydrogen bond with the physiological ligands that regulate MAT activity in plants. The other possible acceptors of the hydrogen bonds might be Asp321 (residue which differs plant MATs from human MAT), Asp330, or Asn178. Therefore, the cavity seems to be well suited to accommodate linear compounds with multiple hydrogen bond donors, which could be symmetrically bound in the regulatory site. Since polyamines are known to allosterically regulate the activity of various proteins [41,42] and they structurally and chemically fit to this cavity, the close relationship between SAM synthesis and polyamine metabolism brings these linear polycations as potential regulators of plant MATs. To test this hypothesis, we have performed isothermal titration of *A*MAT1

with spermidine (Fig. S6). The experiment indicated a single spermidine binding site per *AtMAT1* subunit (two per dimer). Obtained dissociation constant, K_d of $654 \text{ nM} \pm 256 \text{ nM}$ has a relatively large error which can be attributed to low heat effect or a cooperativity effect.

3. Conclusions

The structures of plant MATs presented in this work support the two-step mechanism of SAM biosynthesis in plants and reveal the initial position of precatalytic Met inside the active site, ruling out its substantial conformational shift proposed earlier. Also, the structures highlight two binding sites which may play essential role in the activity regulation of these enzymes in plants. Having that in mind, the existence of multiple MAT isoforms with similar properties may reflect specificity of MAT isoforms not in kinetics, but in the association with enzymes that use SAM [15]. Moreover, sometimes even small changes in sequence and structure of an enzyme can modify its allosteric sites to acquire novel metabolic or regulatory functions [43]. Now, the structures of plant MATs being available, the question about MAT specialization may be more rationally addressed by thorough functional studies in the future. Such information could shed more light on different functions of MAT isoforms in epigenetics, ethylene production, or polyamine biosynthesis.

4. Materials and methods

4.1. Cloning, overexpression, and purification of plant MATs

Complementary DNA (cDNA) of *M. truncatula* and *A. thaliana* was obtained with the use of SuperScript II reverse transcriptase (Life Technologies), as well as oligo dT (15 and 18) primers and total RNA isolated from leaves with an RNeasy Plant Mini Kit (Qiagen). This protocol was also successfully used by us to clone other plant proteins [44,45]. The open reading frames of *MtMAT3a*, *AtMAT1*, and *AtMAT2*, were cloned by a ligase-independent cloning [46] protocol into pMCSG68 vector (Midwest Center for Structural Genomics). The vector is constructed in a way that the expressed target proteins carry the N-terminal His₆-tag followed by the tobacco etch virus (TEV) protease cleavage site. The His₆-tag cleavage leaves three residues at the N-termini of the proteins: Ser, Asn and Ala.

BL21 Gold *Escherichia coli* competent cells (Agilent Technologies) were transformed with the vectors with appropriate MAT genes and then were cultured at 37 °C in lysogeny broth medium with 150 µg/ml ampicillin until OD₆₀₀ reached value 1.0. Then, the cultures were cooled to 10 °C for 1 h before the induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside. The overexpression was carried out at 18 °C for 16 h and then the cultures were cooled to 4 °C. Cells were pelleted by centrifugation at 3500 ×g for 20 min and then suspended in 35 ml of the binding buffer (50 mM HEPES pH 7.5; 500 mM NaCl; 20 mM imidazole; 1 mM tris(2-carboxyethyl)phosphine, TCEP), and frozen at –80 °C. Isolation of the protein started with sonication of the cells in an ice/water bath. The sonication was carried out with four-second bursts every 30 s; total sonication time was 4 min. In the next step the cell debris was removed by centrifugation at 25,000 ×g for 30 min at 4 °C and the soluble fraction was decanted to the column packed with 5 ml of HisTrap HP resin (GE Healthcare) connected to Vac-Man (Promega). The resin was then

washed five times with 40 ml of the binding buffer. The proteins were eluted with 20 ml of elution buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 400 mM imidazole, 1 mM TCEP) and subjected to the His₆-tag cleavage by the His₆-tagged TEV protease at the concentration of 0.1 mg/ml. This step was carried out simultaneously with the overnight dialysis at 4 °C against the buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 1 mM TCEP. Then, the samples were applied on the fresh HisTrap HP resin in order to separate them from the cleaved His₆-tag and His₆-tagged TEV protease. The final step of the purification procedure concerned the size exclusion chromatography on a HiLoad Superdex 200 16/60 column (GE Healthcare) connected to the AKTA FPLC system (Amersham Biosciences). The running buffer was: 50 mM HEPES pH 7.5, 100 mM KCl, 50 mM NaCl, 1 mM TCEP. The proteins were concentrated with Amicon concentrators (Millipore) to the final concentration of 19 mg/ml (*M*MAT3a), 10 mg/ml (*A*MAT1), and 18 mg/ml (*A*MAT2). The concentration was determined by the absorbance measurement at 280 nm using sequence-based extinction coefficients calculated in ProtParam [47]. The concentrated protein samples were used for crystallization or stored in −80 °C for later use.

4.2. Crystallization and data collection

All protein samples were supplemented with 30–50 mM of MgCl₂ prior to the crystallization setup. Proteins were crystallized with a sitting drop method using the following crystallization screens: Index (Hampton Research), BCS, and Morpheus (both Molecular Dimensions). Afterwards, when the initial crystals appeared, the crystallization was carried with a hanging drop method in the following conditions: *M*MAT3a (0.01 M CoCl₂, 0.2 M MgCl₂, 0.1 M Bis-Tris propane at pH 8.0, 6.25% PEG 3350, 6.25% PEG 4000, 6.25% PEG 2000, 6.25% mmPEG 5000); *A*MAT1 [1.8 M (NH₄)₂SO₄, 0.08 M Bis-Tris at pH 5.5]; *A*MAT2 [0.12 M Alcohols (1,6-hexanediol; 1-butanol; 1,2-propanediol; 2-propanol; 1,4-butanediol; 1,3-propanediol), 0.1 M HEPES and MOPS buffer at pH 7.5, 20% mmPEG500, 10% PEG 20000]. Crystals were cryoprotected as follows: 25% ethylene glycol (*M*MAT3a), 25% glycerol (*A*MAT1), and 10% mmPEG550 (*A*MAT2).

Complex of *A*MAT1 with 5'-methylthioadenosine (*A*MAT1-MTA) was obtained by cocrystallization of *A*MAT1 with MTA (10 mM). The complex of *A*MAT2 with bound SAM was obtained by cocrystallization of the protein with 10 mM Met and ATP. The complex of *A*MAT2 with bound SAM and (diphosphono)aminophosphonic acid (PPNP) was obtained by cocrystallization of *A*MAT2 with Met and adenosine 5'-(β,γ-imido)triphosphate (AMPPNP), both at 10 mM concentration. In both complexes SAM was synthesized *in vitro* with the simultaneous hydrolysis of ATP and AMPPNP, respectively. The complex of *A*MAT2 with bound α,β-methyleneadenosine 5'-triphosphate (AMPCPP) and Met was obtained by consecutive soaking of unliganded *A*MAT2 crystals with ligands. In details, AMPCPP (10 mM) was first added to the crystallization drop with *A*MAT2 crystals. After 25 min, couple of crystals were transferred for 2 min to the drop containing crystallization buffers supplemented with 10 mM Met and 10% mmPEG550 as cryoprotectant.

The diffraction data were collected at the SER-CAT 22-ID and SBC 19-ID beamlines at the Advanced Photon Source (APS), Argonne National Laboratory, USA. *XDS* [48] and

HKL-3000 [49] were used for data processing and scaling. Details of the data collection and data statistics are presented in Table 1.

4.3. Structure determination and refinement

The first determined structure was *MtMAT3a*. It was solved by molecular replacement in *Phaser* [50] using the structure of human MAT2A (PDB ID: 5A1I) [34] as a search model. Then, the model was re-built in *PHENIX AutoBuild* [51] and refined in *Coot* [52] and *Refmac* [53]. The refined model of *MtMAT3a* was used for the determination of the structures of *AtMAT1* and *AtMAT2*. The structures of unliganded *AtMAT1* and *AtMAT2* were used as initial models for the refinement of the structures with ligands.

TLS parameters [54,55] were applied at the later stages of the structure refinement. All ligands were refined using standard CCP4 libraries [56]. The quality of refined structures was controlled by R_{work} , R_{free} factors [57] and geometric parameters. *PROCHECK* [58] and *MolProbity* [59] were used for evaluation of the final models. The final refinement statistics are given in Table 1.

Inspection of the electron density of *AtMAT2*-SAM and *AtMAT2*-SAM-PPNP structures showed that the crystallized protein carries the mutation (caused by deletion close to the stop codon), which was confirmed in the second plasmid sequencing. Therefore, crystallized *AtMAT2* has five additional residues on the C-terminus. Unfortunately, crystallization trials of the protein with original sequence did not succeed with well-diffracting crystals.

4.4. Small-angle X-ray scattering measurement

SAXS experiments with in-line size exclusion chromatography (SEC-SAXS) were conducted at the BioCAT 18-ID beamline [60] at the APS. The concentration of *AtMAT1* and *AtMAT2* was 5 mg/ml and 7.5 mg/ml, respectively. Samples were additionally purified before the SAXS analysis on a WTC-015S5 column (Wyatt Technologies) connected to an Infinity II HPLC (Agilent Technologies) in order to remove aggregates from the sample. Directly after the size-exclusion chromatography, samples were analyzed with the Agilent UV detector, a Multi-Angle Light Scattering (MALS) detector, a Dynamic Light Scattering (DLS) detector (DAWN Helios II, Wyatt Technologies), and an RI detector (Optilab T-rEX, Wyatt). MALS and DLS data were used to calculate molecular weights and hydrodynamic radii, respectively, using the ASTRA 7 software (Wyatt). Afterward, the sample was sent to the SAXS flow cell, a 1.5 mm quartz capillary. Scattering data was collected at 1.03 Å wavelength at room temperature, with 0.5-second exposures every 2 s on a Pilatus3 1 M detector (Dectris) placed 3.5 m from the capillary (collected q -range was 0.007–0.36 Å⁻¹). Data reduction and analysis were performed by *BioXTAS RAW* 1.5.1 [61]. Several frames corresponding to the elution peak of the chromatogram were averaged to increase the signal-to-noise ratio. In order to obtain the final SAXS curves (Fig. 4), we have subtracted the buffer signal (averaged frames immediately proximal to the peak) from the sample scattering. The R_g values calculated from the Guinier and distance distribution analysis (Fig. 4) were 26.5 Å (*AtMAT1*) and 26.8 Å (*AtMAT2*). The calculated maximum dimensions of the particles (D_{max}) were 90 Å (*AtMAT1*) and 86 Å (*AtMAT2*). The qR_g limits for further calculations were 0.20–1.30. *Ab initio* envelopes with twofold symmetry restraint

were calculated in *DAMMIF* [62], averaged with *DAMAVAR* [63], refined with *DAMMIN* [64], and filtered with *DAMFILT*.

4.5. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) titration was carried out at 30 °C using a VP-ITC microcalorimeter (Micro Cal Inc., Northampton, MA) in the running buffer. The concentration of *AMAT1* was 35 μ M and the concentration of spermidine was 0.6 mM. In all experiments, volume of the first injection into the perfusion vessel was 2 μ l; the following injections of 10 μ l were applied with 190 s intervals upon constant mixing. The ITC data were analyzed with Origin 7.0. The parameters such as stoichiometry (n), association constant (K_a), and the changes in the enthalpy (ΔH) and entropy (ΔS) during the complexation reaction were determined (Fig. S6).

4.6. Other software used

Molecular illustrations were created with UCSF *Chimera* [65]. Ramachandran plot was calculated in *RamPage* [66]. The secondary structure was recognized with ProMotif [67] within the PDBsum server [68]. Sequence alignments were performed in CLUSTAL W [69] and MUSCLE [70] and edited in BioEdit [71]. Sequence similarity network was generated using EFI-ESN webserver [25]. After filtering, 775 unique sequences from *Viridiplantae* between 370 and 430 residues long were analyzed (alignment score of 210). The graph was created in Cytoscape 3.3 [72].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The coordinates and structure factors of the structures described in this work were deposited in the Protein Data Bank under the following accession codes: 6VCW, 6VCX, 6VCY, 6VCZ, 6VD0, 6VD1, 6VD2.

Abbreviations:

ATP

adenosine triphosphate

At

Arabidopsis thaliana

cDNA

complementary DNA

PPNP

(diphosphono)aminophosphonic acid

cMAT

Escherichia coli S-adenosylmethionine synthase

AMPPNP

5'-(β,γ -imido)triphosphate

Mt

Medicago truncatula

MTA

5'-methylthioadenosine

AMPCPP

α,β -methyleneadenosine 5'-triphosphate

MOPS

3-morpholinopropane-1-sulfonic acid

PEG

polyethylene glycol

dc-SAM

S-adenosylmethioninamine

SAM

S-adenosylmethionine

MAT

S-adenosylmethionine synthase

sMAT

Sulfolobus solfataricus S-adenosylmethionine synthase

TEV

tobacco etch virus

TCEP

tris(2-carboxyethyl)phosphine

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Fig. 1.

Sequence alignment of A-MATs and M-MATs. The following sequences were used: A-MAT1 (At1g02500, UniProt ID: P23686), A-MAT2 (At4g01850, UniProt ID: P17562), A-MAT3 (At2g36880, UniProt ID: Q9SJL8), A-MAT4 (At3g17390, UniProt ID: Q9LUT2), M-MAT1 (MTR_7g110310, UniProt ID: A4PU48), M-MAT2 (MTR_4g123810, UniProt ID: G7JQ29), M-MAT3a (MTR_7g102120, UniProt ID: G7L3W1), M-MAT3b (MTR_1g063060, UniProt ID: A0A072VKL5), M-MAT4 (MTR_2g046710, UniProt ID: A0A072V8Q4); sequence position above the alignment refers to the sequence of A-MAT1.

Fig. 2. Architecture of the subunit of plant MAT. The superposition of *MtMAT3a*, *AtMAT1*, and *AtMAT2* subunits from the unliganded MAT structures (PDB IDs: 6VCW, 6VCX, 6VCZ, respectively).

Fig. 3. Biological assembly of plant MATs. (A) the crystallographic dimer of type I plant MAT, *AtMAT2* in the bound state (in complex with SAM and PPNP, PDB ID: 6VD1); (B) illustration of the difference in the subunit orientation within the dimers of type I (*AtMAT2* PDB ID: 6VCZ) and II (*MtMAT3a*, PDB ID: 6VCW) MATs; when single subunits of both proteins are superposed, the other subunits (shown in the figure) display ~13° relative rotation.

Fig. 4. SAXS data for *AtMAT1* (A) and *AtMAT2* (B). The plots present: SAXS experimental curves (top) Guinier plot of the scattering curve (middle) with the best fit (dashed black line), and pair-distance distribution functions (bottom); the *ab initio* SAXS envelopes (mesh representation) superposed with crystallographic MAT dimers are shown on the right of each panel.

Fig. 5. Active site of MAT. (A) Conformation of the gate loop in closed conformation in the *A-MAT2*-AMPCPP structure (PDB ID: 6VD0); (B) interactions of the bound ligands inside the active site of *A-MAT2* before the catalytic event (PDB ID: 6VD0). An apostrophe denotes residues from the other MAT subunit of the dimer.

Fig. 6. Ligands bound in the active site of MAT structures. The polder omit maps at 3.5σ around the ligands and metal ions bound in (A) unliganded *A-MAT2*, PDB ID: 6VCZ, (B) *A-MAT1-MTA*, PDB ID: 6VCY, (C) *A-MAT2-AMPCPP*, PDB ID: 6VD0, (D) *A-MAT2-SAM-PPNP*, PDB ID: 6VD1, (E) *A-MAT2-SAM*, PDB ID: 6VD2.

Fig. 7. Regulatory site. (A) comparison of the conformation of PEG molecule bound in A-MAT2-SAM-PPNP structure (PDB ID: 6VD1) with the N-terminus of the hMATβ2 domain bound to human MATα2 (PDB ID: 4NDN) and PF-9366, allosteric inhibitor of human MATα2, bound to human MATα2 (PDB ID: 5UGH), green mesh represents polder omit maps [73] at 3 σ around the PEG molecule (B) Comparison of the key residues in the allosteric site in A-MAT2-SAM-PPNP structure (top, PDB ID: 6VD1) with the structure of human MATα2-PF-9366 (bottom, PDB ID: 5UGH).

Table 1

Data collection and refinement statistics.

Structure	MMAT3a	AMAT1	AMAT1-MTA	AMAT2	AMAT2-AMPCPP	AMAT2-SAM-PPNP	AMAT2-SAM
Data collection							
Beamline	19-ID	22-BM	22-ID	22-BM	22-BM	22-ID	22-ID
Wavelength (Å)	0.979	1.0	1.0	1.0	1.0	1.0	1.0
Temperature (K)	100	100	100	100	100	100	100
Space group	$C2$	$I2_12_12_1$	$I2_12_12_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Unit cell parameters							
a b c (Å)	111.60 62.04 102.38	57.72 68.10 209.70	58.28 69.16 210.45	62.80 99.28 86.93	101.94 84.72 119.50	61.21 101.48 84.33	61.98 101.35 84.71
β	90.02			109.74	95.67	99.37	99.80
Oscillation range (°)	0.5	0.5	0.5	0.25	0.25	0.25	0.25
Resolution (Å)	48.99–1.40 (1.48–1.40)	48.78–1.10 (1.17–1.10)	49.25–1.82 (1.93–1.82)	42.44–1.52 (1.61–1.52)	39.83–2.00 (2.07–2.00)	47.09–1.32 (1.40–1.32)	45.72–1.97 (2.09–1.97)
Reflections collected/unique	624,301/130,457 (90,554/19,394)	1,182,621/160,169 (182,051/24,752)	139,464/37,699 (22,450/5,980)	635,086/154,106 (98,228/24,808)	438,591/136,621 (32,559/13,511)	778,010/232,535 (126,163/37,677)	229,937/71,629 (38,326/11,496)
Completeness (%)	94.2 (87.0)	96.2 (92.8)	97.8 (97.3)	99.7 (99.3)	99.8 (99.1)	97.8 (98.2)	97.7 (97.4)
Multiplicity	4.79 (4.67)	7.38 (7.36)	3.70 (3.75)	4.12 (3.96)	3.21 (2.41)	3.35 (3.35)	3.21 (3.33)
R_{merge} (%)	6.6 (76.2)	4.9 (35.1)	7.1 (77.4)	5.4 (60.9)	11.6 (37.5)	5.6 (58.5)	6.3 (45.0)
$\langle I/\sigma(I) \rangle$	14.48 (2.39)	20.51 (4.91)	12.53 (1.84)	16.26 (2.15)	8.78 (2.13)	12.45 (2.03)	10.70 (2.60)
Refinement							
R_{free} reflections	1305	1602	1131	1542	2672	1163	1075
No. of atoms (non-H)							
Protein	5918	3148	3003	5988	12,105	6254	6106
Ligands	10	41	49	95	252	191	160
Solvent	966	551	352	1022	1201	1223	616
$R_{\text{work}}/R_{\text{free}}$ (%)	11.5/15.9	11.1/13.5	15.9/20.9	16.0/18.0	22.2/25.1	14.2/17.3	19.1/24.0
Mean ADP ^a (Å ²)	21.0	14.0	31.0	20.3	28.0	17.0	38.0
RMSD from ideal geometry							
Bond lengths (Å)	0.02	0.01	0.02	0.02	0.01	0.01	0.01
Bond angles (°)	1.9	1.8	1.9	2.0	1.7	1.8	2.0
Ramachandran statistics (%)							

Structure	MtMAT3a	AtMAT1	AtMAT1-MTA	AtMAT2	AtMAT2-AMPCPP	AtMAT2-SAM-PPNP	AtMAT2-SAM
Favored	98	98	98	98	97	98	97
Allowed	2	2	2	2	3	2	3
Outliers	0	0	0	0	0	0	0
PDB code	6VCW	6VCX	6VCY	6VCZ	6VD0	6VD1	6VD2

Values in parentheses refer to the highest-resolution shell.

^aADP = atomic displacement parameter.