Cytoplasmic Serine Hydroxymethyltransferase Mediates Competition between Folate-dependent Deoxyribonucleotide and S-Adenosylmethionine Biosyntheses*

Received for publication, May 21, 2002, and in revised form, July 24, 2002
Published, JBC Papers in Press, August 2, 2002, DOI 10.1074/jbc.M205000200

Katherine Herbig‡, En-Pei Chiang§, Ling-Ru Lee§, Jessica Hills‡, Barry Shane§, and Patrick J. Stover‡‡

From the ‡Cornell University, Division of Nutritional Sciences, Ithaca, New York 14853 and the ¶Department of Nutritional Sciences and Toxicology, University of California, Berkeley, California 94720

Folate-dependent one-carbon metabolism is required for the synthesis of purines and thymidylate and for the remethylation of homocysteine to methionine. Methionine is subsequently deaminated to S-adenosylmethionine (SAM), a cofactor that methylates DNA, RNA, proteins, and many metabolites. Previous experimental and theoretical modeling studies have indicated that folate cofactors are limiting for cytoplasmic folate-dependent reactions and that the synthesis of DNA precursors competes with SAM synthesis. Each of these studies concluded that SAM synthesis has a higher metabolic priority than dTMP synthesis. The influence of cytoplasmic serine hydroxymethyltransferase (cSHMT) on this competition was examined in MCF-7 cells. Increases in cSHMT expression inhibit SAM concentrations by two proposed mechanisms: (1) cSHMT-catalyzed serine synthesis competes with the enzyme methylenetetrahydrofolate reductase for methylenetetrahydrofolate in a glycine-dependent manner, and (2) cSHMT, a high affinity 5-methyltetrahydrofolate-binding protein, sequesters this cofactor and inhibits methionine synthesis in a glycine-independent manner. Stable isotope tracer studies indicate that cSHMT plays an important role in mediating the flux of one-carbon units between dTMP and SAM syntheses. We conclude that cSHMT has three important functions in the cytoplasm: (1) it preferentially supplies one-carbon units for thymidylate biosynthesis, (2) it depletes methylenetetrahydrofolate pools for SAM synthesis by synthesizing serine, and (3) it sequesters 5-methyltetrahydrofolate and inhibits SAM synthesis. These results indicate that cSHMT is a metabolic switch that, when activated, gives dTMP synthesis higher metabolic priority than SAM synthesis.

Folate is present in cells as a family of coenzymes that carry one-carbon units and function in both the mitochondrial and cytoplasmic compartments (1–5). Mitochondrial folate metabolism is necessary for the conversion of serine to glycine and formate (a one-carbon unit) (1, 3), whereas cytoplasmic folate metabolism utilizes mitochondria-derived formate for the biosynthesis of purines (supplies the #2 and #8 carbons of the purine ring), thymidine (conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) and for the generation of methionine from homocysteine (1, 3) (see Fig. 1 below). Methionine, in turn, can be converted to S-adenosylmethionine (SAM),1 a cofactor for many methylation reactions, including the methylation of proteins, phospholipids, neurotransmitters, RNA, and DNA (4, 5). Serine hydroxymethyltransferase (SHMT) catalyzes the reversible transfer of the hydroxymethyl group of serine to tetrahydrofolate (THF) to form methyleneTHF and glycine (6). The enzyme is present in both the mitochondria and cytoplasm (7, 8). This reaction is a major source of THF-activated one-carbon units in mammalian cells (6). Loss of mitochondrial SHMT (mSHMT) function cannot be rescued by the activity of the cytoplasmic SHMT (cSHMT) isozyme in cultured cells; some evidence suggests that cSHMT may be a serine synthase in the cytoplasm (7, 9).

Cellular folate derivatives are sequestered by a variety of proteins collectively called folate-binding proteins (2, 10). The cellular concentration of folate-binding proteins exceeds that of folate cofactor (12, 13). This competition for folate cofactors is most pronounced for reactions that utilize methyleneTHF, a derivative that serves as a cofactor in three known enzymatic reactions in the cytoplasm (see Fig. 1). It is required for the conversion of dUMP to dTMP, catalyzed by thymidylate synthase (TS); for the conversion of glycine to serine, catalyzed by cSHMT; and for the synthesis of 5-methylTHF, catalyzed by methylenetetrahydrofolate reductase (MTHFR), a reaction that commits one-carbon units to the methionine cycle. Because the MTHFR reaction is virtually irreversible in vivo (1, 2), methionine synthase (MS) activity is essential for recycling 5-methylTHF to other folate cofactor forms. Otherwise, 5-methylTHF accumulates at the expense of all other folate derivatives, impairing folate-dependent deoxyribonucleotide synthesis. This phenomenon is known as the “methyl trap,” a state of functional folate deficiency and impaired DNA synthesis (3, 13) (see Fig. 1). SAM inhibits MTHFR and thereby provides feedback regulation that protects against a folate methyl trap (see Fig. 1) and ensures that, during methionine repletion, folate-activated one-carbon units are spared for DNA

1 The abbreviations used are: SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; mSHMT, mitochondrial SHMT; cSHMT, cytoplasmic SHMT; THF, tetrahydrofolate; TS, thymidylate synthase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; HCF, heavy chain ferritin; MEM, minimal essential medium; SAH, S-adenosylhomocysteine; DHA, dehydroalanine; GNMT, glycine N-methyltransferase; CD₃, one-carbon unit with one deuterium; CD₄, one-carbon unit with two deuteriums.
Serine Hydroxymethyltransferase

precursor synthesis. During B_{12} deficiency, apo-MS cannot convert 5-methylTHF to THF, resulting in the accumulation of cytoplasmic folate as 5-methylTHF, homocysteine accumulation and in decreased SAM synthesis (4, 14). Depleted SAM leads to an increase in MTHFR activity and accelerated 5-methylTHF synthesis, thereby exacerbating the metabolic dysfunction.

Although inhibition of MTHFR by SAM certainly contributes to the prevention of methyl trapping under normal conditions, there is evidence that competition among folate-dependent enzymes also regulates the supply of one-carbon units to the methionine cycle. Fowler et al. (15) examined the formation of both methionine and serine in human fibroblasts. In fibroblasts where MS activity was deficient due to a variety of genetic mutations, serine formation was low compared with control cells, consistent with the formation of a folate methyl trap. Moreover, mutant fibroblasts with diminished MTHFR activity exhibited normal to high serine formation, indicating that MTHFR deficiency increased the availability of 5,10-methyleneTHF for both methionine and serine in human fibroblasts. In fibroblasts lacking NaCl but containing 200 mM KCl to inhibit transport by GlyT, the accumulation of [2-3H]glycine was determined using a liquid scintillation counter. Intracellular amino acid pools were determined as previously described (16).

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Media**—Fetal bovine serum and α-minimal essential media (αMEM) lacking sodium bicarbonate, ribosomes, ribotides, deoxyribosides, and deoxyribonucleotides were obtained from HyClone Laboratories, Inc. (Logan, UT). Stable isotypes were obtained from Cambrex Isotope Laboratories, Woburn, MA. Human MCF-7 mammary adenocarcinoma cells (ATCC, #HTB22) were cultured in αMEM, 2.5 g/liter NaHCO3, 11.2% (v/v) fetal calf serum, and maintained at 37 °C in a 5% CO2 atmosphere.

**Generation of Cell Lines**—The MCF/HGF cell line, which expresses rat heavy chain ferritin, and the MCF/hSHMT2T cell line, which expresses the human cSHMT cDNA, were described previously (16). MCF/GlyT cells were developed by subcloning the murine glycine transporter cDNA (GlyT1, obtained from Dr. Nathan Nelson, Roche Institute of Molecular Biology) (17) into the EcoRI/XhoI site of the vector pcDNA3. The GlyT1 construct was transfected into MCF-7 cells (2 × 10^6 cells) by electroporation (0.22 kV, 950 microfarads) and cultured until stable G418 clones formed and isolated.

**GlyT1 Transport Assay**—GlyT1 activity is dependent on the presence of Na+ and Cl-. (17). MCF/GlyT cells (1 × 10^6) in mid- to late-log phase were incubated with 100 mM NaCl, 100 mM KCl, 10 mM HEPES (pH 7.5), 0.1 mM of [2-3H]glycine (PerkinElmer Life Sciences), and 1 mM unlabeled glycine. Control cells were incubated with a similar buffer lacking NaCl but containing 200 mM KCl to inhibit transport by GlyT. Transport was allowed to proceed for 30 min at room temperature; then the cells were pelleted by centrifugation and washed three times with 100 volumes of cold phosphate-buffered saline. The cell pellets were lysed with 0.5% Triton X-100 in 10 mM potassium phosphate (pH 7.5), and the accumulation of [2-3H]glycine was determined using a liquid scintillation counter. Intracellular amino acid pools were determined as previously described (18).

**Determination of Folate Cofactor One-carbon Distribution**—Cells were cultured to 60–80% confluence in 100-mm plates and exposed to experimental culture medium 24 h prior to labeling with [3H]folic acid (Moravek). The culture medium (αMEM-modified, HyClone), 2.5 g/liter NaHCO3, 11.2% (v/v) dialyzed fetal bovine serum (HyClone), 0.1 mM methionine) lacked ribo- and deoxyribo nucleosides, hypoxanthine, thymidine, folic acid, and serine unless otherwise noted. For all experiments, 20 nM [3H]folic acid was added to the medium, and glycine was added at concentrations ranging from 0 to 50 mM. After 24 h, media were removed from cells, and cells were washed twice with 2 ml of ice-cold phosphate-buffered saline. Cells were lifted from the plate by incubation with 0.75–1 ml of trypsin-EDTA at room temperature for 10 min. The cell suspension was pelleted, and the cells were washed with 0.25 ml of ice-cold phosphate-buffered saline. The supernatant, the cell pellet was frozen at −80 °C. The relative distribution of the folate-one-carbon forms was quantified by high-performance liquid chromatography as described previously (18).

**Stable Isotope Tracer Studies**—Cells were plated in 100-mm plates at 30–50% confluence in treatment media (10 g/liter HyQ αMEM-modified (HyClone), 2.5 g/liter NaHCO3, 11.2% (v/v) dialyzed fetal bovine serum, 0.05 μM folic acid, 10 μM methionine, glycine at concentrations ranging from 0 to 50 mM, 26 mg/liter L-[5,5,5-2H3]leucine, but lacking riboflavin, lacking ribo- and deoxyribo nucleosides, hypoxanthine, thymidine and serine). Media were supplemented with either [14C]-formate (250 μM) or [2-3,3-2H3]l-serine (250 μM) and were refreshed every 48–72 h. Cells were passaged for 2 to 4 population doublings. Media were removed and cells were washed twice in 2 ml of ice-cold phosphate-buffered saline. Cellular protein was isolated by lysing whole cell pellets with 600 μl of 5% trichloroacetic acid and pelleting the protein by centrifugation at 4000 × g for 30 min at 4 °C. The supernatant was removed, and the supernatant and pellet were stored separately at −80 °C for further analyses. Total genomic DNA was isolated using a QIAamp DNA Blood Mini kit (Qiagen).

Protein pellets were suspended in 6 x HCl (100 μl) in vacuum dried, and heated at 100 °C for 20 h. The amino acids were purified by cation exchange chromatography (21–23). Amino acids were converted to heptfluorobutyryl α-propyl ester derivatives (21) and were separated on an HP-SMS column (30 μm × 0.25 mm). Isotopic enrichment was determined in electron capture negative ionization mode by gas chromatography-mass spectrometry using a model 6890 gas chromatograph and model 5973 mass spectrometer (Hewlett-Packard Corp., Palo Alto, CA). Selected ion monitoring was conducted on a mass-to-charge ratio of 519–523 for serine, m/z 305–308 for dehydroxylamine (DNA), m/z 349–353 for leucine, m/z 367–370 for methionine, and m/z 293–295 for glycine.

DNA samples were dried under nitrogen and suspended in formic acid (1 ml) and hydrolyzed at 150 °C for 45 min in vacuum-dried hydroxyls tubes. After drying at 55 °C under nitrogen, the bases were dissolved in 0.2 ml of a 1:1 mixture of N,O-bis [trimethylsilyl] trifluoroacetamide/1%
Glycine uptake by transfectant MCF-7 cells
Transfected MCF-7 cells expressing the Na⁺/Cl⁻-dependent glycine transporter were assayed for [2-³H]glycine uptake. Cells (1 × 10⁶) were incubated in 10 mM HEPES (pH 7.5), 0.1 mM of [2-³H]glycine, 1 mM L-glutamine, 100 mM KCl, 100 mM NaCl for 30 min at room temperature. Control reactions lacked NaCl but contained an additional 100 mM KCl. Following the incubation period, the accumulation of radiolabeled glycine was determined.

| Cell line | NaCl | Radioactivity (cpm) | Increase in uptake (fold) |
|-----------|------|-------------------|--------------------------|
| MCF-7     | 0    | 1,570             | <0                       |
| MCF-7     | +    | 1,222             |                          |
| MCF GlyT Clone 1 | 0    | 2,990             | 9.15                     |
| MCF GlyT Clone 1 | +    | 27,359            |                          |
| MCF GlyT Clone 2 | 0    | 1,519             | 10.65                    |
| MCF GlyT Clone 2 | +    | 16,155            |                          |

trimethylchlorosilane (Pierce) and acetonitrile, and heated at 140 °C for 30 min. The trimethylsilane-base derivatives were separated on a HP-5MS column. Isotopic enrichment was determined in positive ionization mode by gas chromatography-mass spectrometry using a model 6890 gas chromatograph and model 5973 mass spectrometer (Hewlett-Packard Corp., Palo Alto, CA). Selected ion monitoring was conducted at a mass-to-charge ratio m/z 255–257 for thymine, m/z 280–283 for adenosine, and m/z 368–371 for guanine.

**RESULTS**

**Isolation and Characterization of MCFGlyT Cells**—Previous studies indicate that cellular glycine levels influence serine synthesis and that folate-dependent serine synthesis comes at the expense of methionine synthesis. Activation of cSHMT in neuroblastoma by removal of its endogenous inhibitor 5-formylTHF depletes 5-methylTHF pools, impairs homocysteine remethylation, elevates cellular serine levels, and increases the methionine requirement for maximal cell growth (18). In other studies, Penafiel et al. (24) observed that adult mice injected with high levels of glycine display a 14-fold increase in hepatic glycine concentrations and a 6-fold rise in hepatic serine, suggesting that high levels of glycine were able to drive the direction of the SHMT reaction toward serine synthesis. Additionally, an isotope labeling study by Petzke et al. (25) showed that the rate of glycine-to-serine conversion is increased in the hepatocytes of rats that are fed glycine-rich diets. Indeed, the clinical manifestations of nonketotic hyperglycinemia may be partly explained by the influence of glycine on cSHMT activity and utilization of methyleneTHF for serine synthesis. Measurements of metabolites in cerebrospinal fluid from nonketotic hyperglycinemia patients revealed elevated glycine and homocysteine, but low normal methionine concentrations (26). However, no one has previously systematically examined the effects of cellular glycine levels on folate-dependent one-carbon metabolism.

To study the effects of cellular glycine on cSHMT activity and homocysteine remethylation, the cDNA encoding a glycine transporter, GlyT, was transfected into human MCF-7 cells. The transporter is Na⁺- and Cl⁻-dependent and functions to clear glycine from synaptic clefts. Because it is a reversible transporter, GlyT is effective in cells with a high glycine concentration. The glycine transporter accumulates ~10-fold more [³H]glycine when cultured in the presence of NaCl, whereas nontransfected MCF-7 cells do not display any increased glycine accumulation in the presence of NaCl (Table I). Additionally, the transporter can deplete intracellular glycine in cells cultured in the absence of glycine: the concentration of free cellular glycine in MCF-7 cells that express the transporter is reduced by 85% compared with untransfected cells when cultured in the absence of glycine (Table II).

**Effects of Glycine on the Cellular Distribution of Folate Derivatives**—The effect of intracellular glycine on cellular folate one-carbon metabolism was determined in MCF-7 and MCF-GlyT cells (Fig. 2). 5-methylTHF levels respond to changes in medium glycine concentrations in all cells (Fig. 2A) and are highest at 0 mM glycine and decrease with increasing concentrations of medium glycine. For MCF-7 cells, 5-methylTHF comprises about 40% of total cellular folate when cultured without glycine and decreases to about 25% of total folate at 1 mM glycine in the culture medium. The 5-methylTHF levels are not affected by increases in medium glycine from 1 to 10 mM in these cells. In contrast, 5-methylTHF accounts for up to 55% of total cellular folate in MCF GlyT cells when cultured without glycine, and 5-methylTHF levels decrease nearly linearly as a function of increasing medium glycine from 0 to 10 mM. All decreases in 5-methylTHF levels are accompanied by increases in 10-formylTHF relative concentrations of similar magnitude (Fig. 2B). The relative concentration of unsubstituted THF did not change with exogenous glycine and was not different between the transfected and nontransfected cells (data not shown). These observations indicate that glycine alters the distribution of cellular one-carbon substituted folate derivatives but does not limit the availability of one-carbon units for cycloprolasic metabolism.

The glycine-induced decrease in cellular 5-methylTHF levels indicates that glycine increases cSHMT-catalyzed serine synthesis and thereby increases the enzyme’s effectiveness in competing with MTHFR for one-carbon units in the form of 5,10-methylenetetrahydrofolate (Fig. 1). Relative 5-methylTHF levels reach a minimum threshold in wild type cells between 1 and 2 mM exogenous glycine, whereas 5-methylTHF levels decrease nearly linearly in cells expressing GlyT over the range of glycine concentrations tested. This indicates the MCF-7 cells are capable of sequestering, accumulating, and regulating cellular glycine and that the expression of GlyT disrupts the ability of these cells to concentrate glycine. In both MCF-7 and GlyT-expressing cell lines, the highest relative 5-methylTHF levels were observed at 0 mM glycine, further indicating that cSHMT activity is influenced by mass action and that glycine is critical in preventing the accumulation of folate as 5-methylTHF. At the pharmacological concentration of 50 mM exogenous glycine, 5-methylTHF levels were reduced to 2.5–3.0% of total folate in all cell lines (data not shown). This relationship suggests that the regulation of cellular glycine can be disrupted when cells are exposed to superphysiological concentrations of glycine. If the relative 5-methylTHF levels are reflective of cellular glycine concentrations and cells expressing GlyT do precisely equalize cellular and medium glycine concentrations, then Fig. 2A indicates that MCF-7 cells maintain intracellular glycine levels between 8 and 10 mM when cultured between 1.0 and 10 mM glycine.

**Effects of Glycine on Cellular SAM and SAH**—The determination of SAM and SAH concentrations provides direct meas-

| Cell line | Glycine/valine | Serine/valine |
|-----------|---------------|---------------|
| MCF-7     | 2.15 ± 0.2    | 0.75 ± 0.2    |
| MCF GlyT (clone 1) | 0.33 ± 0.1 | 0.25 ± 0.04 |

**Table II**

Free amino acid levels were determined in MCF-7 cells cultured in αMEM, 2.5 g/liter NaHCO₃, 11.2% (v/v) dialyzed fetal bovine serum (HyClone), 0.1 mM methionine lacking ribo- and deoxyribonucleosides, hypoxanthine, thymidine, glycine, and serine. αMEM contains 145 mM Na⁺ and 106 mM Cl⁻. Free amino acids were isolated and quantified as described elsewhere (18) and normalized to cellular valine concentrations. All values are expressed as the mean and standard deviation of three independent experiments.
Measurement of the methylation capacity of the cell. The effects of increasing exogenous glycine on the cellular concentrations of SAM and SAH are presented for MCF-7 and GlyT-expressing cells (Fig. 3, A and B). SAM concentrations in MCF-7 cells decreased by 50% between 0 and 10 mM medium glycine; similarly, there is more than a 3-fold decline in SAM in GlyT-expressing cells from 2 to 10 mM glycine. The lack of SAM at 0 mM glycine in GlyT-expressing cells may be explained by a shortage of adenosine, because adenosine synthesis requires glycine as a precursor. Although there appears to be a slight increase in SAH concentrations as medium glycine is increased (Fig. 3B), the magnitude of the change is dramatically less than that observed for SAM. This difference may be explained by the activity of SAH hydrolase, which catabolizes SAH to adenosine and homocysteine, and by efficient efflux of homocysteine from these cells. Kinetic data have demonstrated that SAH hydrolase activity favors the formation of SAH, but in the cellular environment, where homocysteine and adenosine are removed, the equilibrium favors hydrolysis (27). Fig. 3 (A and B) indicates that the glycine-induced decrease in 5-methylTHF levels affects the methionine cycle primarily through depletion of available methyl groups.

SAM levels decrease in MCF-7 cells over the range of 2–10 mM medium glycine even though 5-methylTHF levels are constant over this range. This continued reduction in SAM may reflect the activity of glycine N-methyltransferase (GNMT), a SAM-dependent enzyme that converts glycine to sarcosine. GNMT activity is stimulated by increasing glycine concentrations and is inhibited by 5-methylTHF (2, 10). However, GNMT expression is tissue-specific, and its activity has been detected in liver and pancreas only (2). A more likely explanation involves feedback regulation of MTHFR activity by SAM, an allosteric inhibitor of MTHFR (Fig. 1) (4). As SAM concentrations continue to decrease as a function of increasing glycine levels, MTHFR activity increases such that 5-methylTHF synthesis is stimulated. In this manner, SAM’s effect on MTHFR activity may buffer cytosolic 5-methylTHF levels even in the presence of increasing glycine concentrations. This buffering of

FIG. 1. Folate-dependent one-carbon metabolism. Folate metabolism in the cytoplasm incorporates mitochondria-derived formate into purines, thymidylate, and methionine. MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; cSHMT, cytoplasmic serine hydroxymethyltransferase; mSHMT, mitochondrial serine hydroxymethyltransferase; TS, thymidylate synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine. SAM is a feedback inhibitor of MTHFR.

FIG. 2. Distribution of select folate derivatives as a function of exogenous glycine concentration. MCF-7 cells (squares) and two clones of MCF-7 cells expressing GlyT (triangle, clone 1; inverted triangle, clone 2) were cultured in MEM with 100 μM methionine but lacking hypoxanthine, thymidine, and serine and at varying glycine concentrations ranging from 0 to 10 mM, and intracellular folate one-carbon distribution was determined. 5-methylTHF levels (A) and 10-formylTHF levels (B) are expressed as percentages of total cellular folate. Values are shown as the mean and standard error of duplicate measurements.
5-methylTHF concentrations may also serve to ensure cSHMT is inhibited during SAM depletion. In summary, these data demonstrate that glycine influences the distribution of folate derivatives and the availability of methyl groups for SAM-dependent methylation reactions in MCF-7- and GlyT-expressing cells. These observations support the proposition that serine and methionine syntheses are competitive pathways.

Effects of cSHMT Expression on the Distribution of Folate Derivatives and Cellular Methylation Capacity—The cSHMT protein is known to sequester 5-methylTHF as a tight-binding inhibitor (28). Therefore, it is predicted that increased expression of cSHMT would affect the relative level of 5-methylTHF in cultured mammalian cells. We have previously reported an MCFHCF2 cell line and reported that expression of rat heavy chain ferritin (HCF) results in a 5- to 10-fold increase in cSHMT protein concentrations (16). We have also developed MCFcSHMT21 cells, which express the human cSHMT cDNA and contain 2- to 4-fold increased cSHMT protein levels (16). 5-methylTHF levels were determined in all cell lines as a function of medium glycine concentrations (Fig. 4). Compared with MCF-7 cells, cellular 5-methylTHF levels are elevated nearly 2-fold for all cell lines with elevated cSHMT expression when cultured in medium lacking glycine (Fig. 4). Because nearly 50% of total cellular folate is localized in mitochondria, which do not accumulate 5-methylTHF (3, 29), it is likely that nearly all cytoplasmic folate accumulates as 5-methylTHF in MCF-7 cells expressing the cSHMT or HCF cDNA. Increasing medium glycine above 1 mM decreases 5-methylTHF levels in HCF-expressing cells to levels found in MCF-7 cells cultured in the same medium, whereas cells expressing the cSHMT cDNA retain elevated 5-methylTHF levels when medium glycine concentrations are maintained between 0 and 2.0 mM.

Effect of Glycine on Thymidylate Synthesis—Previous studies from our laboratory have demonstrated that increased cSHMT expression stimulates de novo synthesis of thymidine precursor, presumably by supplying methyleneTHF for this pathway (16) and that cSHMT activity is rate-limiting for de novo thymidine biosynthesis. The effect of medium glycine on thymidylate biosynthesis was determined for MCF-7 cells and MCF-7 cells that express GlyT (Fig. 5). Over a physiological range of medium glycine levels, we did not observe a decreasing trend in de novo thymidylate synthesis for either cell line. In fact, thymidylate synthase was stimulated when medium glycine concentrations were increased from 0.2 to 0.5 mM. This indicates that the stimulation of thymidylate biosynthesis by cSHMT (16) is not inhibited by glycine.
MCF-7 cells, stimulates [13C]formate incorporation into methionine by elevations in cSHMT is glycine-dependent. For MCF-7 cells with increased cSHMT expression, [13C]formate incorporation into methionine is increased by about 70% compared with untransfected cells when cultured without glycine but by only 30% or less when cells are cultured with 2 mM glycine. Elevated medium glycine diminishes the ability of cSHMT-derived one-carbon units to compete with exogenously supplied [13C]formate (Fig. 1).

The data in Table 4C indicate that most of the serine that is used for protein synthesis (and presumably most of the serine in the cell) is synthesized from folate-dependent one-carbon metabolism in these cells. Assuming that the 10-formylTHF one-carbon pool is in equilibrium with the methylenelTHF pool, the mass isotopomer distribution (determined from the ratio of the M+1 and M+2 isomers of dA and dG) can be used to calculate the enrichment of formate into serine and methionine (Table IVC). Note that this calculation includes one-carbon units that are derived from unlabeled serine that become incorporated into the one-carbon pool. These data indicate that 69% of serine used for protein synthesis in MCF-7 cells was derived from the one-carbon pool in cells cultured without glycine, and this value increases to 97% in the presence of 2 mM medium glycine. The methionine enrichment reflects competition between methionine derived from the methyleneTHF pool and exogenous methionine present in the culture medium. Consequently, the extent of folate-dependent methionine synthesis can be calculated from the met/mass isotopomer distribution ratio. This calculation indicates that 19–21% of methionine in MCF-7 cells is remethylated independently of medium glycine concentrations, whereas cells with increased expression of cSHMT displayed decreased homocysteine remethylation when medium glycine concentrations were increased (Table IVC).

Cells were also labeled with [3,3,3-2H3]serine to establish the contribution of cSHMT-derived one-carbon units into the thymidine and methionine pools (Fig. 1). The data in Table V quantify the direct incorporation of the hydroxymethyl group of [3,3,3,3-2H4]serine into methionine or thymidine by cSHMT as a percentage of all one-carbon units derived from exogenous [3,3,3,3-2H4]serine. MethyleneTHF that is supplied by cSHMT and incorporated directly into methionine or thymidine is expected to retain the two deuterium atoms (CD2) that are present on the hydroxymethyl group of serine. Alternatively, if the [3,3,3-2H3]serine enters the mitochondria and the hydroxymethyl group is released from the mitochondria as formate, it should contain a single deuterium atom (CD1). Previous studies have shown that up to 90% of one-carbon units used for cytosolic one-carbon metabolism in mammalian cells are derived from formate generated by mitochondrial serine metabolism.2 During derivatization of serine for GC analysis, most of the serine is converted to dehydroalanine (DHA), with loss of the proton on the C2 position. Consequently, the m/z distribution of DHA isotopes gives the isotopic distributions at the C3 position of serine.

Table V shows that about 70% of the isotopically labeled cellular serine pool that provided serine for protein synthesis retained both deuterium atoms on the C3 carbon (as assessed by DHA labeling), and this percentage did not differ significantly under the different culture conditions. The ~30% of serine that contained one deuterium in this position is due to serine resynthesized from glycine using a CD1-THF pool. If the methionine and dTMP one-carbon units were derived directly from cSHMT only, the mass +2 species of these metabolites should also be about 70% of the labeled species. The data in Table V show that the proportions are much lower, indicating

**Effects of Glycine and cSHMT on the Flux of Formate between the Thymidylate and Methionine Biosynthetic Pathways**—The incorporation of exogenously supplied [13C]formate into methionine and serine (present in cellular protein) and purines (present in nuclear DNA) was determined in MCF-7 cells as a function of cSHMT and glycine concentration (Table IVA). The one-carbon precursor pools used for synthesis of these metabolites were derived from the exogenous labeled formate or from endogenously synthesized unlabeled serine metabolites. Increasing medium glycine concentrations from 0 to 2 mM enhances exogenous [13C]formate incorporation into methionine and serine in MCF-7 cells (Table IVA). The increase in [13C]formate incorporation into methionine indicates endogenous formate synthesis is depressed when glycine is elevated, presumably by shifting the equilibrium of the mSHMT reaction toward serine synthesis (Fig. 1). [13C]Formate incorporation into serine is also increased because of increased serine synthesis by the mSHMT and/or cSHMT enzymes (Fig. 1). Table IVB demonstrates that glycine increases the ability of exogenous formate to enrich the 10-formylTHF precursor pool used for purine synthesis, confirming that glycine inhibits the endogenous synthesis of formate in MCF-7 cells. The effect of glycine on formate production is most pronounced in the MCFHGlyT cells. When these cells are cultured without glycine, only 20% of one-carbon units that are incorporated into purines are derived from [13C]formate. At 2 mM medium glycine, 40% are derived from [13C]formate; at 10 mM medium glycine, 50% are derived from [13C]formate. Therefore, elevations of cellular glycine in MCF-7 cells have a general effect of depleting the supply of formate that is available for DNA precursor and SAM synthesis.

Elevated cSHMT expression, as seen in MCFcSHMT21 and MCFHCF2 cells, stimulates [13C]formate incorporation into methionine but has no effect on the incorporation of [13C]formate into purines (Table IV). Previously, we demonstrated that increased cSHMT expression stimulates thymidylate biosynthesis (16). Because TS and MTHFR compete for methyleneTHF, the cSHMT-mediated stimulation of [13C]formate incorporation into methionine probably results from decreased demand of [13C]formate-derived methyleneTHF for thymidine precursor synthesis, because TS seems to prefer methyleneTHF supplied from the cSHMT enzyme (Fig. 1). The data in Table IV demonstrate that the stimulation of [13C]formate incorporation into methionine by elevations in cSHMT is glycine-dependent. For MCF-7 cells with increased cSHMT expression, [13C]formate incorporation into methionine is increased by about 70% compared with untransfected cells when cultured without glycine but by only 30% or less when cells are cultured with 2 mM glycine. Elevated medium glycine diminishes the ability of cSHMT-derived one-carbon units to compete with exogenously supplied [13C]formate (Fig. 1).

![Fig. 5. Radioisotope competition assay for incorporation of [3H]thymidine into DNA as a function of exogenous glycine concentrations. MCF-7 cells (squares) and MCF-7 cells expressing GlyT (triangle, clone 1) were cultured with and without deoxyuridine at varying glycine concentrations ranging from 0 to 5 mM. Incorporation of exogenously supplied [3H]thymidine into DNA was quantified by scintillation counting. Deoxyuridine suppression is defined as (cpm/ng of DNA in cells cultured without deoxyuridine)/(cpm/ng of DNA in cells cultured with deoxyuridine). Values are normalized to 1 at 0 mM glycine and are represented as the mean of duplicate measurements; error bars indicate the standard error of the mean.

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2 S. Myong, S. Cho, and B. Shane, unpublished data.
that most of the one-carbon units used for cytosolic methionine and dTMP synthesis were derived from mitochondrial serine metabolism.

Cells with increased cSHMT expression show increased incorporation of CD2 into both methionine and dTMP, indicating that cSHMT can deliver single carbons to both pathways (Table V). However, the data also show that cSHMT preferentially directs methyleneTHF to dTMP synthesis, as evidenced by the higher percentage of CD2 in dTMP compared with methionine. This indicates that there are two methyleneTHF pools in the cytoplasm: one generated from serine through cSHMT (CD1), the other generated from mitochondria-derived formate (CD2) (Fig. 1). MethyleneTHF derived from cSHMT is preferentially directed toward dTMP synthesis, but can also be incorporated into methionine, indicating that the two methyleneTHF pools are in equilibrium. Increasing the medium glycine concentration from 0 to 2 mM enhanced the incorporation of CD2 into both methionine and thymidine, consistent with glycine inhibiting formate production in mitochondria (Table IV). In MCF-7 cells, CD2 incorporation into dTMP was further enhanced when the concentration of glycine in the culture medium was increased to 10 mM glycine, whereas incorporation of CD2 into methionine was depressed by greater than 40% when medium glycine concentration was increased from 2 to 10 mM. These data indicate that cSHMT contributes one-carbon units to both methionine and thymidine biosynthesis but that the incorporation of CD2 into methionine is inhibited by elevated glycine concentrations, whereas glycine does not impair the incorporation of CD2 into thymidine, consistent with the deoxuryridine suppression data (Fig. 4). The stimulation of CD2 incorporation, catalyzed by cSHMT, into both methionine and thymidine biosynthesis at 0 and 2 mM exogenous glycine may also result from glycine-induced reductions of cellular 5-methylTHF, which is an inhibitor of cSHMT catalytic activity (Fig. 2).

Increased cSHMT expression also enhanced the incorporation of cSHMT-derived one-carbon units into methionine and thymidylate at 0 and 2 mM exogenous glycine, and increasing the medium glycine concentration from 0 to 2 mM stimulated the incorporation of CD2 into thymidine and methionine in all cells lines. It should be noted that the CD2 proportions shown

### Table IV

**Effect of cSHMT and glycine on formate metabolism in MCF-7 cells**

Cells were cultured in medium containing 0.05 μM folinic acid, 10 μM methionine, 26 μM leucine, 250 μM [1-13C]formate but lacking ribo- and deoxyribonucleosides, hypoxanthine, and thymidine. Isotopic enrichment of [13C]formate in serine and methionine derived from cellular protein and purines derived from DNA was determined as described under “Experiment Procedures.”

| Cell line | Glycine (mM) | 0 mM | Met/Ser | Glycine (mM) | 2 mM | Met/Ser | Glycine (mM) | 10 mM | Met/Ser |
|-----------|-------------|------|---------|-------------|------|---------|-------------|-------|---------|
| MCF-7     | Met/Ser     |      |         | Met/Ser     |      |         | Met/Ser     |      |         |
| MCFHCF2   | 0.098       | 0.359 | 0.132   | 0.611       | 0.136 | 0.608   |
| MCFcSHMT21| 0.165       | 0.378 | 0.169   | 0.631       |      |         |             |      |         |

### Table V

**Effect of cSHMT and glycine on 3-2H3serine metabolism in MCF-7 cells**

Human MCF-7 cells and cells expressing the cSHMT or HCF cDNA were cultured in media containing 0.05 μM folinic acid, 10 μM methionine, 26 μM leucine, 250 μM [1,2,3-3H]serine but lacking ribo- and deoxyribonucleosides, hypoxanthine, and thymidine. Isotopic enrichment of [1,2,3-3H]serine into methionine (Met) and dehydroalanine (DHA, derived from serine) residues present within cellular protein, and into thymidine (dT) present within DNA, was determined at various glycine concentrations. All values are expressed as a ratio of carbons containing one or two deuterium atoms. Values in parentheses represent the percent change in experimental values relative to control values determined in MCF-7 cells, which are given an arbitrary value of 100%.

| Cell line | Glycine (mM) | 0 mM | dA/dG | Glycine (mM) | 2 mM | dA/dG | Glycine (mM) | 10 mM | dA/dG |
|-----------|-------------|------|-------|-------------|------|-------|-------------|-------|-------|
| MCF-7     | dA/dG       |      |       | dA/dG       |      |       | dA/dG       |      |       |
| MCFHCF2   | 0.519       | 0.459 | 0.623 | 0.598       | 0.658 | 0.624 |
| MCFcSHMT21| 0.216       | 0.212 | 0.375 | 0.361       | 0.500 | 0.482 |

### Notes

a Enrichment relative to labeled leucine enrichment in protein.
b Determined from the ratio of the M + 1 and M + 2 isomers of dA and dG. A value of 1.0 would indicate that 100% of the C-2 and C-8 carbons of the purine ring were derived from [13C]formate.
c Determined from enrichments shown in A, and enrichments of 10-formyl-THF pool (MIA) as judged by adenine labeling in B.
in Table V underestimate the direct contribution of cSHMT-derived one-carbon units to methionine and dTMP synthesis by about 50%, as the CD2 proportion of the labeled serine precursor was about 70%.

**DISCUSSION**

Several studies have investigated the competition among folate-dependent enzymes. Based on observations of monkeys rendered vitamin B12-deficient through long term nitrous oxide administration, Scott et al. (13) proposed that limited methyl group availability, caused by either folate or methionine deficiency, shifts the flux of one-carbon units among folate-dependent pathways such that folate cofactors are preferentially shuttled to the methionine cycle to protect methylation reactions and thereby suppress DNA synthesis. Similarly, Green et al. (27) made use of the known affinities of several relevant enzymes that utilize 5,10-methyleneTHF and predicted that folate coenzymes are preferentially directed toward SAM-dependent methylation reactions at low cellular folate concentrations. Additionally, these authors projected that MTHFR enzyme would be insensitive to changes in methyleneTHF availability, whereas TS activity would be highly dependent on them. This model assumed that these two enzymes directly compete for a common cellular pool of methyleneTHF. Neither of these studies directly measured the utilization of one-carbon units through these competing pathways.

More recent studies have included some measurements of the flow of folate one-carbon units through these pathways, and this work has confirmed that changes in a single biosynthetic pathway influence the shuttling of folate cofactors through other pathways. Fell and Selhub (30) demonstrated that Raji cells cultured in high methionine (7 mM) or homocystine (0.7 mM) display decreased de novo thymidylate synthesis and increased utilization of 5,10-methyleneTHF for serine synthesis. Furthermore, evidence from studies of cultured fibroblasts from patients suffering from either MS or MTHFR genetic deficiencies also supports competition for 5,10-methyleneTHF between MTHFR and cSHMT (15). MTHFR-deficient cells have reduced concentrations of 5-methylTHF (31, 32) and increased thymidine synthesis by supplying or perhaps channeling methyleneTHF to TS. Because this pool of cSHMT would not be in equilibrium with the bulk phase, it would be insensitive to changes in equilibrium levels of 5,10-methyleneTHF. Data also show that this pool of cSHMT is insensitive to cellular glycine concentrations. Our model further predicts a second pool of cSHMT enzyme that competes with MTHFR for 5,10-methyleneTHF in a glycine-dependent manner and competes with MS for 5-methylTHF in a glycine-independent manner. Our model does not account for the data in Tables IV and V that indicate that cSHMT associated with dTMP synthesis is insensitive to glycine concentrations, whereas the cSHMT pool that competes with MTHFR is sensitive to cellular glycine concentrations. Additional studies are required to account for these data.

Previous studies have indicated that folate metabolism is regulated such that SAM synthesis has metabolic priority over thymidylate biosynthesis (13, 27). We propose that, under certain conditions, cSHMT acts as a switch to increase DNA synthesis at the expense of homocysteine remethylation. This regulation is accomplished in two ways. Deoxyribonucleotide biosynthesis is enhanced by providing 5,10-methyleneTHF to TS for thymidylate synthesis as observed by Oppenheim et al. (16) and by increasing the cytoplasmic availability of THF for conversion to 10-formylTHF and use in purine synthesis (Fig. 2B). Simultaneously, cSHMT inhibits homocysteine remethylation by two mechanisms: (1) by decreasing the availability of 5,10-methyleneTHF to MTHFR (at elevated glycine concentrations) and (2) by sequestering 5-methylTHF and depleting cellular levels of SAM. The cSHMT-induced depletion of SAM concentrations may result directly from 5-methylTHF sequestration, but this mechanism was not conclusively demonstrated in this study.

The two most common biomarkers for impaired folate metabolism are elevated tissue and serum homocysteine (4, 33) and increased uracil content in DNA (34). These biochemical markers are risk factors for certain cancers, vascular disease, and neural tube defects (4, 35). Therefore, the ability of cSHMT to affect these pathways indicates a potential role for this enzyme in the etiology of homocysteine- and uracil-related diseases. Additionally, cSHMT expression and activity is regulated by a number of factors, including retinoic acid (36), ferritin, and developmental stage (16). Further studies are required to determine the effects of altered cSHMT activity on these folate-sensitive biomarkers in animal models and its role in folate-related pathologies.

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Serine Hydroxymethyltransferase

38389
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Katherine Herbig, En-Pei Chiang, Ling-Ru Lee, Jessica Hills, Barry Shane and Patrick J. Stover

J. Biol. Chem. 2002, 277:38381-38389.
doi: 10.1074/jbc.M205000200 originally published online August 2, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205000200

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