Lack of Catalytic Activity of a Murine mRNA Cytoplasmic Serine Hydroxymethyltransferase Splice Variant: Evidence against Alternative Splicing as a Regulatory Mechanism†

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ABSTRACT: Mammalian serine hydroxymethyltransferase (SHMT) is a tetrameric, pyridoxal phosphate-dependent enzyme that catalyzes the reversible interconversion of serine and tetrahydrofolate to glycine and methylenetetrahydrofolate. This reaction generates single-carbon units for purine, thymidine, and methionine biosynthesis. Cytoplasmic SHMT (cSHMT) has been postulated to channel one-carbon substituted folates to various folate-dependent enzymes, and alternative splicing of the cSHMT transcript may be a mechanism that enables specific protein−protein interactions. The cytoplasmic isozyme is expressed from species-specific and tissue-specific alternatively spliced transcripts that encode proteins with modified carboxy-terminal domains, while the mitochondrial isozyme is expressed from a single transcript. While the full-length mouse and human cSHMT proteins are 91% identical, their alternatively spliced transcripts differ. The murine cSHMT gene is expressed as two transcripts. One transcript encodes a full-length 55 kDa active enzyme (cSHMT), while the other transcript encodes a 35 kDa protein (McSHMTtr). The McSHMTtr protein present in mouse liver and kidney does not bind 5-formyltetrahydrofolate, nor does it oligomerize with the full-length cSHMT enzyme. While recombinant cSHMT−glutathione *S*-transferase fusion proteins form tetramers and are catalytically active, McSHMTtr−glutathione *S*-transferase fusion proteins are catalytically inactive, do not form heterotetramers, and do not bind pyridoxal phosphate. Analysis of the murine cSHMT crystal structure indicates that the active site lysine that normally binds pyridoxal phosphate in the cSHMT protein is exposed to solvent in the McSHMTtr protein, preventing stable formation of a Schiff base with pyridoxal phosphate. Modeling studies suggest that the human cSHMT proteins expressed from alternatively spliced transcripts are inactive as well. Therefore, channeling mechanisms enabling specific protein−protein interactions of active enzymes are not based on cSHMT alternative splicing.

Serine hydroxymethyltransferase (SHMT,[1] EC 2.1.2.1) is a pyridoxal phosphate-dependent enzyme that supplies single-carbon units in the form of methyleneTHF for purine and thymidine biosynthesis, and for the remethylation of homocysteine to methionine (*1*). These single-carbon units are derived from serine. SHMT catalyzes the reversible conversion of serine and tetrahydrofolate (THF) to glycine and 5,10-methyleneTHF (reaction 1).

$$\text{serine} + \text{THF} \leftrightarrow \text{glycine} + \text{5,10-methyleneTHF} \quad (1)$$

When catalyzing the reaction in the direction of serine cleavage, this reaction generates single-carbon units for folate-dependent anabolic reactions. When catalyzing serine synthesis, the enzyme depletes the pool of folate-activated one-carbon units. In mammals, SHMT is present in both the mitochondria (mSHMT) and cytoplasm (cSHMT), and the enzymes are encoded by separate genes (*2*, *3*). The metabolic roles of the SHMT isozymes are not completely understood, although mSHMT is the major source of glycine in mammalian cells (*4*). The human mSHMT message is present at similar levels in all tissues (*3*). The human cSHMT message, while present in all tissues, is highly enriched in kidney, liver, and skeletal muscle (*3*). The human mitochondrial and cytoplasmic isozymes share highly similar catalytic properties, and their amino acid sequences are 63% identical (*5*).

Previously, we have demonstrated that the human cSHMT hnRNA undergoes tissue specific alternative splicing. Three transcripts are produced: one that encodes a full-length protein, one that lacks exon 9, and another that lacks exons 9 and 10 (*3*). The peptide sequence encoded in exon 9 of the human cSHMT protein is 80% identical to the sequence contained within the human mSHMT enzyme, and the peptide sequence encoded by exons 9 and 10 within the

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[1] Abbreviations: THF, (6*R*)-tetrahydrofolate; PLP, pyridoxal 5′-phosphate; cSHMT, cytoplasmic serine hydroxymethyltransferase; McSHMTtr, murine alternatively spliced cytoplasmic SHMT protein with a deleted carboxy-terminal domain; mSHMT, mitochondrial serine hydroxymethyltransferase; GST, glutathione *S*-transferase.

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human cSHMT protein is 66% identical with the corresponding sequence in the mSHMT protein. This high degree of sequence conservation suggests similar roles for these residues in the structure or catalytic activity of both the full-length mSHMT and cSHMT enzymes. However, alternative splicing of the hnRNA transcript occurs only with cSHMT. It has been proposed that tissue-specific alternative splicing may be an important mechanism used by cells to manipulate cSHMT activity, reaction specificity, or metabolic function (*3*). Several distinct metabolic roles have been demonstrated for the cytoplasmic SHMT isozyme in addition to interconverting serine and glycine. The enzyme possesses a second catalytic activity, the irreversible conversion of methenylTHF to 5-formylTHF (*6*). 5-FormylTHF does not serve as a metabolic cofactor but rather is an inhibitor of several folate-dependent enzymes (*7*, *8*). Once synthesized, 5-formylTHF remains bound to SHMT enzymes as a slow, tight-binding inhibitor (*8*, *9*). The cytoplasmic isozyme has also been proposed to channel THF cofactors to other folate-dependent enzymes, including 10-formyltetrahydrofolate synthetase (*10*). There is also evidence that the cSHMT enzyme can accelerate the rate of dissociation of tightly bound THF from the enzyme 10-formyltetrahydrofolate dehydrogenase (*11*). If cSHMT physically interacts with several folate-dependent enzymes, alternative splicing is a potential mechanism for tailoring the enzyme for specific protein−protein interactions (*3*).

Recently, we have reported the X-ray structure of the murine cSHMT enzyme complexed with glycine and 5-formylTHF (*12*). This structure represents a transition or intermediate state analogue of the catalytically competent cSHMT−serine−THF ternary complex and enables predictions regarding the effect of cSHMT alternative splicing on cSHMT structure and catalytic function. In this paper, using both experimental and modeling approaches, we investigate the role of alternative splicing in murine cSHMT structure and catalytic function.

EXPERIMENTAL PROCEDURES

Materials. [^{32}P]dCTP and [^{32}P]dATP (800 Ci/mmol) were obtained from New England Nuclear. Restriction and modifying enzymes were obtained from Boehringer Mannheim, Promega, or New England Biolabs. *Taq* DNA polymerase was from Perkin-Elmer; *Pfu* DNA polymerase was from Stratagene. All other materials were of high quality and obtained from various commercial vendors.

Characterization of Murine cSHMT Alternative Splicing. To determine if the McSHMTtr transcript resulted from an alternative splicing event, genomic DNA was used as a template and primers generated from exon 8 (5′-CACAAGACCCTGAGAGACTGCC-3′) and exon 9 (5′-AGTTGATGAGGGACTCCAGTTCATA-3′) were used to amplify intron 8. The cycling conditions were 94 °C for 45 s, 62 °C for 45 s, and 70 °C for 6 min for 30 cycles. The PCR product was sequenced and found to contain a nucleotide sequence identical to the insertion present in the McSHMTtr transcript.

Partial Affinity Purification of the cSHMT Protein in Mouse Tissue. Two mouse livers or four mouse kidneys were homogenized in 25 mL of extraction buffer containing 10 mM potassium phosphate (pH 7.0), 50 mM glycine, 0.1 μM PLP, 1 mM EDTA, 5 mM DTT, and 1 mM PMSF. The homogenate was clarified by centrifugation (15000*g*) for 20 min. Two milliliters of the supernatant was applied to a 2 cm × 4 cm 5-formylTHF affinity column. The synthesis of the 5-formylTHF agarose affinity column has been described elsewhere (*13*). The column was washed with 10 column volumes of extraction buffer, and the protein was eluted with 10 mL of extraction buffer containing 500 μM (6*RS*)-5-formylTHF. The eluted protein was quantified by the method of Lowry (*14*).

Cloning and Expression of the Recombinant Murine McSHMTtr Protein. The cloning and expression of the full-length murine cSHMT cDNA (cSHMT) was described previously (*12*). The cSHMT cDNA was generated by RT-PCR using the high-fidelity *Pfu* polymerase. Mouse liver (strain 129/sv) cDNA was generated from total RNA (Purgene, Inc.) and an oligo dT primer, and then used as a template for PCR. The murine McSHMTtr splice variant open reading frame was amplified using the forward primer 5′-GCGCCATATGGCAGACAGGGATGCCAC-3′ and the reverse primer 5′-GCGCAAGCTTTTATTTCTTCCTGAGCTCTTCC-3′. The primers contain the *Nde*I and *Hind*III sites, respectively. Following RT-PCR, a 0.93 kb fragment was isolated. The nucleotide sequence of three independently derived cDNA clones was determined by nucleotide sequencing at the Cornell Biotechnology Center. For McSHMTtr, all three nucleotide sequences were found to be identical with each other and with the published nucleotide sequences (*15*). The cDNA was ligated into the *Nde*I and *Hin*dIII sites of expression vectors pET-22b and pET-28a (Novagen), and then expressed in *Escherichia coli* BL21-(DE3) pLysS (Novagen). For the GST fusion protein, the cDNA was ligated into pGEX-4T, and expressed in *E. coli* BL21(DE3) pLysS (Novagen).

Purification of the McSHMTtr−GST Fusion Protein. Unlike the murine cSHMT enzyme, recombinant McSHMTtr is not expressed as a soluble protein in *E. coli.* Attempts to express recombinant human cSHMT splice variants in *E. coli* were unsuccessful[2] as well. The recombinant murine full-length cSHMT protein and GST fusion McSHMTtr protein were coexpressed in *E. coli* BL21(DE3) pLys. Cells were grown at 37 °C to an OD_{550} of 0.6. Protein expression was induced by the addition of 0.2 mM IPTG at 25 °C, and the cells were cultured for an additional 3 h. The bacteria were pelleted and suspended in a minimal volume of 10 mM potassium phosphate buffer, and the cSHMT protein was extracted from the bacteria by sonication. The GST fusion protein was purified by passing the extract over a glutathione-Sepharose 4B affinity column following the manufacturer's instructions (Amersham Pharmacia Biotech). The recombinant murine cSHMT protein was purified on a metal affinity column as described by the manufacturer.

Western Analysis. Extracts from *E. coli* expressing the murine cSHMT cDNA or mouse tissue extracts were incubated at 100 °C for 10 min in a buffer containing 60 mM Tris (pH 6.8) and 2% SDS. SDS−polyacrylamide gel electrophoresis was carried out using a 5% stacking gel and a 10% separating gel in a slab gel apparatus (Hoefer, San Francisco, CA) with the discontinuous buffer system of Laemmli. Proteins were transferred overnight to a poly-

[2] E. Oppenheim and P. J. Stover, unpublished results.

(vinylidene difluoride) membrane (Millipore) in a Bio-Rad Transblot apparatus at 30 V with a limit of 0.2 mA. The membrane was rinsed with PBS before being incubated in 5% nonfat dry milk and 1% Nonidet P-40 in phosphate-buffered saline for 11.5 h at 4 °C. The sheep anti-murine cSHMT antiserum was obtained from Chiron Mimotopes (San Diego, CA) using a synthetic peptide (SDAEVYSI-IKKESNRQRVGL) representing amino acids present in the amino-terminal domain of the enzyme. This peptide shares no identity with the mitochondrial SHMT primary sequence. The serum was diluted 1:1000 in blocking buffer, and the membrane was incubated in the buffer for 14 h at 4 °C. The membrane was rinsed eight times with 0.1% Tween 20 in PBS and incubated for 14 h at 4 °C with blocking buffer containing the horseradish peroxidase-conjugated mouse anti-sheep IgG antibody in a 1:10000 dilution. The membrane was visualized using the SuperSignal chemiluminescent horseradish peroxidase substrate system from Pierce (Rockford, IL).

Northern Blot Analyses. The tissue distribution of murine cSHMT mRNA was determined by Northern analysis using a "Multiple Tissue Northern" blot (Clontech) with each tissue sample containing 2 μg of mRNA. For cSHMT mRNA detection, [^{32}P]dATP-labeled probes were generated from a nucleotide sequence comprising the 5′-UTR of murine cSHMT or intron 8 of murine cSHMT genomic DNA. The Northern blot was prepared, probed, and washed following the manufacturer's protocols. All data were recorded and quantified using the Molecular Imager System (Bio-Rad).

Refolding of the cSHMT Splice Variant. Murine McSHMTtr was expressed in *E. coli* BL21(DE3) pLys at 25 °C as described above. The pelleted bacteria were suspended in a minimal volume of 100 mM HEPES (pH 7.6) and incubated with 10 μg/mL lysozyme, followed by sonication in 50 mM potassium phosphate (pH 7.5), 5 mM DTT, and 2 mM EDTA. The solution was clarified by centrifugation (15000*g*). The pellet, which contains the McSHMTtr protein inclusion bodies, was washed twice in sonication buffer, and then with 2% (w/v) sodium deoxycholate with 0.05 M NaCl. The pellet was dissolved in lysis buffer containing 0.4% (w/v) sarkosyl and incubated at room temperature for 30 min. The protein was renatured by slowly diluting the unfolded protein into a 10-fold excess of lysis buffer at 4 °C.

Gel Filtration Chromatography. The molecular mass of native McSHMTtr was determined by HPLC using a 300 mm × 7.8 mm Biosep Sec-S3000 column (Phenomenex) inline with a 75 mm × 7.8 mm Biosep Sec-3000 guard column (Phenomenex). The elution time of the McSHMTtr protein was determined using a mobile phase of 10 mM HEPES buffer (pH 6.8) and 2 mM 2-mercaptoethanol, and the molecular mass was determined relative to protein standards. Protein standards were obtained from Bio-Rad and reconstituted in 500 μL of sterile water following the instructions provided by the manufacturer. All experiments were performed in duplicate.

SHMT Activity Assays. The catalytic activities of murine cSHMT and McSHMTtr were determined by measuring the rate of allothreonine cleavage as described previously (*8*).

Molecular Modeling. The structure of full-length murine cSHMT, as determined by single-crystal X-ray diffraction (*12*), was used as a starting point for modeling McSHMTtr and the human alternatively spliced cSHMTs. The full length of the polypeptide chain is visible in the crystal structure. Residues are numbered on the basis of alignment of the murine and human sequences; using this convention, murine cSHMT comprises residues 8−484. The programs O (*16*) and RasMol (*17*) were used to identify contacts involving regions that are deleted in the alternatively spliced forms, and to model the active site of McSHMTtr.

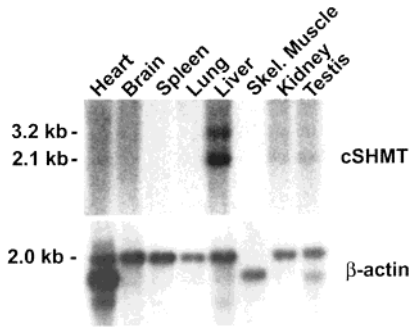


FIGURE 1: Tissue distribution and alternative splicing of murine cSHMT mRNA. A murine multitissue Northern blot purchased from Clontech was hybridized with probes complementary to the 5′-untranslated region of cSHMT messages (top). Following exposure, the blot was stripped and visualized with probes complementary to β-actin mRNA (bottom).

RESULTS AND DISCUSSION

Identification and Characterization of a cSHMT Variant in Mouse Tissue. The murine cSHMT isozyme was recently identified as a retinoic acid responsive gene in murine embryonic carcinoma cells (*15*). In that study, two partial cSHMT transcripts were identified; one transcript was similar to the human cSHMT message that encodes a full-length cSHMT protein (cSHMT), while the second message contained a nucleotide insertion (McSHMTtr). Neither the origin nor the functions of the second transcript have been determined, nor has the expression of the predicted 35 kDa protein been verified. While the human cSHMT hnRNA is alternatively spliced to form one full-length and two shorter transcripts by exon excision, no human cSHMT transcript has ever been reported to contain a nucleotide insertion. This indicates that human and mouse species splice cSHMT hnRNA by different mechanisms.

The presence of the McSHMTtr message in mouse tissue was confirmed by Northern blot analysis (Figure 1). While human Northern blots probed with cSHMT antisense oligonucleotides reveal up to four distinct bands (*3*), murine tissue contains only two cSHMT transcripts. These two transcripts are similar in size to the cSHMT transcripts present in cultured murine P19 cells (*15*). The murine cSHMT messages are enriched in liver, although prolonged exposure of the blot demonstrates that both McSHMTtr and cSHMT messages are present in all tissues. The ratio of the McSHMTtr to cSHMT transcript number does not appear to display tissue specific differences as seen for the human cSHMT splice variants (*3*).

The full-length mammalian cSHMT proteins are homotetramers, with each monomer containing two domains (*18*, *19*). The amino-terminal domain of the human enzyme is encoded by exons 3−8, with exon 8 encoding the lysine that binds the active site pyridoxal phosphate. The carboxy-terminal domain is encoded by exons 9−13. The human cSHMT hnRNA is alternatively spliced by exon excision,

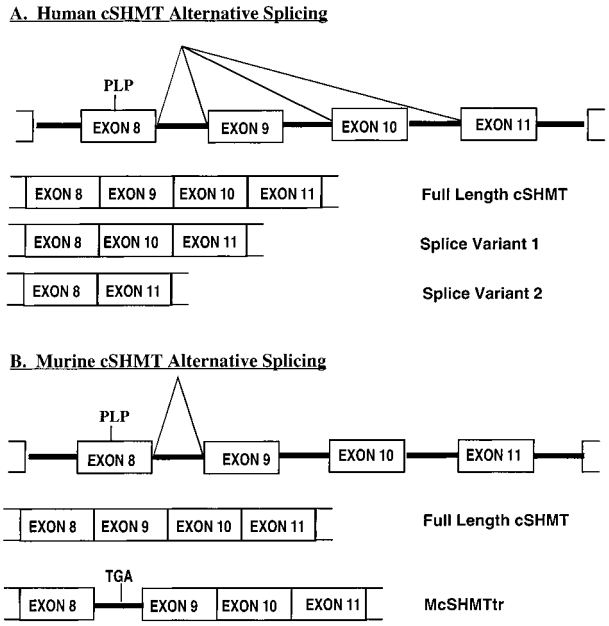


FIGURE 2: Alternative splicing of the murine and human cSHMT message. The nucleotide sequence that encodes the active site lysine that binds pyridoxal phosphate is designated PLP, and the in-frame stop codon present within intron 8 is designated TGA. (A) Alternative splicing of the human hnRNA within the ORF proceeds to produce one full-length transcript, and two smaller transcripts resulting from excision of exon 9, and exons 9 and 10, respectively. (B) Alternative splicing of the murine cSHMT hnRNA proceeds to produce two transcripts, a full-length transcript (cSHMT) and a second transcript containing intron 8 (McSHMTtr). Exon numerical assignments for the murine cSHMT gene correspond to the previously reported human cSHMT gene assignments.

generating three transcripts: a full-length transcript, one lacking exon 9, and another lacking exons 9 and 10 (Figure 2). Alternative splicing of the human cSHMT transcript occurs with retention of the open reading frame, and therefore, all human cSHMT transcripts encode cSHMT proteins with selective deletions within the carboxy-terminal domain (*3*).

The cDNAs encoding the murine McSHMTtr and cSHMT proteins were cloned by RT-PCR and sequenced. The murine McSHMTtr transcript contains a 1.1 kb nucleotide insertion located at the exon 8−intron 8 splice junction (*3*). The nucleotide sequence of intron 8 in the murine cSHMT gene was determined following PCR amplification of murine genomic DNA using primers complementary to nucleotide sequences present within exon 8 and exon 9. The nucleotide sequences of the PCR products were identical to the nucleotide sequence of the insert present in the McSHMTtr transcript, confirming that the McSHMTtr transcript is derived from an alternative splicing event (Figure 2). Hybridization of the Northern blot shown in Figure 1 with probes complementary to the nucleotide sequence present in intron 8 resulted in the appearance of only the upper band (3.2 kb), confirming the presence of McSHMTtr mRNA in mouse tissues. Murine intron 8 contains an in-frame stop codon; hence, the McSHMTtr transcript contains an open reading frame that encodes a 35 kDa cSHMT protein lacking the carboxy-terminal domain present in the full-length cSHMT protein, a 55 kDa protein. For the McSHMTtr protein, the 212 amino acids in the carboxy-terminal domain of the cSHMT protein are replaced with a 43-amino acid

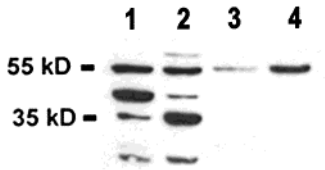


FIGURE 3: Western blot analyses of cSHMT protein in murine tissue. Crude soluble tissue extracts (150 μg/lane) or partially purified extracts (1 μg/lane) were run on a 10% SDS−PAGE gel, and the cSHMT protein was visualized by Western blot analysis. The cSHMT protein appears as a 55 kDa band and the McSHMTtr protein as a 35 kDa band. Crude extracts were generated from clarified tissue homogenates, while partially purified cSHMT extracts were obtained after application of the crude extracts to a 5-formylTHF affinity column: lane 1, crude liver extract; lane 2, crude kidney extract; lane 3, affinity-purified liver extract; and lane 4, affinity-purified kidney extract.

peptide encoded within intron 8. This peptide does not share any primary sequence identity with the carboxy-terminal domain of the cSHMT protein. Although the splicing mechanisms differ between the mouse and human cSHMT transcripts, both alternatively splice at the exon 8−intron 8 boundary and produce transcripts that encode cSHMT proteins with modified carboxy-terminal domains (Figure 2).

Expression of cSHMT Splice Variants in Mouse Tissues. Expression of the McSHMTtr and cSHMT transcripts in mouse tissue was confirmed by Western blot analysis using an antibody specific for amino acid residues in the amino-terminal domain of the enzyme (Figure 3). Both kidney and liver contain immunoreactive bands at the expected molecular masses for cSHMT (55 kDa) and McSHMTtr (35 kDa). An additional band with a molecular mass of 48 kDa is also visualized. This band may represent a degradation product of the cSHMT protein, as the intensity of the band increases at the expense of the cSHMT band when protease inhibitors are excluded from the homogenization buffer. The ability of the McSHMTtr protein to bind 5-formylTHF was investigated by passing the crude tissue extract over a 5-formylTHF affinity column prior to the Western analysis. 5-FormylTHF is a potent transition or intermediate state inhibitor of full-length cSHMT enzymes, and binds to the cSHMT protein with an affinity of ~10 μM (*8*, *12*). Following affinity purification, neither the 35 nor the 48 kDa band was visualized, indicating that the McSHMTtr protein does not effectively bind 5-formylTHF with high affinity and therefore likely does not catalyze folate-dependent reactions. Additionally, the lack of the McSHMTtr protein in the partially purified extract indicates that the McSHMTtr protein does not oligomerize with the catalytically active cSHMT protein.

Expression and Characterization of the Recombinant McSHMTtr and cSHMT Proteins. The catalytic potential of the McSHMTtr protein was further investigated by expressing recombinant cSHMT and McSHMTtr proteins in *E. coli*. While the cSHMT protein can be expressed in a soluble and active form in *E. coli*, the McSHMTtr protein is expressed as insoluble inclusion bodies. Manipulation of the culture temperature or medium does not result in the expression of the soluble protein, but the inclusion bodies can be purified and refolded in a soluble form as described in Experimental Procedures.

The full-length cSHMT protein is a PLP-dependent protein, and exhibits the characteristic spectral properties of all PLP-dependent enzymes (Scheme 1). The cSHMT

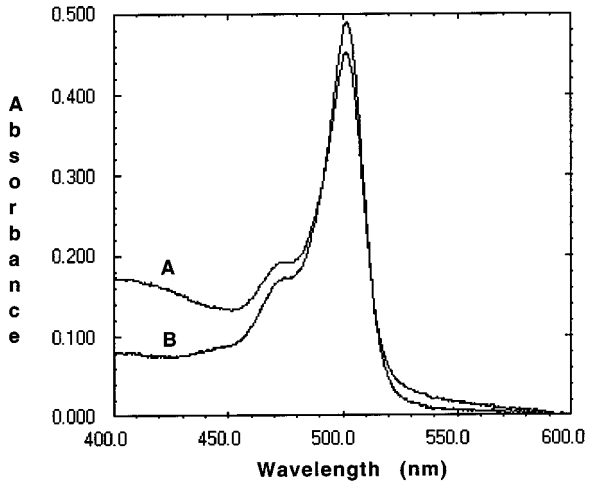


FIGURE 4: Spectra of the cSHMT enzyme. The absorbance spectra of the cSHMT and cSHMT−GST fusion protein were determined in the presence of 5-formylTHF (200 μM) and glycine (50 mM): (A) 12.5 μM recombinant cSHMT protein and (B) 12.5 μM recombinant cSHMT−GST fusion protein.

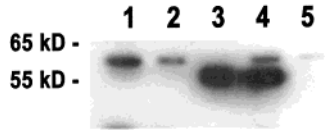


FIGURE 5: Western blot of recombinant cSHMT protein in bacterial extracts. Crude soluble bacterial extracts (20 μg/lane) were separated by 10% SDS−PAGE, and the cSHMT protein was visualized by Western blot analysis. The cSHMT protein appears as a 55 kDa band and the McSHMTtr−GST fusion protein as a 65 kDa band. Crude extracts were purified on a GST affinity column, and the cSHMT protein was visualized in the purified extract by Western blot analysis (1 μg/lane): lane 1, crude extract from *E. coli* expressing a McSHMTtr−GST fusion protein; lane 2, GST-purified recombinant McSHMTtr−GST fusion protein; lane 3, crude extract from *E. coli* expressing murine cSHMT protein; lane 4, crude extract from *E. coli* coexpressing the McSHMTtr−GST fusion protein and the murine cSHMT protein; and lane 5, GST-purified crude extract from *E. coli* coexpressing the McSHMTtr−GST fusion protein and the murine cSHMT protein.

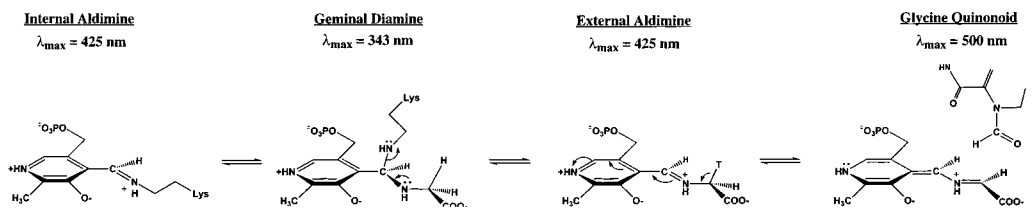
enzyme contains an active site PLP cofactor bound as an internal aldimine (λ_{max} = 425 nm). Upon binding substrates, the enzyme displays distinct spectral properties that are associated with specific reaction intermediates. When glycine and 5-formylTHF are bound, the PLP cofactor exists in equilibrium between the *geminal* diamine (λ_{max} = 343 nm), external aldimine (λ_{max} = 425 nm), and glycine quinonoid (λ_{max} = 502 nm) forms. Formation of the quinonoid structure from the external aldimine is associated with a conformational change in the enzyme (*8*). Dialysis of the solubilized, refolded McSHMTtr protein in 10 mM potassium phosphate (pH 7.0) and 10 μM PLP does not result in the formation of a PLP internal aldimine. Inclusion of glycine or glycine and 5-formylTHF in the dialysis buffer also does not result in the binding of the PLP cofactor. These results indicate that the McSHMTtr protein does not bind PLP, although we cannot exclude the possibility that the protein has not attained its proper conformation during the in vitro renaturation and folding.

Expression of McSHMTtr and cSHMT proteins with an N-terminus fusion does result in the synthesis of soluble proteins. Additionally, the cSHMT−GST fusion protein retains its catalytic function. Figure 4 shows the absorbance spectra of the cSHMT proteins with glycine and 5-formylTHF bound. The cSHMT protein (spectrum A) exhibits an absorbance peak at 502 nm, consistent with the formation of the glycine anion quinonoid species. This is a key catalytic intermediate associated with serine and glycine interconversion, and its formation indicates that the protein contains catalytic activity. The cSHMT−GST fusion protein also exhibits absorbance spectral properties consistent with the formation of the quinonoid intermediates (spectrum B). This indicates that the GST tag does not disrupt the cSHMT activity or ability to oligomerize. The purified McSHMTtr−GST fusion protein does not contain bound PLP and therefore does not display spectral properties associated with cSHMT reaction intermediates. Additionally, the purified McSHMTtr−GST protein does not bind PLP when dialyzed in buffer containing 10 μM PLP. The catalytic activity of the splice variants was also directly determined. In addition to catalyzing the THF-dependent cleavage of serine to glycine, SHMT can also catalyze the THF-independent cleavage of allothreonine. Neither the McSHMTtr−GST fusion protein nor the McSHMTtr refolded protein was capable of catalyzing the cleavage of allothreonine to glycine in buffer containing 10 μM PLP.

The recombinant McSHMTtr−GST fusion protein does not oligomerize with the cSHMT protein (Figure 5). The cSHMT and McSHMTtr−GST fusion proteins can be coexpressed in *E. coli*, and the presence of both proteins can be visualized in crude *E. coli* extracts by Western blot analyses. Passage of the crude extract over a GST affinity column results in the purification of the McSHMTtr−GST fusion protein only, without copurification of the cSHMT protein, demonstrating that the McSHMTtr and cSHMT proteins do not form hetero-oligomers. However, gel filtration studies indicate that the refolded McSHMTtr protein can form homodimers. The experimentally determined molecular mass of the native mcSHMTtr protein is approximately 80 kDa.

Structural Analyses of the cSHMT Splice Forms. Recently, the structure of the murine cSHMT enzyme was determined with substrates glycine and 5-formylTHF bound (*12*). The cSHMT−Gly−5-formylTHF ternary complex is an intermediate state analogue of the catalytically competent cSHMT−Gly−methyleneTHF ternary complex (*8*, *12*). The cSHMT enzyme is a tetramer containing four active sites and is best

Scheme 1: Reaction Intermediates Associated with cSHMT Catalysis



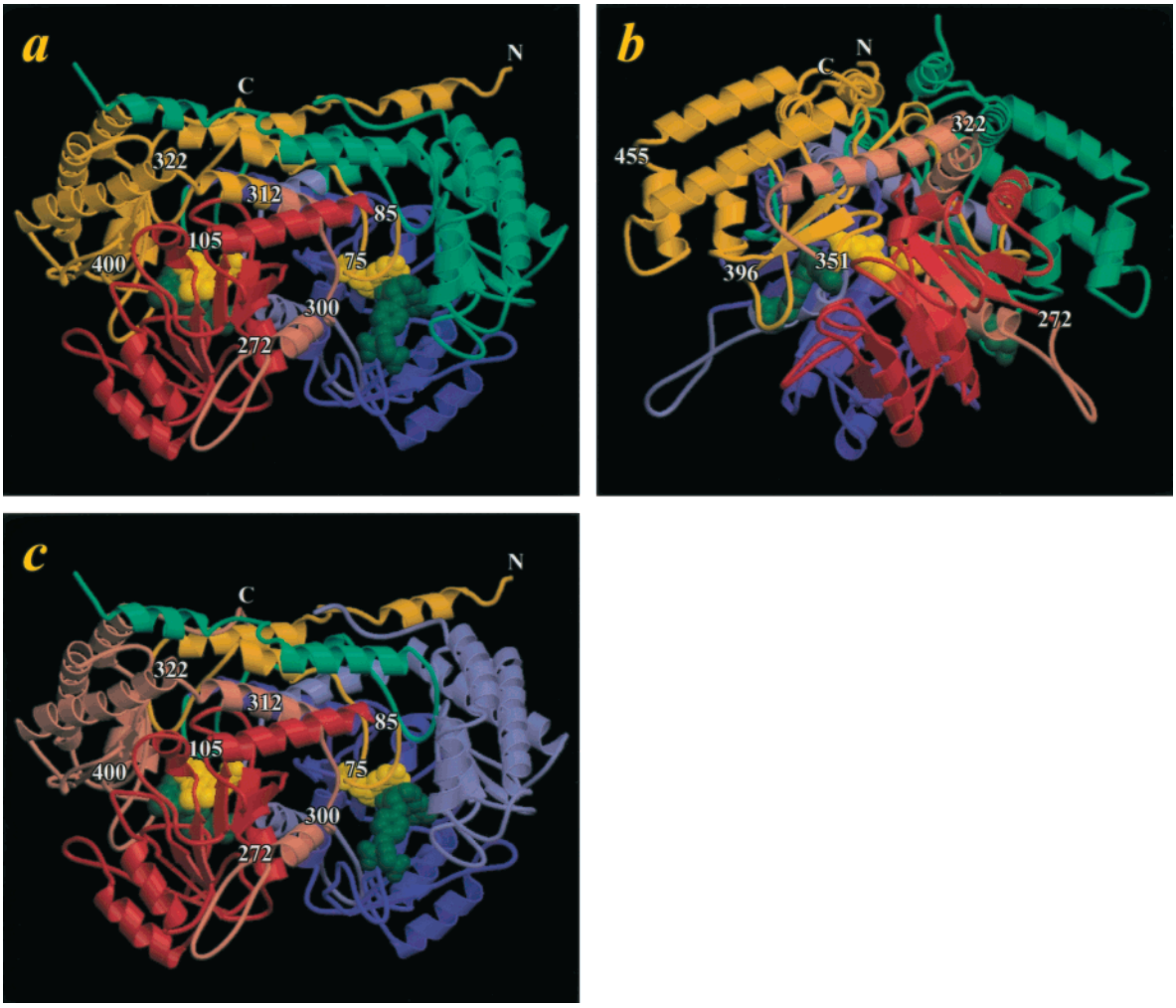


FIGURE 6: Panels a−c show the X-ray structure of a cSHMT dimer with glycine and 5-formylTHF bound. The large domains (residues 86−272) are shown in red (A chain) and blue (B chain). The N-terminal (residues 8−85) and small (residues 273−484) domains are gold (A chain) and turquoise (B chain). The PLP cofactors are shown as yellow space-filling models, and the folate ligands are dark green. Regions that would be deleted by alternative splicing are shown in pink (A chain) and light blue (B chain). Some important residues, in the A chain only, are labeled. (a) Exon 9 is deleted as in a human cSHMT splice variant. (b) Exons 9 and 10 deleted as in another human cSHMT splice variant. (c) Chain truncated at residue 272 as in the murine cSHMT splice variant (the 43-residue insertion in McSHMTtr is not modeled). The molecule in panel b is rotated approximately 90° about a vertical axis, relative to panels a and c. This figure was prepared using MOLSCRIPT (*21*) and Raster3D (*22*).

described as a dimer of obligate dimers (*18*, *19*). Within an obligate dimer, there are two active sites with amino acid residues from each monomer contributing to both active sites (*12*, *18*, *19*). Interestingly, the obligate dimers within the cSHMT tetramer are not equivalent. Only the active sites in one of the dimers bind 5-formylTHF tightly, while the active sites in the other dimer do not bind 5-formylTHF or bind the cofactor in a disordered conformation (*12*).

Figure 6 depicts the structure of the cSHMT obligate dimer. Highlighted in pink (monomer A) and light blue (monomer B) are residues that are lost due to alternative splicing. In Figure 6a, these are the residues, encoded by exon 9, which are missing in one of the human cSHMT splice variants. Deletion of exon 9 (residues 273−312) is expected to disrupt substrate binding properties as well as the overall structure of the protein. This deletion opens up one side of the folate-binding pocket, with loss of the loops centered around Pro298 and Gly303; these loops are involved in binding THF and PLP, respectively (*12*). Deletion of these residues from the cSHMT model has little effect on the central β-sheets and most of the surrounding α-helices. Likewise, contacts between the large and small domains are little affected. Hence, the overall conformation of each monomer is likely to be unperturbed by the deletion of exon 9. However, the model suggests that some local intramonomer contacts may be lost as a result of the deletion, potentially disrupting the structure of the small, carboxy-terminal, domain. The helix of residues 305−322 is almost certain to be disrupted by the deletion. The loop of residues 75−85, which normally makes contact with the deleted region around residue 300, would appear to adopt a different conformation, thereby altering the position of the helix of residues 86−104. Because this helix is in contact with the N-terminal arm of the second monomer in the tight dimer of the full-length crystal structure, some change is expected in the relative disposition of the two monomers of the splice variant upon oligomerization. This postulation is further supported by the loss of intermolecular contacts in the loop region containing residue 300. Residue Asp90 is conserved in all SHMT primary sequences, and its mutation results in an equilibrium mixture of cSHMT dimers and tetramers (*20*). This indicates that this residue is important in forming

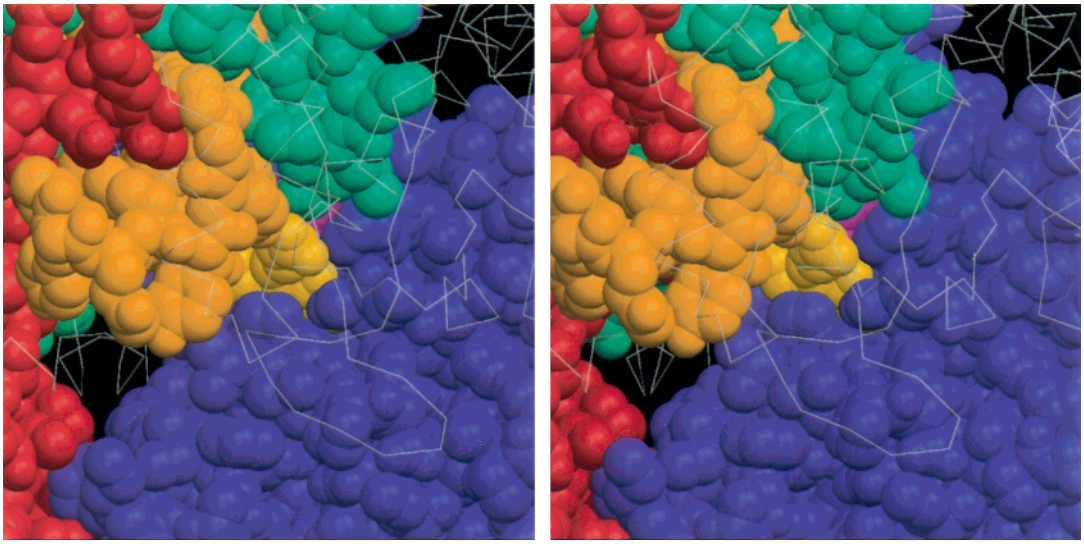


FIGURE 7: View of the cSHMT active site as it might exist in the McSHMTtr splice variant. Monomer A is shown in red and gold and monomer B in blue and turquoise, as in Figure 6. The viewpoint is from the lower right of Figure 6c. The gold and turquoise regions, which are part of the N-terminal domain (residues 8−85), are likely to differ from this model, as some refolding of this region is expected when its several contacts with the (deleted) C-terminal region are removed. PLP is yellow, and residue Lys257 (just barely visible to the upper right of the PLP) is magenta. The deleted chain, which normally covers the PLP, is shown as a white backbone trace. This figure was prepared using MOLSCRIPT (*21*) and Raster3D (*22*).

cSHMT tetramers from SHMT obligate dimers; hence, the alternative positioning of the helix of residues 86−104 in McSHMTtr is likely to disrupt the cSHMT tetramer interface.

The crystal structure also suggests that the deletion of exon 10 (residues 313−351) as well as exon 9 (Figure 6b), as in another splice variant of human cSHMT, results in the loss of a helix involved in interdomain contacts within a monomer, and further reduces contacts with the N-terminal arm of the second monomer in the tight dimer. Hence, the interdomain and intermonomer contacts are expected to be perturbed to a greater degree in this variant. These perturbations may affect the formation of the tetrameric complex.

Truncation of the chain at residue 272, as occurs in the mouse alternatively spliced variant, removes the entire small domain of each monomer (Figure 6c). The conformation of the 43-residue insertion which replaces this domain is not known, and this part of the polypeptide chain is not shown in the figure. The 43-mer is predominantly hydrophilic and probably forms irregular loops on the surface of the protein. Wherever these loops are located, they clearly cannot fill up the volume normally occupied by the 210-residue small domain. In this model, the active site environment has been opened up by the loss of folate-ligating residues Asn387 and Arg402, and the loop centered around Leu396. The N-terminal portion of the chain, residues 8−85, has lost the majority of its contacts with the rest of the molecule, and probably refolds completely. With the large domains expected to be little altered, the McSHMTtr monomers have the potential to dimerize, but the dimer interface is likely to be substantially different from that in the full-length molecule. This is supported by the experimental evidence that indicates that these McSHMTtr monomers do not dimerize with the cSHMT protein (Figures 3 and 5).

Structural analysis of the full-length cSHMT also yields evidence as to why neither the recombinant McSHMTtr−GST fusion protein nor the refolded McSHMTtr protein binds PLP. Figure 7 displays one of the PLP binding sites, as it might appear in truncated murine SHMT. The polypeptide backbone of the deleted amino acids (273−484) is traced in white. It is clear that, with this chain removed, the active site lysine 257 (shown in magenta) and the PLP are accessible to solvent. N-Terminal motion could easily lead to direct solvent exposure of the contact between PLP and K257, in which case a Schiff base linkage there would not be stable and PLP would not bind, as experimentally demonstrated (Figure 5). We cannot rule out the possibility that the carboxy-terminal amino acid residues encoded within murine intron exon 8 take over the functions of the amino acid residues encoded by exons 9−13 in the full-length cSHMT protein. However, this is not supported by the data outlined in Figures 3−5.

CONCLUSIONS

In this study, we show that the alternative splicing of cSHMT in mice and humans occurs by different mechanisms. The location of the alternative splicing is positionally conserved between these species, suggesting that this modification of cSHMT is important for cell function. Alternatively spliced messages are found in both human and murine cells (*3*, *15*), and in this study, we provide the first evidence that the murine alternatively spliced variant, McSHMTtr, is translated to produce the McSHMTtr protein with a modified carboxy-terminal domain. The experimental evidence indicates that the McSHMTtr protein does not bind either PLP or 5-formylTHF and does not contain SHMT catalytic activity. These data are confirmed by analyses of the X-ray structure of the cSHMT−Gly−5-formylTHF ternary complex, which suggest that truncated forms of the protein lack critical residues necessary to bind PLP and folate cofactors, and most likely are not capable of oligomerizing with the full-length cSHMT protein. Additionally, we can infer from these studies that the human cSHMT splice variants are catalytically inactive as well. Deletion of exon 9 disrupts critical residues involved in both PLP and 5-formylTHF binding as occurs with the murine McSHMTtr protein.

The function of the alternatively spliced variants, if any, remains unknown. The characterization and analyses reported here suggest that they do not function in folate metabolism as catalysts. The possibility of their serving as inhibitors of the full-length protein is also unlikely. Although the experiment outlined in Figure 3 cannot distinguish conclusively between (1) the inability of McSHMTtr and cSHMT to oligomerize and (2) the inability of McSHMTtr−cSHMT oligomers to bind 5-formylTHF (species that do not bind 5-formylTHF tightly cannot be purified on a 5-formylTHF affinity column), the data shown in Figure 5 directly demonstrate that recombinant McSHMTtr and cSHMT proteins do not physically associate. Hence, a mechanism whereby the conformation of one SHMT dimer in a tetramer influences the conformation of the second dimer, reducing its affinity for the substrate (*12*), is not feasible in this case. We conclude that cSHMT proteins with modified carboxy-terminal domains resulting from alternative splicing of hnRNA are expressed but are not functional SHMT enzymes and likely do not interact with catalytic full-length SHMT proteins. However, the positional conservation of cSHMT alternative splicing suggests that these proteins do play some important role in cellular functions. Further studies are needed to elucidate this role.

REFERENCES

1. Shane, B. (1990) *Vitamins and Hormones 45*, 273.
2. Stover, P., Chen, L., Suh, J. R., Stover, D., Keyomarsi, K., and Shane, B. (1997) *J. Biol. Chem. 272*, 1842.
3. Girgis, S., Nasrallah, I., Suh, J. R., Oppenheim, E. and Stover, P. (1998) *Gene*, *210*, 315.
4. Appling, D. R. (1991) *FASEB J. 5*, 2645.
5. Garrow, T. A., Brenner, A. A., Whitehead, V. M., Chen, X., Duncan, R. G., Korenberg, J. R. and Shane, B. (1993) *J. Biol. Chem. 268*, 11910.
6. Stover, P. and Schirch, V. (1990) *J. Biol. Chem*. *265*, 14227.
7. Stover, P. and Schirch, V. (1993) *Trends in Biochem. Sci.*. *18*, 102.
8. Stover, P. and Schirch, V. (1991) *J. Biol. Chem. 266*, 1543.
9. Girgis, S., Suh, J. R., Jolivet, J. and Stover, P. J. (1997) *J. Biol. Chem. 272*, 4729.
10. Schirch, V and Strong, W. B. (1989) *Arch. Biochem. Biophys*. *269*, 371.
11. Kim, D. W., Huang, T., Schirch, D. and Schirch, V. (1996) *Biochemistry 35*, 15772.
12. Szebenyi, D. M. E., Liu, X., Kriksunov, I., Stover P. J., Thiel, D. (2000) *Biochemistry 39*, 13313−13323.
13. Jones, C. W. and Priest, D. G. (1976) *Arch. Biochem. Biophys. 174*, 305.
14. Bensadoun, A. and Weinstein, D. (1976) *Anal. Biochem*. *70*, 241.
15. Nakshatri, H., Bouillet, P., Bhat-Nakshatri, P., and Chambon, P. (1996) *Gene*, *174*, 79.
16. Jones, T. A. (1982) in Computational Crystallography (sayre, D., Ed.) p 303, Clarendon Press, Oxford. Web site: http://www.imsb.au.dk/~mok/o.
17. RasMol Version 2.7.1, original program by Roger Sayle, modifications by Herbert Bernstein. Web site: http://www.bernstein-plus-sons.com/software/rasmol/.
18. Renwick, S. B., Snell, S., and Baumann, U. (1998) *Structure 6*, 1105.
19. Scarsdale, J. N., Kazanina, G., Radaev, S., Schirch, V. and Wright, H. T. (1999) *Biochemistry*, *38*, 8347.
20. Krishna Rao, J. V., Jagath, J. R., Sharma, B., Appaji Rao, N., and Savithri, H. S. (1999) *Biochem. J. 343*, 257.
21. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946.
22. Merritt, E. A. and Bacon, D. J. (1997) *Methods Enzymol. 277*, 505.

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