CHARACTERIZATION OF THE PLASTIDIAL SERINE HYDROXYMETHYL-TRANSFERASE FROM ARABIDOPSIS THALIANA

By

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CHARACTERIZATION OF THE PLASTIDIAL SERINE HYDROXYMETHYL-TRANSFERSASE FROM ARABIDOPSIS THALIANA

Abstract

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One-carbon (C₁) metabolism is crucial to all living organisms. Tetrahydrofolate (H₄PteGluₙ) –bound C₁ units are needed in biosynthesis of purines, thymidylate, methionine, and N-formylmethionine, in the methylation of DNA, proteins, and phospholipids, and in biosynthesis of important plant secondary metabolites such as lignin, alkaloids, and betaines.

Folates can only be synthesized de novo by plants and prokaryotes. Folate deficiency in humans has been linked to cardiovascular disease and cancer. To fight the problem of folate deficiency, wheat products have been fortified with synthetic folic acid. Dietary folate intake could also be increased by consuming folate-enriched plants; this could potentially be accomplished by engineering C₁ metabolism.

Serine hydroxymethyltransferase (SHMT) catalyzes reversible conversion of H₄PteGluₙ and serine to 5,10-CH₂-H₄PteGluₙ and glycine and is one of the three enzymes that derivatize folates with C₁ units. Understanding of SHMT features such as subcellular localization and kinetic properties for polyglutamylated H₄PteGluₙ is necessary
groundwork for plant C$_1$ engineering. However, such knowledge is still lacking although
SHMTs from several plant species have been studied in the past.

Sequencing of the *Arabidopsis* genome revealed seven SHMTs predicted to be
localized in mitochondria, plastids, the cytosol, and the nuclei. As a part of the effort to
investigate all seven SHMTs in *Arabidopsis*, this study presents the first report on the
characterization of a putative plastid SHMT (AtSHMT3). The localization of AtSHMT3
in plastids was verified with GFP fusions. Additionally, the presence of SHMT and
10-formyltetrahydrofolate synthetase in plastids of pea and barley was demonstrated. In
order to assay SHMT at low concentrations of H$_4$PteGlu$_n$, we developed a sensitive
HPLC-based fluorometric assay. By using this HPLC assay, we obtained kinetic
parameters for H$_4$PteGlu$_{n=1-8}$ showing that AtSHMT3 has increased substrate affinity and
catalytic efficiency as $n$ grows from 1 to 8. This work also showed that both
5-CH$_3$-H$_4$PteGlu$_{1,5}$ and 5-HCO-H$_4$PteGlu$_{1,5}$ inhibit AtSHMT3-catalyzed
hydroxymethyl-group transfer from serine to H$_4$PteGlu$_6$ with the pentaglutamylated
inhibitors being more effective. Calculations revealed that these pentaglutamylated
inhibitors could lead to little reduction in AtSHMT3 activity under estimated folate
concentrations in plastids.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER ONE</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1. One-carbon Metabolism</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Folates in Plant Cells</td>
<td>6</td>
</tr>
<tr>
<td>1.3. C₁ Unit Formation in Plant Cells</td>
<td>8</td>
</tr>
<tr>
<td>1.3.1. FTHFS in C₁ Metabolism</td>
<td>11</td>
</tr>
<tr>
<td>1.3.2. SHMT in C₁ Metabolism</td>
<td>13</td>
</tr>
<tr>
<td>1.4. Research Goals</td>
<td>16</td>
</tr>
<tr>
<td>1.5. References</td>
<td>18</td>
</tr>
<tr>
<td>CHAPTER TWO</td>
<td></td>
</tr>
<tr>
<td>AN HPLC-BASED FLUOROMETRIC ASSAY FOR SERINE HYDROXYMETHYLTRANSFERASE</td>
<td>23</td>
</tr>
<tr>
<td>2.1. Abstract</td>
<td>24</td>
</tr>
<tr>
<td>2.2. Introduction</td>
<td>24</td>
</tr>
</tbody>
</table>
CHAPTER THREE
ONE-CARBON METABOLISM IN PLANTS: CHARACTERIZATION OF A PLASTID SERINE HYDROXYMETHYLTRANSFERASE

3.1. Abstract...........................................................................................................37
3.2. Introduction.........................................................................................................38
3.3. Experimental Procedures....................................................................................41
  3.3.1. Materials and Plant Growth Conditions...................................................41
  3.3.2. Phylogenetic Analysis..............................................................................42
  3.3.3. cDNA Cloning, Constructs, Sequence Analysis, and Expression in
         E. coli cells.......................................................................................................42
  3.3.4. Recombinant Protein Purification............................................................44
  3.3.5. Extraction of Proteins from Leaves and Chloroplasts of Pea and
         Barley............................................................................................................45
  3.3.6. Transient Expression of EGFP-fused Proteins in A. thaliana
CHAPTER FOUR

CONCLUSIONS ........................................................................................................ 81

References ............................................................................................................. 85
LIST OF TABLES

Page

CHAPTER TWO

2.1. SHMT activity in protein extracts from *Arabidopsis* leaves as measured by the HPLC-based assay and a radioassay............................................................35

CHAPTER THREE

3.1. Kinetic parameters of AtSHMT3 for H$_4$PteGlu$_{1-8}$.........................................................79

3.2. Inhibition of AtSHMT3 with 5-CH$_3$-H$_4$PteGlu$_{1&5}$ and 5-HCO-H$_4$PteGlu$_{1&5}$.....80
LIST OF FIGURES

CHAPTER ONE

1.1. Structures of tetrahydrofolate and its C1 derivatives............................................4
1.2. Structure of tetrahydrofolate polyglutamates.......................................................5
1.3. Schematic representation of folate-dependent C1 metabolic network in
    plant cells...............................................................................................................10

CHAPTER TWO

2.1. Chromatogram of (6R,6S)-H4PteGlu and (6R,6S)-5-CH3-H4PteGlu under
    same HPLC conditions........................................................................................33
2.2. Standard curve for 5-CH3-H4PteGlu and linearity of the reaction rate with
    time and enzyme concentration.........................................................................34

CHAPTER THREE

3.1. Schematic representation of major reactions in plant one-carbon
    metabolism............................................................................................................70
3.2. Phylogenetic tree of SHMTs from representative plant species.........................71
3.3. SHMT, FTHFS, MTHFR, fumarase, and glyceraldehyde-3-phosphate
    dehydrogenase activities in chloroplast and leaf extracts of pea and barley.....72
3.4. Purification of recombinant AtSHMT3 from E. coli cells....................................73
3.5. Plots of steady-state kinetic data for AtSHMT3................................................74
3.6. Inhibition of AtSHMT3 with 5-CH3-H4PteGlu1&5 and 5-HCO-H4PteGlu1&5.....75
3.7. Effects of enzyme dilution on AtSHMT3 activity with $\text{H}_4\text{PteGlu}_{n=1-3,5}$............76

3.8. Localization of AtSHMT3 in *A. thaliana* chloroplasts........................................77

3.9. *In silico* analysis of AtSHMT3 expression in *A. thaliana*.................................78
To My Parents and My Wife
CHAPTER ONE

INTRODUCTION

1.1 One-carbon Metabolism

The metabolism of one-carbon (C₁) groups is crucial to all living organisms, as they are needed in many biochemical reactions of primary and secondary metabolism. C₁ metabolism involves the generation, interconversion, and transfer of C₁ groups. These C₁ units exist in various oxidation states, commonly formyl-, methenyl-, methylene-, and methyl groups. These groups are bound to a family of cofactors that contain a pteridine functional group that are called tetrahydrofolates (H₄PteGluₙ, Fig. 1.1). H₄PteGluₙ and its C₁ derivatives are also referred to as folates.

Plant C₁ metabolism can be split into two sectors: folate-dependent C₁ metabolism and folate-independent C₁ metabolism. Folate-dependent C₁ metabolism is involved in biosynthesis of purines, thymidylate (dTMP), methionine, N-formylmethionine, serine, and other important molecules (Luo et al., 1997; Hanson et al., 2000; Ravanel et al., 2004; Zrenner et al., 2006). Folate-dependent C₁ metabolism is also central to photorespiration in C₃ plants. The folate-independent C₁ metabolism contains the Activated Methyl Cycle. In this cycle, the methyl group of methionine is activated by addition of an adenosyl group from ATP to form S-adenosylmethionine (AdoMet). AdoMet is the ultimate methyl donor for a great number of methylation reactions including those of DNA, proteins, phospholipids, and neurotransmitters (Chiang et al., 1996; Grillo and Colombatto, 2008). Furthermore, in plants, methyl groups from
AdoMet are required in biosynthesis of important secondary metabolites such as lignin, alkaloids, and betaines (Kutchan, 1995; Whetten and Sederoff, 1995; Rathinasabapathi et al., 2001; Tabuchi et al., 2005).

C1 metabolism in mammals has been well studied, but studies of C1 metabolism in plants have lagged behind those in mammals. In addition, the existence of plastids in plant cells adds to the complexity of plant C1 network. For these reasons, there are some plant C1 enzymes and pathways remaining to be investigated. Serine hydroxymethyltransferase (SHMT) is one such enzyme requiring additional investigation. SHMT catalyzes the formation of 5,10-methylenetetrahydrofolate (5,10-CH2-H4PteGlu₂) and is a principal C1 entry point of C1 units into the C1-H4PteGlu₂ pool in plant cells. In light of this fact, SHMT may regulate carbon flux into the C1 network. 5,10-CH2-H4PteGlu₂ is required for thymidylate synthesis and is the immediate precursor of 5-methyltetrahydrofolate (5-CH3-H4PteGlu₂), which is needed for biosynthesis of methionine. Despite the importance of SHMT, knowledge of its key features is still lacking; these features include subcellular localizations and kinetic parameters for its physiological folate substrates and inhibitors. Obtaining such information is necessary for a deeper understanding of its physiological functions.

In addition to the fundamental scientific significance, studies of SHMT and other poorly characterized C1 enzymes are also important to applications such as folate fortification and methionine enhancement in plants. Folate deficiency is a known world issue and is thought to contribute to human diseases such as cardiovascular disease and cancer (Stanger, 2002; Stover, 2004). To fight the problem of folate deficiency, countries
including the United States have initiated programs to fortify folic acid (PteGlu₁) in flour and cereals (Nathoo et al., 2005). This problem could also be resolved by modifying plant folate synthesis pathways to increase folate content in plants. Folate enhancement in tomato and rice has been accomplished by overexpressing foreign genes encoding folate synthetic enzymes (Díaz de la Garza et al., 2004; Díaz de la Garza et al., 2007; Storozhenko et al., 2007). In contrast, methionine enhancement in engineered plants has not been very successful. Only small increases in total methionine have been reported in two noteworthy attempts to modify the first step of methionine synthesis (Zeh et al., 2001; Shen et al., 2002). A new strategy for methionine enrichment is to overexpress two C₁ enzymes (methionine synthase and 5,10-methylenetetrahydrofolate reductase, MTHFR) in plastids. Methionine could overaccumulate in plastids because methionine synthesis employs methyl groups generated by MTHFR and would not be affected by feedback regulation if taking place in plastids. Since SHMT produces 5,10-CH₂-H₄PteGluₙ that is needed by MTHFR in the methionine synthesis pathway, understanding of mechanistic features of SHMT will give us information on how C₁ flux is controlled by SHMT at this step of the pathway and provide a basis for planning and interpretation of SHMT-related C₁ engineering in plants.
Figure 1.1. Structures of tetrahydrofolate and its C\textsubscript{1} derivatives.
**Figure 1.2.** Structure of tetrahydrofolate polyglutamates
1.2 Folates in Plant Cells

Folates have three distinct subcomponents, a pteridine moiety, a para-aminobenzoic acid (pABA) moiety, and a polyglutamate tail linked by a peptide bond to the pABA carboxyl group (Fig. 1.2). The polyglutamate tail in natural folates may contain up to eleven glutamate residues (Scott et al., 2000). Reducing the pteridine ring by adding four hydrogen atoms to position 5, 6, 7, and 8 produces H₄PteGluᵣ, the active form of folate and the C₁ carrier in living cells. H₄PteGluᵣ is more prone to oxidative degradation than non-reduced folates (Blakley, 1969). As a consequence, a reducing environment is necessary when dissolving H₄PteGluᵣ in solutions. The nitrogen atoms at position 5 and 10 in the H₄PteGluᵣ pteridine ring are where the C₁ modifications take place. PteGlu₁, the synthetic form of folate added to supplement pills and fortified foods, is a non-reduced folate with a monoglutamate tail. Once absorbed in the human body, PteGlu₁ is reduced to dihydrofolate (H₂PteGluᵣ) and then to H₄PteGluᵣ by dihydrofolate reductase (Scott et al., 2000).

In plants, pterin and pABA moieties of H₄PteGluᵣ are synthesized separately by two independent pathways in the cytosol and in chloroplasts, respectively (Rébeillé et al., 2006). Pterin and pABA are then transported into mitochondria, where they are combined and subsequently converted in two steps to tetrahydrofolate monoglutamate (H₄PteGlu₁) (Sahr et al., 2005). H₄PteGlu₁ is the form of folate preferred by plant folate transporters identified in Arabidopsis chloroplasts (Bedhomme et al., 2005; Klaus et al., 2005). This suggests that the H₄PteGlu₁ synthesized in mitochondria can be exported to the cytosol and plastids to participate in C₁ metabolism in those compartments. In each subcellular
compartment, the monoglutamate tails of H₄PteGlu₁ are elongated by adding more glutamate residues in a reaction catalyzed by the folylpolyglutamate synthetases found in mitochondria, plastids, and the cytosol (Ravanel et al., 2001). In pea leaves, it was estimated that mitochondria contain ~50% of the total folates, while plastids contain ~10% and the rest of the folates are in vacuoles and the cytosol (Neuburger et al., 1996; Orsomando et al., 2005).

In plant cells, the dominant folate species contain four-to-six glutamate residues in their tails (Besson et al., 1993; Orsomando et al., 2005). These polyglutamate tails help stabilize the folates and sequester them inside each subcellular organelle (Rébeillé et al., 1994). Another important role of the polyglutamate tails is to increase the binding affinity of folate-utilizing enzymes for folates; the affinity increases with the number of polyglutamates (Schirch and Strong, 1989). Most of the evidence relating the affinity of these enzymes for polyglutamate folates and the length of the folate substrate’s glutamate tail was generated with animal enzymes and there are a few reports of this relationship for plant-derived C₁ enzymes. The $K_m$ values of pea 10-formyltetrahydrofolate synthetase (FTHFS) was reported as 40 µM for H₄PteGlu₁ and 3 µM for H₄PteGlu₅ (Kirk et al., 1994). Besson et al. showed that SHMT and glycine decarboxylase (GDC) from pea leaf mitochondria have a higher affinity (lower $K_m$ values) for H₄PteGlu₂ than for H₄PteGlu₁ (Besson et al., 1993) but, due to the lack in sensitivity of SHMT assay, kinetic parameters of pea mitochondrial SHMT for H₄PteGluₙ with tails of more than two glutamates were not determined.

Folate species in plant cells are predominantly tetra-, penta-, and
hexaglutamylated, so it is necessary to know how kinetic properties of $C_1$ enzymes for these physiological substrates change as a function of glutamylation. The low sensitivity of existing SHMT assays has been a major obstacle for conducting enzymatic studies of this enzyme, and the effects of folate polyglutamylation on the kinetic parameters of these $C_1$ enzymes are unknown in plants. Obviously, this makes it difficult to estimate the physiological importance of these enzymes, including those for SHMT, in cellular metabolism. In Chapter Two, I describe a new fluorometric assay that extends the range of potential substrate concentrations substantially. This assay will facilitate characterization of $C_1$ enzymes at meaningful and appropriate folate concentrations.

### 1.3 $C_1$ Unit Formation in Plant Cells

Plant $C_1$ metabolism is made up of a network of enzymes that generate, interconvert, and donate $C_1$ units. These $C_1$ units come from glycine, serine, and formate: 5,10-CH$_2$-H$_4$PteGlu$_n$ is formed by GDC from glycine and H$_4$PteGlu$_n$ or by SHMT from serine and H$_4$PteGlu$_n$; 10-formyltetrahydrofolate (10-HCO-H$_4$PteGlu$_n$) is generated by FTHFS from formate and H$_4$PteGlu$_n$. These $C_1$-generating enzymes exist in more than one subcellular location to meet demands for $C_1$ units in each subcellular compartment (Fig. 1.3). Both the cytosol and plastids have FTHFS and SHMT, while mitochondria contain FTHFS, SHMT and GDC.

All five $C_1$ derivatives of H$_4$PteGlu$_n$ exist in each plant subcellular compartment. They range from the most oxidized form, 5-formyl-tetrahydrofolate (5-HCO-H$_4$PteGlu$_n$)
and 10-HCO-H₄PteGluₙ, to the most reduced form, 5-CH₃-H₄PteGluₙ (Fig. 1.3). These C₁ groups on H₄PteGluₙ can be interconverted between different oxidation states via reactions catalyzed by a group of enzymes: SHMT, 5,10-methenyltetrahydrofolate cyclohydrolase–5,10-methylene tetrahydrofolate dehydrogenase (MTHFC–MTHFD), MTHFR, and 5-formyltetrahydrofolate cycloligase (Fig. 1.3). MTHFR is confined to the cytosol. However, there is 5-CH₃-H₄PteGluₙ in plastids and mitochondria. Therefore, it is thought that 5-CH₃-H₄PteGluₙ is imported from the cytosol by folate transporters (Hanson et al., 2000).

In plant cells, C₁ derivatives of folates are not evenly distributed among subcellular compartments. In the cytosol, folates are mainly associated with methyl groups, which are required for methionine synthesis (Chen et al., 1997). In mitochondria, most folates are 5-formyl derivatives, with H₄PteGluₙ being the second most dominant species (Orsomando et al., 2005; Rébeillé et al., 2006). In agreement with this, H₄PteGluₙ is synthesized in mitochondria, and 5-formyltetrahydrofolate cycloligase, which converts 5-HCO-H₄PteGluₙ to 5,10-methenyltetrahydrofolate (5,10-CH⁺-H₄PteGluₙ), is only present in mitochondria (Roje et al., 2002). The role of 5-HCO-H₄PteGluₙ in C₁ metabolism is not clear, although it may serve as a regulator of SHMT activity in C₁ metabolism and photorespiration (Stover and Schirch, 1993; Roje et al., 2002).
Figure 1.3. Schematic representation of folate-dependent C₁ metabolic network in plant cells. FDH, formate dehydrogenase; MTHFC–MTHFD, 5,10-methenyltetrahydrofolate cyclohydrolase–5,10-methylenetetrahydrofolate dehydrogenase; 5-FCL, 5-formyltetrahydrofolate cycloligase; SHMT, serine hydroxymethyltransferase; GDC, glycine decarboxylase; FTHFS, 10-formyltetrahydrofolate synthetase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MS, methionine synthase; TS, thymidylate synthase.
1.3.1 FTHFS in C\textsubscript{1} Metabolism

FTHFS catalyzes the formation of 10-HCO-H\textsubscript{4}PteGlun\textsubscript{n} from formate, H\textsubscript{4}PteGlun\textsubscript{n}, and ATP. The resulting 10-HCO-H\textsubscript{4}PteGlun\textsubscript{n} can be either used in purine synthesis or further reduced to 5,10-CH\textsubscript{2}-H\textsubscript{4}PteGlun\textsubscript{n} and 5-CH\textsubscript{3}-H\textsubscript{4}PteGlun\textsubscript{n}, which are engaged in the biosynthesis of thymidylate and methionine. Using $^{13}$C nuclear magnetic resonance in Arabidopsis, carbons from formate were shown to be integrated into serine, presumably through the pathway of FTHFS and SHMT, (Prabhu et al., 1996). This finding confirms that formate is a source of C\textsubscript{1} units in plants. A FTHFS cDNA has been cloned from spinach leaves (Nour and Rabinowitz, 1992), and a FTHFS from pea cotyledons has been isolated with $K_m$ values for mono- and pentaglutamylated H\textsubscript{4}PteGlun\textsubscript{n} reported (Kirk et al., 1994). No other work on characterization of isolated plant FTHFS is available.

In yeasts and animals, FTHFS is part of a trifunctional C\textsubscript{1}-tetrahydrofolate synthase, which also contains the domain for MTHFC-MTHFD activities (Cossins and Chen, 1997; Christensen and MacKenzie, 2006). MTHFC–MTHFD catalyzes the conversion between 10-HCO-H\textsubscript{4}PteGlun\textsubscript{n}, 5,10-CH\textsuperscript{\text{\textdegree}}-H\textsubscript{4}PteGlun\textsubscript{n}, and 5,10-CH\textsubscript{2}-H\textsubscript{4}PteGlun\textsubscript{n}. In plants, FTHFS remains separate from MTHFC–MTHFD (Kirk, 1995). This separation could lead to differential expression of FTHFS and MTHFC–MTHFD in different tissues or under different environments (Chen et al., 1997). Activities of MTHFC–MTHFD from mitochondria of etiolated pea seedlings remained unchanged when the seedlings were exposed to light, whereas FTHFS activity decreased ~70%, suggesting that formate is a minor source of 10-HCO-H\textsubscript{4}PteGlun\textsubscript{n} in mitochondria (Chen et al., 1997).

In pea cotyledons, FTHFS activity was mainly associated with the cytosolic
fraction (Kirk et al., 1994). FTHFS activity was also detected in pea mitochondria (Kirk et al., 1994; Chen et al., 1997). In pea chloroplasts, the existence of FTHFS was implied by feeding experiments in which $^{14}$C-formate was incorporated into serine, probably under the catalysis of FTHFS and SHMT (Shingles et al., 1984). However, FTHFS activity or protein could not be detected in pea chloroplasts in another study (Chen et al., 1997). To clarify its existence in chloroplasts, I show in Chapter Three the ability to detect FTHFS activity in isolated chloroplasts of pea and barley. This evidence supports that formate is a $C_1$ source in chloroplasts. Even though FTHFS activity has been found in all these subcellular compartments, cytosolic FTHFS accounts for the majority of activity in plant cells (Kirk et al., 1994; Chen et al., 1997), suggesting that formate is an important source of $C_1$ units in the cytoplasm.

Formate in plants is considered to be a by-product of photorespiration through the non-enzymatic degradation of glyoxylate (Igamberdiev, 1999). Other possible routes for formate formation are oxidation of formaldehyde and cleavage of 10-HCO-H$_4$PteGlu$_{\text{a}}$. Plant cells, however, contain a very small amount of formate (usually below 1 $\mu$mol g$^{-1}$ fresh weight), even with added exogenous formate (Prabhu et al., 1996; Hanson and Roje, 2001). Under normal conditions, formate is readily degraded to CO$_2$ by formate dehydrogenase (FDH) with the formation of NADH. In Arabidopsis leaves, both the FDH enzyme activity and mRNA levels increased following the addition of methanol, formaldehyde, or formate (Olson et al., 2000). The above evidence supports the role of FDH in formate degradation. However, the discovery of the new location of FDH in Arabidopsis chloroplasts implies a new role of this enzyme in generating formate from CO$_2$. Olson and colleagues proposed that in chloroplasts, where there are high energy
electrons, FDH could act in the direction of reducing CO₂ to formate (Olson et al., 2000). Formate formed by the reduction can be removed by formate-utilizing enzymes such as FTHFS, which has been confirmed in chloroplasts in this work.

1.3.2 **SHMT in C₁ Metabolism**

SHMT is a pyridoxal phosphate-dependent enzyme that catalyzes the reversible conversion of H₄PteGlu₉ and serine to 5,10-CH₂-H₄PteGlu₉ and glycine. In the presence of glycine, SHMT also converts 5,10-CH⁺-H₄PteGlu₉ to 5-HCO-H₄PteGlu₉, a potent inhibitor of SHMT’s primary catalytic activity. 5,10-CH₂-H₄PteGlu₉ is directly involved in thymidylate synthesis and is an important intermediate for biosynthesis of other essential molecules. In animal cells, SHMT activity generates about 70% of the C₁ groups needed for biosynthesis of thymidylate, purines, choline, and methionine (Stover and Schirch, 1991). In non-photosynthetic sycamore cambial cells, cytosolic SHMT produces 5,10-CH₂-H₄PteGlu₉ that is further reduced to 5-CH₃-H₄PteGlu₉ and primarily used in methionine biosynthesis (Mouillon et al., 1999).

SHMT activity has been found in all major plant subcellular fractions: plastids, mitochondria, the cytosol, and the nuclei (Besson et al., 1995; Neuburger et al., 1996). The *Arabidopsis* genome contains seven SHMT genes which encode proteins targeted to plastids, mitochondria, the cytosol, or the nuclei. One of the two putative *Arabidopsis* mitochondrial SHMTs, SHMT1, was shown to be localized in mitochondria (Jamai et al., 2009), while locations of the other six SHMTs remain to be confirmed. The subcellular localization of the putative plastid SHMT is verified by GFP fusions in this work.
Mitochondrial SHMT activity is crucial to plant photorespiration. In plant leaf mitochondria, the SHMT-catalyzed reaction moves in the direction of serine formation by consumption of 5,10-CH$_2$-H$_4$PteGlu$_a$ and glycine. This direction is driven by the large amount of 5,10-CH$_2$-H$_4$PteGlu$_a$ generated by the high activity of GDC during photorespiration (Rébeillé et al., 1994). In close cooperation with GDC, mitochondrial SHMTs play a major role in recycling carbons from glycolate-2-phosphate generated during photorespiration. An early study characterizing *Arabidopsis* photorespiration mutants showed that these mutants lacked mitochondrial SHMT activity (Somerville and Ogren, 1981). In one *Arabidopsis* SHMT mutant (*shm1-1*), the photorespiratory phenotype could not be rescued by overexpression of *shm2*, the gene of another mitochondrial SHMT. This means that *shm1* and *shm2* are not functionally redundant (Voll et al., 2006). Recently, a study demonstrated that *in vivo* interaction is required between SHMT1 and the mitochondrial ferredoxin-dependent glutamate synthase for photorespiration in *Arabidopsis* (Jamai et al., 2009). Interestingly, Moreno et al. found that another *shm1* mutant, *shmt1-1*, showed increased expression of stress-related genes under abiotic stress and were more susceptible to biotic infections than wild-type controls (Moreno et al., 2005). But significance of the rest SHMTs remain unclear.

Some plant SHMTs, including a plastid SHMT from spinach, have been isolated and partially characterized (Mitchell et al., 1986; Henricson and Ericson, 1988; Besson et al., 1993; Rébeillé et al., 1994; Besson et al., 1995). However, their properties for polyglutamylated folate substrates and inhibitors were not investigated. The only report available is on pea mitochondrial SHMT for diglutamylated folates. This SHMT has a $K_m$ value of 13.5 µM for H$_4$PteGlu$_2$ and a higher $K_m$ value of 37.5 µM for H$_4$PteGlu$_1$ (Besson
et al., 1993). Similarly, another study showed that SHMT from pig liver has increased affinity for \( \text{H}_4\text{PteGlu}_n \) with three glutamates (Matthews et al., 1982). The report on the pea mitochondrial SHMT also shows that \( V_{\text{max}} \) of this enzyme decreased only 30% while \( K_m \) dropped from 37.5 to \( \leq 3.7 \) \( \mu \text{M} \), a ten fold decrease, when \( n \) of \( \text{H}_4\text{PteGlu}_n \) increased from 1 to 6 (Besson et al., 1993). As a result, the predicted \( k_{\text{cat}}/K_m \) values, which indicate catalytic efficiency, would go up sharply with increasing of \( n \). However, this hypothetical high affinity and efficiency of plant SHMT has not been tested for \( \text{H}_4\text{PteGlu}_n \) with more than three glutamates. Furthermore, \( \text{H}_4\text{PteGlu}_n \) with three-to-six glutamates are dominant \( \text{H}_4\text{PteGlu}_n \) species in plant cells (Imeson et al., 1990; Neuberger et al., 1996; Orsomando et al., 2005). Therefore, assaying SHMT with these polyglutamylated \( \text{H}_4\text{PteGlu}_n \) would have more physiological significance than with mono- or diglutamylated ones. With the assumed high substrate affinity, SHMT could use polyglutamylated \( \text{H}_4\text{PteGlu}_n \) at very low concentrations. Indeed, estimated total folate concentration in pea protoplasts is around 4 \( \mu \text{M} \) as calculated from the published data (200 pmol mg\(^{-1}\) protein) (Orsomando et al., 2005), and the amount of \( \text{H}_4\text{PteGlu}_n \) is < 10% of total folates (Orsomando et al., 2005). The low cellular \( \text{H}_4\text{PteGlu}_n \) concentration makes it interesting and necessary to perform SHMT kinetic studies using \( \text{H}_4\text{PteGlu}_n \) with four-to-six glutamates. By using the new fluorometric assay, we were able to get kinetic parameters of an \emph{Arabidopsis} plastid SHMT for \( \text{H}_4\text{PteGlu}_{n=1-8} \). Conceivably, obtaining these parameters would help us understand SHMT-catalyzed reactions under conditions similar to \emph{in vivo} situations.

The mono- and triglutamate forms of both 5-HCO-\( \text{H}_4\text{PteGlu}_n \) and 5-CH\(_3\)-\( \text{H}_4\text{PteGlu}_n \) have been proven to be inhibitors of SHMTs from rabbit (Stover and
Schirch, 1991). Plant SHMT inhibition by 5-HCO-H₄PteGluₙ has been demonstrated in *Arabidopsis* leaves and with pea leaf mitochondria extracts *in vitro* (Roje et al., 2002; Goyer et al., 2005). Information on the inhibitory effects of these two inhibitors in both mono- and polyglutamylated forms for isolated plant SHMTs is still lacking. These two inhibitors have been found in plant mitochondria, plastids, and the cytosol (Orsomando et al., 2005). 5-HCO-H₄PteGluₙ is synthesized in a side reaction of SHMT, and it is not a C₁ donor. Possible roles of this compound are regulation of SHMT activity and a storage form of folates (Kruschwitz et al., 1994). 5-CH₃-H₄PteGluₙ is generated from 5,10-CH₂-H₄PteGluₙ by MTHFR, which is only present in the cytosol. Thus, 5-CH₃-H₄PteGluₙ in mitochondria and plastids are thought to be imported from the cytosol (Hanson et al., 2000). Since these inhibitors in plant cells are also polyglutamylated, inhibition by these folates will show us their effects on SHMT activity under physiologically relevant conditions. Here I report, in Chapter Three, the inhibition of the *Arabidopsis* plastid SHMT by these two inhibitors in both mono- and pentaglutamylated forms. Using polyglutamylated folate substrates and inhibitors in SHMT assays to mimic the *in vivo* cellular folate environment would help us gain insights into how SHMTs function inside plant cells.

1.4 Research Goals

Despite the importance of SHMTs in plant C₁ metabolism, many biochemical properties of plant SHMTs remain to be investigated, such as those for polyglutamylated folate substrates, mono-, and polyglutamylated inhibitors. Plant mitochondrial SHMTs
have been the focus of SHMT studies due to their vital roles in photorespiration. However, there is still much to learn about the physiological functions of plant SHMTs in other subcellular compartments. The availability of seven SHMT sequences in the *Arabidopsis* genome enabled us to undertake a comprehensive study on all seven SHMT isoforms. This study will allow us to better understand SHMT functions with respect to C\textsubscript{1} flux regulation and folate utilization in different compartments of plant cells. As part of this project, this thesis is focused on characterization of the plastid SHMT from *Arabidopsis*.

Chapter Two contains the content of a paper published in Analytical Biochemistry, showing the method development of an HPLC-based SHMT assay that uses as low as 10\textsuperscript{-8} M (6\text{R},6\text{S})-H\textsubscript{4}PteGlu\textsubscript{n}. The HPLC detection method in SHMT assay was also adapted to quantify products in FTHFS and MTHFR assays with enhanced detection ability. Chapter Three shows FTHFS and SHMT activities found in pea and barley chloroplast extracts, which clarifies the occurrence of these two C\textsubscript{1} providers in monocotyledon and dicotyledon plants and suggests that formate and serine are C\textsubscript{1} sources in plastids. Chapter Three also describes a detailed characterization of the *Arabidopsis* plastid SHMT, including gene cloning, recombinant protein purification, subcellular localization, kinetic properties, and inhibition. The data in Chapter Three presents a first-time report of SHMT properties for polyglutamylated substrates with one-to-eight glutamates and for inhibitors with one and five glutamates. This report furthers our understanding of plastid SHMT in C\textsubscript{1} metabolism and it also serves for comparisons with studies on other SHMTs that are being or to be investigated.
1.5 References


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AN HPLC-BASED FLUOROMETRIC ASSAY FOR SERINE HYDROXYMETHYLTRANSFERASE

Yi Zhang, Kehan Sun, and Sanja Roje

This chapter was adapted from a paper published in *Analytical Biochemistry* (2008), 375: 367-369. Sanja Roje and I developed and optimized the assay. Kehan Sun contributed to the tests of DTT concentrations in samples before loading to HPLC.

Abbreviations: SHMT, serine hydroxymethyltransferase; HCHO, formaldehyde; PLP, pyridoxal 5'-phosphate; DTT, dithiothreitol; NaBH₄, sodium borohydride.
2.1 Abstract

We have developed a novel HPLC-based fluorometric assay for serine hydroxymethyltransferase activity. In this assay, the 5,10-CH₂-H₄PteGlu formed by serine hydroxymethyltransferase activity is reduced to 5-CH₃-H₄PteGlu using NaBH₄. Then, the fluorescent assay components are separated by reversed-phase chromatography under isocratic conditions and 5-CH₃-H₄PteGlu is quantified by comparison with standards. We show that this assay can be used to measure serine hydroxymethyltransferase activity at 10⁻⁸ to 10⁻³ M (6R,6S)-H₄PteGlu.

2.2 Introduction

Serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) catalyzes reversible conversion of serine and (6S)-H₄PteGluₙ to glycine and (6S)-5,10-CH₂-H₄PteGluₙ in eukaryotes and prokaryotes. A radioassay and a spectrophotometric assay to measure SHMT activity toward formation of glycine and 5,10-CH₂-H₄PteGluₙ have been described previously (Taylor and Weissbach, 1965; Schirch and Gross, 1968; Geller and Kotb, 1989). The radioassay measures formation of 5,10-[methylene⁻¹⁴C]CH₂-H₄PteGluₙ from L-[3⁻¹⁴C]serine and H₄PteGluₙ. In one adaptation of the radioassay (Taylor and Weissbach, 1965), [¹⁴C]formaldehyde (HCHO) released from 5,10-[methylene⁻¹⁴C]CH₂-H₄PteGluₙ is derivatized into a dimedone adduct, which is then extracted into toluene and measured by scintillation counting. In another adaptation (Geller and Kotb, 1989), an aliquot of the reaction is deposited onto DEAE cellulose
paper, L-[3-\textsuperscript{14}C]serine is removed by a water wash, and 5,10-[methylene-\textsuperscript{14}C]CH\textsubscript{2}-H\textsubscript{4}PteGlu\textsubscript{a} bound to the paper is counted.

The spectrophotometric assay for SHMT activity is a coupled assay. In this assay, 5,10-CH\textsubscript{2}-H\textsubscript{4}PteGlu\textsubscript{a} formed by the activity of SHMT is oxidized to 5,10-CH\textsuperscript{=}+H\textsubscript{4}PteGlu\textsubscript{a} by the activity of 5,10-CH\textsubscript{2}-H\textsubscript{4}PteGlu\textsubscript{a} dehydrogenase in the presence of NADP\textsuperscript{+} (Schirch and Gross, 1968). Formation of NADPH from NADP\textsuperscript{+} is measured spectrophotometrically at 340 nm. 5,10-CH\textsubscript{2}-H\textsubscript{4}PteGlu\textsubscript{a} dehydrogenase required for this assay is not commercially available and so must be purified from an expression clone (Fu et al., 2003).

SHMT also catalyzes exchange of the pro-2S proton of glycine with solvent protons (Chen and Schirch, 1973). This side reaction of SHMT accelerates by approximately three orders of magnitude in the presence of H\textsubscript{4}PteGlu\textsubscript{a} and has been used accordingly to develop a sensitive radioassay to measure SHMT activity (Elsea et al., 1995; Kim et al., 1997; Stover et al., 1997). However, this assay is not suitable for determining kinetic constants of SHMTs for the physiologically relevant substrates serine and H\textsubscript{4}PteGlu\textsubscript{a}.

High $K_m$ values for serine and low $K_m$ values for H\textsubscript{4}PteGlu\textsubscript{a} in some SHMTs complicate biochemical characterization of these enzymes using the standard radioassay. For example, radioassays with SHMT from pea leaf mitochondria revealed $K_m$ values below $3.7 \times 10^{-6}$ M for H\textsubscript{4}PteGlu\textsubscript{a}=3–6 and $K_m$ values at 1 to $2 \times 10^{-3}$ M for serine in the presence of H\textsubscript{4}PteGlu\textsubscript{a}=1–6 (Besson et al., 1993). The $K_m$ values for H\textsubscript{4}PteGlu\textsubscript{a}=3–6 could not be determined because of the unfavorable signal/background ratio in the assays at low
H₄PteGluₙ concentrations. Hence, a method suitable for assaying SHMTs at high (>10⁻³ M) serine and low (<10⁻⁵ M) H₄PteGluₙ concentrations is needed to determine the $K_m$ values for H₄PteGluₙ for some SHMTs. Our goal was to develop such a method.

2.3 Experimental Methods

2.3.1 Reagents and Plant Materials

L-[3⁻¹⁴C]-serine was obtained from American Radiolabeled Chemicals (St. Louis, MO); (6R,6S)-H₄PteGlu, (6R,6S)-5,10-CH₂-H₄PteGlu, and (6R,6S)-5-CH₃-H₄PteGlu were from Schircks Laboratories (Jona, Switzerland); L-serine was from Sigma–Aldrich (St. Louis, MO); PD-10 column and Biodegradable Counting Scintillant were from GE Healthcare (Piscataway, NJ).

Wild type *Arabidopsis* plants, ecotype Columbia, were grown under normal conditions in the IBC Plant Growth Facility at Washington State University.

2.3.2 Protein Extraction from Arabidopsis

To prepare the protein extract, 1 g of *Arabidopsis* leaf tissue was frozen in liquid nitrogen, ground into a fine powder using a mortar and pestle, and resuspended in 3 ml of buffer A (0.2 M potassium phosphate [pH 7.5], 10% glycerol, 10⁻³ M EDTA, 10⁻³ M tris(hydroxypropyl)phosphine, and 10⁻⁵ M pyridoxal 5’-phosphate [PLP]). After centrifuging the sample at 10,000g for 15 min to remove the cellular debris, the supernatant was desalted on a PD-10 column equilibrated with buffer B (0.05 M
potassium phosphate [pH 7.5], 10% glycerol, 10^{-3} M tris(hydroxypropyl)phosphine, and 10^{-5} M PLP). Plant extracts contain enzymes, such as thymidylate synthase and 5,10-CH_{2}-H_{4}PteGlu dehydrogenase, which consume 5,10-CH_{2}-H_{4}PteGlu. We desalted the crude plant extracts to remove cosubstrates and coenzymes required by these enzymes, thereby preventing consumption of the newly formed 5,10-CH_{2}-H_{4}PteGlu.

2.3.3 SHMT Radioassay

The radioassay was essentially as described by Taylor and Weissbach (Taylor and Weissbach, 1965). Each 100-µl assay mixture contained 10^{-4} or 10^{-3} M (6R,6S)-H_{4}PteGlu (0.5 × 10^{-4} or 0.5 × 10^{-3} M, respectively, of the active substrate (6S)-H_{4}PteGlu), 2 × 10^{-3} M serine, 1 nCi L-[3-^{14}C]serine, 4 × 10^{-3} M tris(hydroxypropyl)phosphine, and 2.5 × 10^{-4} M PLP in 0.05 M potassium phosphate buffer (pH 7.5). A reducing agent, tris(hydroxypropyl)phosphine, was added to the assays to prevent oxidative cleavage of H_{4}PteGlu. After incubating at 30 °C for 20 min, the reactions were chilled on ice and serine was added to the blanks. Then, 75 µl of 1 M sodium acetate, 50 µl of 0.1 M HCHO, and 75 µl of 0.4 M dimedone in 50% ethanol were added to the assays. Formation of the dimedone–HCHO adduct (visible as a yellow precipitate) was driven to completion by boiling the samples for 5 min. After centrifuging at 20,800g for 8 s and adding 1 ml of toluene, the sample tubes were shaken for 10 min at maximum speed in a vortexer with a multitube attachment. The samples were centrifuged again at 20,800g for 10 min. To quantify the extracted HCHO–dimedone adduct, 0.5 ml of the toluene phase was mixed with 5 ml of Biodegradable Counting Scintillant and counted in a Tri-Carb 2100 TR liquid scintillation analyzer (PerkinElmer, Waltham, MA, USA). Extraction efficiency
was determined from those assays in which [14C]HCHO replaced L-[3-14C]serine.

2.3.4 SHMT HPLC-based Assay

To assay SHMT activity using the HPLC procedure we developed, the samples were prepared and incubated as described above for the radioassay except that L-[3-14C]serine was omitted and each 50-µl assay mixture contained 10^{-8} to 10^{-3} M (6R,6S)-H_{4}PteGlu (0.5 \times 10^{-8} to 0.5 \times 10^{-3} M of the active substrate (6S)-H_{4}PteGlu). The reactions were stopped by adding 25 µl of 0.1 M dithiothreitol (DTT) and 50 µl of 0.1 M NaBH_{4}. The NaBH_{4} solution was freshly prepared before use. The reactions were incubated at 37 °C for 15 min to drive formation of 5-CH_{3}-H_{4}PteGlu to completion. Adding more DTT before the borohydride reduction step lowered reduction efficiency, whereas adding less DTT raised blank values (not shown). The derivatized samples were boiled for 3 min and centrifuged at 20,800g for 5 min to pellet the precipitated protein. To prevent product decomposition, 25 µl of 0.6 M DTT was added to the supernatant before HPLC separation. The 5-CH_{3}-H_{4}PteGlu formed was stable at 30 °C for at least 24 h.

The fluorescent compounds were separated by reversed-phase chromatography using a Waters Alliance 2695 separation module equipped with a SunFire C_{18} column (4.6 × 150 mm, 3.5 µm) and a SunFire C_{18} guard column and were measured by a Waters 2475 fluorescence detector using an excitation wavelength of 280 nm and an emission wavelength of 359 nm. The separation was done isocratically with 5% acetonitrile and 95% phosphoric acid (3.3 \times 10^{-2} M) at 0.8 ml/min for 10 min at 30 °C. Sample injection was 5 µl unless otherwise indicated.
2.4 Results and Discussion

In the method we developed, the 5,10-CH₂-H₄PteGlu formed by SHMT activity is reduced to 5-CH₃-H₄PteGlu using NaBH₄. Then the fluorescent assay components are separated by reversed-phase chromatography under isocratic conditions and 5-CH₃-H₄PteGlu is quantified by comparison with standards. Borohydride is widely used to prepare reduced folate derivatives that are needed as substrates for enzyme assays (Whitfield et al., 1970; Matthews, 1986), and it is also used for analyses of folates in biological samples to reduce 5,10-CH₂-H₄PteGlu, 5,10-CH⁺-H₄PteGlu, 5-HCO-H₄PteGlu, and 10-HCO-H₄PteGlu to 5-CH₃-H₄PteGlu (Horne, 2001; Ndaw et al., 2001). To separate and measure H₄PteGlu and 5-CH₃-H₄PteGlu, we modified a previously published procedure (Gregory et al., 1984; Konings, 1999). A similar method has been used to separate and measure products of methionine synthase and methylenetetrahydrofolate reductase activities (Huang et al., 2001).

Under the chromatographic conditions described here, 5,10-CH₂-H₄PteGlu decomposes to H₄PteGlu and HCHO. The retention time of H₄PteGlu was approximately 6.7 min, and that of 5-CH₃-H₄PteGlu was approximately 8.5 min (Fig. 2.1). 5-CH₃-H₄PteGlu formed in the assay was quantified by comparison with standards (Fig. 2.2A). The efficiency of borohydride reduction was approximately 95% as judged by a comparison of a standard curve obtained using reduced 5,10-CH₂-H₄PteGlu with that obtained using authentic 5-CH₃-H₄PteGlu (not shown). The reaction rate was linear with the time and enzyme concentration (Fig. 2.2B).

SHMT activity measured using the HPLC-based assay was similar to that
measured using the radioassay at $10^{-4}$ or $10^{-3}$ M (6R,6S)-H$_4$PteGlu (Table 2.1). Consuming approximately 15% of (6S)-H$_4$PteGlu resulted in a signal/blank ratio of 1.8 at $10^{-4}$ M (6R,6S)-H$_4$PteGlu. Lower signal/blank ratios precluded the use of the radioassay at lower substrate concentrations, in agreement with the published evidence (Besson et al., 1993). Adding more L-[3-$^{14}$C]serine did not increase the radioassay sensitivity because the blank value increased proportionally to the amount of the radiolabel in the assay (not shown). Our HPLC-based assay yielded measurable SHMT activity with as low as $10^{-8}$ M (6R,6S)-H$_4$PteGlu (Table 2.1). In conclusion, the HPLC-based enzyme assay we developed is suitable for measuring SHMT activity at low H$_4$PteGlu and high serine concentrations. This assay is expected to enable determination of kinetic parameters for SHMTs with a low $K_m$ value for H$_4$PteGlu and a high $K_m$ value for serine.

2.5 Acknowledgment

This work was supported by grant MCB-0429968 from the National Science Foundation.
2.6 References


Figure 2.1. Chromatogram of (6R,6S)-H₄PteGlu and (6R,6S)-5-CH₃-H₄PteGlu under same HPLC conditions. The chromatograms show H₄PteGlu produced by decomposition of 5,10-CH₂-H₄PteGlu and 5-CH₃-H₄PteGlu produced by borohydride reduction of 5,10-CH₂-H₄PteGlu. 5,10-CH₂-H₄PteGlu (0.5 pmol) was separated by reversed-phase chromatography and measured by fluorescence detection before and after reduction as described in the text. Peak identity was confirmed by comparison with H₄PteGlu and 5-CH₃-H₄PteGlu standards (not shown). FAU, fluorescence in arbitrary units.
Figure 2.2. Standard curve for 5-CH$_3$-H$_4$PteGlu (A) and linearity of the reaction rate with time and enzyme concentration (B). The standard curve was prepared by reducing, isocratically separating, and fluorometrically measuring a series of samples containing 0.05 to 5 pmol of 5,10-CH$_2$-H$_4$PteGlu, followed by linear regression analysis of the associated peak areas. Data are means ± SD of three replicates. Error bar not shown is smaller than the symbol.
Table 2.1. SHMT activity in protein extracts from *Arabidopsis* leaves as measured by the HPLC-based assay and a radioassay

<table>
<thead>
<tr>
<th>(6R,6S)-H$_4$PteGlu (M)</th>
<th>SHMT activity (nmol min$^{-1}$ mg$^{-1}$)</th>
<th>HPLC-based assay</th>
<th>Radioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-5}$</td>
<td>58.045 ± 1.630</td>
<td>60.572 ± 1.170</td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>26.829 ± 1.350</td>
<td>36.456 ± 1.460</td>
<td></td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>8.0877 ± 0.2730</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>1.4917 ± 0.0350</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>0.1177 ± 0.0060</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>0.0137 ± 0.00045</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* SHMT activity was measured at various concentrations of (6R,6S)-H$_4$PteGlu at 2 × $10^{-3}$ M serine using our HPLC-based assay and a radioassay. Sample injection volumes were 100 µl for $10^{-8}$ M H$_4$PteGlu, 50 µl for $10^{-7}$ M H$_4$PteGlu, and 5 µl for the remaining assays. Data are means ± SD of three replicates. –, not determined.
CHAPTER THREE

ONE-CARBON METABOLISM IN PLANTS: CHARACTERIZATION OF A PLASTID SERINE HYDROXYMETHYLTRANSFERASE

Yi Zhang, Kehan Sun, Francisco J. Sandoval, Katherine Santiago, and Sanja Roje

This chapter was adapted from a manuscript submitted to the Journal of Biological Chemistry. I conducted protein purification, subcellular localization, and kinetic characterization of AtSHMT3. I also performed enzyme assays of proteins extracts of chloroplasts from pea and barley. Kehan Sun participated in assaying 5-methylene-tetrahydrofolate reductase. Francisco J. Sandoval isolated chloroplasts from pea and barley. Katherine Santiago participated in the protein purification of recombinant AtSHMT3. Sanja Roje cloned the cDNA of AtSHMT3.

Abbreviations: EGFP, enhanced green fluorescent protein; FPLC, fast protein liquid chromatography; THP, tris(hydroxypropyl)phosphine; PLP, pyridoxal 5′-phosphate; CHES, 2-(cyclohexylamino)ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography.
3.1 Abstract

Serine hydroxymethyltransferases (SHMTs, EC 2.1.2.1) catalyze reversible hydroxymethyl-group transfer from serine to tetrahydrofolate ($\text{H}_4\text{PteGlu}_n$), yielding glycine and $5,10\text{-CH}_2\text{H}_4\text{PteGlu}_n$. In plastids, SHMTs are thought to catalytically direct the hydroxymethyl moiety of serine into the metabolic network of $\text{H}_4\text{PteGlu}_n$-bound one-carbon units. Genes encoding putative plastid SHMTs were found in the genomes of an alga (*Chlamydomonas reinhardtii*), a moss (*Physcomitrella patens*), and dicotyledon flowering plants, but not in the genomes of monocotyledon flowering plants. SHMT activity was detected in chloroplasts in pea (*Pisum sativum*) and, unexpectedly, in barley (*Hordeum vulgare*), suggesting that plastid SHMTs exist in all flowering plants. Enzyme activity of 10-formyltetrahydrofolate synthetase, which directs formate into the one-carbon metabolic network, was detected in chloroplasts in pea and barley, suggesting that plastids in all flowering plants use both serine and formate as sources of one-carbon units. The *Arabidopsis thaliana* genome encodes one putative plastid SHMT (AtSHMT3). Its cDNA was cloned by reverse transcription-PCR and the encoded recombinant protein was produced in *Escherichia coli*. Evidence for plastid targeting of AtSHMT3 was found by confocal microscopy of *A. thaliana* protoplasts transformed with proteins fused to enhanced green fluorescent protein. Characterization of recombinant AtSHMT3 revealed that substrate affinity for and the catalytic efficiencies of $\text{H}_4\text{PteGlu}_{n=1-8}$ increase with $n$, and that $\text{H}_4\text{PteGlu}_{n=1-8}$ inhibit AtSHMT3. $5\text{-CH}_3\text{H}_4\text{PteGlu}_{1&5}$ and $5\text{-HCO-H}_4\text{PteGlu}_{1&5}$ inhibited AtSHMT3-catalyzed hydroxymethyl-group transfer from serine to $\text{H}_4\text{PteGlu}_6$, with the pentaglutamylated inhibitors being more effective. Calculations revealed
inhibition with 5-CH$_3$-H$_4$PteGlu$_n$ or 5-HCO-H$_4$PteGlu$_n$ resulting in little reduction in AtSHMT3 activity under folate concentrations estimated for plastids.

### 3.2 Introduction

Tetrahydrofolate (H$_4$PteGlu$_n$) and its one-carbon (C$_1$) derivatives (folates) are essential cofactors and co-substrates for C$_1$ transfer reactions in plants and other organisms. One-carbon H$_4$PteGlu$_n$ derivatives are required in plastids for biosynthesis of methionine (Ravanel et al., 2004), N-formylmethionine (Hanson et al., 2000), thymidine nucleotide (Luo et al., 1997; Hanson et al., 2000), and the inosine monophosphate precursors formylglycinamide ribonucleotide and 5-formaminoimidazole-4-carboxamide ribonucleotide (Hanson et al., 2000; Zrenner et al., 2006) (Fig. 3.1). Also, 5,10-methenyltetrahydrofolate (5,10-CH$^+$-H$_4$PteGlu$_n$) serves as light-harvesting cofactor in a plastid-localized cryptochrome (Huang et al., 2006).

Living cells conjugate folates with a varying number of glutamate residues, which are linked into a chain through the $\gamma$-carboxyl group. Folates having up to eleven glutamate residues have been found in a diverse range of organisms including plants (Qi et al., 1999; Scott et al., 2000). Folate polyglutamylation is catalyzed by folylpolyglutamate synthetase. This enzyme resides in plastids, mitochondria, and the cytosol in plants (Ravanel et al., 2001). Physiological roles of folate polyglutamylation in plants are not known. Studies in the yeast *Saccharomyces cerevisiae* (Cherest et al., 2000) and in Chinese hamster ovary cells (Osborne et al., 1993) found that folate
polyglutamylation is essential for biosynthesis of methionine, adenine, and thymidine, as well as for the stability of mitochondrial DNA. Accordingly, it is plausible that polyglutamylation also plays important physiological roles in plants. Understanding how the degree of the substrate and inhibitor folate polyglutamylation affects catalytic properties of $C_1$ enzymes is thus essential to understanding the roles of folate polyglutamylation in plant metabolism.

Two enzymes which catalyze derivatization of $H_4PteGlu_n$ to its $C_1$ derivatives are thought to operate in plastids: serine hydroxymethyltransferase (SHMT) catalyzes reversible hydroxymethyl-group transfer from serine to $H_4PteGlu_n$, yielding glycine and 5,10-methylenetetrahydrofolate (5,10-CH$_2$-$H_4PteGlu_n$) (Besson et al., 1995; Hanson et al., 2000); and formate-tetrahydrofolate ligase (10-formyltetrahydrofolate synthetase, FTHFS) catalyzes ATP-driven formylation of $H_4PteGlu_n$, yielding 10-formyltetrahydrofolate (10-HCO-$H_4PteGlu_n$), ADP, and phosphate (Shingles et al., 1984; Hanson et al., 2000). These two enzymes are thought to provide $C_1$ units for biosynthesis of 5,10-CH$_2$-$H_4PteGlu_n$, 5,10-CH$^+$-$H_4PteGlu_n$, and 10-HCO-$H_4PteGlu_n$; 5-methyltetrahydrofolate (5-CH$_3$-$H_4PteGlu_n$) is probably imported into plastids from the cytosol (Hanson et al., 2000) (Fig. 3.1). Thus, SHMT and FTHFS activities in plastids are potentially vital for photoreception and for biosynthesis of purines, pyrimidines, and $N$-formylmethionine. Plastid SHMTs are the main focus of this study.

Multiple SHMTs are encoded in plant genomes; bioinformatic evidence suggests the presence of seven enzymes in Arabidopsis thaliana (McClung et al., 2000; Hanson and Roje, 2001) and five in rice (Oryza sativa) (Ohyanagi et al., 2006). Previous studies
have found SHMT activity in mitochondria, plastids, the nuclei, and the cytosol in various plant species (Somervilie and Ogren, 1981; Shingles et al., 1984; Gardeström et al., 1985; Besson et al., 1995; Neuburger et al., 1996; Moreno et al., 2005; Schjoerring et al., 2006; Voll et al., 2006). The gene for a mitochondrial SHMT from pea has been cloned, and the encoded enzyme has been detected in mitochondria using antibodies raised to the recombinant protein (Turner et al., 1992). Also, the gene for a mitochondrial SHMT in which mutation causes a photorespiratory phenotype in *A. thaliana* has been identified (Voll et al., 2006). Though SHMTs have been isolated and in some cases partially characterized from several plant species (Prather and Sisler, 1966; Mazelis and Liu, 1967; Rao and Rao, 1982; Mitchell et al., 1986; Henricson and Ericson, 1988; Besson et al., 1993; Rébeillé et al., 1994; Besson et al., 1995), the kinetic properties of those enzymes against folylpolyglutamate substrate species need to be investigated. A study in pea (*Pisum sativum*) found increased affinity for folylpolyglutamate substrates by SHMT from leaf mitochondria (Besson et al., 1993); however, the enzyme’s *K_m* values for H_4PteGlu_n>2 were not determined because of the lack in assay sensitivity. Studies in mammals found increased affinity for folylpolyglutamate substrates in SHMTs from rabbit liver (Strong et al., 1989; Strong and Schirch, 1989), mouse L1210 cells (Strong et al., 1990), and pig liver (Matthews et al., 1982).

Studies on inhibition of individual SHMTs by 5-CH_3-H_4PteGlu_n and 5-HCO-H_4PteGlu_n are also lacking. 5-CH_3-H_4PteGlu_n species inhibit SHMTs from rabbit (Schirch and Ropp, 1967; Stover and Schirch, 1991), and they bind SHMTs from pig and *Escherichia coli* (Matthews et al., 1983; Schirch et al., 1985), but it is not known whether 5-CH_3-H_4PteGlu_n bind or inhibit plant SHMTs. 5-HCO-H_4PteGlu_n species inhibit SHMTs
from rabbit and pea mitochondria (Schirch and Ropp, 1967; Stover and Schirch, 1991; Roje et al., 2002), and they bind SHMTs from human and zebrafish (Fu et al., 2005; Chang et al., 2006; Chang et al., 2007). It is not known whether 5-HCO-H₄PteGluₙ bind or inhibit SHMTs present in plastids and the cytosol in plants.

Here we report biochemical evidence for SHMT activity in plastids in both A. thaliana and barley (Hordeum vulgare), contradicting bioinformatic prediction suggesting this enzyme resides only in dicot plastids. We also report biochemical evidence for FTHFS activity in chloroplasts in pea and barley, suggesting that plastids in all flowering plants use both serine and formate as sources of one-carbon units. Also, we describe cloning, recombinant expression, and biochemical characterization of a putative plastid SHMT from A. thaliana (AtSHMT3), and show evidence that AtSHMT3 resides in plastids from fluorescence microscopy of EGFP-fused protein constructs. The significance of these new findings for the overall understanding of C₁ metabolism is discussed.

3.3 Experimental Procedures

3.3.1 Materials and Plant Growth Conditions

(6R,6S)-H₄PteGlu₁, (6R,6S)-5,10-CH₂-H₄PteGlu₁, (6R,6S)-5,10-CH⁺-H₄PteGlu₁, (6R,6S)-5-HCO-H₄PteGlu₁·₅, (6R,6S)-5-CH₃-H₄PteGluₙ=1–₅, and PteGluₙ=1–₈ were purchased from Schircks Laboratories (Jona, Switzerland); Benzonase nuclease and recombinant enterokinase were from Novagen (Madison, WI); oligonucleotides were
from MWG (High Point, NC); Percoll was from GE Healthcare; serine was from Sigma; KBH₄ was from Acros Organics (Morris Plains, NJ).

*A. thaliana* plants, ecotype Columbia, were grown in potting soil at 12-h light intervals (100–120 µE m⁻² s⁻¹) for 3 weeks at 22 °C. Pea, cv. Progress 9, and barley, cv. Bob, were respectively grown in coarse vermiculite and potting soil at 12-h light intervals (75 µE m⁻² s⁻¹) for 7–10 days at 22 °C during the day and at 18 °C during the night.

### 3.3.2 Phylogenetic Analysis

Putative SHMT protein sequences were obtained from the GenBank™ database using the BLASTP and TBLASTN prediction programs with AtSHMT3 as a query sequence. The phylogenetic analysis included SHMT protein sequences from four plant species: *A. thaliana* (At4g37930, At5g26780, At4g32520, At4g13930, At4g13890, At1g22020, At1g36370), *O. sativa* (Os03g0738400, Os11g0455800, Os12g0409000, Os01g0874900, Os05g0429000), *Physcomitrella patens* (Phypadraft_195007, Phypadraft_220881, Phypadraft_197330, Phypadraft_168438, Phypadraft_141291, Phypadraft_117570, Phypadraft_129878), and *Chlamydomonas reinhardtii* (Chlredraft_196354, Chlredraft_196400, Chlredraft_194461). The phylogenetic tree was made with MEGA 4.0 (Tamura et al., 2007). The neighbor-joining method included Poisson correction, complete deletion, and (1000) bootstrap replication.

### 3.3.3 cDNA Cloning, Constructs, Sequence Analysis, and Expression in E. coli

The AtSHMT3 cDNA (At4g32520) was cloned by reverse transcription-PCR. Total *A. thaliana* RNA was isolated and reverse-transcribed as described before
(Sandoval et al., 2008). The AtSHMT3 open reading frame (ORF) was amplified using Taq2000 DNA polymerase (Stratagene, La Jolla, CA) and the primer pair 5′–GACGACGACAAGATGCAAGCTTGTTGTGG–3′ (AtSHMT3 Forward) and 5′–GAGGAGAAGCCCGGTTTAAACGCAGGAATGGGAA–3′ (AtSHMT3 Reverse). Note that the vector-specific sequences (underlined), needed for cloning into the pET Ek/LIC expression vectors (Novagen), flank the gene-specific sequences of the primers. The amplified ORF was purified with a Wizard PCR spin column (Promega, Madison, WI), and then cloned into the pGEM-T Easy vector (Promega) to generate the pGEM-AtSHMT3 construct.

The AtSHMT3 ORF, excluding the region coding for the putative organellar targeting peptide, was amplified from pGEM-AtSHMT3 using Pfu DNA polymerase (Stratagene) and the primer pair 5′–GACGACGACAAGATGAGAGCATTCTTCAGT–3′ (AtSHMT3n Forward) and AtSHMT3 Reverse. The resulting PCR fragment was purified with a Wizard PCR column, treated with T4 DNA polymerase (Promega), and then inserted into the pET-44 Ek/LIC vector. All procedures were done in accordance with the manufacturer’s protocols. All constructs were verified by DNA sequencing.

The expression vectors were introduced into the Rosetta strain of E. coli (Novagen) to produce the recombinant protein. Bacteria carrying the expression vector were cultured at 37 °C in LB medium containing 100 µg ml⁻¹ kanamycin and 34 µg ml⁻¹ chloramphenicol until absorbance at 600 nm (A₆₀₀) reached 0.6–1. After addition of isopropyl-β-D-thiogalactopyranoside (200-μM final concentration), the culture was further incubated overnight at 15 °C.
3.3.4 Recombinant Protein Purification

Induced *E. coli* cells expressing recombinant AtSHMT3 were harvested by centrifugation (7,500 × g, 10 min, 4 °C). Bacterial cells were resuspended in 1 ml of buffer A plus 25 U ml⁻¹ Benzonase nuclease, and mechanically broken with 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK). (Buffer A: 50 mM Tris-HCl, pH 8.5, 1 mM THP, 0.01 mM PLP, 10% glycerol.) Protein extracts were cleared by centrifugation (20,800 × g, 15 min, 4 °C), followed by filtering supernatants through a 0.45-µm polyvinylidene fluoride membrane. The recombinant protein was purified by anion exchange chromatography using an ÄKTA FPLC system equipped with a Mono Q 5/50 GL column (GE Healthcare); bound proteins were eluted with a linear gradient of buffer A to buffer A plus 1 M NaCl over 20 column volumes. Fractions containing the recombinant protein were desalted with PD-10 Desalting columns (GE Healthcare) equilibrated with buffer A, and then digested with recombinant enterokinase. The digested recombinant enzyme was further purified on a Mono Q 5/50 GL column as described above; untagged AtSHMT3 eluted in the flow-through. Fractions containing the recombinant enzyme were frozen in liquid N₂ and stored at −80 °C until use. The recombinant enzyme for activity assays was desalted with 50 mM CHES-HEPES-citric acid buffer, pH 8.5 (Newman, 2004), containing 1 mM THP, 0.25 mM PLP, and 10% glycerol.

3.3.5 Extraction of Proteins from Leaves and Chloroplasts of Pea and Barley

To isolate soluble leaf proteins, pea or barley leaves were pulverized in liquid N₂,
and the material was suspended in 1.25 ml of buffer B per 0.5 g of leaf material. (Buffer B: 200 mM potassium phosphate, pH 7.5, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 10% glycerol.) Leaf protein extracts were mixed briefly by stirring, mixtures were cleared by centrifugation (20,800 × g, 30 min, 4 °C), and then the supernatant was desalted with a PD-10 column equilibrated with buffer B without PMSF.

Chloroplasts from pea or barley were isolated by centrifugation on Percoll density gradients using published procedures (Cline, 1986; Brock et al., 1993; Schulz et al., 2004). Isolated chloroplasts were resuspended in buffer B, and broken by four cycles of freezing and thawing. Chloroplast protein extracts were cleared by centrifugation (20,800 × g, 20 min, 4 °C), followed by desalting supernatants with Zeba Desalt spin columns (Pierce Biotechnology, Rockford, IL) equilibrated with buffer B without PMSF. The plastid marker glyceraldehyde-3-phosphate dehydrogenase and the mitochondrial marker fumarase were assayed as described before (Roje et al., 2002). 5,10-methylenetetrahydrofolate reductase (MTHFR) was assayed to evaluate contamination of isolated chloroplasts with the cytosol (Roje et al., 1999).

MTHFR activity was assayed as described before (Roje et al., 1999) but using unlabeled assay components and HPLC-fluorescence detection. Assay mixtures (20-µl final volume) were incubated at 30 °C for 20 min. Assay blanks had the enzyme added after the incubation. The reactions were stopped by addition of 1 M β-mercaptoethanol (10 µl), followed by boiling reaction mixtures for 4 min; denatured proteins were pelleted by centrifugation (20,800 × g, 30 min, 4 °C). Reaction products were separated isocratically on a Waters XTerra C18 column (4.6 × 100 mm, 5 µm), and monitored with
280 nm excitation and 359 nm emission wavelengths. The mobile phase contained 27 mM phosphoric acid and 7% methanol. The length of separation was 6 min. Authentic (6R,6S)-5-CH₃-H₄PteGlu₁ was used as standard for quantification.

Leaf or chloroplast protein extracts were frozen in liquid N₂ and stored at −80 °C until use. Protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as standard.

3.3.6 Transient Expression of EGFP-fused Proteins in A. thaliana Protoplasts

The full-length AtSHMT3-coding sequence was amplified with primers 5′−AAAAAGCAGGCTATGCAAGCTTGTTGTGGTG−3′ (AtSHMT3 GFPF) and 5′−AGAAAGCTGGGTGAACGCCAGGAATGGAAA−3′ (AtSHMT3 GFPR), and then reamplified with the attB adapter primer pair (Invitrogen). Resulting PCR fragments were purified with Wizard PCR columns, and then inserted into the pDNOR 221 vector (Invitrogen) using BP recombination. The cloned sequences were then fused to the N terminus of the EGFP sequence segment in the p2GWF7 destination vector using LR recombination. The construct in which the putative plastid targeting peptide of AtSHMT3 (MQACC GGNSMASLQQPGRVQG SVFPIMPVVTKFSQQLKFNISKPFRSSFLKRN LVSEMRA SSVSLPNVEISS) was fused to the N terminus of the EGFP sequence segment in the p2GWF7 destination vector was purchased from GenScript (Piscataway, NJ).

Leaf protoplasts were isolated from 4-week-old A. thaliana plants, and transformed with the EGFP-containing vectors using a published procedure (Sheen, 2001). Fluorescence was monitored using an LSM 510 confocal laser scanning
microscope (Carl Zeiss MicroImaging, Thornwood, NY). EGFP fluorescence was monitored with 488 nm excitation and 505–530 nm emission wavelengths, and chlorophyll fluorescence with 488 nm excitation and >650 nm emission.

3.3.7 Synthesis of (6R,6S)-H₄PteGluₙ=2−8

PteGluₙ=2−8 were reduced to (6R,6S)-H₄PteGluₙ=2−8 with KBH₄ (Scrimgeour and Vitols, 1966). (6R,6S)-H₄PteGluₙ=2−8 were then purified on a Mono Q 5/50 GL column; bound folates were eluted with a linear gradient of 0.13–2.0 M sodium acetate, pH 6.9 plus 0.2 M β-mercaptoethanol over 20 column volumes as described before (Besson et al., 1993). For use in enzyme activity assays, (6R,6S)-H₄PteGluₙ=2−8 were further purified with solid phase extraction on a Chromabond C₁₈ Hydra column (Macherey-Nagel, Düren, Germany). The column was washed with pure methanol and water prior to loading the samples; (6R,6S)-H₄PteGluₙ=2−8 were eluted with 10 mM THP. Fractions containing (6R,6S)-H₄PteGluₙ=2−8 were pooled, frozen in liquid N₂, and then stored at −80 °C until use. (6R,6S)-H₄PteGluₙ concentration was determined spectrophotometrically at A₂₉₈ (Temple and Montgomery, 1984).

3.3.8 SHMT Enzyme Assays

SHMT activity was measured using an HPLC-based fluorometric assay as described before (Zhang et al., 2008). CHES-HEPES-citric acid buffer, pH 8.5, replaced Tris-HCl in the assay mixture as preliminary results indicated artifacts when assaying in Tris-HCl. Measurements were made using an Alliance 2695 separations module equipped with a 2675 fluorescence detector (Waters, Milford, MA). Substrates were saturating, and
product formation was proportional to time and enzyme concentration. Less than 10% of the substrates were typically consumed. Product formation was determined after subtraction of a blank, wherein serine was added after incubation.

Reaction products were separated by reversed-phase chromatography on a Waters X Terra C18 column (4.6 × 100 mm, 5 µm), and were measured by fluorescence detection with 280 nm excitation and 359 nm emission wavelengths. The mobile phase contained 27 mM phosphoric acid and respectively 7%, 9%, or 10% methanol when substrates were (6R,6S)-H₄PteGluₙ=1–4, (6R,6S)-H₄PteGluₙ=5, or (6R,6S)-H₄PteGluₙ=6–8 and the inhibitors were absent; the length of the isocratic separation varied (6–15 min). Reaction products were separated isocratically when measuring inhibition with (6R,6S)-5-CH₃-H₄PteGlu₁&₅. The mobile phase contained 27 mM phosphoric acid and respectively 10% methanol or 4% acetonitrile for the assays with added (6R,6S)-5-CH₃-H₄PteGlu₁ or (6R,6S)-5-CH₃-H₄PteGlu₅. The length of separation was 9 min for (6R,6S)-5-CH₃-H₄PteGlu₁ and 20 min for (6R,6S)-5-CH₃-H₄PteGlu₅. Reaction products were separated under gradient conditions when measuring inhibition with (6R,6S)-5-HCO-H₄PteGlu₁&₅. The mobile phase contained 27 mM phosphoric acid and varied methanol content. For the assays with added (6R,6S)-5-HCO-H₄PteGlu₁, methanol was held at 11% for 10 min, increased to 20% over 2 min, and then decreased to 11% for 2 min. For the assays with added (6R,6S)-5-HCO-H₄PteGlu₅, methanol was held at 9% for 13 min, increased to 20% over 2 min, and then decreased to 9% for 2 min.

The (6R,6S)-5-CH₃-H₄PteGlu₁ standards were produced by reducing authentic (6R,6S)-5,10-CH₂-H₄PteGlu₁ using NaBH₄ under conditions identical to those used for
the assay products (Zhang et al., 2008). (6R,6S)-5,10-CH₂-H₄PteGlu₄=2–8 are commercially unavailable. Thus, these assay products were quantified by first comparing the peak areas associated with the reduced products with that of the (6R,6S)-5-CH₃-H₄PteGlu₁ standard, and then multiplying with the ratio of the respective peak areas for (6R,6S)-5-CH₃-H₄PteGlu₄=2–5 and (6R,6S)-5-CH₃-H₄PteGlu₁. (6R,6S)-5-CH₃-H₄PteGlu₄=6–8 are commercially unavailable. Thus, the (6R,6S)-5-CH₃-H₄PteGlu₄=6–8 to (6R,6S)-5-CH₃-H₄PteGlu₁ ratios were assumed to be 1 as deduced from the experimental data available for (6R,6S)-5-CH₃-H₄PteGlu₄=4&5. The concentrations of (6R,6S)-H₄PteGlu₄, (6R,6S)-5-CH₃-H₄PteGlu₄, and (6R,6S)-5,10-CH₂-H₄PteGlu₁ were determined spectrophotometrically using published molar extinction coefficients (Temple and Montgomery, 1984).

Initial reaction rates were measured at pH 8.5 with varied (6R,6S)-H₄PteGlu₄ concentrations and constant serine concentration. To measure inhibition with (6R,6S)-5-CH₃-H₄PteGlu₁&₅ or (6R,6S)-5-HCO-H₄PteGlu₁&₅, (6R,6S)-H₄PteGlu₆ was used as substrate. Serine (5 mM) and two (6R,6S)-H₄PteGlu₆ (2 and 4 µM) concentrations were held constant while the inhibitor concentrations were varied.

Apparent values for the kinetic parameters (Kₘ, Kᵢ, Vₘₐₓ) were found by fitting measured initial reaction rates against substrate or substrate and inhibitor concentrations to suitable enzyme inhibition models in the Enzyme Kinetics Module 1.2 for SigmaPlot 9.01. The coefficient of determination, R², helped single out the inhibition model (and derived parameters) that best fit one set of measurements.
3.3.9 Molecular Weight Determination and Enzyme Dilution Analysis

Molecular weight of AtSHMT3 was estimated by gel filtration chromatography using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 50 mM CHES-HEPES-citric acid buffer, pH 8.5, containing 1 mM THP, 0.25 mM PLP, and 10% glycerol. Reference proteins were ovalbumin (43,000), conalbumin (75,000), aldolase (158,000), and ferritin (440,000).

The effect of enzyme dilution on AtSHMT3 activity was checked in assays with (6R,6S)-H4PteGlu1, (6R,6S)-H4PteGlu2, (6R,6S)-H4PteGlu3, and (6R,6S)-H4PteGlu5 at 970, 100, 60, and 20 µM, respectively. Calculated AtSHMT3 activities were plotted against enzyme amounts used in the assays. Native AtSHMT3 oligomerization under buffer conditions similar to those in AtSHMT3 assays was examined by gel filtration chromatography using a Superose 6 10/300 GL column (GE Healthcare) equilibrated with the same buffer as used in standard SHMT assay plus 5 mM serine. AtSHMT3 was diluted using the same buffer before loading to the column. Eluate was collected in 250 µl fractions. Each fraction was then assayed for SHMT activity using the HPLC assay with 4 µM (6R,6S)-H4PteGlu5 and 5 mM serine.

3.3.10 FTHFS Enzyme Assays

FTHFS activity was assayed in 100 mM triethanolamine buffer, pH 7.5, containing 100 mM ammonium formate, 200 mM KCl, 2.5 mM MgCl2, 1 mM (6R,6S)-H4PteGlu1, and 2 mM ATP. Assay mixtures (50-µl final volume) were incubated at 30 °C for 20 min after addition of ammonium formate. Assay blanks had ammonium
formate added after the incubation. The reactions were stopped by addition of 0.36 M HCl (50 µl) (Tan et al., 1977), followed by incubation at room temperature for 10 min to reduce (6R,6S)-10-HCO-H₄PteGlu₁ to (6R,6S)-5,10-CH⁺-H₄PteGlu₁; denatured proteins were pelleted by centrifugation (20,800 × g, 30 min, 4 °C). Reaction products were separated on a Waters XTerra C₁₈ column (4.6 × 100 mm, 5 µm), and monitored with 360 nm excitation and 460 nm emission wavelengths. The mobile phase contained 27 mM phosphoric acid and 15% methanol. Authentic (6R,6S)-5,10-CH⁺-H₄PteGlu₁ was used as standard for quantification.

3.3.11 In Silico Expression Analysis

Gene expression was analyzed in silico using the Meta Analyzer tool of the GENEVESTIGATOR software package (www.genevestigator.ethz.ch), and the publicly available Affymetrix microarray data for A. thaliana (Zimmermann et al., 2004).

3.4 Results

3.4.1 Bioinformatic Sequence Analysis

Seven putative SHMT genes were previously found in the fully sequenced genome of A. thaliana; one gene was predicted to encode a plastid protein (Hanson and Roje, 2001). In addition, protein sequences from a monocot (rice, O. sativa), a moss (P. patens), and a green alga (C. reinhardtii) were retrieved from GenBank databases for use in the phylogenetic analysis (Fig. 3.2). The complete genomes of the four species used in
The SHMT protein sequences clustered into two main groups on the phylogenetic tree (Fig. 3.2). The first group divides into three sub-groups. The first two sub-groups contain SHMTs predicted to be localized in the nucleus or the cytosol in *A. thaliana*, *O. sativa*, and *P. patens*. The third sub-group contains an SHMT from *C. reinhardtii*. This sequence distribution within the first group suggests that plants evolved separate SHMTs for the nucleus and the cytosol after the evolution of algae, and before the evolution of mosses. The second group divides into two sub-groups, with SHMTs predicted to be localized in plastids or in mitochondria; this suggests that organellar SHMT isoforms evolved before the speciation of vascular plants. Thus, SHMTs from the same plant species but putatively targeted to different subcellular compartments are less similar to each other than they are to SHMTs from other plant species putatively targeted to the same organelle.

We found no SHMTs from rice that clustered with plastid SHMTs from the three other plant species, nor did we find candidates for putative plastid SHMTs from other monocot species after searches of DNA and protein databases.

Based on the bioinformatic analysis presented above and on previous paper reporting no SHMT activity in wheat chloroplasts (Gardeström et al., 1985), we hypothesized that monocot plastids do not contain SHMTs, but instead draw C₁ moieties from the formate pool via a reaction catalyzed by FTHFS. In support of this hypothesis, the rice genome contains two genes encoding putative FTHFSes (Os09g0446800 and AK110334), and the protein encoded by Os09g0446800 contains an N-terminal extension
predicted by ChloroP to be a plastid targeting peptide.

3.4.2 SHMT and FTHFS Activities in Chloroplasts

SHMT and FTHFS activities were examined in plastids of pea, a representative dicot, and barley, a representative monocot, with leaf extracts serving as positive assay controls. Chloroplasts of both species were found to have nearly equal levels of SHMT and FTHFS activity (Fig. 3.3), thus contradicting bioinformatic prediction suggesting lack of SHMT activity in monocot plastids.

3.4.3 cDNA Cloning and Recombinant Expression of AtSHMT3

Full-length cDNA for AtSHMT3 was cloned by reverse transcription-PCR using mRNA isolated from *A. thaliana* stems as template. The resulting cDNA fragment encoding the putative mature protein was amplified by PCR, subcloned into the pET-44 Ek/LIC vector, and then functionally expressed in *E. coli*.

3.4.4 Purification and Biochemical Characterization of AtSHMT3

Recombinant AtSHMT3 carrying Nus and hexahistidine tags at the N terminus was purified by ion exchange chromatography. The purified protein was then digested with recombinant enterokinase to cleave the tags. The cleaved Nus tag and the uncleaved fusion protein were removed by ion exchange chromatography (Fig. 3.4). Untagged recombinant enzymes were used in all subsequent work.

Primary plots of steady-state kinetic data for AtSHMT3 are shown in Fig. 3.5. AtSHMT3 was assayed for activity in the presence of varying H₄PteGluₙ=1−8
concentrations and a constant serine concentration. Kinetic parameters calculated from these data are presented in Table 3.1. These data show that $K_m$ values decreased and the catalytic efficiency ($k_{\text{cat}}/K_m$) increased with \( n \) for \( H_4\text{PteGlu}_{n=1-4} \), and that those two values remained nearly unchanged for \( H_4\text{PteGlu}_{n=4-8} \). Increased polyglutamylation resulted in smaller relative decreases in the $K_i$ values than in the $K_m$ values; the $K_i/K_m$ value increased about 18-fold for \( H_4\text{PteGlu}_{2&3} \), and about 54-fold for \( H_4\text{PteGlu}_{n=4-8} \) when compared to that for \( H_4\text{PteGlu}_1 \) (Table 3.1).

AtSHMT3 activity was inhibited by 5-CH$_3$-$H_4$PteGlu$_{1&5}$ and 5-HCO-$H_4$PteGlu$_{1&5}$ (Fig. 3.6 and Table 3.2). The pentaglutamate folates were more effective inhibitors than the monoglutamate folates (Table 3.2). This is consistent with previous studies showing that enzyme affinity for folylpolyglutamate inhibitors increases with the number of glutamates in mammalian SHMTs (Matthews et al., 1983; Stover and Schirch, 1991; Huang et al., 1998; Fu et al., 2005).

### 3.4.5 AtSHMT3 Molecular Weight Determination and Enzyme Dilution Analysis

Molecular weight of AtSHMT3 was estimated by gel filtration chromatography. AtSHMT3 peaks were identified by UV detection at 280 nm with apparent molecular weight value of 210,900 ± 320 (mean ± S.E., \( n = 3 \)). By comparing it to the theoretical value of 51,600, we concluded that AtSHMT3 is a tetramer under the specific condition used in the gel filtration experiment.

The effect of enzyme dilution on AtSHMT3 activity was investigated using \( H_4\text{PteGlu}_n \) with one, two, three, or five glutamates (Fig. 3.7). AtSHMT3 activities did not change significantly with enzyme dilutions when \( H_4\text{PteGlu}_1 \) and \( H_4\text{PteGlu}_2 \) were used as
substrates, but the activities of highly diluted AtSHMT3 decreased ~30% and ~40%, respectively, when H₄PteGlu₃ and H₄PteGlu₅ were used as substrates. We hypothesized that the decrease of activity is caused by deoligomerization when AtSHMT3 is highly diluted, and that polyglutamylated H₄PteGluₙ could help maintain this enzyme’s tetramer structure. To further check the oligomerization of AtSHMT3 under diluted concentrations, we loaded diluted AtSHMT3 to a Superose 6 column. When AtSHMT3 was diluted about fifty times (5-fold diluted AtSHMT was further diluted ten times in the column), the activity of AtSHMT3 migrated with apparent molecular weight of ~196,000, indicating a tetramer of AtSHMT3. However, we were unable to determine the elution volume of AtSHMT3 due to unreliable assay signals when AtSHMT3 was estimated to be diluted five-hundred times. The oligomerization of highly diluted AtSHMT3 remains to be explored by future studies.

3.4.6 Transient Expression of EGFP-fused Proteins in A. thaliana Protoplasts

Subcellular localization of AtSHMT3 was examined by fusing the full-length protein or its putative N-terminal targeting peptide to EGFP. Green fluorescence produced by expression of these fusion proteins in A. thaliana protoplasts colocalized with the red autofluorescence of chloroplasts (Fig. 3.8). Green fluorescence was also visible throughout the cytoplasm in control protoplasts expressing EGFP alone (Fig. 3.8). These findings thus establish that SHMT3 has a functional N-terminal peptide for targeting to plastids.
3.4.7 In Silico Expression Analysis

Organ- and development-specific expression of AtSHMT3 was studied in silico using publicly available microarray data and the GENEVESTIGATOR software package. AtSHMT3 was expressed in all plant organs and at all developmental stages examined; the expression level in germinated seeds was about two-fold higher than those in all other organs (Fig. 3.9). These findings suggest that AtSHMT3 is a housekeeping enzyme needed by all plant organs throughout development.

3.5 Discussion

In plastids, the C₁ metabolic network provides C₁ moieties for biosynthesis of methionine, N-formylmethionine, thymidylate, and purines (Hanson et al., 2000; Ravanel et al., 2004; Zrenner et al., 2006). The prevalent model is that 5-CH₃-H₄PteGlu₁, the methyl group donor for methionine synthesis, is imported into plastids from the cytosol (Fig. 3.1). The remaining one-carbon H₄PteGluᵣ derivatives are formed endogenously in plastids. C₁ moieties which originate from serine or formate are respectively directed into the plastid C₁ network via reactions catalyzed by SHMT or FTHFS. The H₄PteGluᵣ–bound C₁ moieties are then interconverted via reactions catalyzed by bifunctional 5,10-methylenetetrahydrofolate cyclohydrolase–5,10-methenyltetrahydrofolate dehydrogenase (Hanson et al., 2000) (Fig. 3.1).

The first evidence of SHMT activity in dicot plastids came from a study showing formation of ¹⁴C-serine in pea chloroplasts fed ¹⁴C-formate (Shingles et al., 1984). This
study suggested that the most likely route for the $^{14}$C-serine formation is via the reactions catalyzed in sequence by FTHFS, 5,10-methylenetetrahydrofolate cyclohydrolase–5,10-methenyltetrahydrofolate dehydrogenase, and SHMT (Fig. 3.1). An SHMT was subsequently isolated from spinach chloroplasts, but the corresponding gene remains to be cloned (Besson et al., 1995). However, SHMT activity was not detected in wheat chloroplasts (Gardeström et al., 1985), raising the question whether SHMTs exist in plastids of all flowering plants.

We found no bioinformatic evidence for the presence of genes encoding plastid SHMTs in the fully sequenced rice genome, nor did we find evidence for the presence of putative plastid SHMTs by searching protein and DNA sequences from other monocot species. We detected SHMT activity, however, in Percoll-isolated chloroplasts from pea (a representative dicot) and barley (a representative monocot) (Fig. 3.3). These data provide evidence that monocot plastids do contain SHMTs. An explanation to the search prediction failure is that the encoded proteins are more related to SHMTs that are putatively targeted to another subcellular compartment in dicots, mosses, and algae.

Nearly equal levels of FTHFS activity were found in Percoll-isolated chloroplasts from pea and barley (Fig. 3.3). This is consistent with previous work showing formation of $^{14}$C-serine in pea chloroplasts fed $^{14}$C-formate (Shingles et al., 1984), and with bioinformatic prediction of a plastid FTHFS in rice. Contrary to biochemical evidence presented here (Fig. 3.3) and published before (Shingles et al., 1984), we were unable to find bioinformatic evidence for the existence of plastid FTHFSes in dicots. The A. thaliana genome contains a single gene for FTHFS encoding a putatively cytosolic
enzyme. No evidence of alternative splicing at the N terminus of this protein was found in searches of the *A. thaliana* EST database. Searches of genome and EST databases from other dicot species yielded similar results, suggesting that plastid FTHFSes from dicots may lack typical peptides for targeting to these organelles. Import into plastids of proteins lacking typical targeting peptides is known to occur in plants (Rathinasabapathi et al., 1994). Alternatively, dicot plastids could contain a novel type of FTHFS.

Thus, our results support the premise that plastids of monocots and dicots can draw C₁ moieties from the endogenous serine and formate pools (Fig. 3.1).

Plastid folates are predominantly polyglutamylated, the most abundant forms being tetra-, penta-, and hexaglutamates (Imeson et al., 1990; Orsomando et al., 2005). We found decreases in the $K_m$ value and increases in the $k_{cat}/K_m$ value of AtSHMT3 for H₄PteGluₙ=1–4 species, and no significant changes in those two values for H₄PteGluₙ=4–8 species. Thus, if one assumes that in plastids the distribution of H₄PteGluₙ=1–8 species is nearly equal to that of the entire plastid folate pool reported before (Imeson et al., 1990; Orsomando et al., 2005), then AtSHMT3 is equally efficient at utilizing the three most abundant H₄PteGluₙ species present in plastids (H₄PteGluₙ=4–6).

In *A. thaliana* chloroplasts, H₄PteGluₙ was found to be below the detection limit, and the total folate content was estimated to be around 40 pmol mg⁻¹ protein (Klaus et al., 2005). Protein concentration in chloroplast lumen and stroma in spinach was estimated to be >20 mg ml⁻¹ (Kieselbach et al., 1998). Assuming that the protein concentrations in chloroplasts in *A. thaliana* and spinach have about equal values, we estimated that total folate concentration in *A. thaliana* chloroplasts is about 0.8 µM. If we assume that
H₄PteGluₙ is <10% of the total folate pool in A. thaliana chloroplasts, we can then estimate that H₄PteGluₙ concentration is <0.08 µM in these organelles. A published value for folate concentration in pea chloroplasts is 1.7 µM (Neuburger et al., 1996). Based on this value and on consideration that H₄PteGluₙ is about 10% of the total folate pool (Orsomando et al., 2005), we estimated that H₄PteGluₙ concentration in pea chloroplasts is about 0.17 µM, which is close to that estimated for A. thaliana chloroplasts. Thus, H₄PteGluₙ concentration values in chloroplasts of both species are well below the $K_i$ values we determined for H₄PteGluₙ (Table 3.1). Therefore, inhibition of AtSHMT3 activity by H₄PteGluₙ species likely lacks physiological significance.

Effects of polyglutamylation on the $K_m$ values of plant SHMTs have been studied only in an enzyme isolated from pea mitochondria (Besson et al., 1993). The $K_m$ values for this enzyme are reported as 37.5 µM for H₄PteGlu₁, 13.5 µM for H₄PteGlu₂, and ≤3.7 µM for H₄PteGluₙ=3–6. The $K_m$ values for H₄PteGluₙ≥3 could not be determined because of lack in assay sensitivity. Likewise, a decrease in $K_m$ values with increase in polyglutamylation (56 µM for H₄PteGlu₁, 3.9 µM for H₄PteGlu₂, and 1.7 µM for H₄PteGlu₃) was found in an SHMT isolated from pig liver (Matthews et al., 1982). We are unaware of previous work reporting $K_m$ values of SHMTs for H₄PteGluₙ≥3, though $K_m$ values for H₄PteGlu₁ are known for several SHMTs (Mazelis and Liu, 1967; Schirch and Peterson, 1980; Schirch et al., 1985; Mitchell et al., 1986; Strong et al., 1990; Besson et al., 1995; Kruschwitz et al., 1995; Capelluto, 1999; Capelluto et al., 2000; Fu et al., 2005; Mukherjee et al., 2006; Vatsyayan and Roy, 2007). Nor are we aware of previous work reporting $K_i$ values of SHMTs for H₄PteGluₙ, though inhibition of SHMT activity by
H₄PteGlu₁ has been described for enzymes from *Crithidia fasciculata* (Capelluto, 1999), *Trypanosoma cruzi* (Capelluto et al., 2000), and *Leishmania donovani* (Vatsyayan and Roy, 2007). Thus, the data we report in Table 3.1 represent to our knowledge the first set of kinetic parameters for H₄PteGluₙ₋₁–₈ species of an SHMT from any organism.

5-CH₃-H₄PteGluₙ and 5-HCO-H₄PteGluₙ are known to bind and inhibit SHMTs from several organisms (Schirch and Ropp, 1967; Matthews et al., 1983; Schirch et al., 1985; Mitchell et al., 1986; Stover and Schirch, 1991; Roje et al., 2002; Fu et al., 2005; Chang et al., 2006; Chang et al., 2007). It is also known that plastids contain these folates (Klaus et al., 2005; Orsomando et al., 2005). Applying the reasoning presented above to estimate the concentration of H₄PteGluₙ, we arrive at about 0.22 µM 5-CH₃-H₄PteGluₙ and 0.14 µM 5-HCO-H₄PteGluₙ in chloroplasts in *A. thaliana* (Klaus et al., 2005).

Current evidence supports import of 5-CH₃-H₄PteGlu₁ into plastids from the cytosol, where this folate is formed in a reaction catalyzed by MTHFR (Roje et al., 1999), and its polyglutamylation in plastids, which is catalyzed by the resident folylpolyglutamate synthetase (Ravanel et al., 2001). Folate transporters have been found in plastids (Bedhomme et al., 2005; Klaus et al., 2005).

5-HCO-H₄PteGluₙ can be formed from 5,10-CH⁺-H₄PteGluₙ by catalysis in a side reaction of SHMT in the presence of glycine (Stover and Schirch, 1990; Stover and Schirch, 1992; Fu et al., 2005), or by chemical hydrolysis at acidic pH (Baggott, 2000). The stromal pH of illuminated chloroplasts is mildly basic (Heldt et al., 1973), which is unfavorable for chemical formation of 5-HCO-H₄PteGluₙ from 5,10-CH⁺-H₄PteGluₙ. Thus, the plastid 5-HCO-H₄PteGluₙ pool is most likely formed by catalysis.
5-CH$_3$-H$_4$PteGlu$_n$ and 5-HCO-H$_4$PteGlu$_n$ exist predominantly as tetra-, penta-, and hexaglutamates (Imeson et al., 1990; Orsomando et al., 2005). Thus, we consider monitoring inhibition of AtSHMT3 activity with H$_4$PteGlu$_6$ as substrate, and 5-CH$_3$-H$_4$PteGlu$_5$ or 5-HCO-H$_4$PteGlu$_5$ as inhibitor, to provide physiologically relevant data pertaining to substrate and inhibitor species that are abundant in plastids \textit{in vivo}. The difference between the $K_i$ values obtained using the pentaglutamate inhibitors and those obtained using monoglutamate inhibitors (Table 3.2), which are not abundant in plant cells, underscores the importance of assaying SHMTs using the polyglutamylated folate species.

Using the $K_i$ values we determined for 5-CH$_3$-H$_4$PteGlu$_5$ (1.94 $\mu$M) and 5-HCO-H$_4$PteGlu$_5$ (4.96 $\mu$M), the assumption that the $K_i$ values for tetra-, penta- and hexaglutamylated inhibitors are similar, and also using the folate concentrations estimated above, we calculate by applying the equations for mixed tight (5-CH$_3$-H$_4$PteGlu$_n$) or mixed (partial) (5-HCO-H$_4$PteGlu$_n$) enzyme inhibition that AtSHMT3 would be 3–10% inhibited by these metabolites under the folate concentrations estimated for plastids. Thus, our data suggest that 5-CH$_3$-H$_4$PteGlu$_n$ and 5-HCO-H$_4$PteGlu$_n$ would not significantly inhibit SHMT activity in plastids, not acting as important \textit{in vivo} regulators of C$_1$ metabolism in these organelles.

\textbf{3.6 Acknowledgement}

We thank Dr. Matthew Willmann from the University of Pennsylvania for advice
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Figure 3.1. Schematic representation of major reactions in plant one-carbon metabolism. FDH, formate dehydrogenase; MTHFC–MTHFD, 5,10-methylene-tetrahydrofolate cyclohydrolase–5,10-methenyltetrahydrofolate dehydrogenase; FGAR, formylglycinamide ribonucleotide; FAICAR, 5-formaminoimidazole-4-carboxamide ribonucleotide; MS, methionine synthase; TS, thymidylate synthase. The dotted arrow denotes import of 5-CH$_3$-H$_2$PteGlu$_n$. 
Figure 3.2. Phylogenetic tree of SHMTs from representative plant species. The sequence alignment for the phylogenetic tree included SHMT protein sequences of plant species *A. thaliana* (At), *O. sativa* (Os), *P. patens* (Phypa), and *C. reinhardtii* (Chlre) as described under “Experimental Procedures.” The phylogenetic tree was made with MEGA 4.0. The neighbor-joining method included Poisson correction, complete deletion, and (1000) bootstrap replication.
**Figure 3.3.** SHMT, FTHFS, MTHFR, fumarase, and glyceraldehyde-3-phosphate dehydrogenase activities in chloroplast and leaf extracts of pea and barley. Enzyme activity values from one of three independent determinations for representative enzyme activities. Assay conditions were as described under “Experimental Procedures.” Data points are mean ± S.E. of three triplicate independent determinations. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PC, pea chloroplast; PL, pea leaf; BC, barley chloroplast; BL, barley leaf.
Figure 3.4. Purification of recombinant AtSHMT3 from *E. coli* cells. The recombinant enzyme was purified as described under “Experimental Procedures.” Samples were separated by SDS-PAGE on a 10% NuPAGE BisTris gel and stained with Coomassie Blue. *Lane 1*, 10 μg of crude *E. coli* extract expressing AtSHMT3; *lane 2*, 10 μg of partially purified tagged AtSHMT3 after Mono Q ion-exchange chromatography; *lane 3*, 2.5 μg of purified AtSHMT3 after a second passage on Mono Q column; *lane 4*, 5 μg of molecular mass standard. Molecular masses of the standards (kDa) are indicated at the right.
Figure 3.5. Plots of steady-state kinetic data for AtSHMT3. AtSHMT3 activity was measured at pH 8.5 with constant serine and variable H₄PteGlu₉₋₁₈. Substrate concentrations were 5 mM for serine and 0.3–960 μM for the physiological substrate (6S)-H₄PteGlu₉₋₁₈. Assay conditions were as described under “Experimental Procedures.” Data points are mean ± S.E. of three-to-six triplicate independent determinations. All curves are best fits to a model of uncompetitive substrate inhibition (SigmaPlot 9.01).
Figure 3.6. Inhibition of AtSHMT3 with 5-CH$_3$-H$_4$PteGlu$_{1&5}$ and 5-HCO-H$_4$PteGlu$_{1&5}$. AtSHMT3 activity was measured at pH 8.5 with H$_4$PteGlu$_6$ as substrate and 5-CH$_3$-H$_4$PteGlu$_{1&5}$ and 5-HCO-H$_4$PteGlu$_{1&5}$ as inhibitors. Concentrations were 5 mM for serine, 1 (diamonds) and 2 µM (triangles) for the physiological substrate (6S)-H$_4$PteGlu$_6$, and 0.5–200 µM for inhibitors. Assay conditions were as described under "Experimental Procedures." Data points are mean ± S.E. of three-to-four triplicate independent determinations. Curves for inhibitors 5-HCO-H$_4$PteGlu$_{1&5}$ and 5-CH$_3$-H$_4$PteGlu$_3$ are best fits to a model of mixed (partial) inhibition, and the curve for inhibitor 5-CH$_3$-H$_4$PteGlu$_3$ is best fit to a model of mixed tight inhibition (SigmaPlot 9.01).
**Figure 3.7.** Effects of enzyme dilution on AtSHMT3 activity with $H_4PteGlu_{1-5}$. Relative enzyme dilution, 1-$\mu$l original enzyme in 50-$\mu$l assay was defined as 1; mAU, arbitrary unit of fluorescence.
Figure 3.8. Localization of AtSHMT3 in *A. thaliana* chloroplasts. N-terminal EGFP fusions to full-length AtSHMT3 (FL) or to putative organellar targeting peptide (TP) were transiently expressed in *A. thaliana* protoplasts. EGFP expressed from the pUC18-GFP5T-sp plasmid was used as a control for targeting to the cytosol. Subcellular localization was analyzed by fluorescence microscopy. AC, EGFP fluorescence; DF, chlorophyll autofluorescence; GI, merged images.
In silico analysis of AtSHMT3 expression in *A. thaliana*. Public Affymetrix expression analysis microarray data were analyzed using the GENEVESTIGATOR reference expression data base and meta-analysis system. GENEVESTIGATOR obtains the signal intensity values from raw array data by normalization using the Affymetrix MAS5 algorithm. Error bars denote standard errors.
Table 3.1. Kinetic parameters of AtSHMT3 for H₄PteGlu₁₋₈.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kₘ (µM)</th>
<th>Kᵢ (µM)</th>
<th>kₜₐₜ (s⁻¹)</th>
<th>kₜₐₜ/Kₘ (s⁻¹ µM⁻¹)</th>
<th>Kᵢ/Kₘ (µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₄PteGlu₁</td>
<td>217.73 ± 3.35</td>
<td>85.2 ± 2.3</td>
<td>15.8 ± 0.7</td>
<td>0.07 ± 0.00</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>H₄PteGlu₂</td>
<td>13.30 ± 0.78</td>
<td>93.9 ± 4.0</td>
<td>7.6 ± 0.7</td>
<td>0.57 ± 0.06</td>
<td>7.06 ± 0.51</td>
</tr>
<tr>
<td>H₄PteGlu₃</td>
<td>3.06 ± 0.03</td>
<td>22.0 ± 0.3</td>
<td>8.7 ± 0.2</td>
<td>2.84 ± 0.07</td>
<td>7.19 ± 0.12</td>
</tr>
<tr>
<td>H₄PteGlu₄</td>
<td>0.83 ± 0.03</td>
<td>15.7 ± 1.6</td>
<td>4.3 ± 0.2</td>
<td>5.18 ± 0.31</td>
<td>18.92 ± 2.05</td>
</tr>
<tr>
<td>H₄PteGlu₅</td>
<td>0.64 ± 0.07</td>
<td>13.3 ± 2.2</td>
<td>3.5 ± 0.4</td>
<td>5.47 ± 0.87</td>
<td>20.78 ± 4.12</td>
</tr>
<tr>
<td>H₄PteGlu₆</td>
<td>0.69 ± 0.05</td>
<td>15.0 ± 1.4</td>
<td>3.5 ± 0.4</td>
<td>5.07 ± 0.69</td>
<td>21.74 ± 2.57</td>
</tr>
<tr>
<td>H₄PteGlu₇</td>
<td>0.67 ± 0.11</td>
<td>13.9 ± 3.4</td>
<td>3.8 ± 0.2</td>
<td>5.67 ± 0.98</td>
<td>20.75 ± 6.11</td>
</tr>
<tr>
<td>H₄PteGlu₈</td>
<td>0.55 ± 0.04</td>
<td>11.8 ± 2.2</td>
<td>3.3 ± 0.6</td>
<td>6.00 ± 1.17</td>
<td>21.45 ± 4.29</td>
</tr>
</tbody>
</table>

Kinetic parameters are best fits to a model of uncompetitive substrate inhibition (SigmaPlot 9.01). Assay conditions were as described under “Experimental Procedures.” Data points are mean ± S.E. of three-to-six triplicate independent determinations.
Table 3.2. Inhibition of AtSHMT3 with 5-CH₃-H₄PteGlu₁&₅ and 5-HCO-H₄PteGlu₁&₅.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-CH₃-H₄PteGlu₁</td>
<td>49.69 ± 16.55</td>
</tr>
<tr>
<td>5-CH₃-H₄PteGlu₅</td>
<td>1.94 ± 0.31/0.59 ±0.20</td>
</tr>
<tr>
<td>5-HCO-H₄PteGlu₁</td>
<td>50.22 ± 25.54</td>
</tr>
<tr>
<td>5-HCO-H₄PteGlu₅</td>
<td>4.96 ± 0.63</td>
</tr>
</tbody>
</table>

Kinetic parameters are best fits to a model of mixed (partial) inhibition for 5-HCO-H₄PteGlu₁&₅ and 5-CH₃-H₄PteGlu₁, and to a model of mixed tight inhibition for 5-CH₃-H₄PteGlu₅ (SigmaPlot 9.01). Inhibition by 5-CH₃-H₄PteGlu₅ was biphasic; $K_i$ values for 5-CH₃-H₄PteGlu₅ were determined from two phases of inhibition. Assay conditions were as described under “Experimental Procedures.” Data points are mean ± S.E. of three-to-four triplicate independent determinations.
CHAPTER FOUR

CONCLUSIONS

Plant SHMTs exist in chloroplasts, mitochondria, nuclei, and the cytosol of plant cells as shown by biochemical and bioinformatic evidence (Besson et al., 1995; Hanson and Roje, 2001). SHMTs serve as important C1 providers and may regulate the entry of C1 units into the plant C1 pool. Plant mitochondrial SHMTs have been studied in the past for their importance in photorespiration. Some mitochondrial SHMT mutants have been identified and characterized, and mitochondrial SHMT activity was found to be essential to maintaining proper photorespiration functions in plants (Somerville and Ogren, 1981; Moreno et al., 2005; Voll et al., 2006). Additionally, a recent study characterizing a new photorespiratory mutant of Arabidopsis shows that in vivo physical interaction between a mitochondrial SHMT (AtSHMT1) and the ferredoxin-dependent glutamate synthase is required for AtSHMT1 to be functioning in photorespiration (Jamai et al., 2009). A cDNA encoding a pea mitochondrial SHMT isoform has been cloned (Turner et al., 1992) and a partial steady-state kinetic characterization of purified SHMT from pea mitochondria was reported (Besson et al., 1993).

However, there is little known about physiological functions of plant SHMTs in the cytosol and plastids. Kinetic characterization of these SHMTs is also lacking. Most biochemical studies on SHMTs from plants, animals, and prokaryotes conducted SHMT assays with H4PteGlu1, having no physiological relevance and thus not useful to understanding of SHMT’s function under in vivo conditions. Two published reports,
where SHMT assays employed \( \text{H}_n\text{PteGlu}_{n=1-2} \) (Besson et al., 1993) and \( \text{H}_n\text{PteGlu}_{n=1-3} \) (Matthews et al., 1982), show decreased \( K_m \) values of SHMTs for \( \text{H}_n\text{PteGlu}_n \) when \( n \) increased from 1 to 2, or 1 to 3. \( K_m \) values of SHMTs for \( \text{H}_n\text{PteGlu}_{n>3} \) are still missing, and so are \( k_{\text{cat}} \) values of SHMTs for \( \text{H}_n\text{PteGlu}_{n>1} \).

The lack of such characterization is caused by the difficulty to assay SHMT with its physiological substrates, \( \text{H}_n\text{PteGlu}_{n=4-6} \). The expected \( K_m \) values of pea mitochondrial SHMT for \( \text{H}_n\text{PteGlu}_{n>3} \) are to be no higher than 3.7 \( \mu \)M (Besson et al., 1993), and we think that \( K_m \) values of SHMT from pig liver for \( \text{H}_n\text{PteGlu}_{n>4} \) would be lower than 1.7 \( \mu \)M (\( K_m \) value for \( \text{H}_4\text{PteGlu}_3 \)). The predicted low \( K_m \) values of SHMT for \( \text{H}_n\text{PteGlu}_{n>3} \) warrant the lowest \( \text{H}_n\text{PteGlu}_n \) concentration in SHMT assays to be less than 1 \( \mu \)M. However, existing SHMT assays either do not meet such requirements (see Chapter Two for detail) or need MTHFD, which is not commercially available. Therefore, a more sensitive and practical SHMT assay is needed for such characterization.

In this present work, we describe the development of a very sensitive (10\(^{-8} \) M \( \text{H}_n\text{PteGlu}_n \)) SHMT assay based on HPLC separation and fluorescence detection. By using this new assay, we were able to assay a plastid SHMT from \textit{Arabidopsis} (AtSHMT3) with \( \text{H}_n\text{PteGlu}_{n=1-8} \). Kinetic parameters such as \( K_m \), \( k_{\text{cat}} \), and \( k_{\text{cat}}/K_m \) of AtSHMT3 are reported. Analysis of these parameters shows that the substrate affinity and catalytic efficiency of AtSHMT3 for \( \text{H}_n\text{PteGlu}_{n=1-4} \) increase when \( n \) increases from 1 to 4, and that the substrate affinity and catalytic efficiency of AtSHMT3 for \( \text{H}_n\text{PteGlu}_{n=4-8} \) are similar when \( n \) varies from 4 to 8, suggesting that AtSHMT3 is equally efficient at using these physiological substrates. In our study, \( \text{H}_4\text{PteGlu}_n \) also displayed substrate inhibition.
However, the substrate inhibition should have no physiological effect on AtSHMT3 activity, considering the high $K_i$ values and very low $H_4PteGlu_n$ concentrations in Arabidopsis plastids we estimated from published results (<0.08 µM) (Klaus et al., 2005).

Another feature of SHMT enzyme is the inhibition by two inhibitors, 5-CH$_3$-$H_4PteGlu_n$ and 5-HCO-$H_4PteGlu_n$. Inhibition of SHMTs from rabbit liver by these inhibitors was demonstrated (Schirch and Ropp, 1967; Stover and Schirch, 1991). Inhibition of SHMT from pea mitochondrial extracts by 5-HCO-$H_4PteGlu_n$ was also reported (Roje et al., 2002). But inhibition of isolated plant SHMTs by these inhibitors remains to be investigated. 5-CH$_3$-$H_4PteGlu_n$ and 5-HCO-$H_4PteGlu_n$ have been detected in Arabidopsis plastids (Klaus et al., 2005), so it would be interesting to know how AtSHMT3 are affected by these two inhibitors. In our study, we performed inhibition assays of AtSHMT3 with these inhibitors and found that both of them were inhibitory, in their mono- and pentaglutamylated forms. Furthermore, the pentaglutamylated inhibitors are more effective than their monoglutamylated counterparts, indicating the same relationship of high affinity of AtSHMT3 for polyglutamylated folates and the polyglutamate tail in these folates as demonstrated by the kinetic properties of AtSHMT3 for $H_4PteGlu_n=1–8$.

Because we had obtained kinetic parameters for $H_4PteGlu_n=1–8$ and inhibition constants for 5-CH$_3$-$H_4PteGlu_{1&5}$ and 5-HCO-$H_4PteGlu_{1&5}$ of AtSHMT3, it was possible for us to estimate AtSHMT3 activity under physiological conditions. By using equations of the best-fit models, kinetic parameters of $H_4PteGlu_6$, 5-CH$_3$-$H_4PteGlu_5$, and 5-HCO-$H_4PteGlu_5$, and estimated concentrations of $H_4PteGlu_n$, 5-CH$_3$-$H_4PteGlu_n$, and
5-HCO-H₄PteGluₙ in Arabidopsis plastids (Klaus et al., 2005), we found that AtSHMT3 was inhibited about 10% by 5-CH₃-H₄PteGluₙ or about 3% by 5-HCO-H₄PteGluₙ. These estimated results suggest that both 5-CH₃-H₄PteGluₙ and 5-HCO-H₄PteGluₙ are not important \textit{in vivo} regulators of AtSHMT3 activity in \( C_1 \) metabolism in plastids. \textit{In vivo} evidence of SHMT inhibition by 5-HCO-H₄PteGluₙ was shown in Arabidopsis mutants of genes encoding 5-formyltetrahydrofolate cycloligase or 10-formyltetrahydrofolate deformylase, respectively (Goyer et al., 2005; Collakova et al., 2008). In the mutants of the genes for 10-formyltetrahydrofolate deformylase, 5-HCO-H₄PteGluₙ level in leaves was about 6-fold of those in wild-type plants, and the accumulated 5-HCO-H₄PteGluₙ presumably caused photorespiratory phenotype by inhibiting SHMT and GDC activities (Collakova et al., 2008). However, in the mutants of the gene encoding 5-formyltetrahydrofolate cycloligase, the accumulated 8-fold higher 5-HCO-H₄PteGluₙ in leaf mitochondria did not lead to photorespiratory phenotype under ambient CO₂ conditions (Goyer et al., 2005). These results suggest that there are unknown factors involved in the inhibition of SHMT by 5-HCO-H₄PteGluₙ.

In this work, we also confirmed the predicted subcellular localization of AtSHMT3 in plastids by GFP fusions. Additionally, we provide biochemical evidence that activities of SHMT and FTHFS were detected in chloroplasts of pea and barley. This evidence supports the existence of these two enzymes in plastids so that both of them are present to provide \( C_1 \) units. This finding also clarifies confusion caused by contradicting biochemical evidence of FTHFS in pea chloroplasts reported previously (Shingles et al., 1984; Chen et al., 1997).
References


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