Pyridoxal-Phosphate Inhibits Dynamic Subunit Interchange among Serine Hydroxymethyltransferase Tetramers

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Abbreviations: 5-formylTHF, 5-formyltetrahydrofolate; PLP, pyridoxal 5’-phosphate; SHMT, serine hydroxymethyltransferase; cSHMT, cytoplasmic serine hydroxymethyltransferase; DNcSHMT, dominant-negative cytoplasmic serine hydroxymethyltransferase, SDS-PAGE; denaturing polyacrylamide electrophoresis.
Summary

Cytoplasmic serine hydroxymethyltransferase (cSHMT) is a tetrameric, pyridoxal-phosphate (PLP)-dependent enzyme that catalyzes the reversible interconversion of serine and tetrahydrofolate to glycine and methylenetetrahydrofolate. The enzyme has four active sites and is best described as a dimer of obligate dimers. Each monomeric subunit within the obligate dimer contributes catalytically important amino acid residues to both active sites. To investigate the interchange of subunits among cSHMT tetramers, a dominant-negative human cSHMT enzyme (DNcSHMT) was engineered by making three amino acid substitutions: K257Q, Y82A, and Y83F. Purified recombinant DNcSHMT protein was catalytically inactive and did not bind 5-formyltetrahydrofolate. Coexpression of the cSHMT and DNcSHMT proteins in bacteria resulted in the formation of heterotetramers with a cSHMT/DNcSHMT subunit ratio of one. Characterization of the cSHMT/DNcSHMT heterotetramers indicates that DNcSHMT and cSHMT monomers randomly associate to form tetramers and that cSHMT/DNcSHMT obligate dimers are catalytically inactive. Incubation of recombinant cSHMT protein with recombinant DNcSHMT protein did not result in the formation of hetero-oligomers, indicating that cSHMT subunits do not exchange once the tetramer is assembled. However, removal of the active site PLP cofactor does permit exchange of obligate dimers among preformed cSHMT and DNcSHMT tetramers, and the formation of heterotetramers from cSHMT and DNcSHMT homodimers does not affect the activity of the cSHMT homodimers. The results of these studies demonstrate that PLP inhibits dimer exchange among cSHMT tetramers and suggests that cellular PLP concentrations may influence the stability of cSHMT protein \textit{in vivo}. 
INTRODUCTION

Serine hydroxymethyltransferase (EC 2.1.2.1) (SHMT) is a pyridoxal-phosphate (PLP)-dependant enzyme that catalyzes the reversible interconversion of serine and tetrahydrofolate (THF) to glycine and methylenetetrahydrofolate (methyleneTHF) (1,2). This reaction is a primary source of single carbons that are required for cytoplasmic one-carbon metabolism. SHMT is present in the cytoplasm (cSHMT) and mitochondria of eukaryotic cells, and different genes encode the two SHMT proteins (3-5). Both SHMT isozymes are sources of one-carbon units for cytoplasmic one-carbon metabolism (1,3). In the cytoplasm, folate-activated one-carbon units are required for the de novo synthesis of purines and thymidylate, and for the remethylation of homocysteine to methionine (1,6). Mitochondrial one-carbon metabolism is necessary for the conversion of serine to formate (7) and the mitochondrial SHMT enzyme catalyzes the first step in this pathway by converting serine and THF to glycine and methyleneTHF (6,7). Formate enters the cytoplasm where it is incorporated into the folate one-carbon pool (1,3,4). The mitochondrial SHMT gene is expressed at similar levels in most mammalian tissues, whereas the cSHMT gene exhibits a dynamic range of tissue-specific expression (5).

Mammalian SHMT enzymes are 55 kD homotetramers with four active sites per tetramer. High-resolution structures are available for the human, mouse, and rabbit cSHMT enzymes, some with amino acid and folate substrates bound (8-10). All solved cSHMT structures reveal that the enzyme is best described as a dimer of tight, obligate dimers. Each obligate dimer contains two active sites, and catalytically essential amino acid residues from each monomer contribute to both active sites. Tetramer formation results from the relatively weak association
of two obligate dimers. Analysis of the mouse cSHMT structure reveals that the tetramer contact surface is small, involving residues 135-137; 154-157, 168-171 and 189-194 of each monomer (9). Prokaryotic SHMT enzymes lack residues that lie at the tetramer interface and are catalytically active as obligate dimers in solution (11,12). Mammalian SHMT isozymes are tetramers in solution, but form mixtures of dimers and tetramers in the absence of bound PLP (13). The dissociation of cSHMT tetramers into obligate dimers in the absence of bound PLP suggests that the catalytic site of the enzyme communicates with amino residues at the tetramer interface. Site-directed mutations that alter amino acid residues near the tetramer interface site, or that decrease the affinity of PLP for the enzyme weaken the interactions between two obligate dimers (13,14). For example, recombinant D89N cSHMT from sheep has decreased catalytic activity and is a mixture of dimers and tetramers in solution (14). Recombinant H134N cSHMT from sheep has decreased affinity for PLP and is present in solution as a mixture of tetramers and dimers (13). The H134N cSHMT dimers are active, but the specific activity of the enzyme reduced by 75% compared to the nonmutated enzyme under conditions of saturating PLP. This study indicates that tetramer formation is not necessary for cSHMT catalytic activity (13).

To better understand the stability of cSHMT tetramers and the interaction of subunits within cSHMT tetramers, and to further examine the relationship between tetramer formation and cSHMT activity, we engineered a catalytically inactive, dominant-negative cSHMT enzyme (DNcSHMT). The results from these studies provide evidence that neither monomers nor obligate dimers exchange among preformed cSHMT tetramers in the presence of PLP. However, loss of cSHMT-bound PLP permits exchange of obligate dimers, but not monomers, among preformed cSHMT tetramers. Furthermore, we show that cSHMT and DNcSHMT
heterodimers are catalytically inactive, indicating that the DNcSHMT monomer effectively inactivates endogenous cSHMT activity.
EXPERIMENTAL PROCEDURES

Materials. 5-formylTHF, allothreonine, alcohol dehydrogenase, NADH, isopropyl β-D-thiogalactopyranoside, lysozyme and pyridoxal 5-phosphate were obtained from Sigma Chemical. All other chemicals were reagent grade. Restriction enzymes were obtained from Promega and Gibco BRL. The pET22b and pET28a vectors were obtained from Novagen. TOPO vector, TOP 10 competent cells and BL21* competent cells were obtained from Invitrogen.

Generation and expression of the DNCsSHMT cDNA. The DNCsSHMT cDNA was constructed using the human cSHMT cDNA as a template. Site mutations were incorporated into the cSHMT cDNA using the following primers. The forward primer was 5’-tctagggtaccggccagagcctttggcggactgag-3’ with the Kpn I site underlined and the altered nucleotides in bold which result in Y83A and Y82F codon substitutions in the recombinant protein. The reverse primer was 5’-ttgggatccacacttctttcctgtagaagatcatgccagctcggcagcctcgcaggtgagtgg-3’ with the Bam HI site underlined and the altered nucleotides shown in bold resulting in a K257Q codon substitution in the human protein. The region of the cSHMT cDNA that encodes the targeted amino acid residues was amplified by PCR: 30 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 90 sec with a 10 min extension at 72°C. The DNCsSHMT cDNA was generated by replacing the KpnI-BamHI fragment within the human cSHMT cDNA with the PCR product that contains the three codon substitutions. The DNCsSHMT cDNA was subcloned into the NdeI and NotI restriction sites of the pET22b expression vector, which confers kanamycin resistance. The cSHMT cDNA was subcloned into pET28a expression vector in frame with the N-terminus
polyhistidine tag using the NdeI and NrlI restriction sites, and this vector confers ampicillin resistance. The mutated cDNAs were sequence verified. The expression vectors containing the cSHMT and DNcSHMT cDNAs were transformed into competent BL21* bacteria both singly and in combination. One liter cultures of BL21* cells expressing the cSHMT, DNcSHMT or co-expressing cSHMT and DNcSHMT cDNAs were grown to mid log phase and protein synthesis was induced with isopropyl β-D-thiogalactopyranoside for 8 hrs at room temperature. Cell pellets were harvested and stored at -80°C until purification.

Purification of the cSHMT and DNcSHMT proteins. Cell pellets were lysed in a buffer containing 40 mM Kphosphate, pH 7.0, 10 mM 2-mercaptoethanol and 100 nM PLP using a French press and the insoluble material was removed by centrifugation at 12,000 rpm. For purification of the DNcSHMT protein, the clarified supernatant was applied directly to a CM sepharose ion exchange column (Clontech) and the protein purified to homogeneity as described previously (15). Recombinant SHMT from either bacteria that expressed the cSHMT protein, or that coexpressed cSHMT and DNcSHMT proteins, was purified by affinity chromatography. Cell suspensions were centrifuged at 12,000 rpm for 20 min at 4°C to pellet insoluble material. The cSHMT protein, which contains a N-terminal polyhistidine tag, was purified from the clarified sample on a Talon® metal affinity resin following the manufacturer’s instructions (Clontech). The purity of all proteins was determined by SDS-polyacrylamide gel electrophoresis and protein concentrations were determined by a modified Lowry assay (16). Purified protein was stored at -80°C.
SHMT activity assay. Michaelis-Menten constants were determined for the cSHMT and DNcSHMT-catalyzed cleavage of allothreonine using the coupled enzyme assay with alcohol dehydrogenase as described previously (17). The rate of absorbance loss at 340 nm was recorded after the addition of 300-2000 pmol of SHMT to a 1 ml cuvette containing 25 mM HEPES, pH 7.2, 10 mM 2-mercaptoethanol, allothreonine, alcohol dehydrogenase, and 0.15 mM NADH in a spectrophotometer (Shimadzu UV-2401PC).

Affinity of the recombinant DNcSHMT protein for 5-formylTHF. The affinity of recombinant DNcSHMT protein for 5-formylTHF was determined by a previously described competitive binding assay (18). The binding of reduced folates to cSHMT results in the formation of a PLP-glycine-quinonoid intermediate, which has an absorption maximum at 502 nm ($\varepsilon = 40,000$) (19). The DNcSHMT does not form the quinonoid intermediate and therefore did not exhibit an increase in absorbance at 502 nm upon binding 5-formylTHF. For the competitive binding assay, recombinant human cSHMT protein (10 $\mu$M) was added to cuvette that contained 1 ml of the reaction buffer (200 mM glycine, 50 mM HEPES, pH 7.3 and 10 $\mu$M (6S) 5-formylTHF (a value equal to the $K_d$)). The absorbance spectrum was recorded from 550 to 400 nm. Recombinant DNcSHMT was added (to a final concentration of 25 $\mu$M) and the spectrum was recorded. To quantify the affinity of DNcSHMT for 5-formylTHF, the loss of absorbance at 502 nm was recorded as a function of DNcSHMT added to the cuvette as described previously (18).

Monomer exchange studies. The ability of SHMT monomeric subunits to exchange among preformed cSHMT tetramers was determined by incubating purified, recombinant cSHMT
protein (5 µM) in a solution containing 200 mM glycine, 50 mM HEPES, pH 7.3 and 200 µM (6RS) 5-formylTHF. The absorbance spectrum of the protein was recorded from 550 to 450 nm. Then DNcSHMT was added to a final concentration of 20 µM and spectra were recorded following 10 min, 1 h, and 24 h incubations at 37°C. Loss of absorbance at 502 nm indicates that recombinant cSHMT monomers are exchanging with DNcSHMT monomers. To determine the effect of glycine or 5-formylTHF on monomer exchange, glycine and 5-formylTHF were omitted from the incubation solution. Following incubation, glycine (to 200 mM) and 5-formylTHF (to 200 µM) were added to the protein solution and the absorbance spectrum was recorded immediately. For all experiments, the absorbance at 502 nm was recorded for the protein that underwent the exchange reaction, and compared to the absorbance at 502 nm for purified cSHMT protein that did not undergo the exchange reaction.

Subunit interchange with apocSHMT enzyme. PLP was removed from the cSHMT active site by the addition of L-cysteine, which reacts with the bound PLP to form a thiazolidine complex (20). L-cysteine (16 mg/ml) was added to a 2 ml solution that contained 3 mg of cSHMT (with an N-terminal polyhistidine tag), 20 mg of DNcSHMT, and 100 mM 2-mercaptoethanol. The solution was incubated at room temperature for 15 min. The protein was precipitated by the addition of ammonium sulfate to 70% saturation, incubated on ice for 5 min, and then centrifuged at 4300 rpm for 20 min. The precipitated protein pellet was suspended in 2 ml of 100 mM L-cysteine, 100 mM 2-mercaptoethanol. This cycle was repeated 3 times and the protein was incubated at 37°C for 5 min. The procedure lasted 3 h in duration. The protein was then dialyzed for 24 h against 2 l of 20 mM Kphosphate, pH 7.2, 2.5 mM 2-mercaptoethanol, 100 mM glycine and 100 nM PLP at 4°C. A control reaction contained 3 mg of cSHMT (with an
N-terminal polyhistidine tag), 20 mg of DNcSHMT, and 100 mM 2-mercaptoethanol and was incubated at room temperature for the duration of the procedure described and stored at 4°C for the duration of the dialysis described above. After 24 h, both the experimental and control proteins were dialyzed against a buffer containing 10 mM Tris-Cl, pH 7.0, 50 mM NaCl, 50 mM glycine, and 100 nM PLP for 1 hr at 4°C. The proteins were each purified using the batch/gravity flow protocol for the Talon® metal affinity resin (Clontech) with the following buffers: extraction/wash buffer - 10 mM Tris-Cl, pH 7.0, 50 mM NaCl, 50 mM glycine, and PLP; stringent wash buffer - 20 mM Tris-Cl, pH 7.0, 50 mM NaCl, 50 mM glycine, PLP, and 12.5 mM imidazole; elution buffer – 20 mM Tris-Cl, pH 7.0, 100 mM NaCl, 50 mM glycine, PLP, and 200 mM imidazole. Each protein was dialyzed overnight at 4°C in 20 mM Kphosphate, pH 7.2, 100 mM glycine, 3 mM 2-mercaptoethanol, 100 nM PLP, and then analyzed for exchange by SDS-PAGE. Protein bands were quantified using ChemiImager 4400 from Alpha Innotech Corp. (San Leandro, CA). This densitometry method was validated by analyzing a series of gels that contained from 0.5 to 10 µg cSHMT protein per lane. The optical density values increased linearly as a function of cSHMT concentrations from 0.5 to 4 µg cSHMT/lane.

**SDS-polyacrylamide gel electrophoresis.** Purified proteins (1-3 µg) were suspended in buffer containing 2% SDS, 62.5 mM Tris, pH 6.8, 100 mM dithiothreitol and 10% glycerol and then incubated at 100°C for 10 min. The purified proteins were then run on a mini-SDS-PAGE using a 5% stacking gel and 12% separating gel in a slab gel apparatus (Biorad) with the discontinuous buffer system of Laemmli.
RESULTS

Design of a dominant negative SHMT protein. To study the assembly of cSHMT tetramers and the occurrence of dynamic interchange of cSHMT subunits among cSHMT tetramers, a human DNcSHMT protein was designed using information derived from previous studies of mutated cSHMT proteins, as well as information derived from the murine cSHMT protein crystal structure (9). The murine cSHMT structure was solved with glycine and 5-formylTHF bound at the active site. The cSHMT-gly-5-formylTHF ternary complex is an intermediate state analog of the cSHMT-ser-THF catalytic complex (9), and this structure was used to design rationally a dominant-negative SHMT protein that can inactivate cSHMT activity.

The DNcSHMT protein was designed to: (1) oligomerize with and inactivate recombinant cSHMT monomeric subunits by inhibiting serine and allothreonine cleavage activity, (2) have decreased affinity for folate and (3) retain affinity for PLP. Modeling studies indicated that three amino acid substitutions on a single cSHMT polypeptide were needed to achieve these goals, and these three amino acids are conserved in all known SHMT enzymes (Figure 1, Panel A). K257 is the active site lysine in the murine and human cSHMTs that forms a Schiff base with the PLP cofactor. Mutation of this active site K to Q inactivates the *E. coli* cSHMT. The mutated protein can catalyze only a single turnover; this mutation does not allow the expulsion of the amino acid product and therefore subsequent turnover is inhibited. However, this mutant retains affinity for folate cofactors and purifies with a PLP and amino acid bound (20) (Table 1). Y83 in the human and mouse cSHMT forms a hydrogen bond with the carboxylate of the amino acid substrate, and mutation of the analogous residue in the *E. coli* SHMT to F decreases the specific activity of the protein by greater than 99% and increases the enzyme’s affinity for tetrahydrofolate (Table 1) (21). Y83 and K257 from the same polypeptide function in different active sites within the
obligate dimer, and therefore dimerization between cSHMT and SHMT monomers that contain
the double mutation, Y83F/K257Q, would be expected to lack catalytic activity in both subunits,
but retain folate binding in both subunits (Figure 1). A third mutation was designed to eliminate
folate binding to the DNsSHMT protein. The crystal structure of the murine SHMT protein
shows that Y82 forms a stacking interaction with the \( p \)-aminobenzoylglutamate moiety of THF
that is predicted to be essential for THF binding (Figure 1), although this has not been tested
experimentally (9).

The DNsSHMT monomer was engineered by making 3 amino acid substitutions;
K257Q/Y82A/Y83F (Figure 1, Panel B). Purified recombinant DNsSHMT is expected retain
high affinity for PLP in the presence of amino acid substrates, but lack serine cleavage catalytic
activity and affinity for folate cofactors. Oligomerization of the DNsSHMT monomer with a
cSHMT monomer will generate two active sites (Figure 1, Panels C and D). Active site C lacks
K257 and therefore should lack catalytic activity, but retain affinity for folate cofactors. Site D
lacks Y82 and Y83, and therefore is anticipated to have less than 1% serine cleavage activity and
decreased affinity for folate cofactors.

Expression and characterization of the DNsSHMT recombinant protein. The DNsSHMT and
cSHMT proteins were coexpressed in E. coli to test the ability of the DNsSHMT monomers to
oligomerize with and inactive cSHMT protein as illustrated in Figure 1. The human recombinant
cSHMT cDNA was engineered with an N-terminal polyhistidine tag to enable affinity
purification of this protein; the DNsSHMT cDNA lacked a coding sequence for the polyhistidine
tag. Figure 2A, lanes 1 and 2 show that both the cSHMT and DNsSHMT proteins can be
expressed in E. coli, and that the cSHMT protein can be separated from the DNsSHMT protein
by SDS-PAGE because of its increased molecular mass resulting from the polyhistidine tag.
Figure 2A, lane 3 shows that the cSHMT protein is more abundant in crude extracts of *E. coli* that coexpress the cSHMT and DNcSHMT proteins. Following purification, all recombinant proteins were greater than 95% pure (Figure 2B, lanes 1-3). As expected, affinity purification of cSHMT protein from *E. coli* that coexpressed the cSHMT and DNcSHMT proteins also resulted in the purification of DNcSHMT protein, indicating that the cSHMT and DNcSHMT subunits associate with one another. Additionally, the ratio of cSHMT to DNcSHMT monomeric subunits in purified cSHMT/DNcSHMT protein was 1.0, despite the higher concentration of cSHMT protein compared to DNcSHMT protein in the crude extracts. This indicates that cSHMT monomers may have higher affinity for DNcSHMT monomers than other cSHMT monomers.

**Prediction of subunit assembly.** Two models were derived to predict the assembly and catalytic activity of cSHMT/DNcSHMT heterotetramers (Figure 3). Model I is a random association model whereby cSHMT and DNcSHMT monomers randomly associate to form heterodimers and homodimers, that randomly associate to form homotetramers and heterotetramers (Figure 3, Model I). Model 2 predicts that cSHMT and DNcSHMT monomers can only form homodimers, but that homodimers can randomly associate to form homotetramers and heterotetramers. Assuming equal concentrations of cSHMT and DNcSHMT monomers (as shown in Figure 2), the expected frequency of each potential tetramer was calculated for Models I and II. Additionally, the activity of each tetramer was predicted by assuming that both DNcSHMT homodimers and DNcSHMT/cSHMT heterodimers are inactive, and that the formation of tetramers from obligate dimers does not influence the activity of either obligate dimer. The expected specific activity of the purified cSHMT/DNcSHMT protein will be the average of the specific activity for each tetrameric isoform, after correcting for its relative abundance. If the
random association model is correct (Model I), we anticipate that the specific activity of the cSHMT/DNcSHMT tetrameric protein will be reduced by 75% compared to tetrameric cSHMT protein. For Model II, the cSHMT/DNcSHMT tetrameric protein is predicted to exhibit a 50% decrease in specific activity compared to cSHMT protein.

*Spectral properties of cSHMT.* The cSHMT protein is a PLP-dependent enzyme, and the reaction intermediates associated with catalysis have distinct spectral properties. PLP binds to cSHMT through a Schiff base with K257 forming an intermediate known as the internal aldimine (Figure 4, structure 1). Binding of amino acid substrates results in the formation of the geminal diamine (Figure 4, structure 2), which is a tetrahedral intermediate that results from partial displacement of K257 by the incoming amino acid. Full displacement of K257 results in a Schiff base between the amino acid substrate and the active site PLP, an intermediate known as the external aldimine (Figure 4, structure 3). Loss of the pro-2S proton of glycine, or the hydroxymethyl group of serine, results in the formation of a highly conjugated glycine quinonoid intermediate (Figure 4, structure 4).

Figure 5, panel A shows the spectrum of the cSHMT-gly binary complex (spectrum 1), and the cSHMT-gly-5-formylTHF ternary complex (spectrum 2). The absorbance spectrum of the cSHMT-glycine binary complex shows the presence of the external aldimine ($\lambda_{\text{max}}$ 425 nm) and the glycine quinonoid ($\lambda_{\text{max}}$ 492 nm). The addition of 5-formylTHF shifts the equilibrium of the enzyme-bound PLP to the glycine quinonoid ($\lambda_{\text{max}}$ 502 nm). Panel B shows the spectrum of the DNcSHMT-gly binary complex (spectrum 1) and DNcSHMT-gly binary complex in the presence of 5-formylTHF (spectrum 2). Previous studies have demonstrated that the K to Q mutation does not permit formation of the internal aldimine or geminal diamine (20), and the concentration of the glycine quinonoid associated with the cSHMT-gly-5-formylTHF ternary
complex is reduced to less than 0.1% compared to the cSHMT protein (Table 1). Other studies have shown that the Y to F mutation eliminates the formation of the glycine quinonoid (Table 1) (21). The spectra of the DNcSHMT-gly binary complex in the presence and absence 5-formylTHF are consistent with previous studies because no quinonoid intermediate was seen. Panel C shows the spectra of the cSHMT/DNcSHMT-gly binary complex (spectrum 1) and the cSHMT/DNcSHMT-gly-5-formylTHF ternary complex (spectrum 2). The concentration of the quinonoid intermediate associated with the cSHMT/DNcSHMT-gly-5-formylTHF ternary complex was decreased by 75% compared to the concentration of the quinonoid intermediate in the cSHMT-gly-5-formylTHF ternary complex. The formation of a quinonoid intermediate is a measure of catalytic competence, and the 75% reduction in the concentration of the quinonoid intermediate in the cSHMT/DNcSHMT-gly-5-formylTHF complex is consistent with random association of cSHMT and DNcSHMT monomeric subunits within the tetramer (Figure 2, Model I). This result indicates that the K257Q/Y82A/Y83F mutations inactivate the cSHMT enzyme and that DNcSHMT inactivates cSHMT protein as predicted.

Affect of Y82A on folate binding. The Y82A mutation in the DNcSHMT protein is predicted to reduce cSHMT’s affinity for 5-formylTHF. The ability of DNcSHMT to bind folate was investigated using a competitive binding assay described elsewhere (Figure 6) (18). In this assay, a solution containing cSHMT protein (10 µM), saturating concentrations of glycine (200 mM), and 5-formylTHF (10 µM, the concentration equal to the K_d) is titrated with the DNcSHMT. Loss of absorbance at 502 nm following the addition of DNcSHMT would indicate that DNcSHMT binds 5-formylTHF. The intensity of the glycine quinonoid intermediate was not diminished by the addition of up to 25 µM DNcSHMT to this solution, indicating that DNcSHMT does not have high affinity for folate cofactors (Figure 6).
A similar experiment was performed to determine if cSHMT monomers exchange among preformed cSHMT and DNcSHMT tetramers. The competitive binding experiment described above was repeated with 2 alterations: (6RS) 5-formylTHF was added in saturating concentrations (200 µM) and the incubation time for the reaction was extended to 24 h at 37°C. After 24 h, no decrease in the absorbance at 502 nm occurred, indicating that cSHMT and DNcSHMT monomers do not exchange from preformed cSHMT and DNcSHMT tetramers. To determine if monomer exchange occurred from preformed cSHMT and DNcSHMT tetramers in the absence of glycine and 5-formylTHF, a solution containing cSHMT (10 µM) and DNcSHMT (20 µM) tetramers was incubated at 37°C for 24 h, then glycine and 5-formylTHF were added to the reaction and the absorbance intensity at 502 nm was determined. The absorbance at 502 nm was identical to that observed for a solution of cSHMT (10 µM) incubated without DNcSHMT, indicating that cSHMT and DNcSHMT monomers do not exchange from preformed tetramers in the presence or absence of glycine and 5-formylTHF.

Effect of PLP on subunit interchange among cSHMT and DNcSHMT tetramers. In this study, the affect of PLP on exchange of cSHMT subunits between preformed cSHMT and DNcSHMT tetramers was investigated as described in experimental procedures. Incubation of purified, recombinant cSHMT protein with a 7-fold molar excess DNcSHMT followed by affinity purification of the cSHMT protein did not result in the co-purification of DNcSHMT, indicating that cSHMT subunits do not exchange between preformed cSHMT and DNcSHMT tetramers when PLP is bound (Figure 7, lane 1). However, when cSHMT and DNcSHMT proteins that lack bound PLP are incubated together, subunit exchange must have occurred because DNcSHMT copurified with cSHMT on the affinity column (Figure 7, lane 2). The ratio of
cSHMT to DNcSHMT monomers in the purified protein was 63% to 27% respectively, indicating that the subunit exchange had not reached equilibrium. Incubation beyond 3 h was not possible because of the formation of insoluble precipitate, suggesting that cSHMT dimers are unstable.

Because cSHMT monomers have never been isolated and loss of PLP results in the dissociation of tetramers to dimers, it is assumed that loss of PLP permits exchange of obligate homodimers between cSHMT and DNcSHMT tetramers (Figure 3, model II). To test this hypothesis, absorbance spectra of the purified protein that underwent the subunit exchange reaction were recorded (Table 2). The addition of glycine and 5-formylTHF to this purified protein resulted in the formation of the glycine quinonoid intermediate, with an A_502 nm that was 30% less than that observed for the cSHMT homotetramer (Table 2), indicating that the predicted catalytic competency of the cSHMT enzyme had decreased 30% following the exchange reaction. This 30% reduction in cSHMT predicted catalytic competency represents the percentage of DNcSHMT subunits in the tetramer after the exchange reaction (Figure 7), indicating that these cSHMT/DNcSHMT heterotetramers are comprised of cSHMT and DNcSHMT homodimers (Figure 3, Model II). It should be noted that the dimer exchange reaction did not go to completion, and that a 50% decrease in predicted catalytic competency would be the maximum expected if the reaction went to completion. Monomer exchange could not have occurred because no dominant negative effect was seen, thus the results are only consistent with Model II. This study demonstrates that the activity of cSHMT obligate dimers is not affected by tetramerization with DNcSHMT obligate dimers, a primary assumption in our models (Figure 3).

**Catalytic activity of cSHMT and DNcSHMT proteins.** The catalytic activities of the cSHMT, DNcSHMT and DNcSHMT/cSHMT tetramers were determined to verify that quinonoid
formation was an adequate indicator of cSHMT catalytic potential. The results shown in Table 2 verify that the catalytic activity \( (k_{\text{cat}}) \) of the enzymes parallel their ability to form glycine quinonoid intermediates, and that the DNcSHMT protein can only inactive cSHMT catalytic function when it dimerizes with cSHMT monomers.
Conclusions

The generation of a DNcSHMT protein enabled a thorough investigation into the dynamic exchange of subunits among cSHMT tetramers, and the ability of amino acids, folates and PLP to affect subunit exchange among preformed cSHMT tetramers. Furthermore, the study of cSHMT/DNcSHMT heterotetramers gives new insight into the stoichiometry of folate binding and communication among active sites within the tetramer.

*Pyridoxal phosphate inhibits dynamic subunit exchange.* A polymorphism in the cSHMT gene is associated with altered serum homocysteine levels (a risk for birth defects and certain cancers) (22) and decreased risk for leukemia (23). Therefore, understanding the factors that regulate cSHMT activity is important to elucidate the mechanisms whereby altered folate metabolism influences risk for diseases and birth defects (1). SHMT activity is decreased in the livers of vitamin B6-deficient rats, and it is presumed that the decreased activity results from the loss of PLP cofactor from the active site and formation of apoenzyme (24). However, the effect of vitamin B-6 deficiency on SHMT turnover rates has not been investigated. Whereas other studies have demonstrated that loss of PLP from the SHMT active site weakens the interactions of the obligate dimers within the tetramer, this study demonstrates that cSHMT tetramers are very stable and do not exchange subunits unless they lack bound PLP because PLP inhibits this exchange by stabilizing the tetramer. Further investigation is required to determine if cellular PLP deficiency results in increased rates of cSHMT protein turnover resulting from dissociation of cSHMT tetramers.

*Communication among cSHMT monomers and obligate dimers.* Previous titration calorimetry studies have demonstrated that only half of the active sites within the cSHMT tetramer bind reduced folates (25). There are three mechanisms that may account for half-site occupancy.
within the cSHMT tetramer: (1) half-site occupancy within the obligate dimer, (2) asymmetric obligate dimers with one obligate dimer saturated with folate, the other dimer lacking folate or (3) random binding of two folate molecules per SHMT tetramer. Mechanism 1 implies that only active sites within the obligate dimer communicate, whereas mechanism 2 and 3 assume all four active sites within a tetramer communicate. Mechanism 1 is supported by the report of the *Bacillus stearothermophilus* SHMT crystal structure (12). This enzyme is an obligate dimer in solution because it lacks the amino acid residues required for tetramer formation. This enzyme was crystallized with glycine and 5-formylTHF bound, and only one of the active sites within the obligate dimer contained 5-formylTHF. In contrast, the structure of the *E. coli* cSHMT that was solved with 5-formylTHF and glycine bound does not support mechanism 1 (11); this structure showed 5-formylTHF tightly bound in both active sites of the obligate dimer (11). Mechanism 2 is supported by the report of the *E. coli* (described above) and murine cSHMT structures that were obtained from crystals grown in the presence of glycine and 5-formylTHF (9). The mouse structure had only two 2 equivalents of 5-formylTHF bound tightly, with one obligate dimer displaying full occupancy of the active sites, and no or disordered folate binding in the other obligate dimer (9), indicating negative cooperativity between the obligate dimers within the tetrameric enzyme. There are no data to support mechanism 3.

The inability of obligate dimers to communicate with each other (mechanism 1) within the cSHMT tetramers was an underlying assumption of our assembly models (Figure 3), and these assumptions were supported by the experimental data presented. The data presented here also support mechanism 1 with respect to stiochiometry of folate binding within the tetramer. Loss of PLP results in exchange of obligate dimers between preformed cSHMT and DncSHMT tetramers (Figure 3, Model 2) and a decrease in the specific activity of the cSHMT protein (table
2). The 34% decrease in the specific activity associated with cSHMT/DNcSHMT heterotetramer formation is fully accounted for by the inclusion of inactive DNcSHMT obligate dimers within the tetramers (Figure 7), thereby diluting the specific activity of the cSHMT homodimers that exchanged with DNcSHMT homodimers by 50%. If mechanism 2 for folate binding is correct, the concentration of the quinonoid should be the same in tetramers that are formed from two cSHMT homodimers and from cSHMT/DNcSHMT tetramers formed from cSHMT homodimers and DNcSHMT homodimers (Figure 3, Model 2). The replacement of an “inactive cSHMT obligate dimer” (that cannot bind folate) within the cSHMT homotetramers with an inactive DNcSHMT obligate dimer should not alter the intensity of the quinonoid within the tetramer. Furthermore, “inactive cSHMT obligate dimers” should become “activated” following tetramer formation with DNcSHMT homodimers. Therefore, these studies seem to eliminate mechanism 2 with respect to folate binding to cSHMT tetramers in solution. We recognize that this mechanism is not consistent with results from the *E. coli* and murine SHMT structures, but these results are supported by the structure of the *Bacillus stearothermophilus* cSHMT enzyme (12). Also, we recognize that the ability of cSHMT obligate dimers to communicate within the tetramer may have been lost as a result of minor structural perturbations resulting from the three mutations in the DNcSHMT protein.

**DNcSHMT inactivates cSHMT activity.** The data presented here demonstrate that the DNcSHMT monomers can effectively and randomly associate with cSHMT monomers and inactivate them, indicating that this construct may be effective in inhibiting cSHMT function and one-carbon metabolism *in vivo*. The data also indicate that the DNcSHMT protein can only inhibit cSHMT activity by forming heterodimers and therefore the DNcSHMT cannot inhibit the activity of preformed cSHMT tetramers. Previously, we have demonstrated that cSHMT plays a
key role in one-carbon metabolism by accelerating \textit{de novo} thymidylate biosynthesis and also by inhibiting homocysteine remethylation in MCF-7 cells (1). We are currently generating cancer cell lines and transgenic mice that express the DNcSHMT protein to further understand the metabolic role of cSHMT. This approach could also be used to inactive the mitochondrial SHMT isozyme because these three mutated residues within the DNcSHMT protein are conserved in all SHMT proteins.
Literature Cited


*Biochemistry* **39**, 13313-13323


*Biochemistry* **38**, 8347-8358


*Biochem J* **343 Pt 1**, 257-263


Figure Legends.

Figure 1: Schematic representation of the cSHMT active sites that result from association of cSHMT and DNeSHMT monomers. Amino acid residues that contribute to the active site and were the targets for mutagenesis are shown in boxes. Residues that contribute to an active site that originate from the opposite monomer within the obligate dimer are indicated with a prime. Panel A: active site of a cSHMT homodimer with PLP, glycine and 5-formylTHF bound. Panel B: active site of a DNeSHMT homodimer with three mutations in the active site. Panels C and D, the two active sites that result from heterodimer formation between a cSHMT and DNeSHMT monomer.

Figure 2: SDS-PAGE gels of recombinant human cSHMT and DNeSHMT proteins. Panel A is a crude protein extract from E. coli expressing: Lane 1: the cSHMT protein with an N-terminal polyhistidine tag; Lane 2: the DNeSHMT protein without a polyhistidine tag; Lane 3: both the cSHMT and DNeSHMT proteins. Panel B shows the purified SHMT proteins: Lane 1: cSHMT protein with a polyhistidine tag (purified on Talon® affinity column); Lane 2: DNeSHMT protein that lacks a polyhistidine tag (purified on CM sephadex); Lane 3: coexpressed DNeSHMT and cSHMT proteins (purified on a Talon® affinity column). Approximately 0.85 μg of purified protein was run on a 12% mini-SDS-PAGE using the discontinuous buffer system of Laemmli. The gel was stained with SimplyBlue (Invitrogen) to visualize the proteins.
Figure 3: Models that predict the assembly and activity of cSHMT/DNcSHMT obligate dimers and tetramers. The cSHMT monomers are shown as open circles, the DNcSHMT monomers are shown as gray circles. WT denotes a nonmutated cSHMT monomer, DN denotes a DNcSHMT monomer. Activity predictions assume that cSHMT/DNcSHMT dimers are inactive, and that obligate dimers do not communicate or influence each other within the tetramer.

Figure 4: Structures of catalytic intermediates associated with the SHMT serine retroaldol cleavage mechanism.

Figure 5: Spectral properties of the cSHMT enzymes. The absorbance spectra of the SHMT enzymes (25 µM) was determined in the presence of 200 mM glycine (Spectrum 1) and 200 mM glycine, 200 µM (6RS) 5-formylTHF (Spectrum 2). Panel A: recombinant human cSHMT homotetramers; Panel B: recombinant human DNcSHMT homotetramers; Panel C: Recombinant human cSHMT/DNcSHMT heterotetramers.

Figure 6: Affinity of the DNcSHMT homotetramers for 5-formylTHF. Purified recombinant cSHMT protein (10 µM) was incubated in a solution of 20 mM Kphosphate, pH 7.2, 200 mM glycine and 10 µM (6S) 5-formylTHF (equal to the value of Kd) and spectrum 1 was recorded. The DNcSHMT protein was added to a final concentration of 20 µM and spectrum 2 was recorded after a 60 min incubation at 28°C. No decrease in the absorbance at 502 nm was observed following the addition of DNcSHMT protein, indicating that the
DNcSHMT protein does not bind folate. The increase in the absorbance at 502 nm following the 60 min incubations occurred independent of the addition of DNcSHMT protein.

**Figure 7: Effect of PLP on SHMT subunit exchange.** The cSHMT (3 mg) and DNcSHMT (20 mg) proteins were co-incubated for 3h, then the cSHMT protein was purified on a Talon® affinity column and the presence of DNcSHMT determined by SDS-PAGE as described in *experimental procedures*. Lane 1: Purified cSHMT following incubation of cSHMT and DNcSHMT protein; Lane 2, Purified cSHMT protein following incubation of apocSHMT and apoDNcSHMT protein that lacks PLP; Lane 3, Purified cSHMT protein from *E. coli* that coexpressed DNcSHMT and cSHMT proteins. Approximately 1-3 µg of purified protein was run on a 12% mini-SDS-PAGE using the discontinuous buffer system of Laemmli. The gel was stained with SimplyBlue (Invitrogen) to visualize the proteins.
Table 1: Predicted properties of the DNcSHMT based on studies of the *E. coli* SHMT.

<table>
<thead>
<tr>
<th>Ability to form reaction intermediates</th>
<th>K257Q&lt;sup&gt;(20)&lt;/sup&gt;</th>
<th>Y83F&lt;sup&gt;(21)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geminal Diamine</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>External Aldimine</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Quinonoid</td>
<td>0.1%</td>
<td>no</td>
</tr>
<tr>
<td>Activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allo-threonine Cleavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>K</em>&lt;sub&gt;m&lt;/sub&gt;</td>
<td>n.d.</td>
<td>reduced 19-fold</td>
</tr>
<tr>
<td><em>k</em>&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>0</td>
<td>decreased 98.6%</td>
</tr>
<tr>
<td>Serine Cleavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>K</em>&lt;sub&gt;m&lt;/sub&gt; (ser)</td>
<td>n.d.</td>
<td>reduced 435-fold</td>
</tr>
<tr>
<td><em>K</em>&lt;sub&gt;m&lt;/sub&gt; (THF)</td>
<td>n.d.</td>
<td>reduced 3-fold</td>
</tr>
<tr>
<td><em>k</em>&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>0</td>
<td>decreased &gt; 99%</td>
</tr>
<tr>
<td>Glycine Proton Exchange (with tetrahydrofolate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>k</em>&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>n.d.</td>
<td>decreased &gt; 99%</td>
</tr>
</tbody>
</table>

n.d. – not determined

Table 2. Effect of DNcSHMT on cSHMT catalytic activity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Activity (Predicted)</th>
<th>Allo-threonine <em>k</em>&lt;sub&gt;cat&lt;/sub&gt; s&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Quinonoid Formation&lt;sup&gt;3&lt;/sup&gt; (<em>A</em>&lt;sub&gt;502&lt;/sub&gt;/10 µM SHMT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cSHMT</td>
<td>100%</td>
<td>1.20 (100%)</td>
<td>0.4 (100%)</td>
</tr>
<tr>
<td>DNcSHMT</td>
<td>0%</td>
<td>&lt; 0.02 (0%)</td>
<td>0.0 (0%)</td>
</tr>
<tr>
<td>cSHMT/DNcSHMT&lt;sup&gt;1&lt;/sup&gt;</td>
<td>25%</td>
<td>0.24 (20%)</td>
<td>0.10 (25%)</td>
</tr>
<tr>
<td>cSHMT/DNcSHMT&lt;sup&gt;2&lt;/sup&gt;</td>
<td>63%</td>
<td>0.79 (66%)</td>
<td>0.28 (70%)</td>
</tr>
</tbody>
</table>

<sup>1</sup>cSHMT/DNcSHMT heterotetramers purified from bacteria coexpressing cSHMT and DNcSHMT monomers (Model I).

<sup>2</sup>cSHMT/DNcSHMT heterotetramers formed from obligate dimer exchange in the absence of PLP (Model II).

<sup>3</sup>10 µM enzyme was incubated in 10 mM HEPES, pH 7.2, 200 mM glycine, and 200 µM (6RS) 5-formylTHF and the *A*<sub>502</sub> recorded.
Figure 1

A  Wild Type (cSHMT)

B  Dominant Negative (DNcSHMT)

C  Wild Type-Dominant Negative (cSHMT/DNcSHMT) Site I

D  Wild Type-Dominant Negative (cSHMT/DNcSHMT) Site II
Figure 2
Model I

**Monomer** →

\[
\begin{array}{c}
\text{WT} \quad \text{DN} \\
1/2 \\
\end{array}
\]

**Obligate Dimer** →

\[
\begin{array}{c}
\text{WT} \quad \text{WT} \\
\text{WT} \quad \text{DN} \\
\text{DN} \quad \text{DN} \\
1/4 \\
2/4 \\
1/4 \\
\end{array}
\]

**Tetramer** →

\[
\begin{array}{c}
\text{WT} \quad \text{WT} \\
\text{WT} \quad \text{DN} \\
\text{DN} \quad \text{DN} \\
\text{DN} \quad \text{DN} \\
1/16 \\
4/16 \\
2/16 \\
4/16 \\
4/16 \\
1/16 \\
\end{array}
\]

**Expected Activity** → 100 %

**Activity x Abundance** → 6.25 %

**Specific Activity** = 25%

Model II

**Obligate Dimer** →

\[
\begin{array}{c}
\text{WT} \quad \text{WT} \\
1/2 \\
\text{DN} \quad \text{DN} \\
1/2 \\
\end{array}
\]

**Tetramer** →

\[
\begin{array}{c}
\text{WT} \quad \text{WT} \\
\text{WT} \quad \text{DN} \\
\text{DN} \quad \text{DN} \\
\text{DN} \quad \text{DN} \\
1/4 \\
2/4 \\
1/4 \\
\end{array}
\]

**Expected Activity** → 100 %

**Activity x Abundance** → 25 %

**Specific Activity** = 50 %

Figure 3
Structure 1: Internal Aldimine ($\lambda_{max} = 425$ nM)

Structure 2: Geminal Diamine ($\lambda_{max} = 343$ nM)

Structure 3: External Aldimine ($\lambda_{max} = 425$ nM)

Structure 4: Glycine Quinonoid ($\lambda_{max} = 502$ nM)

Figure 4
Figure 5
Figure 7
Pyridoxal phosphate inhibits dynamic subunit interchange among serine hydroxymethyltransferase tetramers
Krista A. Zanetti and Patrick J. Stover

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