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

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ABSTRACT

The reversible reaction catalyzed by serine hydroxymethyltransferase (SHMT) is the major one-carbon unit source for essential metabolic processes. The *Arabidopsis thaliana* genome encodes seven SHMT isozymes localized in mitochondria, plastids, nuclei, and the cytosol. Knowledge of the biochemical properties of each isozyme is central to understanding and manipulating one-carbon metabolism in plants. We heterologously expressed and purified three recombinant SHMTs from *A. thaliana* (AtSHMTs) putatively localized in mitochondria (two) and the cytosol (one). Their biochemical properties were characterized with respect to the impact of folate polyglutamylation on substrate saturation kinetics. The two mitochondrial AtSHMTs, but not the cytosolic one, had increased turnover rates at higher (>0.4 ng/μL) enzyme concentrations in the presence of monoglutamylated folate substrates, but not in the presence of pentaglutamylated 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.

Introduction

Serine hydroxymethyltransferase (SHMT; EC 2.1.2.1)[2] catalyzes the reversible reaction L-serine + (6S)-H_4PteGlu$_n$ (Fig. 1) → glycine + (6S)-5,10-CH_2–H_4PteGlu$_n$ [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 (6*S*)-5,10-CH_2–H_4PteGlu$_n$, which is then reduced to (6*S*)-5-CH_3–H_4PteGlu$_n$ by 5,10-CH_2–H_4PteGlu$_n$ reductase (EC 1.5.1.20) or oxidized sequentially by 5,10-CH_2–H_4PteGlu$_n$ dehydrogenase (EC 1.5.1.5) and 5,10-CH=H_4PteGlu$_n$ cyclohydrolase (EC 3.5.4.9), respectively yielding (6*S*)-5,10-CH=H_4PteGlu$_n$ and (6*S*)-10-HCO–H_4PteGlu$_n$ [17]. These folate derivatives are essential for nucleotide and amino acid biosyntheses, methyl group biogenesis, and vitamin metabolism [14,17,18]. During photorespiration in mitochondria of C_3 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 formed during photorespiration into one molecule of serine [11,18,21]. A functional photorespiratory pathway is essential to plants, and mutants in this pathway have to be grown under elevated CO_2 concentrations to suppress photorespiration [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 hexaglutamylat-

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[2] *Abbreviations used:* (6*S*)-5-CH_3–H_4PteGlu$_n$, 5-methyltetrahydrofolate; (6*S*)-5,10-CH=H_4PteGlu$_n$, 5,10-methenyltetrahydrofolate; (6*S*)-5,10-CH_2–H_4PteGlu$_n$, 5,10-methylenetetrahydrofolate; (6*S*)-10-HCO–H_4PteGlu$_n$, 10-formyltetrahydrofolate; (6*S*)-H_4PteGlu$_n$, tetrahydrofolate; EGFP, enhanced green fluorescent protein; Ni–NTA, nickel–nitrilotriacetic acid; PLP, pyridoxal 5′-phosphate; SHMT, serine hydroxymethyltransferase; THP, tris-(3-hydroxypropyl)phosphine.

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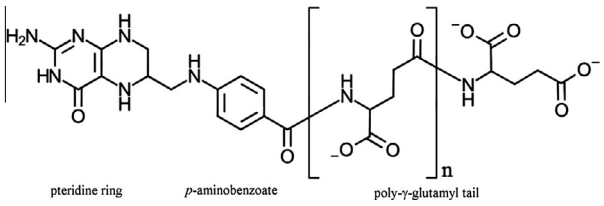

Fig. 1. Chemical structure of H4PteGlun.

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 *A. thaliana* methionine synthase shows a higher preference for polyglutamylated over monoglutamylated 5-CH_3–$H_4PteGlu_n$ [30,31]. The glycine decarboxylase complex and the SHMT from pea leaf mitochondria have lower K_m values for polyglutamylated than for monoglutamylated $H_4PteGlu_n$ [24,32]; the catalytic constants for the $H_4PteGlu_{n>2}$ could not be determined for the SHMT because of limitations in assay sensitivity. We previously demonstrated that polyglutamylated folates are better substrates and better inhibitors than the monoglutamylated species for the reaction in the direction of glycine formation for the plastid AtSHMT3 from *A. thaliana* [10]. Catalytic properties remain to be determined for the remaining AtSHMT isozymes.

Here we report cDNA cloning, recombinant expression, purification, and biochemical characterization of the mitochondrial AtSHMT1 and AtSHMT2 [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]. AtSHMT1 and AtSHMT2, but not AtSHMT4, displayed an increase in catalytic activity at higher (>0.4 ng/μL) 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

$PteGlu_5$, (6*R*,*S*)-$H_4PteGlu_1$, and (6*R*,*S*)-5,10-CH_2–$H_4PteGlu_1$ 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® nuclease, recombinant enterokinase, and Ni–NTA His•Bind® 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 (ABCR), The Ohio State University, for *AtSHMT1* (*At4g37930*, ABRC clone 135G2), *AtSHMT2* (*At5g26780*, ABRC clone C104687), *AtSHMT4* (*At4g13930*, 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 (complementary to ReverseA) 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•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 recoded 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 ReverseD (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 *PfuTurbo* DNA polymerase (Stratagene, 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-