Serine Hydroxymethyltransferase Isoforms Are Inhibited by Leucovorin: Characterization and Comparison of Recombinant Zebrafish Serine Hydroxymethyltransferases

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ABSTRACT:

Serine hydroxymethyltransferase (SHMT) provides activated one-carbon units required for the biosynthesis of nucleotides, protein, and methyl group by converting serine and tetrahydrofolate to glycine and N5,N10-methylenetetrahydrofolate. It is postulated that SHMT activity is associated with the development of methotrexate resistance and the in vivo activity of SHMT is regulated by the binding of N5-CHO-THF, the rescue agent in high-dose methotrexate chemotherapy. The aim of this study is to advance our understanding of the folate-mediated one-carbon metabolism in zebrafish by characterizing zebrafish mitochondrial SHMT. The cDNA encoding zebrafish mitochondrial SHMT was cloned, overexpressed in Escherichia coli, and purified with a three-step purification protocol. Similarities in structural, physical, and kinetic properties were revealed between the recombinant zebrafish mitochondrial SHMT and its mammalian orthologs. Surprisingly, leucovorin significantly inhibits the aldol cleavage of serine catalyzed by zebrafish cytosolic SHMT but inhibits to a lesser extent the reaction catalyzed by the mitochondrial isozyme. This is, to our knowledge, the first report on zebrafish mitochondrial folate enzyme as well as the differential inhibition of leucovorin on these two SHMT isoforms. Western blot analysis revealed tissue-specific distribution with the highest enrichment present in liver for both cytosolic and mitochondrial SHMTs. Intracellular localization was confirmed by confocal microscopy for both mitochondrial and cytosolic SHMTs. Unexpectedly, the cytosolic isofrom was observed in both nucleus and cytosol. Together with the previous report on zebrafish cytosolic SHMT, we suggest that zSHMTs can be used in in vitro assays for folate-related investigation and antifolate drug discovery.

Folates carry the chemically activated single carbons at N5 and/or N10 positions and are required for the biosynthesis and metabolism of nucleic acid, protein, amino acid, methyl group, neurotransmitter, and vitamins. Its vital role in nucleotide biogenesis has led to the development of many anticancer drugs targeting folate-requiring enzymes. Among them, methotrexate (MTX) is one of the most widely used anticancer agents to date. It blocks de novo nucleotide synthesis by inhibiting dihydrofolate reductase (DHFR) and thymidylate synthase (Fig. 1, enzymes 2 and 3, respectively). However, resistance to MTX often emerges and becomes the major impediment to its curative potential.

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ABBREVIATIONS: MTX, methotrexate; SHMT, serine hydroxymethyltransferase; zmSHMT and zcSHMT, zebrafish mitochondrial and zebrafish cytosolic SHMT; hsSHMT and rcSHMT, human cytosolic and rabbit cytosolic enzymes; THF or H4PteGlu, tetrahydropteroylglutamate; N5-CHO-H4PteGlu, N5-formyl-tetrahydropteroylglutamate; N5,N10-CHO-H4PteGlu, N5,N10-methylene; N5-CHO-H4PteGlu, N5-CH2-THF, N5,N10-methyl; PLP, pyridoxal-5'-phosphate; DHFR, dihydrofolate reductase; RT-PCR, reverse transcription-polymerase chain reaction; EGFP, enhanced green fluorescent protein; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ZLE, zebrafish liver epithelial; RACE, rapid amplification of cDNA ends; UPM, Universal Primer Mix; NUP, Nested Universal Primer; bp, base pair(s); kb, kilobase(s); (F), forward; (R), reverse; GFP, green fluorescent protein; SUMO, small ubiquitin-like modifier.
catalyzed by serine hydroxymethyltransferase (SHMT). $N^5$-CHO-THF also acts as a tight-binding inhibitor of the cytosolic form of SHMT (Stover and Schirch, 1991). SHMT is a pyridoxal-5'-phosphate (PLP)-dependent enzyme and reversibly converts serine and THF to glycine and $N^5,N^{10}$-CH$_2$-THF, the principal pathway of one-carbon unit incorporation in cells. One-carbon metabolism is compartmentalized with folate coenzymes equally distributed in cytosol and mitochondria (Appling, 1991). In higher organisms more than one SHMT isoform is often present: a cytosolic isoform and an organellar-associated form, usually mitochondria. Although the physiological functions of these two isoforms remain unclear, it is suggested that cytosolic SHMT (cSHMT) tends to function in the direction of serine synthesis, whereas the production of glycine and $N^5$-CHO-THF tends to function in the direction of serine aldol cleavage catalyzed by mitochondrial serine hydroxymethyltransferase.

Materials and Methods

Materials. Polymerase chain reaction (PCR) primers were ordered from MDBio, Inc. (Taipei, Taiwan). The SMART RACE Amplification Kit was purchased from Clontech, Inc. (Mountain View, CA). PCR Master Mix was purchased from ABgene House (Epsom, Surrey, UK). Restriction enzymes used for cloning procedures were purchased from either Invitrogen (Carlsbad, CA) or New England BioLabs (Ipswich, MA). The clone expressing rabbit

The folate-mediated one-carbon metabolism involving both cytosolic and mitochondrial serine hydroxymethyltransferases. Folate coenzymes are equally distributed in cytosolic and mitochondrial compartments. Three cycles are involved in this pathway and are responsible for thymidylate (A), purine (B), and methionine biosynthesis (C). The enzymes participating in this pathway are serine hydroxymethyltransferase (1), dihydrofolate reductase (2), thymidylate synthase (3), glycaminide ribonucleotide transformylase (GAR) and 5-amino-4-imidazolecarboxamide ribotide transformylase (AICAR) (4), $N^5,N^{10}$-methylene-tetrahydrofolate cyclohydrolase (5), $N^5,N^{10}$-methyl-10-formyltetrahydrofolate dehydrogenase (6), $N^5,N^{10}$-methylene-tetrahydrofolate reductase (7), and methionine synthase (8). A parallel pathway also exists in mitochondria, where the activated one-carbon unit is generated in the serine-aldol cleavage catalyzed by mitochondrial serine hydroxymethyltransferase.
protein expression. The pET43.1a plasmid and all the materials and expression were obtained from Novagen (Madison, WI). The materials and methods for the general cloning procedures were as described previously (Chang et al., 2006).

Zebrafish (Danio rerio, AB strain) were bred and maintained in a 14 h/10 h light/dark diurnal cycle according to the standard condition described by Westerfield (2000). Embryos were staged according to the method of Kimmel et al. (1995). Total RNA isolation and cDNA library construction from zebrafish embryos were performed with RNAzol B reagent (Tel-Test Inc., Friendswood, TX) and the SMART RACE cDNA Amplification Kit (Clontech, Inc.) as described previously (Chang et al., 2006).

**Fish Care and Preparation of cDNA Library from Zebrafish Embryos.** Zebrafish (Danio rerio, AB strain) were bred and maintained in a 14 h/10 h light-dark diurnal cycle according to the standard condition described by Westerfield (2000). Embryos were staged according to the method of Kimmel et al. (1995). Total RNA isolation and cDNA library construction from zebrafish embryos were performed with RNAzol B reagent (Tel-Test Inc., Friendswood, TX) and the SMART RACE cDNA Amplification Kit (Clontech, Inc.) as described previously (Chang et al., 2006).

**Bacterial Strains, Plasmids, and General Cloning Procedures.** The E. coli strain XL1 Blue (recA1, endA1, gyrA96, thi-1, hsdR17(rK- mK+)) (supE44, relA1, lac-) was used for the construction of clones. The E. coli strains HMS174(DE3) (F’ recA r122 m12-3) and Rosetta (DE3) (F’ recA r122 m12-3), which contain the T7 RNA polymerase gene, were used for protein expression. The pET21a plasmid and all the E. coli strains for cloning and expression were obtained from Novagen (Madison, WI). The materials and methods for the general cloning procedures were as described previously (Chang et al., 2006).

**Cloning of zmSHMT from Zebrafish cDNA Library by PCR-Based Cloning Strategy.** A PCR-based approach with degenerate primers was used for the amplification and cloning of SHMT-encoding sequences from a zebrafish cDNA mixture. Two degenerate primers (5’-TGGGGGTNAAYGT-NCA-3’ and 5’-WDATRTGNGCCATRTC-3’), corresponding to the conserved regions of SHMT amino acid sequences (WGVNVQ and DMAHIS), were designed for PCR with the following conditions: a denaturation of 94°C for 5 min followed by 55 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. The resultant products were cloned and sequenced. The deduced amino acid sequences of the amplified products fell into two categories and shared 70 to 90% identity with the corresponding regions of SHMT. GenBank BLAST search revealed 100% and 61% identity between these two sequences and zcSHMT cDNA (zebrafish shmt1, accession number NM_201046). Based on the sequence information of the 61% identity fragment, we proceeded with the isolation of prospective zebrafish mitochondrial SHMT cDNA.

The cloning of full-length zmSHMT cDNA was accomplished by the rapid amplification of cDNA ends (RACE) method using zmSHMT gene-specific primers designed on the basis of the sequence information of the cloned fragment (Scheme 1). The reverse primer SHMT II-1(R) (5’-AGGATGTGAGTTTGGGTCGTA-3’) and the UPM primer provided in the SMART RACE cDNA Amplification Kit (Clontech, Inc.) were used in the first-round PCR, with the 5’ RACE cDNA mixture as template. The resultant bands were TA-cloned and sequenced. Based on the sequence information, two primers, SHMT II-2(F) (5’-AGGATCGGCGGGGGCTGTCATTTA-3’) and SHMT II-4(R), were designed on the basis of the sequence information of the cloned fragments. The assembling of subsequent PCR amplifications. The SMART 3’-RACE cDNA mixture was used as template in the second-round PCR with primer pairs SHMT II-2(F) and UPM. The third round of PCR was conducted using the second-round PCR product as template and the primer pairs SHMT II-3(F) and NUP provided in the kit. The all of the above amplifications were performed by touchdown PCR. The cycling conditions were 5 cycles of 94°C for 30 s and 72°C for 3 min; 5 cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min; 40 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. The resulting 2700-bp fragment was identified by restriction mapping and sequencing. The assembling of 5’-RACE and 3’-RACE sequences revealed the prospective full-length zmSHMT cDNA. The final amplification of the complete 1.5-kb encoding sequence was accomplished by PCR with the 5’-RACE cDNA library prepared from 3-day post-fertilization embryos as template and the primer pair SHMT II-1(R), 5’-CCCTGATGAATTCGTTTAATGGTCGTG-3’ (forward) and SHMT II-4(R), 5’-CCCTGATGAATTCGTTTAATGGTCGTG-3’ (reverse). To simplify the cloning procedure, two restriction enzyme sites, NdeI and EcoRI (underlined), were introduced into the primers. The PCR-amplified product was cloned into the expression vector pET43.1a.
Successful cloning was confirmed by both restriction enzyme digestion and DNA sequencing.

Removal of signal peptide and introduction of the first methionine residue was accomplished by PCR using zmSHMT coding sequence as template with the primer pair 5'-GCGCTCCTCCCATATGGAGATCTCCCATATG-3' (forward) and zmSHMT(DeSig) (reverse) followed by cloning into the pET43a.1 vector, yielding the clone zmSHMT(DeSig). The resultant constructs were transformed into an E. coli host cell Rosetta (DE3) for enzyme expression and purification.

Zebrafish SHMT-EGFP fusion plasmids were constructed by PCR cloning with the zmSHMT or zmSHMT plasmids and the primers designed to abolish stop codons and introduce BglII, EcoRI, or SalI restriction enzyme sites. The primers were 5'-GGGTGAGATCTCCCATATG-3' (forward) and 5'-CCCGGGGAATTCTCGACGCTGCGGAATCC-3' (reverse) for zmSHMT(DeSig); 5'-GGGTGAGATCTCCCATATG-3' (forward) and 5'-CCCGGGGAATTCTCGACGCTGCGGAATCC-3' (reverse) for zmSHMT(DeEGFP-N1). Expression and Purification of Recombinant zmSHMT. All buffers described below for the purification of zmSHMTs and kinetic studies contained 5 mM 2-mercaptoethanol, 0.2 mM EDTA, and 2 μM PLP unless otherwise stated. Similar purification procedures for zmSHMT were applied to the purification of zmSHMT(DeSig) with minor modifications indicated below (Chang et al., 2006). In brief, E. coli containing the desired plasmid was grown to log phase and induced at 25°C with 0.08 mM IPTG for 3 h. Cells were harvested and lysed with lysozyme and chromatin was removed by protamine sulfate precipitation. After a 30% to 50% ammonium sulfate fractionation and desalting on a P-6DG column, zmSHMT(DeSig) was loaded onto a CM-Sephadex column (2.5 × 5.0 cm) and eluted with the linear salt gradient of 50 ml of equilibrating buffer and 50 ml of 500 mM potassium phosphate, pH 7.25. The purified enzyme was stored at −20°C or −80°C in the presence of 10% glycerol. Protein from each step of the purification was examined by SDS-PAGE for purity.

Determination of Physical Properties. Apo-SHMTs were prepared by the removal of thiazolidine formed between t-Cys and active site PLP. The same principle was used to determine the stoichiometry of PLP bound per molecule of enzyme with an extinction coefficient of 5580 M⁻¹ cm⁻¹ for thiazolidine (Ulevitch and Kallen, 1977). The quaternary structure was determined on a Superdex 200 size-exclusion column as described previously (Chang et al., 2006). The molar absorptivity coefficient was determined as stated previously by Gill and von Hippel (1989).

Enzyme Assays and Inhibition. The rate of N₅,N₅'-CH₂-THF formation catalyzed by SHMT can be continuously monitored at 340 nm by coupling with excess N₅,N₅'-CH₂-THF dehydrogenase, which converts NADPH to NADP⁺. All kinetic constants were determined in 20 mM potassium phosphate, pH 7.0, containing 0.4 mM NADP⁺, 5 mM 2-mercaptoethanol, and 0.5 mM NAD⁺. Reaction studies include determination of kₐ and kₑ values for substrates, and inhibition by MTX and N⁵-CHO-THF. t-Serine concentrations used in kₐ and kₑ determination varied from 0.07 to 0.75 mM in the presence of 0.15 mM THF. Reactions were initiated by adding 10 μM of SHMT. Inhibition of initial velocity was determined in a 1-ml cuvette containing 20 mM phosphate, pH 7.3, 0.1 μM NADH, 25 μM THF, 10 nM or 50 μM t-serine, saturated NAD⁺, and inhibitors ranging from 0.1 to 100 μM for both N⁵-CHO-THF and metheinoxate.

Determination of Dissociation Constant for Reduced Folates. The dissociation constants for THF and N⁵-CHO-THF of zmSHMT(DelSig) were measured by the formation of quinonoid complex in the presence of saturated NAD⁺ and inhibitors ranging from 2.5 to 54 μM (Strong et al., 1989; Chang et al., 2006). Results were analyzed with Scatchard plots and double-reciprocal plots, yielding kₛ and stoichiometry of bound folates.

Fish Tissue Homogenization. Tissues or organs, including brain, eye, heart, liver, gastrointestinal tract, and muscle, were obtained from adult zebrafish after the animals were euthanized by waterborne exposure to tricaine (ethyl 3-aminobenzoate, methanesulfonic acid; Sigma-Aldrich). Tissues were rapidly isolated, stored in 50 to 200 μl of phosphate-buffered saline, pH 7.2, and kept on ice during the whole process of extraction. Homogenization was carried out in the phosphate-buffered saline lysis buffer containing a protease inhibitor cocktail consisting of AEBSF (aprotinin, leupeptin, bestatin, pepstatin A, and E-64) (Sigma-Aldrich, product number P8340) and RNase inhibitor (Recombinant RNasin; Promega, Madison, WI). Homogenized samples were centrifuged at 10,000 g at 4°C for 10 min to remove particulate matter. Aliquots of the supernatant, about 10 to 30 μl, were subjected to Western blot and RT-PCR.

Western Blot Analysis. Supernatant protein content was determined using the Bradford (1976) and BCA methods. Proteins of 20 μg were separated on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore). After blocking in a solution containing 5% nonfat milk, 0.1% Tween 20 in phosphate-buffered saline overnight, the membrane was probed with anti-zcSHMT or anti-hmSHMT primary antibodies (1:1000–1:5000) and then horseradish peroxidase-conjugated secondary antibody (1:5000). The PVDF membranes were also probed with anti-actin antibody for a loading control. The membrane was visualized using the SuperSignal chemiluminescent horseradish peroxidase substrate system from Pierce on a FUJIFILM LAS-3000 imaging system (Fuji Film, Tokyo, Japan). In the case of the gastrointestinal tract, where the signal for actin was not detectable, Ponceau-S staining was used to verify equal loading.

We used the antibody against human mSHMT instead of zebrafish mSHMT to determine zmSHMT tissue distribution, owing to the concern of possible cross-reaction between zmSHMT and zcSHMT. The human mSHMT peptide sequence is 59% and 76% identical to zmSHMT and zcSHMT, respectively. The identity between zmSHMT and zmSHMT is 61%. Thus, we hoped that the hmSHMT antibody would clearly distinguish zmSHMT from zcSHMT. As expected, no cross-reaction was detected, even when we tested with 1 μg of purified proteins, allowing the uses of the antibodies as described.

RT-PCR Analysis. For RT-PCR determination of SHMT expression, total mRNA was isolated from tissues using a TRizol kit (Invitrogen), following the manufacturer’s instructions. After isolation, 1 μg of total mRNA in each tissue sample was reverse-transcribed with a high-capacity cDNA archive kit (Promega), and 1 μl of the newly synthesized first-strand cDNA library was used as template in the subsequent PCR analysis. The primer sequences are as follows: 5'-GGGAGATCTGGAGATCTCCCATATG-3' (Forward) and 5'-CATTTTGAGCACGTTTCCCTC-3' (Reverse) for zcSHMT (505-bp fragment), 5'-GGGAAGGTTCAACCTC-3' (Forward) and 5'-GGGCCATCTGGAGAAGAC-3' (Reverse) for zmSHMT (523-bp fragment), and 5'-AGACATCAAGGAGAAGCTGTG-3' (Forward) and 5'-TCCGAGCGGATTTAC-3' (Reverse) for β-actin (391-bp fragment) as a control for the RNA isolation and reverse-transcription. The annealing temperatures were 65°C for zcSHMT, 60°C for zmSHMT, and 62°C for β-actin. The PCR condition was 30 cycles of 30 s at 94°C, 30 s at annealing temperature, and 30 s at 72°C. Determination of Intracellular Localization. ZLE cells were cultivated and regularly maintained in Leibovitz’s L-15 medium supplemented with 5% fetal bovine serum at 28°C. For transient transfection, ZLE cells at 1 × 10⁶/ml were subcultured into six-well plates 24 h before transfection with zmSHMTs/PEGFP-N1 fusion plasmids with a si-PORT transfection kit (Ambion, Austin, TX). Cells were incubated for another 24 h and costained with mitochondrial probe MitoTracker Deep Red 633 (Invitrogen) right before examining under a confocal microscope. Confocal microscopy images were acquired on a Leica TCS SP2 microscope.

Results

Cloning and Sequencing Analysis of Recombinant zmSHMT. The sequences of the 370-base pair fragments resulting from PCR amplification with degenerate primers fall into two categories, designated as form I and form II. We had previously reported the cloning and characterization of zmSHMT form I, the prospective zmSHMT, which highly resembles mammalian cytosolic SHMT structurally and functionally (Chang et al., 2006). The full-length zmSHMT form I cDNA isolated is 1479 bp, which encodes a protein of 492 amino acids. Peptide sequence alignment with the known SHMT from other species and the prospective zmSHMT revealed a potential mitochondrial signal peptide cleavage site between residues 20 and 30. Signal peptide prediction using the software SignalP 3.0 (http://www.cbs. dtu.dk/services/) has narrowed the cleavage site down to Ala22 and Val24, and in is agreement with the reports for rabbit and human
mitochondrial SHMTs (Fig. 2) (Martini et al., 1989; Stover et al., 1997). This prospective mitochondrial leader sequence of zSHMT form II (which we now designate as zmSHMT) is rich in arginine, leucine, threonine, and serine, and fulfills the criteria for a mitochondrial targeting sequence that locates at the N terminus of the precursor protein and contains 17 to 35 amino acids rich in positive charge. Both the conserved octapeptide encompassing a stretch of threonine residues and the lysine residue forming the internal aldimine with PLP are necessary for the mitochondrial targeting of zSHMT, do not bind to cation exchange resins under all the growth conditions we had including E. coli expression. Chromatographing on CM-Sephadex separates the endogenous enzyme from our cloned zmSHMT, since most zSHMT and human and rabbit mitochondrial SHMT are 75.4% and 76.0%, respectively. The GenBank accession numbers of the aligned sequences are: human cytosolic SHMT, NP_004160; human mitochondrial SHMT, NP_005403; rabbit cytosolic SHMT, P07511; rabbit mitochondrial SHMT, P14519; zebrafish cytosolic SHMT, NP_957340; and zebrafish mitochondrial SHMT, EF213101.

**Expression and Purification of zSHMT.** The overexpressed full-length zmSHMT with leader peptide resulted in the formation of insoluble precipitate in E. coli at all the growth conditions we had tested, including lower temperatures, reduced inducer concentrations, and addition of cofactor PLP and low molecular weight glycols (data not shown). The attempt to obtain a soluble and fully functional protein succeeded only after we performed a second PCR-based cloning to eliminate the predicted leader peptide that includes the first 23 amino acids. Induction for zmSHMT without signal peptide, designated as zmSHMT(DelSig), reaches an acceptable level with minimal production of insoluble enzyme (Fig. 3). Higher concentrations of IPTG, elevated induction temperature, and/or prolonged induction time were found to increase the ratio of insoluble to soluble zmSHMT(DelSig), although the overall amount of induced enzyme is increased.

A purification protocol similar to those for zcSHMT and human SHMTs was applied to the purification for zmSHMT(DelSig) (Kruschwitz et al., 1995; Chang et al., 2006). After a 30 to 50% ammonium sulfate precipitation, the enzyme was 70% pure, judged from SDS-PAGE, with a 2-fold purification (Table I). We used gel filtration, instead of equilibrium dialysis, to remove ammonium sulfate, because zmSHMT(DelSig) was found to precipitate after overnight dialysis. The zmSHMT(DelSig) bound to CM-Sephadex and was eluted at high salt. Chromatographing on CM-Sephadex separates the endogenous enzyme from our cloned zmSHMT, since most E. coli proteins, including E. coli SHMT, do not bind to cation exchange resins under
results suggest a homotetrameric structure for the recombinant zmSHMT(DelSig).

1-Cysteine forms a thiazolidine compound with the active site PLP, which can be removed by dialysis or precipitation of the protein, providing a simple method for preparing mitochondrial apo-SHMT and determination of PLP binding stoichiometry (Ulevitch and Kallen, 1977). Our results show that one PLP molecule binds to each zmSHMT(DelSig) monomer, as observed for zcSHMT and mostly of the SHMTs studied to date (data not shown).

Steady-State Kinetic Constants and Reduced Folate Affinity. Double-reciprocal plots of initial velocity versus serine concentration permitted the determination of both apparent $K_{m}$ for serine and $k_{cat}$. As shown in Table 2, both the $K_{m}$ of serine and the $k_{cat}$ of zmSHMT(DelSig) are comparable to the values for zcSHMT and rabbit mitochondrial SHMT. The enzyme remains fully active at 37°C for at least 30 min (data not shown).

The quinonoid intermediate formed between reduced folates and the active site PLP absorbs near 500 nm with a molar extinction coefficient of 40,000 M$^{-1}$ cm$^{-1}$ (Schirch et al., 1977). This absorbance shows saturation kinetics with most reduced folate substrates, including $\text{H}_2\text{PteGlu}_4$, $\text{N}^5\text{-CHO-H}_2\text{PteGlu}_4$, and $\text{N}^5\text{-CH}_3\text{-H}_2\text{PteGlu}_4$ (Schirch and Ropp, 1967; Stover and Schirch, 1991). The binding of substrates to rabbit cytosolic SHMT is a sequential random mechanism. Previous studies had confirmed that the $K_{d}$ values determined by this method were essentially the same as the $K_{m}$ for folate determined from kinetic measurements (Schirch et al., 1977; Szepenyi et al., 2004). However, the reported value is an apparent $K_{d}$ because the formation of this complex is at least a two-step process. The lower $K_{d}$ of zmSHMT(DelSig) for THF suggests a higher affinity for this substrate compared with zcSHMT. Both isofoms have comparable affinity for $\text{N}^5$-CHO-THF, as judged from their similar dissociation constants (Table 2).

Inhibition of SHMT Aldol Cleavage Activity. Increasing concentrations of leucovorin ($\text{N}^5$-CHO-THF) inhibit both zmSHMT and hsSHMT activities substantially, yet to a lesser extent than zmSHMT (Fig. 5). The inhibitions of SHMT-catalyzed serine aldol cleavage by leucovorin and MTX were determined for zc-, zm-, and hsSHMT. Approximately 70% and 30% inhibition were observed for zc- and zmSHMT(DelSig) activities, respectively, in the presence of 70 $\mu$M $\text{N}^5$-CHO-THF (Fig. 5A). The $IC_{50}$ of leucovorin is approximately 30 $\mu$M for zmSHMT and higher than 70 $\mu$M for zcSHMT. The differential inhibition is evident with the presence of 10 $\mu$M leucovorin, the concentration estimated in serum in a high-dose leucovorin rescue regimen. A similar pattern of inhibition, but an even larger difference between zc- and zmSHMT was observed when the inhibition was assayed in the presence of 50 $\mu$M serine (Fig. 5B). MTX also represses SHMT activities, but not as significantly as it does zebrafish DHFR activity (Fig. 5C; T. F. Fu, unpublished result). No significant difference was observed when the highest concentrations of leucovorin (70 $\mu$M) and MTX (100 $\mu$M) were added simultaneously to the reaction in a combined assay compared with adding leucovorin alone (data not shown).

Tissue-Specific Distribution of zmSHMT and zcSHMT Isoforms. RT-PCR results showed that zmSHMT mRNA was evenly distributed among tissues, whereas significantly higher levels of zmSHMT mRNA were detected in heart, liver, and ova (Fig. 6C). This result is in agreement with the ubiquitous distribution of human mSHMT message and tissue-specific expression of cSHMT mRNA (Girgis et al., 1998). PCR was also performed using plasmids containing zmSHMT or zmSHMT coding sequences as templates to.
validate the positive signal. No PCR product was observed in this cross-reaction test (Fig. 6A).

Interestingly, we found that the SHMT protein levels did not correspond to their mRNA amounts detected in most of the tissues examined. Strong tissue specificity was observed in protein levels for both zcSHMT and zmSHMT (Fig. 6C). Zebrafish cSHMT protein was predominant in liver and also abundant in ova. Significant zmSHMT protein was detected only in liver and gastrointestinal tract regardless of the evenly distributed mRNA message. We also noted that appreciable amounts of cSHMT, but not mSHMT, were found in unfertilized eggs in both mRNA and protein levels. Equal loading of samples was confirmed by the presence of β-actin, Ponceau-S staining of the membrane, and Coomassie Brilliant Blue-stained SDS-polyacrylamide gel.

Subcellular Localization of Zebrafish SHMTs. The prediction on the recombinant zSHMT’s intracellular localization was confirmed by the site-specific compartmentalization of EGFP-fused SHMTs with confocal microscopy. The overexpressed zmSHMT-EGFP was clearly colocalized with a mitochondrial marker, demonstrating the mitochondrial localization of this enzyme (Fig. 7A). For zcSHMT-EGFP, surprisingly, the fluorescence signal of various intensities was detected in both nucleus and cytosol. No signal corresponding to free GFP was detected in cell extracts prepared from zcSHMT-EGFP transformants, excluding the possibility of artifacts or false signal due to any undesired sample contamination (Fig. 7B).

Discussion

Despite the unknown biological function and significance of SHMTs, studies showed that impairment of SHMT activity resulted in disturbance of homeostasis of the intracellular one-carbon pool and led to pathogeneses including homocysteinemia, cancers, cardiovascular diseases, and neural tube defects, implying a crucial role of SHMT in normal cell growth and functions (Stover and Garza, 2006). The existence of both cytosolic and mitochondrial SHMTs has been known since Nakano et al. (1968) partially purified the enzyme from rat liver mitochondria in 1968. Since then, cytosolic SHMTs of many species have been studied thoroughly, whereas information about the mitochondrial isoform is still very limited.

We report here the cloning and characterization of zebrafish mitochondrial SHMT. The identity of this recombinant protein was confirmed by its serine-aldol cleavage activity and colocalization with MitoTracker Red, a mitochondrial specific dye. The full-length zmSHMT expressed in E. coli forms inclusion bodies. This result was not unexpected since expression of organelle-specific proteins containing signal peptide often leads to formation of insoluble protein. Further characterization of zmSHMT(DelSig) reveals substantial similarities in its structure, physical properties, and kinetics to zcSHMT and mammalian orthologs, adding confidence to using zebrafish as an animal model for folate-related studies. That similar protocols applied to the purification of hcSHMT, rcSHMT, zcSHMT, and zmSHMT(DelSig) suggests comparable surface properties among these isoforms. Surprisingly, no evident cross-reaction between anti-zcSHMT antibody and zmSHMT(DelSig) protein was detected in Western blot analysis, despite a 63% identity being found in their peptide sequences. The possible explanation is that the homologous sequences might be embedded inside and therefore were not exposed to lymphocyte recognition and antibody generation.

It is interesting to note that the aldol cleavage of serine catalyzed by zc- and zmSHMTs was differentially inhibited by N\textsuperscript{5}-CHO-THF (leucovorin), despite both enzymes binding to this compound with similar affinity. To our knowledge, this is the first report on the effect of leucovorin on mitochondrial SHMT. The concentrations of serine and THF used in the inhibition assays encompassed the physiological concentrations of both substrates, implying that the similar inhibition

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Fig. 4. Absorbance of zebrafish SHMTs and its complexes with glycine and folate substrates. A, spectrum of zcSHMT (solid line) and zmSHMT(DelSig)(dotted line) in 20 mM potassium phosphate at pH 7.0. B, spectrum of zmSHMT(DelSig) (curve 1), zmSHMT(DelSig) saturated with glycine (curve 2), zmSHMT(DelSig) saturated with glycine and H\textsubscript{4}PteGlu (curve 3), and zmSHMT(DelSig) saturated with glycine and N\textsuperscript{5}-CHO-H\textsubscript{4}PteGlu (curve 4).
pattern might also occur in vivo (Vinnars et al., 1975). The differential inhibition observed between these two isoforms might be attributed to the lower $K_d$ of zmSHMT than of zcSHMT for THF. Stover and colleagues suggested that $N_5$-CHO-THF binds and modulates cSHMT activity, enabling this enzyme to function as a regulatory switch in one-carbon metabolism. When activated, the cSHMT-derived $N_5,N_{10}$-CH$_2$-THF gives the thymidylate synthetic pathway higher metabolic priority than the homocysteine remethylation cycle (Herbig et al., 2002; Woeller et al., 2007). The latter one generates $S$-adenosyl methionine, the major methyl donor for most intracellular methylation including DNA and protein. Our results add further weight to the notion that zmSHMT is responsible for a stable supply of $N_5,N_{10}$-CH$_2$-THF, whereas cSHMT is sensitive to alteration in nutritional status and functions to regulate the one-carbon flow in a changed environment (Stover and Garza, 2006). In a high-dose MTX combined leucovorin rescue therapy, the differential inhibitory effects of $N_5$-CHO-THF to zc- and zmSHMT might result in a decreased ratio of THF to $N_5,N_{10}$-CH$_2$-THF, and hence, redistribution of the activated one-carbon units between nucleotide biosynthesis and cellular methylation, yielding profound impact in intracellular events, gene activities, and, ultimately, cell survival. Evidence supporting the notion that cSHMT activity might play a role in the development of MTX

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_m$ for serine (mM)</th>
<th>$K_d$ for $H_4PteGlu$ (μM)</th>
<th>$K_d$ for $N_5$-CHO-$H_4PteGlu$ (μM)</th>
<th>Turnover Number (min$^{-1}$)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zebrafish m$^a$</td>
<td>0.43</td>
<td>8</td>
<td>2.8</td>
<td>537</td>
<td>Present report</td>
</tr>
<tr>
<td>Zebrafish c$^b$</td>
<td>0.22</td>
<td>18</td>
<td>2.6</td>
<td>351</td>
<td>Chang et al. (Chang et al., 2006)</td>
</tr>
<tr>
<td>Rabbit m$^c$</td>
<td>0.60</td>
<td>25</td>
<td>10</td>
<td>500</td>
<td>Schirch et al. (Schirch and Peterson, 1980)</td>
</tr>
</tbody>
</table>

$^a$ zebrafish mSHMT(DelSig); $^b$ cytosolic SHMT; $^c$ mitochondrial SHMT

![Graph A](image1.png)

**Fig. 5.** Inhibition of the SHMT-catalyzed serine-aldol cleavage by increasing concentrations of leucovorin ($N_5$-CHO-THF) (A and B) and methotrexate (C). Inhibition on initial velocity was determined at 30°C in the presence of 25 μM $H_4PteGlu$, saturated NADP$^+$, 10 mM (A and C) or 50 μM (B) serine, 0.1 μM SHMT, and inhibitors ranging from 0.1 to 100 μM.
resistance has been reported. In support of this notion are that polymorphism in cSHMT was related to MTX resistance in pediatric patients with acute lymphoblastic leukemia, and overexpression of cSHMT in Leishmania increased resistance to methotrexate in a rich folate-containing medium (de Jonge et al., 2005; Gagnon et al., 2006). The present study adds mSHMT to the picture for possible mechanistic insights and provides clues to further understand the complex relationships between one-carbon metabolism, SHMTs, and the development of MTX resistance.

The MTX and leucovorin concentrations used in the inhibition studies ranged from 0.1 to 100 μM. It was estimated that a concentration of 1 to 10 μM for both MTX and leucovorin is a realistic serum concentration that can be reached in a MTX-leucovorin combined regimen (Widemann and Adamson, 2006). However, we are convinced that the differential inhibition observed in our studies should have reflected what has occurred in vivo, since the polyglutamylation of folate/antifolate substrates will significantly increase their affinities to folate enzymes. Five to seven glutamate residues will be added to the γ-carboxyl group of the internalized MTX and N5-CHO-H4PteGlu5, respectively (Huang et al., 1998; Fu et al., 2005). This result implies that the inhibition mediated by N5-CHO-H4PteGlu5 in cells should be comparable to, if not more significant than, the results observed in vitro studies since polyglutamylation will further potentiate the competitiveness of polyglutamylated leucovorin with tetrahydrofolate polyglutamate.

Tissue-specific expression was evident in the protein level for both SHMT isozymes in zebrafish, with the highest expression in liver. This is, to our knowledge, the first report on the tissue-specific distribution of mitochondrial SHMT. It was documented that human mSHMT mRNA was evenly expressed among tissues (Girgis et al., 1998). Interestingly, our RT-PCR results for zmSHMT were in agreement with the human mSHMT expression pattern and showed equal distribution, suggesting that translational and/or post-translational regulation might play a role in controlling the intracellular concentrations of both enzymes. Protein stabilized by the binding of folate substrates and/or PLP cofactor might also contribute to the different protein levels observed in this study. Support for this notion is very recent, showing that lack of vitamin B6 in cells causes a decrease in SHMT protein but not mRNA level (Perry et al., 2007). Notably, cSHMT is abundant in unfertilized eggs, supporting the view that SHMT is a maternally essential gene (Vatcher et al., 1998). Studies on the cor-
The relationship between the abundance of SHMTs and the potential risk of developing MTX resistance might be rewarded by an understanding of the differential efficacy of MTX observed in various types of cancer.

We were puzzled and vigilant when we observed the presence of zcSHMT in the nucleus, since the nuclear localization of cSHMT had never been reported at that time. A negative result was obtained when the peptide sequence was subjected to a search for a specific nuclear-targeting sequence. Having repeated this experiment carefully and revealed the same results many times prompted us to postulate that the zcSHMT-EGFP might be transported into nucleus via cargo or other component-mediated mechanisms. Interestingly, our observation and hypothesis were found later to be in agreement with what was reported by Woeller et al. (2007) in a very recent study showing that human cSHMT was SUMOylated and nuclear-localized in a cell cycle-dependent manner. Two prospective SUMOylation sites are identified in the zcSHMT peptide sequence, suggesting a possibly similar mechanism for zcSHMT nuclear localization and a resemblance between human and zebrafish SHMTs. The biological significance of the nuclear-localized cSHMT is unknown. Yang and Meier (2003) showed that cSHMT was in some way connected to the nucleolar protein SRP40p and modulated cell cycle and cell size, supporting a noncatalytic function of cSHMT. Enlargement of the cell size was also observed in our zcSHMT-EGFP-transfected cells. The
significance of zcSHMT intracellular localization is currently under investigation. Zebrafish have attracted the interest of many researchers as an animal model in the past two decades. The features of external development, transparent embryo, ease of growth and breeding, economy, and ease of manipulation using well established molecular approaches have made zebrafish an ideal animal model for studying developmental biology and pathogenic mechanisms in a variety of conditions. Especially important for drug discovery is that zebrafish embryos are permeable to small molecules and drugs during organogenesis, providing easy access for drug administration and vital dye staining (Kari et al., 2007). There is no doubt that efforts will be continually invested in the improvement of antifolate drugs, considering the vital role of folates in nucleotides and protein biosynthesis. The search for new targets of antifolate drugs will also be sustained. In addition to its vital role in folate-mediated one-carbon metabolism, the property of being highly expressed in rapidly proliferating cells has made SHMT a potential target for chemotherapy and immunosuppression (Renwick et al., 1998). Our studies conclude that zebrafish SHMTs share high similarity with human isoforms, indicating that zSHMTs, and probably zebrafish as a whole, are appropriate systems for folate-related studies and antifolate drug discovery. Further studies on other folate enzymes should be warranted. In addition, the possible mechanistic insights provided in this study enable us to further understand the complex relationships between one-carbon metabolism, SHMTs, and the development of MTX resistance.

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References


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