Published in final edited form as:

Arch Biochem Biophys. 2007 June 1; 462(1): 21–27.

Effect of Vitamin B₆ Availability on Serine Hydroxymethyltransferase in MCF-7 Cells

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Abstract

Folate-activated one-carbon units are derived from serine through the activity of the pyridoxal-phosphate (PLP)-dependent isozymes of serine hydroxymethyltransferase (SHMT). The effect of vitamin B₆ availability on the activity and expression of the human mitochondrial and cytoplasmic SHMT isozymes was investigated in human MCF-7 cells. Cells were cultured for 6 months in vitamin B₆ replete (4.9 μ M pyridoxine) minimal essential medium (α MEM) or vitamin B₆-deficient medium containing 49 nM, 4.9 nM or 0.49 nM pyridoxine. Total cellular PLP levels and SHMT activity were reduced 72% and 7% respectively when medium pyridoxine was decreased from 4.9 μ M to 49 nM. Cells cultured in medium containing 4.9 nM pyridoxine exhibited 75%, 27% and 60% reduced levels of PLP, SHMT activity and S-adenosylmethionine, respectively compared to cells cultured in α MEM. Cytoplasmic SHMT activity and protein levels, but not mRNA levels, were decreased in cells cultured in vitamin B₆ deficient medium, whereas mitochondrial SHMT activity and protein levels were less sensitive to vitamin B₆ availability. PLP bound to cytoplasmic SHMT with a K_d = 850 nM, a value two orders of magnitude lower than previously reported for the bovine cytoplasmic SHMT isozyme. Collectively, these data indicate that vitamin B₆ restriction decreases the activity and stability of SHMT, and that the cytoplasmic isozyme is more sensitive to vitamin B₆ deficiency than the mitochondrial isozyme in MCF-7 cells.

Keywords

serine hydroxymethyltransferase; vitamin B₆; pyridoxal-phosphate; one-carbon metabolism; homocysteine; folate

Pyridoxal-phosphate is a cofactor for the mitochondrial and cytoplasmic isozymes of serine hydroxymethyltransferase (cSHMT and mSHMT), the P-protein of the glycine cleavage system, cystathionine β -synthase (CBS) and γ -cystathionase, all of which contribute to homocysteine metabolism either through folate-mediated one-carbon metabolism or the transsulfuration pathway (Figure 1). Folate cofactors carry and chemically activate single carbons for the synthesis of purines, thymidylate and methionine (1). The single carbons are derived from serine and glycine through the activity of three PLP- and folate-dependent enzymes: the mSHMT and cSHMT isozymes and the glycine cleavage system (2-4). SHMT catalyzes the reversible interconversion of serine and THF to methyleneTHF and glycine (5). In mitochondria, serine is converted to glycine and formate through a folate-dependent pathway initiated by mSHMT. Formate derived in the mitochondria is a major source of one-carbon

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units for cytoplasmic one-carbon metabolism through its conversion to 10-formylTHF by the enzyme 10-formylTHF synthetase (6). Alternatively, cSHMT can generate folate-activated one-carbon units from serine in the cytoplasm in the form of methyleneTHF (7) (Figure 1).

In the cytoplasm, methionine can be adenylated to form *S*-adenosylmethionine (AdoMet) which serves as a cofactor for cellular methylation reactions, including the methylation of proteins, DNA, RNA, phospholipids and neurotransmitters. *S*-adenosylhomocysteine (AdoHcy), a product of AdoMet-dependent reactions, is hydrolyzed to yield homocysteine, which can be metabolized by the remethylation or transsulfuration pathway (Figure 1). The generation of 5-methylTHF, which is a cofactor for folate-dependent homocysteine remethylation, requires PLP. Likewise, the metabolism of homocysteine by the transsulfuration pathway is initiated by the PLP-dependent enzyme CBS.

Elevated serum homocysteine is a biomarker for folate deficiency and/or impaired folate metabolism, and is an independent risk factor for vascular disease. Vitamin B₆ deficiency is also associated with elevations in serum homocysteine. One study in humans indicated that marginal vitamin B₆ deficiency did not affect homocysteine remethylation or synthesis in the absence of dietary methionine intake (8). Other studies of vitamin B₆-restricted rats indicated that the homocysteine remethylation and the transsulfuration pathways were compromised in rats fed vitamin B₆-deficient diets, thereby indicating that both SHMT and CBS were sensitive to dietary intake of vitamin B₆. PLP binds tightly to CBS ($K_d = 700$ nM; (9) and cystathionase (K_d 1.4 μ M (10). However, the dissociation constant for the interaction of PLP with purified bovine cSHMT was reported to be 27.5 μ M (11). This observation lead Martinez et al. (12) to predict that folate-dependent remethylation of homocysteine may be more sensitive to vitamin B₆ restriction than the transsulfuration pathway.

In this study, the interaction of PLP with the human SHMT isozymes was investigated in MCF-7 cells to determine the influence of PLP availability on mSHMT and cSHMT activity, expression, and on the cellular methylation potential.

Material and Methods

Materials

All reagents were analytical grade unless otherwise noted. Semicarbazide, AdoMet, AdoHcy, glycine, pyridoxine and pyridoxal 5'-phosphate were obtained from Sigma. [³H]Glycine was obtained from Perkin Elmer. 1-Heptanesulfonic acid was obtained from Acros. Sodium Phosphate, phosphoric acid and HPLC grade methanol were purchased from Fisher.

Cell Culture in Pyridoxine Deficient Medium

MCF-7 cells were passaged for 6 months in modified minimal essential medium (α MEM) that lacked nucleotides, nucleosides but contained 10% dialyzed and charcoal-treated fetal bovine serum, 250 μ M serine, 10 μ M methionine, 0.2 mM glycine, 50 nM 5-formylTHF and one of five different concentrations of pyridoxine. Under these culture conditions, functional folate-mediated one-carbon metabolism is essential for cell survival. Cells were cultured either in modified α MEM which contained 1mg/L (4.9 μ M) pyridoxine (referred to as α MEM), α MEM containing 0.01mg/L (49 nM) pyridoxine, α MEM containing 0.001mg/L (4.9 nM) pyridoxine, α MEM containing 0.0001mg/L (0.49 nM) pyridoxine and α MEM containing 0.00001mg/L (0.049 nM) pyridoxine. Intracellular PLP concentrations were monitored during the repletion period to ensure steady-state levels of intracellular PLP were achieved for each culture condition.

Determination of Cellular PLP

Frozen cell pellets (10-30 million cells) were vortexed in 700 μ l of Milli-Q water; 500 μ l of cellular lysate was used for HPLC analysis and the remaining 200 μ l was used for determination of protein concentration (13). A solution of semicarbazide and glycine (250mg/ml) was added to 500 μ l of cellular lysate or PLP standards such that the semicarbazide was in a molar excess of PLP (>10mg/ml) in the sample or standard. The samples or standards were incubated at room temperature for 30 min in the dark. Protein was precipitated with the addition of 70% trichloroacetic acid and the solution was clarified by centrifugation (14,000 rpm for 10 min). The supernatant was collected and 30 - 50 μ l of a 25% sodium hydroxide solution was added to achieve a pH between 3.0 -5.0. Chromatographic separation was performed by HPLC using a C18 Luna phenomenonex column (5 μ 100A°, 25 cm $\times$ 4.6 mm) and an isocratic mobile phase consisting of 60 mM sodium phosphate (pH 6.5), 400 mg/L of EDTA, and 9.5% methanol. The derivatized PLP was quantified using a Shimadzu RF-10A fluorescence detector using excitation and emission wavelengths of 380 and 450nm, respectively (14).

Western Blot Analyses of cSHMT, mSHMT and GAPDH

Cell pellets were homogenized by sonication in 10 mM Tris pH 7.5, 150 mM sodium chloride, 5 mM EDTA, 1% Triton X-100, 20 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. The lysates were cleared by centrifugation and the soluble protein concentration of the supernatant determined (13). Protein extracts (60 μ g/lane) were run on an 8% SDS-PAGE gel, then transferred to a polyvinylidene fluoride microporous membrane (Millipore) using a MiniTransblot apparatus (BioRad). For detection of cSHMT, mSHMT and GAPDH, the membrane was incubated overnight at 4°C with purified polyclonal antibodies generated in sheep (1:10,000 dilution). The membrane was washed in phosphate-buffered saline containing 0.1% Tween-20, then incubated for 2 h with horseradish peroxidase-conjugated rabbit anti-sheep secondary antibody (1:6500 dilution; Pierce). Proteins were visualized using the Super Signal West Pico chemiluminescent detection system (Pierce). The protein bands were quantified using ChemiImager 4400 from Alpha Innotech Corp. (San Leandro, CA).

Determination of S-adenosylmethionine and S-adenosylhomocysteine

Cellular AdoMet and AdoHcy levels were determined by HPLC. All sample and standards were prepared and derivatized as described previously (15). Following derivatization, cell extracts were applied to a 5 μ , 25 cm $\times$ 4.6 mm C8 column and separated using a binary buffer system consisting of: buffer A (25 mM sodium phosphate pH 3.0, 10 mM 1-heptanesulfonic acid and 18% methanol) and buffer B (25 mM sodium phosphate, 10 mM 1-heptanesulfonic acid and 30% methanol). PLP was eluted from the column using a binary gradient follows: 0-10min, 100% of buffer A; 10-20 min, linear gradient of 0% to 100% of buffer B; 20-30 min, 100% of Buffer B; 30-35 min, linear gradient of 100% to 0% buffer B and 35-40 min, equilibration with 100% buffer A. The fluorescent AdoMet and AdoHcy derivatives were detected using a Shimadzu RF-10A fluorescence detector using excitation and emission wavelengths of 270nm and 410nm, respectively. AdoHcy standards and samples eluted between 11 and 16 min, whereas AdoMet eluted between 29 and 33min.

Purification of Mitochondria

5×10^6 MCF-7 cells in 1.0 ml of 10 mM potassium phosphate buffer, pH 7.5, containing 300 mM sucrose were disrupted by 50 strokes of a dounce homogenizer (Wheaton pestle A (tight fitting)), and the cytoplasmic and mitochondrial fractions were prepared as described previously (16). GAPDH and COX IV proteins were used as cytoplasmic and mitochondrial markers respectively, by western blot analyses. COX IV was not present in the cytoplasmic fraction; hence there was no need to correct for mitochondrial breakage. Mitochondrial fractions were determined to be free of GAPDH protein and therefore were not corrected for

cSHMT contamination. Protein concentrations were determined by the method of Lowry as described previously using bovine serum albumin as a standard (13).

Activity Assay

A sensitive radioassay was used to measure mSHMT and cSHMT enzyme activity in cell extracts from as few as 1×10^6 cultured cells as described previously (16,17). The assay is based on the observation that THF accelerates the SHMT-catalyzed exchange of the pro-2S proton of glycine, and that 5-formylTHF inhibits this reaction (18). Isolated mitochondria were lysed in 200 μ l of 20 mM sodium phosphate buffer, pH 7.2, 10 mM 2-mercaptoethanol, 0.5% Triton X-100. mSHMT activity was measured by diluting 40 μ l of the mitochondrial fraction to 500 μ l with 10 mM potassium phosphate buffer, pH 7.5, 10 mM 2-mercaptoethanol, and glycine such that the final glycine concentration was 1 mM with a specific activity of 2×10^6 dpm/ μ mol. The reaction was initiated by the addition of THF (1 mM) and incubated at 37°C for 30-120 min. Control reactions were performed to correct for background exchange by the addition of 5-formylTHF (1mM) in lieu of THF. The reaction was terminated by the addition of 3 ml of 50 mM HCl (4 °C), and the solution was passed through a column containing 0.8 ml of Dowex 50 AG (Bio-Rad) to sequester the radiolabeled glycine. The column was washed with an additional 2 ml of 50 mM HCl, and the tritiated water was collected and quantified by scintillation counting. Control reactions containing 5-formylTHF exhibited less than 4% proton exchange compared with the THF-catalyzed exchange reaction. All assays were performed in duplicate, and all experiments were repeated at least twice.

Message Level Determination

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions. cDNA was prepared using the High-Capacity cDNA Archive Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Briefly, the reaction mixture contained reverse transcriptase (RT) buffer, RT random primers, dNTP mixture, DNase- and RNase-free water, MultiScribe RT enzyme and 8 μ g sample RNA per reaction. The reaction was incubated for 10 min at 25°C followed by 120 min at 37°C. GAPDH and cSHMT mRNA were quantified using FAM-labeled probes purchased from Applied Biosystems (Assay-on-Demand Gene Expression Products). The components of a 25 μ l gene expression assay reaction included 12.5 μ l of a TaqMan Universal PCR Master Mix (AmpErase UNG), 1.25 μ l of 20X FAM-labeled probe, 5 μ l of diluted cDNA and 6.25 μ l of water. The reaction samples were loaded onto an optical reaction plate and covered with optical adhesive cover (Applied Biosystems). The reaction was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The mixture was incubated for 2 min at 50°C and for 10 min at 95°C, followed by 40 cycles of PCR at 95°C for 15 s and at 60°C for 1 min. All samples were performed in triplicate. The expression of GAPDH was used for normalization of all samples.

Purification of Recombinant Human cSHMT Protein

The expression and purification of human recombinant cSHMT was preformed as described previously (19). *E.coli* cell pellets were lysed in a buffer containing 40 mM potassium phosphate, pH 7.0, and 10 mM 2-mercaptoethanol using a French press, and the insoluble material was removed by centrifugation at 12,000 rpm. For purification of the recombinant cSHMT protein, the clarified supernatant was applied directly to a carboxymethyl sepharose ion exchange column, and the protein was purified to homogeneity as described previously (19). The purified recombinant protein was greater than 95% pure as determined by SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined by a modified Lowry assay (13). The purified protein was stored at 80 °C.

Determination of the PLP Binding Affinity

ApoSHMT lacking PLP was prepared as described previously (19). The affinity of PLP for recombinant human apo-cSHMT protein was determined using a modification of a previously described binding assay (19). The binding of reduced folate to SHMT results in the formation of a PLP-glycine-quinonoid intermediate, which has an absorption maximum at 502 nm ($\epsilon = 40,000$) (18). The apoSHMT (lacking bound PLP) does not form the quinonoid intermediate and therefore did not exhibit an increase in absorbance at 502 nm upon binding 5-formylTHF. Recombinant human apo-cSHMT protein (1 μM) was added to a cuvette that contained 1 ml of the reaction buffer (200 mM glycine, 50 mM HEPES, pH 7.3, and 500 μM [6S]-5-formylTHF (a value equal to $20\times K_d$)). After a 30 min incubation, the absorbance spectrum was recorded from 550 to 400 nm. PLP was added incrementally (to achieve final concentrations of 0.5, 1, 2, 4, 8 or 10 μM), and the spectrum was recorded. To determine the affinity of PLP for apo-cSHMT in the absence of glycine and 5-formylTHF, cSHMT and PLP were incubated for 30 min, and the glycine and 5-formylTHF were added immediately before the spectra were recorded. To quantify the affinity of cSHMT for PLP, the absorbance at 502 nm was recorded as a function of PLP added to the cuvette. The data were curve fit by nonlinear regression using prism software.

Statistical Analysis

Differences in culture medium pyridoxine and SHMT activity were analyzed using one-way ANOVA with Newman-Keuls pairwise comparison test using GraphPad software.

RESULTS

Generation of PLP-Deficient Cells

MCF-7 cells were passaged for 6-months in either modified αMEM (4.9 μM pyridoxine) or modified αMEM containing either 49 nM, 4.9 nM, 0.49 nM or 0.049 nM pyridoxine to ensure steady-state levels of intracellular PLP was achieved. Culture medium was refreshed every 48 hours. Cells passaged in medium containing 0.049 nM pyridoxine were not viable after 2 months and were discarded. Cells cultured in medium containing 0.49 nM pyridoxine demonstrated a slight attenuation of growth rate compared to all other cell cultures (less than 25% inhibition of growth rate, data not shown). After 6 months of passage, the cell lines were expanded, harvested, and PLP, AdoMet, AdoHcy, SHMT protein, SHMT activity and SHMT mRNA levels were quantified. The concentrations of vitamin B6 in the culture medium are in the range of that found in human plasma. In a recent large-scale epidemiological study, the average plasma vitamin B6 level was about 50 nM, with a range of 19 to 150 nM (20).

Effect of Vitamin B₆ Availability on Cellular PLP Levels and the Cellular Methylation Potential in MCF-7 cells

Cells cultured in medium with 49 nM pyridoxine contained 0.07 nmoles PLP/ μg protein or approximately 25% of the PLP content present in cells cultured in αMEM (Table 1). Cells cultured in medium containing 4.9 nM or 0.49 nM pyridoxine did not display significant differences in cellular PLP content compared to cells cultured in medium containing 49 nM pyridoxine. The reductions in cellular PLP levels resulted in decreased cellular AdoMet levels by 65% and 75% in cells cultured in medium containing 4.9 nM and 0.49 nM pyridoxine respectively compared to cells cultured in αMEM . AdoHcy levels were not affected by decreasing medium pyridoxine concentrations from 4900 nM to 4.9 nM, although AdoHcy levels do not always reflect cellular homocysteine metabolism because of its export into the culture medium (Table 1). Overall, the 75% decrease in cellular PLP levels resulted in nearly a 65% decrease in the AdoMet/AdoHcy ratio, often referred to as the methylation capacity of the cell (21).

Effect of Vitamin B₆ Availability on SHMT protein, mRNA and Activity

The SHMT isozymes differed in their sensitivity to PLP availability. cSHMT protein levels were sensitive to exogenous pyridoxine availability; cells cultured with 49 nM, 4.9 nM and 0.49 nM pyridoxine exhibited a 10%, 33% and 60% decrease in cSHMT protein levels respectively, compared to cells cultured in α MED (Figure 2). These changes occurred without decreases in cSHMT mRNA levels (Table 2). The mSHMT protein levels were less sensitive than cSHMT protein levels to pyridoxine availability. Cells cultured in medium containing 49 nM and 4.9 nM pyridoxine exhibited a 5% increase in mSHMT protein whereas cells cultured with medium containing 0.49 nM pyridoxine contained 44% less mSHMT protein compared to cells cultured in α MED.

Total SHMT activity in cell extracts appeared to reflect both the effects of vitamin B₆ deficiency on SHMT protein levels and the relative ratio of apoenzyme to holoenzyme. Total SHMT activity in crude cell extracts decreased by 7%, 27% and 58% in α MED containing 49 nM, 4.9 nM and 0.49 nM pyridoxine respectively, compared to extracts from cells cultured in α MED (Figure 2A). Addition of PLP to the assay buffer, which converts all apoenzyme to active holoenzyme, increased total SHMT activity in all cell extracts (referred to as the stimulation index). SHMT activity was stimulated by 1.5 fold in extracts from cells cultured in α MED and α MED containing 49 nM pyridoxine, 1.9-fold in extracts from cells cultured in α MED containing 4.9 nM pyridoxine and 2.4 fold in cells cultured in α MED containing 0.49 nM pyridoxine (Figure 2A). The mSHMT enzyme activity appeared to be less sensitive to vitamin B₆ availability than cSHMT enzyme activity (Figure 2B, 2C). The activity of the mSHMT isozyme was not stimulated by the addition of PLP to the assay in purified mitochondria extracts from cells cultured in α MED, whereas the activity of the cSHMT isozyme was stimulated 1.26 fold in cytosolic extracts. Compared to extracts from cells cultured in α MED, cells cultured in α MED containing 4.9 nM pyridoxine exhibited a 43% reduction in mSHMT activity, but an 89% reduction in cSHMT activity. The addition of PLP in the assay stimulated mSHMT and cSHMT activity in extracts from cells cultured in α MED containing 4.9 nM pyridoxine, but did not restore the activity to levels observed in extracts from cells cultured in α MED; the magnitude of the stimulation was consistent with the observed change in total cSHMT and mSHMT protein levels (Figure 3).

Affinity of Recombinant Human cSHMT for PLP

The dissociation constant for PLP binding to cSHMT was determined in the presence and absence of glycine and 5-formylTHF. The K_d was determined to be 850 nM, and was independent of both glycine and 5-formylTHF (Figure 3).

DISCUSSION

A recent study of rats fed five diets containing a range of vitamin B₆ levels from adequate to deficient resulted in a reduction of plasma PLP from 900 nM to 70 nM and a 30% reduction in liver PLP content over the entire range (22). In that study, both mSHMT and cSHMT activities decreased linearly with reductions in liver PLP concentrations; total unstimulated SHMT activity decreased over the range from adequate to deficient. Although not investigated in that study, decreased protein levels were proposed to account for the reduction in SHMT activity as a function of PLP status. Interestingly, the ratio of apoSHMT/holoSHMT did not change among the 5 dietary groups for either SHMT isozyme; apoenzyme consistently accounted for 50% of total stimulated SHMT activity along the continuum of vitamin B₆ status in rats.

There are three striking differences between the previous study performed in rats and the results from the current study of human MCF-7 cells. First, although total unstimulated SHMT activity

decreased with vitamin B₆ availability, total stimulated SHMT activity decreased only in the cells cultured in the lowest pyridoxine concentrations (α MEM with 0.49 nM pyridoxine). Second, mSHMT activity was not stimulated in crude extracts of MCF-7 cells cultured in α MEM, indicating that all of the mSHMT protein was PLP-bound. Third, the cSHMT and mSHMT isozymes did not exhibit similar decreases in specific activity as PLP levels declined; the cSHMT was more sensitive to PLP availability. This study is also the first to show that SHMT protein levels respond to PLP availability. cSHMT protein levels decreased with reduced PLP availability, whereas mSHMT protein levels were increased in cells cultured in α MEM containing 49 nM and 4.9 nM pyridoxine compared to cells cultured in α MEM. Reductions in cSHMT protein and activity as a function of decreasing pyridoxine availability occurred without decreases in cSHMT mRNA levels, indicating that apocSHMT is vulnerable to turnover.

Cellular AdoMet levels decreased markedly with reductions in pyridoxine in the culture medium (Table 1). Previously, we have shown that cSHMT functions to inhibit the generation of AdoMet in MCF-7 cells by sequestering 5-methylTHF and homocysteine remethylation (23). Therefore, reductions in cSHMT protein are not expected to negatively affect AdoMet levels. The reduction in AdoMet likely reflects the conversion of holomSHMT to apomSHMT and decreased production of formate from serine in the mitochondria; MCF-7 cells do not contain a PLP-dependent glycine cleavage system for formate production. Vitamin B₆ deficiency in rats also lead to depressed AdoMet levels in liver (12).

Finally, this study offers some clarity regarding the affinity of human cSHMT for PLP. Previously, one study determined that cSHMT purified from bovine liver exhibited a K_d of 27 μ M for PLP, whereas cSHMT purified from rabbit liver was reported to exhibit a K_d of 700 nM for PLP (24). In this study, we demonstrated that recombinant human cSHMT binds PLP with a K_d of 850 nM, and this value was independent of bound folate or glycine substrates. Therefore, given the very high sequence identity between the mSHMT and cSHMT isozymes, it seems likely that the PLP-dependent enzymes involved in transsulfuration and one-carbon metabolism exhibit comparable affinities for PLP.

Acknowledgements

This work was supported by PHS HD35687 to PJS

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Figure 1. The role of PLP in folate-mediated one-carbon and homocysteine metabolism

Tetrahydrofolate (THF)-mediated one-carbon metabolism is required for the synthesis of purines, thymidylate and the remethylation of homocysteine to methionine. The hydroxymethyl group of serine is the major source of one-carbon units which are generated in the mitochondria in the form of formate in a reaction initiated by the PLP-dependent enzyme *mSHMT*, or in the cytoplasm through the activity of *cSHMT*. Formate derived in mitochondria can enter the cytoplasm and function as a one-carbon unit for folate metabolism. The transsulfuration pathway is initiated by the PLP-dependent enzyme *CBS*. The one carbon is labeled in “bold”. *MTHFR*, methylenetetrahydrofolate reductase; *cSHMT*, cytoplasmic serine hydroxymethyltransferase; *mSHMT*, mitochondrial serine hydroxymethyltransferase; *TS*, thymidylate synthase; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; PLP, pyridoxal 5' phosphate.

Figure 2. Pyridoxine availability affects SHMT protein levels in MCF-7 cells

Cells were cultured in α MED containing different concentrations of pyridoxine for 6 months and the levels of GAPDH, mSHMT and cSHMT determined by western blot (Figure 2A). The levels of cSHMT and mSHMT were quantified by densitometry and normalized to GAPDH levels (Figure 2B).

Figure 3. Serine Hydroxymethyltransferase activity in MCF-7 cell extracts

The activity of SHMT was quantified in whole cell extracts (Panel A), extracts from purified mitochondria (Panel B) and cytoplasmic extracts (Panel C). Values are mean and standard deviations of 4 independent measurements of extracts. Percentage values shown indicate SHMT activity in cell extracts relative to SHMT activity in cells cultured in α MEM. Numeric values shown above bars indicate fold stimulation resulting from the inclusion of PLP in the assay buffer. SHMT activity in extracts from cells cultured in the presence of varying concentrations of pyridoxine differ: Panel A, $p = .0005$, one-way ANOVA; Panel B and C, $p < .001$, students t-test. SHMT activity in extracts differed as a result of PLP addition to the assay

buffer ($p < 0.001$, student's t-test) except in mitochondrial extracts from cells cultured in medium containing 4900 nM pyridoxine.

Figure 4. The affinity of PLP for cSHMT *in vitro*

The increase in absorbance at 502 nm resulting from the formation of a cSHMT-PLP-Gly-5-formylTHF ternary complex was recorded as a function of PLP in the reaction buffer. A K_d of 850 nM was determined by nonlinear regression (95% confidence interval: 0.5415 to 1.170; $R^2 = 0.9912$).

Table 1**The effect of medium pyridoxine concentration on intracellular PLP, AdoMet and AdoHyc levels in MCF-7 cells**

Cells were grown to 80% confluence in α MEM containing 10 μ M methionine. PLP, AdoMet and AdoHyc were extracted from the cells and levels quantified by HPLC relative to standard curves as described in Materials and Methods. All values represent the average of three independent experiments; variation is expressed as standard deviation.

Media pyridoxine (nM)	PLP (nmoles/ μ g)	AdoMet/AdoHyc	AdoMet	AdoHyc
4900 (α MEM)	0.25 ± 0.02 *	1.7	63.5 ± 4.8	37.1 ± 3.1
49	0.07 ± 0.02 *	--	-----	-----
4.9	0.05 ± 0.005 *	0.6 *	22.4 ± 1.5 *	35.3 ± 2.1 *
0.49	0.04 ± 0.005 *	0.6 *	15.6 ± 3.2 *	25.5 ± 1.6 *

* Values are different from control data obtained in α MEM, $p < 0.01$, student's t-test.

Table 2**The effect of medium pyridoxine concentration on cSHMT mRNA levels in MCF-7 cells**

All values represent the average of three independent experiments; variation is expressed as standard deviation.

Media Pyridoxine (nM)	cSHMT/GAPDH
4900 (α MEM)	1.0 $\pm$ 0.01
49	1.1 $\pm$ 0.01
4.9	1.2 $\pm$ 0.01 *
0.49	1.5 $\pm$ 0.01 *

* Values are different from control data obtained in α MEM, $p < 0.01$, student's t-test.