Folate polyglutamylation eliminates dependence of activity on enzyme concentration in mitochondrial serine hydroxymethyltransferases from *Arabidopsis thaliana*

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Abbreviations used: (6S)-H4PteGlu n, tetrahydrofolate; (6S)-5,10-methenyltetrahydrofolate; (6S)-5-methyltetrahydrofolate; (6S)-5,10-methenyltetrahydrofolate; PteGlu n, 5,10-methyltetrahydrofolate; H4PteGlu n, tetrahydrofolate; EGFP, enhanced green fluorescent protein; Ni–NTA, nickel–nitrilotriacetic acid; PLP, pyridoxal 5′-phosphate; SHMT, serine hydroxymethyltransferase; THP, tris-(3-hydroxypropyl)phosphine.

**Introduction**

Serine hydroxymethyltransferase (SHMT; EC 2.1.2.1) catalyzes the reversible reaction L-serine + (6S)-H4PteGlu (Fig. 1) → glycine + (6S)-5,10-CH2–H4PteGlu [1–5]. In plants, SHMT activity was detected in mitochondria [2,6–9], plastids [2,7,10], the cytosol [2,6,7], and nuclei [7]. The *Arabidopsis thaliana* genome harbors seven genes (*AtSHM1–7*) encoding SHMT isozymes putatively localized in these subcellular compartments [11]. Subcellular localization was confirmed for the isozymes from mitochondria (*AtSHM1* and 2) [12,13] and plastids (*AtSHM3*) [10], but remains to be confirmed for those putatively localized in the cytosol (*AtSHM4* and 5) and nuclei (*AtSHM6* and 7).

In plastids and the cytosol [2,14–16], SHMTs provide one-carbon units to the cellular folate pool by producing (6S)-5,10-CH2–H4PteGlu n, which is then reduced to (6S)-5-CH3–H4PteGlu n by 5,10-CH2–H4PteGlu n reductase (EC 1.5.1.20) or oxidized sequentially by 5,10-CH2–H4PteGlu n dehydrogenase (EC 1.5.1.5) and 5,10-CH=H4PteGlu n cyclohydrolase (EC 3.5.4.9), respectively yielding (6S)-5,10-CH=H4PteGlu n and (6S)-5,10-HCO–H4PteGlu n [17]. These folate derivatives are essential for nucleotide and amino acid biosyntheses, methyl group biogenesis, and vitamin metabolism [14,17,18]. During photosynthesis in mitochondria of *C3* plant cells [16,19,20], SHMTs act in concert with the glycine decarboxylase complex (EC 1.4.4.2) to convert two molecules of glycine into one molecule of serine [11,18,21]. A functional photosynthetic pathway is essential to plants, and mutants in this pathway have to be grown under elevated CO2 concentrations to suppress photosrespiration [22]. A conditional lethal mutant of *A. thaliana*, *shm1–1*, is deficient in the mitochondrial AtSHMT1 [23].

In plants [24,25], as in other organisms [26], polyglutamylated species dominate cellular folate pools. Penta- and hexaglutamylated folate substrates. We found no experimental support for a change in oligomerization state over the range of enzyme concentration studied. Modeling of the enzyme structures presented features that may explain the activity differences between the mitochondrial and cytosolic isozymes.

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ed folates, with tetra- and heptaglutamylated forms following, are the most abundant species in most eukaryotic cells [27]. Polyglutamylated folates comprise over 80% of the folate pool of whole cells, as well as of mitochondria and chloroplasts, in A. thaliana leaves [25]. Polyglutamylated species also dominate the matrix space pool of pea leaf mitochondria, with over 50% of folates having pentaglutamyl or longer tails [24].

Previous studies have shown that polyglutamylation yields efficient substrates for some folate-dependent enzymes by favoring protein binding [28,29]. Dependence of activity on folate polyglutamylation has been investigated in few plant enzymes. The HMT1 and AtSHMT2, but not AtSHMT4, displayed an increase in methionine synthase shows a higher preference for polyglutamylated folates, which are the major folates in the cytosol [30,31]. The glycine decarboxylase complex and the SHMT from spinach [32,33], and the cytosolic AtSHMT4 [11,23,33], and the cytosolic AtSHMT4 [11,33,34]. The second putative cytosolic isozyme, AtSHMT5, was also expressed, but the purified protein was catalytically inactive and, therefore, not studied further.

Subcellular localization of AtSHMT1 and AtSHMT2 has been confirmed [12,13]. We here provide evidence that AtSHMT4 resides in the cytosol using fluorescence microscopy of the enzyme fused to enhanced green fluorescent protein (EGFP). Catalytic properties of AtSHMT1, AtSHMT2, and AtSHMT4 were studied using an HPLC-based fluorometric assay we developed previously [36]. AtSHTM1 and AtSHMT2, but not AtSHMT4, displayed an increase in catalytic activity at higher (>$0.4\text{nmol/\mu L}$) enzyme concentration when assayed in the presence of monoglutamylated folate substrates, but not in the presence of pentaglutamylated folate substrates. Additional experiments did not support the hypothesis that the increased activity at higher enzyme concentration is due to increased dimer-to-tetramer conversion in the enzymes from mitochondria. Modeling of the enzyme structures presented features that may explain the differences in the kinetic parameters between the mitochondrial and cytosolic isozymes.

Materials and methods

**Chemicals and reagents**

PteGlu$_3$, (6R,5S)-H$_4$PteGlu$_1$, and (6R,5S)-5,10-CH$_2$-H$_4$PteGlu$_1$ were obtained from Schircks Laboratories (Jona, Switzerland). NaBH$_4$ was from Sigma–Aldrich. Glutaraldehyde (25% aqueous solution) was from Fisher Scientific. BSA was from EMD Chemicals (Darmstadt, Germany). Benzonase$^\text{TM}$ nuclease, recombinant enterokinase, and Ni–NTA His$^\text{TM}$ superflow resin were from Novagen (Madison, WI). Oligonucleotides were from MWG (High Point, NC). The rabbit polyclonal anti-Spinacia oleracea SHMT antibody was from Agrisera (Vännäs, Sweden).

cDNA cloning and expression in E. coli

The corresponding cDNA clones were ordered from the Arabidopsis Biological Resource Center (ABRC), The Ohio State University, for AtSHMT1 (At4g37930, ABRC clone 135G2), AtSHMT2 (At5g26780, ABRC clone C104687), AtSHMT4 (At4g31390, ABRC clone F1D1T7), and AtSHMT5 (At4g13890, ABRC clone 160C13). For AtSHMT2, two protein sequences are present, AtSHMT2 and AtSHMT2long, the second differing from the first in having a 16-amino acid insertion (Fig. S1). The ABRC clone C104687 encodes AtSHMT2long.

The cDNA sequences encoding the N-terminal regions of AtSHMT1 and AtSHMT2 or AtSHMT2long were recoded by DNA synthesis using a commercial service (GenScript, Piscataway, NJ) to comply with the codon usage bias in E. coli and facilitate expression. The full-length coding sequences were then reconstituted through overlap extension PCR [37]. First, the synthesized recoded sequence of AtSHMT1 (300 bp) was amplified by PCR using the primer pair ForwardA and ReverseA (Table S1). The remaining AtSHMT1 sequence was amplified from clone 135G2 using primers ForwardB and ReverseB (Table S1). The full-length open reading frame of AtSHMT1 was produced using the two amplified sequences as templates and the primer pair AtSHM1 F and R (Table S1). The resulting PCR fragments were purified with Wizard PCR prep mini-columns (Promega, Madison, WI), treated with T4 DNA polymerase (Promega), and then inserted into the pET-43.1 EK/LIC vector (Novagen). All procedures were carried out in accordance with the manufacturer's protocols. Proteins expressed in pET-43.1 EK/LIC vectors have an N-terminal Nus$^\text{TM}$Tag fusion partner, which increases solubility of the recombinant proteins.

The coding sequence for the N-terminal region of AtSHMT2 or AtSHMT2long (300 bp) was amplified using the primer pair ForwardC and ReverseC (Table S1). The coding sequence for the C-terminal region of AtSHMT2long was amplified from clone C104687 using primers ForwardD and ReverseD (Table S1). The full-length recorded open reading frame of AtSHMT2long was produced using the two PCR products as templates and the primer pair AtSHM2 F and R (Table S1).

The coding sequence for the C-terminal region of AtSHMT2 was amplified through another overlap extension PCR to eliminate the sequence encoding the 16-amino acid insertion. The cDNA sequences comprising the segment were amplified from clone C104687 using primer pairs ForwardD and ReverseE, and ForwardF and ReverseF (Table S1). Primers ReverseE and ForwardF (Table S1), which are complementary to each other, introduce the deletion required to reconstitute AtSHMT2. The full-length coding sequence for AtSHMT2 was then amplified using the primer pair AtSHM2 F and R (Table S1), using as templates the PCR products encoding the N- and C-terminal regions of AtSHMT2. The full-length coding sequences for AtSHMT2 and AtSHMT2long were purified and inserted into the pET-30 EK/LIC vector following the manufacturer's protocol. PCR applications used Pfu Turbo DNA polymerase (Strategene, La Jolla, CA).

The F1D1T7 clone containing the AtSHMT4 open reading frame harbored a four-base pair deletion in the middle of the sequence. The deletion was corrected also using overlap extension PCR. The two segments flanking the deletion were amplified respectively with primer pairs ForwardG and ReverseG, and ForwardH and ReverseH (Table S1). Primers ReverseG and ForwardH introduced the four-base pair insertion. Next, the two PCR products were ampli-

![Fig. 1. Chemical structure of H4PteGlu.](image-url)
fied with the primer pair AtSHM4 F and R (Table S1) to produce the full-length coding sequence, adding an NdeI site at the 5' end and an Xhol site at the 3' end. The coding sequence for AtSHMT5 was amplified using the primer pair AtSHMS F and R (Table S1), adding an NdeI site at the 5' end and an Xhol site at the 3' end. PCR applications used PfuTurbo DNA polymerase. The amplified coding sequences for AtSHMT4 and AtSHMT5 were digested with NdeI and Xhol and then inserted into the pET-43.1b(+) vector also digested with those enzymes. Thus, AtSHMT4 and AtSHMT5 were expressed without any fusion tag.

The generated expression vectors were introduced into E. coli NovaBlue competent cells (Novagen), sequence-verified, and then introduced into E. coli Rosetta 2(DE3) (Novagen) for protein production. To express the recombinant proteins, bacteria carrying the expression vectors were incubated at 37 °C in Luria–Bertani medium containing 50 μg/mL chloramphenicol and 50 μg/mL ampicillin or 100 μg/mL kanamycin until the absorbance at 600 nm reached 0.6–1.0. Isopropyl β-D-thiogalactopyranoside (IPTG) (0.6–1.0) was then added, and incubation was continued at 15 °C overnight.

**Protein purification**

Induced E. coli cells from 200 mL cultures were harvested by centrifugation at 7500g for 10 min at 4 °C, resuspended in 5 mL of buffer A (50 mM CHES–HEPES–citric acid buffer (pH 7.5) [38], 1 mM tris-(3-hydroxypropyl)phosphine (THP), 0.25 mM pyridoxal 5’-phosphate (PLP), and 10% glycerol) plus 25 units/mL Benzonase nuclease, and then lysed with 0.1 mm zirconia/silica beads using a Mini-Beadbeater-8 cell disruptor (BioSpec Products, Bartlesville, OK). Soluble protein extracts were cleared by centrifugation at 20000g for 15 min at 4 °C and filtered through a 0.45-μm PVDF membrane.

**Recombinant AtSHMT1, AtSHMT2, and AtSHMT2long were affinity-purified on Ni–NTA His•Bind® resin (Novagen) as instructed by the manufacturer.** Protein preparations were incubated with 1 mL of Ni–NTA resin for 1 h at 8 °C. Binding, wash, and elution buffers contained 50 mM CHES–HEPES–citric acid buffer (pH 7.5), 300 mM NaCl, 10 (binding), 20 (wash), or 150 mM (elution) imidazole, 0.5 mM THP, 0.25 mM PLP, and 10% glycerol. The purified proteins were immediately desalted into buffer A with PD-10 desalting columns (GE Healthcare) and digested with recombinant enterokinase to cleave the fusion tags. The best reaction conditions for enterokinase cleavage were checked by SDS–PAGE using the NuPAGE Novex® Bis-Tris gel system (Invitrogen, Carlsbad, CA). Protein digests were stained with the SimplyBlue SafeStain® (Invitrogen). The fully cleaved enzymes were further purified to remove the tags and undigested enzymes using the AKTA FPLC system (GE Healthcare). Untagged AtSHMT1 was purified into buffer A on a Mono Q 5/50 GL column (GE Healthcare) at 0.75 mL min⁻¹. The purified protein eluted in the flow-through. Untagged AtSHMT2 was purified into buffer A on a Superose 12 10/300 GL column (GE Healthcare) at 0.8 mL min⁻¹. The volume of sample injected was 5 mL. The tagged SHMT2 long was used for characterization because of the inefficiency of tag cleavage for this enzyme.

Recombinant AtSHMT4 and AtSHMT5 were purified using three chromatographic steps. First, protein preparations were loaded onto a HiPrep 16/10 DEAE FF column (GE Healthcare) and eluted with a gradient of 0–0.5 M NaCl in buffer A over 5 column volumes at 1 mL min⁻¹. The purified proteins eluted at ∼0.1 M NaCl. Second, the active fractions were loaded onto a Mono Q 5/50 GL column and eluted with a gradient of 0–0.5 M NaCl in buffer A over 10 column volumes at 0.75 mL min⁻¹. The purified proteins eluted at ∼0.25 M NaCl. Third, the active fractions were purified into buffer A on a Superdex 200 10/300 GL column (GE Healthcare) at 0.5 mL min⁻¹. The volume of sample injected was 5 mL. All purification steps were checked by SDS–PAGE. The purified enzymes were frozen in liquid N2 and stored at −80 °C until use.

**Synthesis of H₄PteGlu₅**

PteGlu₅ was reduced to H₄PteGlu₅ with NaBH₄ [25] for use in the activity assays. The resulting H₄PteGlu₅ was purified as described previously [10]. H₄PteGlu₅ concentration was determined spectrophotometrically at 298 nm with a molar absorption coefficient of 28,400 M⁻¹ cm⁻¹. The purified H₄PteGlu₅ solution was stored in tightly sealed vials at −80 °C.

**SHMT activity assay**

SHMT activity was measured using an HPLC-based fluorimetric assay [36], with modifications as indicated below. Reaction products were analyzed using an Alliance 2995 separations module coupled with a 2475 fluorescence detector (Waters, Milford, MA). The enzymatic reaction contained 50 mM CHES–HEPES–citric acid (pH 7.5), 4 mM THP, 0.25 mM PLP, and 100 ng/μL BSA plus 5 mM l-serine and various (6R,5’)-H₄PteGlu₁₅₈₅ concentrations. The reaction volume was 50 μL. Assay mixtures were pre-incubated on ice for 10 min before addition of serine to start the reaction. Pre-incubation for 30 min had no effect on SHMT activity (not shown). Serine was omitted solely from the assay blanks before incubation. BSA was included in the reaction mixture because preliminary results (not shown) established that SHMT activity increases 5–6-fold in the presence of BSA in all three enzymes.

After incubation at 22 °C for 20 min, the reactions were stopped by simultaneously adding 25 μL of 0.1 M dithiothreitol, which lowers blank values, and 50 μL of 0.1 M NaBH₄. The NaBH₄ solution was freshly prepared before use because it degrades quickly in water. The reaction products were incubated at 37 °C for 15 min to drive (6S)-5-CH₂H₄PteGlu₁₅₈₅ formation to completion, heated at 98 °C for 3 min, and centrifuged at 2000g for 15 min at 4 °C to pellet denatured proteins. To prevent product decomposition, 10 μL of 0.6 M dithiothreitol per 50 μL of supernatant was added to the reaction products. (6S)-5-CH₂H₄PteGlu₁₅₈₅ and (6R,5’)-H₄PteGlu₁₅₈₅ were separated isocratically on a Waters Xterra C₁₈ column (4.6 × 100 mm, 5 μm) and detected fluorometrically at 280 nm excitation and 359 nm emission wavelengths. The mobile phase consisted of 27 mM phosphoric acid and 7 or 9% (v/v) methanol when using mono- or pentaglutamylated folates, respectively. Flow conditions during the isocratic separation were 2 mL min⁻¹ for 6–12 min at 32 °C. The volume of sample injected was 2–20 μL. The (6S)-5-CH₂H₄PteGlu₁₅₈₅ formed during the assays were quantified by comparison with standards. Reaction products increased linearly with time and enzyme concentration in all assay conditions used here. Apparent values for the kinetic parameters were found by fitting initial reaction rates against substrate concentrations to a model of competitive substrate inhibition in the Enzyme Kinetics module 1.2 of SigmaPlot 9.0.1 (Systat Software, San Jose, CA). The rate equation used to fit the data is

\[ v = \frac{V_{\text{max}}}{1 + K_m S + S/K_i} \]

in which v is the initial reaction rate, \( V_{\text{max}} \) is the limiting rate, \( K_m \) is the Michaelis constant, S is the initial substrate concentration, and \( K_i \) is the inhibition constant. To ease data processing and plotting, the reaction rates from three independent assays, each carried out in duplicate, were grouped into a single dataset and simultaneously entered into SigmaPlot 9.0.1 to provide the reported kinetic parameters and standard errors. The standard error for \( k_{\text{cat}}/K_m \) and \( K_i/K_m \) was calculated by error propagation.

**Protein cross-linking and Western blot analysis**

A 10–15 μL sample of SHMT at concentrations corresponding to the range used in the activity assays was cross-linked with glutaraldehyde without direct mixing as described previously [40]. Glutaraldehyde is a volatile liquid, which forms vapor pervading the incubation cell that dissolves in the sample drops. This is a mild
method not requiring direct mixing of the sample with the cross-linker. Proteins were diluted in a buffer solution containing 50 mM CHES–HEPES–citric acid (pH 7.5), 4 mM TBP, and 0.25 mM PLP plus 534 μM (6R,5S)-H₄PteGlu. The cross-linked proteins were separated by SDS–PAGE using the NativePAGE Novex® Bis-Tris gel system (Invitrogen) and analyzed by Western blot with chromogenic detection using the amplified alkaline phosphatase Immun-Blot® assay kit (Bio-Rad, Hercules, CA). The rabbit polyclonal anti-Spinacia oleracea SHMT antibody was diluted 5000 times before use.

Structural modeling of AtSHMT1, AtSHMT2, and AtSHMT4

The crystal structure of a human cytosolic SHMT with bound pyridoxamine 5'-phosphate (PDB ID: 1BJ4) [41] was used to build a molecular model for AtSHMT1, AtSHMT2, and AtSHMT4. The amino acid substitutions were conducted using the coordinates of the human cytosolic SHMT and then regularized using defined geometric constraints for bonds, angles, planes, non-bonded contacts, and torsion constraints in Coot [42]. After geometric regularization, quick global energy minimization was carried out using CNS 1.1 [43], which uses the potential function parameters of CHARMM19. Substrate position was generated using the solid docking module on QUANTA (BioSym/Micron Separations), which is based on conformational space, followed by a quick energy minimization by CNS 1.1.

Transient expression of EGFP-fused AtSHMT4 in A. thaliana protoplasts

The full-length coding sequence for AtSHMT4 was amplified by PCR with forward primer AtSHMT4 GFP and reverse primers AtSHMT4 C-terminus GFP or AtSHMT4 N-terminus GFP (Table S1) for C- or N-terminal EGFP fusion, respectively. The resulting plasmid fragments were re-amplified with the attB adapter primer pair (Invitrogen), purified with Wizard PCR prep mini-columns, and then inserted into the p2GW7 or p2PGW7 vector for C- or N-terminal EGFP fusion, respectively. All procedures were carried out in accordance with the Gateway cloning system (Invitrogen).

Arabidopsis thaliana protoplasts were isolated from leaves of 4-week-old plants and transformed using a polyethylene glycol-mediated transformation method [44] for transient gene expression. Fluorescence was monitored using a TCS SPS confocal laser-scanning microscope (Leica Microsystems, Exton, PA). EGFP fluorescence was excited at 488 nm and detected at 505–530 nm. Chlorophyll fluorescence was excited at 488 nm and detected at >650 nm.

Results

Sequence analysis

Deduced amino acid sequences for AtSHMT1, AtSHMT2, AtSHMT4, and AtSHMT5 were retrieved from The Arabidopsis Information Resource database. Three gene models for AtSHMT2 were present. Two of them, At5g26780.2 and At5g26780.3, encoded the same putative protein (AtSHMT2long) and one, At5g26780.1, encoded a sequence variant (AtSHMT2). AtSHMT2long has a 16-amino acid insertion (amino acids 373–388) relative to AtSHMT2 (Fig. S1). WoLF PSORT prediction suggested that AtSHMT1 and AtSHMT2 have N-terminal extensions for targeting to mitochondria, while AtSHMT4 and AtSHMT5 have no such extensions (Fig. S1) and thus are predicted to be cytosolic. Previously, mitochondrial localization of AtSHMT1 and AtSHMT2 was confirmed respectively by fluorescence microscopy of stable A. thaliana transformants expressing a C-terminal AtSHMT1-GFP fusion [12] and by Western blotting of matrix extracts prepared from purified mitochondria [13].

Purification of recombinant AtSHMTs

Recombinant AtSHMT1, AtSHMT2, and AtSHMT2long were expressed in E. coli from pET-30 or pET-43.1 Ek/LIC. The tag was successfully cleaved from AtSHMT1 and AtSHMT2, but not from AtSHMT2long (Fig. 2). Because AtSHMT2 activity did not change after the tag cleavage (not shown), we used the tagged AtSHMT2long for the activity assays. Preliminary results (not shown) indicated that the tags could not be cleaved from AtSHMT4 or AtSHMT5 expressed from pET-30 and pET-44 Ek/LIC. These two enzymes were therefore expressed and purified without any tags (Fig. 2).

Kinetic characterization of AtSHMTs

AtSHMT1, AtSHMT2, and AtSHMT4 were catalytically active and thus assayed for activity in the presence of mono- or pentaglutamylated folate substrates. Specific activity increased with enzyme concentration in AtSHMT1 and AtSHMT2 when assayed in the presence of monoglutamylated folates (Fig. 3A and C), but not in the presence of pentaglutamylated folates (Fig. 3B and D). Specific activity of AtSHMT4 remained constant with enzyme concentration in the presence of either monoglutamylated (Fig. 3E) or pentaglutamylated (Fig. 3F) folates. Preliminary results (not shown) established that the effect of enzyme concentration on specific activity persisted when BSA was omitted from the assays. An explanation for the observed dependence of specific activity on enzyme concentration for AtSHMT1 and AtSHMT2 is offered in Discussion.

Kinetic parameters derived from nonlinear data fitting to a model of uncompetitive substrate inhibition (SigmaPlot 9.0.1) are presented in Table 1. Substrate saturation curves used to calculate the kinetic parameters are shown in Fig. S2. The Kᵣₑᵥ value for AtSHMT1 and AtSHMT2 could not be determined because of two experimental restrictions. First, the activity of these two enzymes changed with enzyme concentration; second, the enzyme concentration would have to be changed when assaying all substrate concentrations to control substrate consumption and the signal-to-blank ratio. The Kᵣₑᵥ value for AtSHMT1 and AtSHMT2 could be determined only if the enzyme concentration would be held constant across the monoglutamylated folate concentrations assayed. This was not feasible because the enzyme concentration was too high for the lowest substrate concentration points, more than 10% of substrate would be consumed and the rate of the reaction would move too far away from the steady state, or too low for the highest substrate concentration points, requiring past the 50-min incubation time. Our preliminary results showed that the reaction product increases linearly with time for up to 50 min.

The data showed for all three enzymes that the Kᵣₑᵥ values are lower and the catalytic efficiency (kᵣₑᵥ/Kᵣₑᵥ) is higher in the presence of pentaglutamylated folates than in the presence of monoglutamylated folates. Substrate inhibition was also more pronounced for the polyglutamylated substrates. Kᵣₑᵥ of AtSHMT4 is ~190-fold lower for H₄PteGlu than for H₄PteGlu₁. The decrease in Kᵣₑᵥ is relatively larger than the decrease in Kᵣₑᵥ, resulting in a ~5-fold decrease in the Kᵣₑᵥ/Kᵣₑᵥ value. This is different from the previously studied AtSHMT3, for which Kᵣₑᵥ decreases to a lesser degree than Kᵣₑᵥ with the increase in the level of folate polyglutamylation [10].

Oligomerization state of AtSHMT1 and AtSHMT2

Previous studies have shown that SHMTs exist as homotetramers best described as dimers of dimers in eukaryotes such as zebrafish, mouse, rabbit, sheep, and human [41,45–48] and as dimers in prokaryotes such as E. coli, Bacillus subtilis, and Salmonella typhimurium [5,49,50]. However, a dynamic balance between
dimeric and tetrameric forms of some SHMTs has also been reported, and catalytic activity changed with the oligomerization state in these enzymes [51–53]. Therefore, we hypothesized that a concentration-dependent conversion between dimeric and tetrameric forms may be causing the increase in specific activity of AtSHMT1 and AtSHMT2 at higher enzyme concentrations (Fig. 2).

The oligomerization state of AtSHMT1 and AtSHMT2 was studied using mild glutaraldehyde cross-linking in which proteins were not directly mixed with the glutaraldehyde solution [40]. AtSHMT1 and AtSHMT2 were cross-linked at various enzyme concentrations over the range in which the catalytic activity changed. Since the enzyme quantity was too low to visualize by directly staining SDS–PAGE gels, the cross-linked proteins were visualized using antibodies.

The proteins were first cross-linked for 40 min. This reaction time is sufficient for the cross-linking reaction to reach completion. Since the glutaraldehyde cross-linking is an irreversible process that perturbs the equilibrium of different oligomerization states, we checked whether the 40-min cross-linking might have pulled the equilibrium too far toward tetramers. Thus, the proteins were also partially cross-linked for 30 min.

Western blot analysis of partially cross-linked proteins, in combination with the analysis of the band density using MYImageAnalysis software (Pierce, Rockford, IL) suggested no transition from dimer to tetramer with an increase in enzyme concentration (Fig. 4B and C); the tetramer prevailed and the dimer was barely detectable under the conditions tested (Fig. 4A–C). Therefore, our experimental data do not support the hypothesis that the effect of enzyme concentration on specific activity is due to changes in the oligomerization state of these proteins. Non-denaturing PAGE confirmed that AtSHMT1 and AtSHMT2 exist as ~250-kDa homotetramers when the enzyme concentration is high (Fig. 4D). Other methods to explore oligomerization state of SHMTs, namely dynamic/static light scattering, fluorescence polarization, molecular sieve chromatography, and analytical centrifugation could not be used because of method limitations detailed in Discussion.

**Structural modeling of AtSHMT1, AtSHMT2, and AtSHMT4**

Given the high sequence similarity between the human [41] and *A. thaliana* SHMTs, a reliable 3D-model could be built (Fig. 5A) and served as a foundation to explain the differential kinetics of AtSHMT1, AtSHMT2, and AtSHMT4. Most of the participating residues for binding of PLP and pteridine and for catalysis in AtSHMT1, AtSHMT2, and AtSHMT4 were nearly identical (Fig. 5B). Completely conserved among the three isozymes are the residues for formation of internal aldimine and for ion pairing with PLP (Lys286 and Asp257), the residues interacting with external aldimine (Tyr112, His260, and Arg430), the residues interacting with 5′-phosphate of PLP (Tyr102 and His285), and the residues interacting with folate (Asn415 and Tyr111) (Fig. 5B). However, the entry site for folate substrates, which is formed by five loops from the two interfacing subunits, showed substantial heterogeneity between the mitochondrial and cytosolic isozymes, while the sequences of AtSHMT1 and AtSHMT2 were identical in this region (Fig. 5B).

In general, the entry sites of AtSHMT1 and AtSHMT2 had more rigidity than that of AtSHMT4. The side chain of Asp185, locating at the tip of the highly disordered loop (173PHGGHLSHGYQTDTKIKSAVS1194) in both AtSHMT1 and AtSHMT2, formed a salt bridge with Lys251 in another loop constituting the folate entry point (Fig. 5A). The D2 symmetry of the tetramer enabled four such ionic interactions, which could introduce significant rigidity to the entry sites and stability to the quaternary structure of AtSHMT1 and AtSHMT2.
HMT2. The polyglutamyl moiety of the folate substrates might weaken the Asp185-Lys251 salt bridge or might interact with the polar side chains of the entry site, either of which could influence substrate entrance/exit or binding.

In AtSHMT4 the corresponding residue for Asp185 is serine and for Lys251 is glycine, thereby disabling the corresponding salt bridge (Fig. 5B). Also, AtSHMT4 has an inserted glycine after the serine residue (Fig. 5B, dotted box b) forming consecutive glycines in this region. Three implications are clear from the presented results. First, both loops are more disordered and flexible in this enzyme. Second, the peptide backbone of the N-terminal residues (Fig. 5B, dotted box b) accommodates part of the pocket for pteridine binding and thus the effect of consecutive glycines could propagate into this pocket. Third, the constituting residues for the entry site of AtSHMT4 are less polar than those of AtSHMT1 and AtSHMT2 (Fig. 5B). Overall, the size, flexibility, and polarity of the folate entry site are clearly different between the mitochondrial and cytosolic isozymes. It is therefore probable that AtSHMT4 shows the better turnover rate as a result of this flexibility.

Transient expression of EGFP-fused AtSHMT4 in A. thaliana protoplasts

Mitochondrial localization of AtSHMT1 and AtSHMT2 was confirmed previously [12,13]. AtSHMT4 was found to localize in the cytosol [54] or plasma membrane [55,56] by proteomic approaches. We confirmed its cytosolic localization using fluorescence microscopy of the EGFP-fused protein. Transient expression of both the C- and N-terminal fusions of AtSHMT4 to EGFP produced green fluorescence in the cytosol of the transformed A. thaliana protoplasts (Fig. 6). A construct expressing EGFP...

**Table 1**

Kinetic parameters of the AtSHMTs for mono- and pentaglutamylated folate substrates. Kinetic parameters are nonlinear best fits to a model of uncompetitive substrate inhibition (SigmaPlot 9.0.1). The enzymes were assayed for activity with H4PteGlu1 (A) and H4PteGlu5 (B) substrates. In (A), the kinetic parameters for AtSHMT1 and AtSHMT2 are not available due to the dependence of activity on enzyme concentration. Results are means ± SEM of three independent assays, each carried out in duplicate.

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<td>AtSHMT4</td>
<td>17 ± 4</td>
<td>0.74 ± 0.3</td>
<td>4.6 ± 0.4</td>
<td>0.3 ± 0.1</td>
<td>43 ± 19</td>
</tr>
<tr>
<td>B</td>
<td>28.9 ± 0.9</td>
<td>24 ± 11</td>
<td>10.2 ± 3.3</td>
<td>0.4 ± 0.2</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>AtSHMT1</td>
<td>3.0 ± 0.6</td>
<td>10 ± 2</td>
<td>4.5 ± 0.6</td>
<td>1.5 ± 0.4</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td>AtSHMT2</td>
<td>0.5 ± 0.1</td>
<td>4 ± 1</td>
<td>8.3 ± 1.0</td>
<td>15.9 ± 4.4</td>
<td>8.2 ± 2.7</td>
</tr>
</tbody>
</table>

Fig. 3. Dependence of activity on enzyme concentration for AtSHMT1, AtSHMT2, and AtSHMT4. Activities were assayed at pH 7.5 using purified enzymes under native conditions. (A), (C), and (E), when assayed with 267 \(\mu M\) (S)-H4PteGlu. (B), (D), and (F), when assayed with saturating (S)-H4PteGlu\(_5\), which is at 50 \(\mu M\) in (B) and 25 \(\mu M\) in (D) and (F). Results are means ± S.E.M. of three independent assays, each carried out in duplicate.
alone served as a positive control (Fig. 6A, D, and G). The discrepant results of the proteomic studies [54–56] may have been caused by limited purity of the analyzed plasma membrane preparations.

Discussion

SHMTs are the major entry points into the folate-mediated one-carbon metabolism in all organisms [14]. Functional SHMTs have been detected in mitochondria, plastids, nuclei, and the cytosol in plants [2,11,13,23], but only the plastid isozyme from *A. thaliana* has been biochemically characterized [10]. We here report functional expression, purification, and biochemical characterization of SHMTs from mitochondria (AtSHMT1 and AtSHMT2) and the cytosol (AtSHMT4) from *A. thaliana*.

While AtSHMT1 and AtSHMT4 appear to exist as single splicing variants, two splicing variants of AtSHMT2 are present in GenBank®; the longer variant (AtSHMT2long) has a 16-amino acid insertion compared with the shorter one (AtSHMT2). Both variants were expressed and purified following identical procedures, but only AtSHMT2 was catalytically active. A previous study reported that expression of AtSHMT2long under the control of AtSHM1 promoter or CaMV 3SS promoter cannot rescue the photorespiratory phenotype of *shm1* mutant [23]. Taken together, these results sug-
gest that AtSHMT2long is probably catalytically inactive in vivo. Further study is needed to determine whether this variant has an alternative physiological role.

Kinetic characterization showed that AtSHMT4 has the highest catalytic efficiency ($k_{cat}/K_m$) of the three enzymes studied. Unlike the mitochondrial isozymes, AtSHMT4 was also more susceptible to substrate inhibition with the pentaglutamylated folate substrates, which are the most abundant folate derivatives in plant cells. Such differences in catalytic efficiency and sensitivity to substrate inhibition of the AtSHMTs may be interpreted as the adaptation to folate concentrations in mitochondria and the cytosol. A previous study estimated that mitochondria and the cytosol plus nuclei contain respectively 50% and 40–45% of the total folate pool in pea leaves[7]. The volume occupied by mitochondria and the cytosol has respectively been estimated at 2.5–4% and 17–25% of the total cell volume in spinach and barley leaves[57,58]. Assuming these results apply to A. thaliana, we estimated its total folate pool to be at least 7.6-fold higher in mitochondria than in the cytosol. Thus, the higher catalytic efficiency of AtSHMT4 might be an adaption to the relatively low folate concentration in the cytosol.

Published values for folate concentration in pea leaf mitochondria are 0.4–1 mM[7,59]. Assuming further this result applies to A. thaliana, we estimated the total folate concentration at 700 lM in mitochondria and 90 lM in the cytosol. ($S$)-H4PteGlu and ($S$)-5,10-CH2–H4PteGlu together account for <5% of the total folate pool in any subcellular compartment in A. thaliana[25,60]. Therefore, concentration of ($S$)-H4PteGlu plus ($S$)-5,10-CH2–H4PteGlu is expected to be ~35 lM in mitochondria and ~4.5 lM in the cytosol in A. thaliana. These estimates are higher than the corresponding $K_i$ values for the three enzymes assayed with the pentaglutamylated folate substrates (Table 1) Therefore, substrate inhibition may affect the catalytic activity of these SHMTs in vivo.

Fig. 5. Ribbon diagram of the modeled tetrameric AtSHMT2 and amino acid sequence alignment of AtSHMT1, AtSHMT2, and AtSHMT4. (A) One of the H4PteGlu$_n$ entry sites is zoomed up and the four regions (a–d) containing the major differences of AtSHMT1 and AtSHMT2 to AtSHMT4 are highlighted in yellow. H4PteGlu$_n$ and PLP are represented as ball and stick models. The inter-subunit salt bridges unique to both AtSHMT1 and AtSHMT2 are indicated in the ball and stick model. These figures were generated using CCP4MG 2.5.0. (B) The amino acid residues constituting the H4PteGlu$_n$ entry site are marked with dotted boxes. The two residues forming the inter-subunit salt bridge in SHMT1 and SHMT2 are marked with a closed circle (●). The residues for formation of internal aldimine and for ion pairing with PLP (Lys286 and Asp257) are marked with a closed triangle (△). The residues interacting with external aldimine (Tyr112, His260, and Arg430) with a closed square (■), the residues interacting with 5'-phosphate of PLP (Tyr102 and His285) with an open circle (○), and the residues interacting with folate (Asn415 and Tyr111) with an open triangle. The secondary structural elements are indicated in yellow ($\alpha$-helix) and red ($\beta$-strand) on top of the corresponding sequences.

Fig. 6. Transient expression of C- and N-terminal EGFP fusion to AtSHMT4. EGFP expressed from the pUC18-GFP5T-sp plasmid (A, D, and G) served as a positive control for targeting to the cytosol. The full-length AtSHMT4 was expressed as a C-terminal (B, E, and H) or an N-terminal (C, F, and I) fusion to EGFP. (A–C) EGFP fluorescence; (D–F) chlorophyll autofluorescence; (G–I) merged images.
homodimers in prokaryotes [61–63] and as homotetramers in eukaryotes [41,64]. Two exceptions were a *Bacillus stearothermorephilus* SHMT [51] and the Asp89Asn mutant of a sheep liver SHMT [53], both existing as a mixture of dimers and tetramers, with the oligomerization state affecting catalytic activity. In the second case, the proportion of tetramers increased when PLP concentration increased during purification, and the specific activity of the tetramer was over 20-fold higher than that of the dimer. A similar change in specific activity was observed in phosphofructokinases from mammals and yeast, which were more active when concentrated [65,66]. The effect in phosphofructokinases is caused by enzyme aggregation at higher concentrations, which stabilizes the more catalytically active conformation of the enzymes [66,67].

The presented results do not support the hypothesis that the increased specific activity of AtSHMT1 and AtSHMT2 at higher enzyme concentration in the presence of monoglutamylated folate substrates is due to increased oligomerization because the observed oligomerization states did not change over the concentration range studied (Fig. 4). One explanation for not detecting a concentration-dependent change in oligomerization is that the results from glutaraldehyde cross-linking underestimated the dimeric form of the enzymes by pulling dimers into tetramers. To test this possibility, we carried out partial cross-linking experiments over a shorter incubation time (Fig. 4B and C). Analysis of these experiments suggested no conversion between dimeric and tetrameric forms of AtSHMT1 and AtSHMT2.

We considered analyzing the oligomerization state of these enzymes using other independent methods, but found them unsuitable. Gel filtration chromatography could not be used because this method relies on the protein of interest forming a “peak” during the analysis, which is incompatible with the need to control the enzyme concentration precisely. Analytical ultracentrifugation, dynamic light scattering and fluorescence polarization were unsuitable because they lacked the sensitivity needed to study oligomerization at very low protein concentrations. Thus, glutaraldehyde cross-linking was the only method we were able to use considering the need to keep the enzyme concentration constant and very low.

The mechanism responsible for the observed increase in specific activity of AtSHMT1 and AtSHMT2 remains to be determined. In the absence of evidence for a change in the oligomerization state, we speculate that, in the presence of monoglutamylated folate substrates, these two enzymes exist in a balance of two tetrameric conformational states differing in turnover rates, with the more active form being more abundant at higher enzyme concentrations. The existence of enzyme molecules having distinct activity states differing in turnover rates has been described [68]. One may envision a possible scenario where AtSHMT1&2 dimers associate to form two kinds of tetramers: one with lower activity and the other with higher activity. One may further envision the tetramer with the lower activity having also a lower dissociation constant than the tetramer with the higher activity. Under these conditions, a scenario is possible where nearly all of the dimers exist within low-activity tetramers at low enzyme concentrations. As enzyme concentration increases, the high-activity tetramers become more abundant, thus causing an increase in the observed specific activity of the mixture.

The observed effect of enzyme concentration on specific activity was absent in the presence of pentaglutamylated folate substrates. Thus, we hypothesize that the polyglutamyl tail binding stabilizes the tetramers of AtSHMT1 and AtSHMT2 into a single activity state. Investigating the physiological significance of this effect in mitochondria is beyond the scope of the present paper.

Structural modeling and sequence comparison (Fig. 5) suggest that the constituting regions of the entry site for the folate substrates might have less rigidity and polarity in AtSHMT4. Such differences may ease access to the catalytic site in the cytosolic enzyme compared to the mitochondrial ones, which is consistent with the higher catalytic efficiency ($k_{cat}/K_m$) of AtSHMT4. Those regions at the folate entry site may also mediate the dependence of activity on enzyme concentration in AtSHMT1 and AtSHMT2 in the presence of monoglutamylated folate substrates. Binding of polyglutamylated folate substrates might weaken the salt bridges present at the folate entry site in AtSHMT1 and AtSHMT2 (Fig. 5A). In addition, the polyglutamate tail is expected to interact with the polar side chains at the folate entry site. Both modes of interaction of the enzyme with the polyglutamate tail of folate substrates could induce conformational changes responsible for stabilizing the enzymes into a single activity state and eliminating the effect of enzyme concentration on the activities of AtSHMT1 and AtSHMT2.

**Conclusions**

The biochemical characterization of AtSHMT1, AtSHMT2, and AtSHMT4 with respect to the impact of folate polyglutamation on substrate saturation kinetics showed increased turnover rates at higher enzyme concentrations in the presence of monoglutamylated folate substrates, but not in the presence of pentaglutamylated folate substrates, for the two mitochondrial AtSHMTs, but not for the cytosolic one. The oligomerization state did not change over the range of enzyme concentration studied, suggesting that another mechanism is responsible for the observed change in activity. Modeling of the enzyme structures presented features that may explain the activity differences in AtSHMT1, AtSHMT2, and AtSHMT4.

**Acknowledgments**

This work was supported by National Science Foundation grants MCB-0429968 and MCB-1052492 (to S.R.).

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2013.06.004.

**References**
