Role of Mitochondrial and Cytoplasmic Serine Hydroxymethyltransferase Isozymes in *de Novo* Purine Synthesis in *Saccharomyces cerevisiae*†

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ABSTRACT: One-carbon units are essential to a variety of anabolic processes which yield necessary cellular components including purines, pyrimidines, amino acids, and lipids. Serine hydroxymethyltransferase (SHMT) is the major provider of one-carbon units in the cell. The other product of this reaction is glycine. Both of these metabolites are required in *de novo* purine biosynthesis. In *Saccharomyces cerevisiae*, mitochondrial and cytoplasmic SHMT isozymes are encoded by distinct nuclear genes (*SHM1* and *SHM2*). Molecular genetic analyses have begun to define the roles of these two isozymes in folate-mediated one-carbon metabolism [McNeil, J. B., *et al.* (1996) *Genetics 142*, 371−381]. In our study, the *SHM1* and *SHM2* genes were disrupted singly and in combination to investigate the contributions of the two SHMT isozymes to the production of glycine and one-carbon units required in purine biosynthesis. Cell subfractionation experiments indicated that while only 5% of total activity was localized in the mitochondria, the specific activity in that compartment was much higher than in the cytoplasm. Growth and ^{13}C NMR experiments indicate that the two isozymes function in different directions, depending on the nutritional conditions of the cell. When yeast was grown on serine as the primary one-carbon source, the cytoplasmic isozyme was the main provider of glycine and one-carbon groups for purine synthesis. When grown on glycine, the mitochondrial SHMT was the predominant isozyme catalyzing the synthesis of serine from glycine and one-carbon units. However, when both serine and glycine were present, the mitochondrial SHMT made a significant contribution of one-carbon units, but not glycine, for purine synthesis. Finally, NMR data are presented that suggest the existence of at least two sites of *de novo* purine biosynthesis in growing yeast cells, each being fed by distinct pools of precursors.

Tetrahydrofolate (THF)[1] derivatives carrying one-carbon units at different oxidation states participate as coenzymes in many critical single-carbon transfer reactions including purine and pyrimidine biosynthesis and amino acid metabolism and methyl group biogenesis (*1*) (Figure 1). The major source of one-carbon units is serine, derived from glycolytic intermediates (*2*). The 3-carbon of serine is transferred to THF in a reversible reaction catalyzed by serine hydroxymethyltransferase (SHMT) (Figure 1, reaction 4) to generate 5,10-methylene-THF (CH_2-THF). CH_2-THF can then be oxidized to 10-formyl-THF (CHO-THF) by C_1-THF synthase (reactions 3 + 2) for incorporation in two steps of purine biosynthesis. Alternatively, it can be reduced to 5-methyl-THF (CH_3-THF) by methylene-THF reductase (reaction 7) and thus committed to methionine biosynthesis (reaction 8). In addition, CH_2-THF can be utilized for thymidylate synthesis (reaction 9). The other product of the SHMT reaction, glycine, can also serve as a source of one-carbon units (*3*−*6*). It is broken down by the mitochondrially

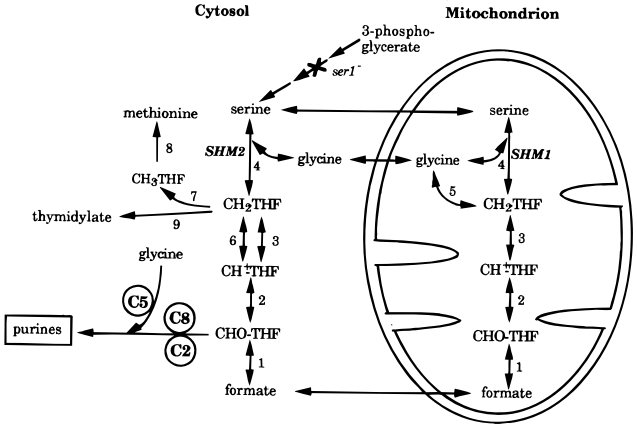

FIGURE 1: Proposed organization of the enzymes of one-carbon metabolism in *Saccharomyces cerevisiae*. Reactions 1, 2, and 3, CHO-THF synthetase (EC 6.3.4.3), CH^+-THF cyclohydrolase (EC 3.5.4.9), and NADP-dependent CH_2-THF dehydrogenase (EC 1.5.1.5), respectively, are catalyzed by cytoplasmic or mitochondrial C_1-THF synthase. Reaction 4 is SHMT (EC 2.1.2.1), present in both compartments. Reaction 5 is the glycine cleavage system (GCV) (EC 2.1.2.10). Reaction 6 is the monofunctional NAD-dependent CH_2-THF dehydrogenase. Reaction 7 is CH_2-THF reductase (EC 1.5.1.20). Reaction 8 is methionine synthase (EC 2.1.1.14). Reaction 9 is thymidylate synthase (EC 2.1.1.45). Carbons 2 and 8 of the purine backbone are derived from CHO-THF, and carbon 5 is derived from glycine.

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[1] Abbreviations: THF, tetrahydrofolate; CH_2-THF, 5,10-methylene-THF; CHO-THF, 10-formyl-THF; CH_3-THF, 5-methyl-THF; SHMT, serine hydroxymethyltransferase; cSHMT, cytoplasmic SHMT; mSHMT, mitochondrial SHMT; GCV, glycine cleavage system; PCR, polymerase chain reaction; BSA, bovine serum albumin; A-H8, adenine hydrogen 8; A-H2, adenine hydrogen 2; G-H8, guanine hydrogen 8; SDS, sodium dodecyl sulfate.

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Table 1: *Saccharomyces cerevisiae* Strains

strain	genotype	relevant phenotype
DAY4	*ser1 ura3-52 trp1 leu2 his4 SHM1 SHM2*	wild-type
EKY1	*ser1 ura3-52 trp1 leu2 his4 shm1::URA3 SHM2*	mSHMT$^-$
EKY2	*ser1 ura3-52 trp1 leu2 his4 SHM1 shm2::LEU2*	cSHMT$^-$
EKY3	*ser1 ura3-52 trp1 leu2 his4 shm1::URA3 shm2::LEU2*	mSHMT$^-$cSHMT$^-$
W3031B	*SER1 ura3-1 trp1-1 ade2-1 his3-11,-15 leu2-3,-112 can1-100 SHM1 SHM2*	wild-type
YMO9	*SER1 ura3-1 trp1-1 ade2-1 his3-11,-15 leu2-3,-112 can1-100 shm1::HIS3 shm2::LEU2*	mSHMT$^-$cSHMT$^-$

localized glycine cleavage system (GCV) (reaction 5), producing CH_2-THF from the 2-carbon of glycine. Formate can also serve as a one-carbon donor, entering the active pool at the level of CHO-THF (reaction 1) (*6*, *7*).

Transport of one-carbon units between mitochondria and cytoplasm occurs via one-carbon donors such as serine, glycine, or formate (*6*, *8*−*10*) since THF derivatives do not cross the mitochondrial membrane to any significant extent (*11*−*13*). Consistent with these metabolite transport constraints, eukaryotic cells contain distinct SHMT isozymes in both the cytoplasm and mitochondria (*2*, *14*−*16*). The existence of isozymes in separate compartments and the reversible nature of the reaction raises questions regarding the roles of each isozyme in providing serine, glycine, and one-carbon units for the cell. For example, mammalian cells with active cSHMT, but lacking mSHMT, are glycine auxotrophs (*14*), implicating mitochondrial SHMT as the primary route of glycine synthesis. The yeast *Saccharomyces cerevisiae* provides a particularly powerful system to study the metabolic roles of these enzymes. Both nuclear genes encoding the two SHMT isozymes have been characterized and mutants have been created by gene disruption at each locus (*16*). Surprisingly, yeast mutants with both SHMT genes inactivated do not require glycine for normal growth (*16*), indicating the existence of an alternative pathway for glycine synthesis. Mutation at a third gene, designated *GLY1*, was required to render the double SHMT-disrupted strain completely auxotrophic for glycine. The *GLY1* gene was recently shown to encode a protein with threonine aldolase activity (*17*, *18*), suggesting threonine as the source of glycine in this pathway. The *GLY1*-dependent pathway, rather than SHMT, appears to be the primary glycine source in yeast (*16*).

Genetic analysis of yeast strains harboring deletions of one or both of the *SHM* genes suggested that mSHMT and GCV are coupled to produce serine from glycine, whereas cSHMT is important in maintaining adequate one-carbon pools in the cytoplasm (*19*). In the present study, we have combined molecular genetics and NMR to examine the roles of the mitochondrial and cytoplasmic SHMT isozymes in the provision of glycine and one-carbon units for purine synthesis. To provide a quantitative description of the contribution of each SHMT isozyme, [2-^{13}C]glycine was used as the main one-carbon source and label incorporation into purines was traced by ^{13}C and ^{1}H NMR. Unlabeled serine was used as a competing source of one-carbon units and glycine through the SHMT reaction. Our results reveal that the role of each isozyme depends on the growth conditions and nutritional requirements of the cells, but in general the two isozymes operate in opposite directions. In addition, analysis of the proton NMR spectra of newly synthesized purines suggests the existence of multiple *de novo* purine synthesizing systems in growing yeast.

EXPERIMENTAL PROCEDURES

Materials. Common reagents were high grade commercial products. [2-^{13}C]Glycine was purchased from Cambridge Isotope Laboratories (Andover, MA). [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from NEN (Boston, MA), and [3-^{14}C]serine was purchased from Amersham (Arlington Heights, IL). (6*R*,*S*)-Tetrahydrofolate was prepared as described previously (*20*, *21*). Oligonucleotide primers were synthesized and purified by Integrated DNA Technologies (Coralville, IA). DIFCO growth media were purchased from Baxter (McGraw Park, IL).

Strains, Plasmids, and Media. Escherichia coli strain XL1-B {endA1, hsdR17 (r_{k-}, m_{k+}), supE44, thi-1, λ-, recA1, gyrA96, relA1, (lac-), [F′, pro′AB, lacI^{q}ZΔM15, Tn10, (tet^r)]} was used as the recipient for the plasmids used in all cloning experiments. *S. cerevisiae* strains DAY4 (*22*) and W3031B and YM09 (*16*) have been described previously. Yeast strains EKY1, EKY2, and EKY3 were constructed by creating disruptions at the *SHM1* and *SHM2* loci using DAY4 as the parent (see below). A description of all the strains used is found in Table 1. Clone pSH36, containing a complete copy of the *SHM1* gene, and clone p3BPS, containing a complete copy of the *SHM2* gene, as well as yeast strains containing disruptions at these loci were kindly provided by A. L. Bognar (University of Toronto).

E. coli strains containing plasmids were grown in 2YT medium (0.16% yeast extract, 0.1% tryptone, and 0.05% NaCl) at 37 °C supplemented with 50 μg/mL ampicillin. Yeast strains were grown on YPD (rich) media (1% yeast extract, 2% bacto-peptone, and 2% glucose) or on synthetic minimal media (0.7% yeast nitrogen base without amino acids, 2% glucose) supplemented with the following nutrients when appropriate (final concentration in mg/L): serine (94, 187, or 375), leucine (30), histidine (20), tryptophan (20), glycine (100), uracil (20), and formate (1000).

Disruption of the SHM1 and SHM2 Loci. A 1.7 kbp insert containing an intact copy of *SHM1* was isolated by PCR on yeast genomic DNA from strain W3031B (*16*) and subcloned in Bluescript vector which lacked an *Eco*RI site in the multiple cloning region. A 100 bp *Nde*I−*Eco*RI fragment was deleted from the middle of *SHM1*, and a 1.1 kbp *Hin*dIII−*Eco*RI fragment containing the *URA3* gene was inserted. A 3 kbp linear fragment containing the *shm1::URA3* disruption was obtained by digestion of the vector with *Pvu*II and used to transform strain DAY4 to uracil prototrophy. The resulting strain was designated EKY1. For disruption of *SHM2*, a 3.5 kbp fragment containing *shm2::LEU2* was amplified by PCR on yeast genomic DNA from strain YMO9 (*16*) and subcloned into the *Eco*RV site of Bluescript vector. *Pvu*II digestion of the vector produced a 4 kbp linear fragment containing *shm2::LEU2* and was used to transform strain DAY4 (producing strain EKY2) or strain EKY1 (producing strain EKY3) to leucine prototrophy.

Transformation, Plasmid Isolation, DNA Modification, Southern Analysis. Transformations in *E. coli* (*23*) and yeast (*24*) were done as described previously. Plasmid isolations were done by a modification of the alkaline lysis method (*25*). Restriction endonuclease digestions and ligations were carried out according to the manufacturer's instructions. Yeast genomic DNA was isolated according to Sherman *et al.* (*26*). DNA blot analysis followed methods described in the Stratagene catalog. Typically, 2−3 μg of genomic DNA from strains DAY4, EKY1, EKY2, and EKY3 was digested with appropriate restriction enzymes for 2 h. DNA fragments were resolved on an agarose gel and blotted onto a Duralon membrane (Stratagene) using a positive pressure system. DNA restriction fragments used as probes were isolated from agarose gels, purified using the Qiaex kit (Qiagen), radiolabeled by the random hexamer method (*27*) with [α-^{32}P]-dCTP using Klenow fragment of *E. coli* DNA polymerase I, and purified from unincorporated nucleotides by passing through a small Sephadex G-50 column. The blot was prehybridized at 42 °C for 1−2 h and hybridized with a radiolabeled probe for 12−18 h at 42 °C. The filter was washed once at room temperature in 1× SSC (0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 1% SDS for 10 min, and then once at 65 °C in 0.1× SSC and 0.1% SDS for 15 min. The blot was exposed to X-ray film overnight at −70 °C with intensifying screens.

Isolation of Mitochondria from Yeast. Mitochondria were isolated as described by Daum *et al.* (*28*). Briefly, yeast were grown aerobically in 1 L of media containing 3 g of yeast extract, 1 g of glucose, 22.5 mL of 85% (w/v) lactic acid , 1 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 0.6 g of $MgSO_4$, and 0.3 mL of 1% $FeCl_3$. The final pH was adjusted to 5.5 with NaOH. Cells were harvested at mid-log and converted to spheroplasts using lyticase (Sigma). Spheroplasts were resuspended in SEM [250 mM sucrose/1 mM EDTA/10 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2] containing 0.2% bovine serum albumin and 1 mM phenylmethanesulfonyl fluoride and homogenized using a tight-fitting Teflon homogenizer. Cell debris was pelleted by centrifugation at 1900*g*, and the supernatant was taken as the whole cell extract. Mitochondria were pelleted at 10000*g* and the supernatant was taken as the cytosolic extract. Mitochondria were lysed by resuspending in SEM containing 0.5% Triton X-100 and assayed immediately.

Enzyme Assays. SHMT activity was measured as previously described (*29*). A typical assay contained 63 mM sodium phosphate (pH 7.4), 10 mM 2-mercaptoethanol, 1.9 mM (6*R*,*S*)-tetrahydrofolate, 0.23 mM pyridoxal phosphate, and 0.27 mM [3-^{14}C]serine (1000 dpm/nmol) and was incubated at 37 °C for 15 min. The radiolabeled 5,10-methylene-THF produced is in equilibrium with formaldehyde. Addition of dimedone traps the formaldehyde into a complex which was extracted in toluene and counted by liquid scintillation. Protein concentration was determined by the method of Bradford (*30*), and specific activities were calculated.

Preparation of extracts for NMR analysis. Yeast (500 mL) was grown in synthetic minimal media as described above, but containing 100 mg/L [2-^{13}C]glycine. Unlabeled serine at 94 or 187 mg/L was used as a competing substrate in some experiments. The cells were pelleted and the purine bases were released by resuspending in 30 mL of 0.3 N HCl and heating over a steam bath for 6−7 h (to reduce the volume to ∼5 mL). The supernatant was evaporated to dryness in a Rotavapor-R evaporator. For ^{13}C NMR, the residue was resuspended in 1−2 mL of deuterated dimethyl sulfoxide. ^{13}C NMR spectra were obtained on a Bruker AMX500 equipped with a 5 mm probe at 125 MHz. A pulse width corresponding to a 90° flip angle was used, and data were collected during a 1.0 s acquisition time with a 4.5 s delay and continuous broad-band proton decoupling. A total of 1200 scans of 32 K data points was acquired over a sweep width of 31 250 Hz. For integration purposes, a line broadening of 2 Hz was used. For ^{1}H NMR, the residue was resuspended in H_2O, neutralized to pH 4.0, applied over a 1−2 cm Chelex-100 column (Sigma), and eluted with 50 mM sodium acetate/acetic acid, pH 4, buffer, and fractions containing A_{260} greater than 4 were collected. The sample was evaporated and resuspended in D_2O twice, and its pH was adjusted to 0.5 using concentrated D_2SO_4. ^{1}H NMR spectra were obtained on Bruker 500 MHz or Varian Unity Plus 500 MHz spectrometers equipped with 5 mm probes. A pulse width corresponding to a 90° flip angle was used, and data were collected during a 3 s acquisition time with a 4 s delay during which the residual water signal was presaturated to avoid dynamic range problems. Typically, 200−500 scans of 54K data points were acquired over a spectral width of 5000 Hz (10 ppm). A total of 260K points was used for Fourier transform. An exponential line broadening of 0.2 Hz was used to improve the signal-to-noise ratio. Chemical shifts were determined using the dioxane signal (3.64 ppm) as an internal standard. Assignment of the purine resonances was based on natural abundance spectra of yeast extracts to which 5 mg of adenine or guanine were added. An additional comment concerning the ^{1}H NMR spectra should be made. It was observed that the resonances of A-H8 (mainly) but also of A-H2 and G-H8 shifted, in some cases considerably, from one spectrum to another. The shift was caused by differences in the ionic strength of the samples due to different volumes of the eluate collected from the Chelex column. This was demonstrated by obtaining ^{1}H NMR spectra of adenine and guanine samples that were identical with regard to the purine concentration (5 mg/mL) and pH (0.5) but varied in terms of the sodium acetate concentration. As the sodium acetate concentration increased from 0.5 to 2.0 M, the A-H8 resonance shifted from 8.78 to 8.66 ppm. The G-H8 and A-H2 resonances also shifted, though only about 0.011 ppm. These shifts did not interfere with quantitation of the resonances.

RESULTS

Three new yeast strains (EKY1, EKY2, and EKY3) were constructed by gene disruption of the parental strain (DAY4) at one or both of the *SHM* loci (Table 1). These strains are all isogenic with each other, except at the *SHM* loci, and all carry the *ser1* mutation. The *ser1* mutation blocks the synthesis of serine from glycolytic intermediates (*31*), forcing these cells to use one or both of the SHMT isozymes to satisfy serine requirements. High levels of glycine (5× glycine; 100 mg/L) can provide both the two-carbon unit and the 5,10-methylene-THF (via GCV) required for serine synthesis via SHMT (*4*, *6*). These four strains were analyzed for enzyme levels, growth characteristics, and metabolic flux by NMR.

Table 2: Cellular Localization of Serine Hydroxymethyltransferase Activity in Wild-Type and Disrupted Strains

strain[a]	fraction	specific activity[b]	total activity[c]
DAY4 (*SHM1 SHM2*)	mitochondria	10.4	20
wild-type	cytosol	4.5	410
EKY1 (*shm1::URA3 SHM2*)	mitochondria	0.08	0.25
lacking mitochondrial SHMT	cytosol	3.8	378
EKY2 (*SHM1 shm2::LEU2*)	mitochondria	8.4	18
lacking cytoplasmic SHMT	cytosol	0.02	2.9
EKY3 (*shm1::URA3 shm2::LEU2*)	mitochondria	0.02	0.06
lacking both SHMT isozymes	cytosol	0.01	1.2

[a] All strains were grown in lactic acid media as described in Experimental Procedures. [b] nmol of product/mg of protein/min. [c] nmol of product/min.

Table 3: Growth Rates of Wild-Type and Mutant Strains

strain (supplement)	serine[a]	serine + formate	5× glycine	5× glycine + formate
		doubling time (h)[b]		
DAY4 (mSHMT$^+$ cSHMT$^+$)	2.8	2.7	8.5	2.1
EKY1 (mSHMT$^-$ cSHMT$^+$)	2	2.1	24	5
EKY2 (mSHMT$^+$ cSHMT$^-$)	6	2.4	7	2.8
EKY3 (mSHMT$^-$ cSHMT$^-$)	21	2.7	>100	>100

[a] Cells were grown in minimal dextrose media supplemented with leucine, histidine, tryptophan, uracil, and the indicated one-carbon donors at the following concentrations: serine (375 mg/L); formate (1000 mg/L); 5× glycine (100 mg/L). [b] Growth experiments were performed 2−3 times for each strain/condition. Doubling times varied less than 20%.

Enzyme Localization. Mitochondrial and cytosolic fractions from strains DAY4, EKY1, EKY2, and EKY3 were isolated and assayed for SHMT activity as described in the Experimental Procedures. The results are shown in Table 2. Strain DAY4 (wild-type) had significant levels of SHMT activity in both compartments, with the specific activity in the mitochondria being twice that in the cytoplasm. However, only 5% of the total activity was mitochondrial. Strain EKY1 (*shm1::URA3*) had wild-type levels of activity in the cytoplasm, but the specific activity in the mitochondria was less than 1% of wild-type. Conversely, strain EKY2 (*shm2::LEU2*) had activity levels comparable to wild-type in the mitochondria but the cytoplasmic specific activity was less than 1% of normal. Loss of one isozyme did not significantly affect the activity level of the remaining isozyme. In the double mutant (EKY3), SHMT activity was barely detectable above background in either fraction. These assays confirm the specificity of the gene disruptions of *SHM1* and *SHM2* and are consistent with previous studies (*3*, *16*).

Growth Studies with Various One-Carbon Donors. Table 3 summarizes the growth rates of the wild-type and mutant strains. All four strains grew with a 2 h doubling time in rich media (YPD, data not shown). The parental strain DAY4 (harboring an active SHMT in both compartments) and EKY1 (lacking mSHMT) grew with doubling times of 2−3 h in minimal media supplemented with serine. In contrast, both the cytoplasmic mutant (EKY2) and the double mutant (EKY3) grew much more slowly on serine and required formate to achieve wild-type growth rates. All four strains grew slower on high glycine (5× glycine) than serine. The cytoplasmic mutant (EKY2) grew at about the same rate as the parental strain (DAY4), whereas the mitochondrial mutant (EKY1) grew much slower. The double mutant (EKY3) did not grow on 5× glycine. Addition of formate to the 5× glycine media stimulated the growth of all of the strains except EKY3. The slow growth of cells on 5× glycine could also be rescued, in certain cases, by adenine as well as formate (data not shown). For example, adenine completely rescued the growth of DAY4, but only partially rescued the growth of EKY1 (from 24 to 9 h doubling time).

NMR Studies. The wild-type (DAY4) and the mutant (EKY1, EKY2, and EKY3) strains were grown to late log phase in minimal media supplemented with the appropriate amino acids and 100 mg/L of [2-^{13}C]glycine as a source of one-carbon units. In certain cases, unlabeled serine was added in addition to labeled glycine, as a competing source of one-carbon units. The competition experiments were used to determine the contributions of the two SHMT isozymes in providing glycine and one-carbon units for purine biosynthesis. Purine bases were extracted and samples prepared for ^{13}C NMR as described in the Experimental Procedures.

Yeast cells grown on [2-^{13}C]glycine as their only source of one-carbon units incorporate label at the C2, C5, and C8 positions of the purine backbone (*32*). The 2-carbon of labeled glycine is converted to labeled CH_2-THF in the mitochondria (by GCV) and exits into the cytoplasm as labeled serine (C3) (via mitochondrial SHMT) or labeled formate (via mitochondrial C_1-THF synthase). Serine and formate are ultimately converted to labeled CHO-THF (via cytoplasmic SHMT and C_1-THF synthase) which is incorporated at the C2 and the C8 positions of the purine ring in the *de novo* pathway. Labeled glycine is also incorporated as an intact molecule at an earlier step of purine biosynthesis, resulting in introduction of ^{13}C at C5 of the purine ring. The top two panels of Figure 2 show ^{13}C NMR spectra of the wild-type strain (DAY4) with and without unlabeled serine as a competing source of one-carbon units. The resonances of carbons 2, 5, and 8 of adenine and carbon 8 of guanine are indicated. SHMT mutant strains (EKY1, EKY2, and EKY3) grown on [2-^{13}C]glycine alone exhibited spectra similar to that of DAY4 (data not shown). As expected, in the wild-type strain, unlabeled serine diluted the ^{13}C label at all three positions, but especially at C2 and C8. In the two single mutants, EKY1 and EKY2, unlabeled serine did not compete as well as in the wild-type strain (Figure 2). Competition by serine was even less in the double mutant EKY3.

Intensities of the three resonances provide a qualitative picture of the relative contributions of the two SHMT isozymes to purine biosynthesis. However, a full quantitative description requires determination of the *absolute* enrichment of both C5 and C8 (or C2) of the purine ring. Absolute enrichments cannot be measured without knowing the size of the unlabeled purine pool, which cannot be observed by ^{13}C NMR. The size of this unlabeled pool can differ substantially from strain to strain and condition to condition. To overcome this limitation, ^{1}H NMR was used. All molecules can be detected by ^{1}H NMR, regardless of whether they contain ^{13}C-labeled carbons. Thus, the hydrogens on C8 (A-H8) and C2 (A-H2) of adenine and C8 of guanine (G-H8) can be clearly seen in ^{1}H NMR spectra of samples prepared as described in the Experimental Procedures. The resonances of these protons are singlets for molecules with all ^{12}C carbons, but they split to doublets by strong carbon−proton spin−spin interaction if the protons are attached to ^{13}C. Moreover, each component of the doublet is split into

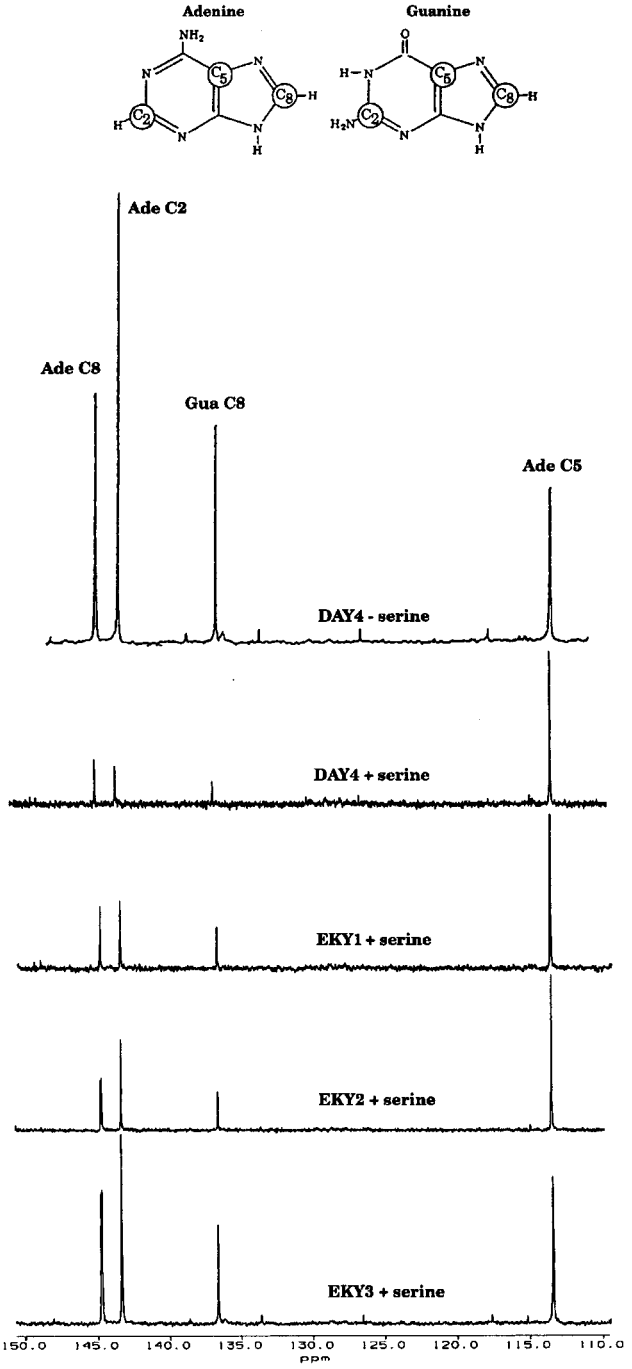

FIGURE 2: ^{13}C NMR spectra of purine resonances in cell extracts of wild-type and SHMT mutant strains grown with [2-^{13}C]glycine as the main one-carbon source. DAY4 is the wild-type, EKY1 lacks mSHMT, EKY2 lacks cSHMT, and EKY3 is the double mutant. The cells were grown in minimal media containing 100 mg/L [2-^{13}C]glycine with or without 187 mg/L unlabeled serine as indicated. The C2, C5, and C8 resonances of adenine and the C8 resonance of guanine are indicated. Spectra are normalized to the height of the A5 resonance in each case.

a second doublet if another carbon (in this case C5) in the same molecule is ^{13}C. Thus, a characteristic splitting pattern exists for each isotopomer. Label incorporation into C2 was equivalent to that into C8 in all cases, indicating that both carbons were derived from the same pool of CHO-THF (see below). Thus, for the purpose of evaluating the glycine and CHO-THF pools, only the labeling at C5 and C8 are considered in the following discussion. There are four possible purine isotopomers under these conditions: molecules containing ^{13}C at both C5 and C8; molecules containing ^{13}C at only one or the other of these two positions; or molecules containing ^{13}C at neither position. For a mixture of isotopomers (as in samples of cells grown on a labeled precursor), the observed ^{1}H spectrum will be the superposition of the individual spectra of each isotopomer present and the size of each resonance can be used to determine the relative proportion of each isotopomer to the total population.

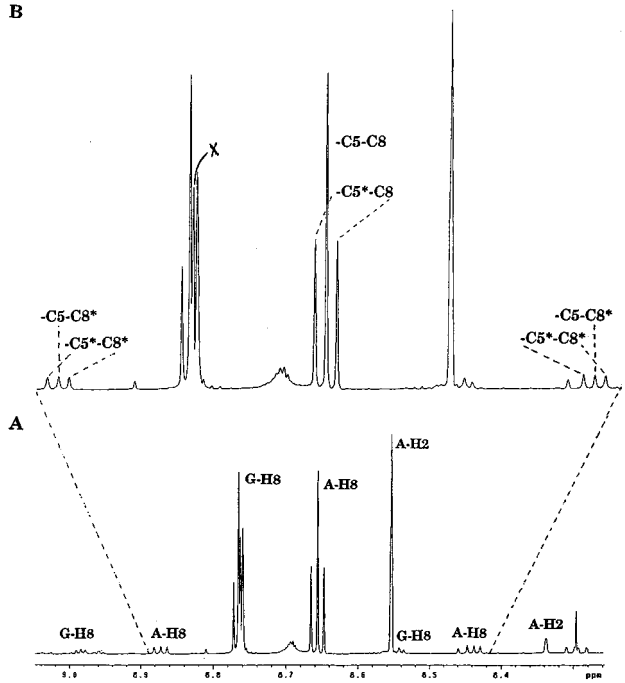

FIGURE 3: ^{1}H NMR spectrum of extract from strain DAY4 (wild-type). (A) Growth on 100 mg/L [2-^{13}C]glycine and 94 mg/L unlabeled serine. Spectra from growth on glycine alone or with the addition of 187 mg/L unlabeled serine were also obtained but are not shown. The resonances of adenine hydrogens 2 and 8 (A-H2, A-H8) and guanine hydrogen 8 (G-H8) are indicated. (B) Expanded spectrum illustrating proton-carbon coupling. Splitting of the A-H8 resonance due to coupling to C5 or C8 is indicated. An asterisk (*) indicates the presence of ^{13}C label. X indicates a non-guanine resonance.

Figure 3 shows the ^{1}H NMR spectrum for an acid extract of strain DAY4 grown in 100 mg/L [2-^{13}C]glycine. The A-H8, A-H2, and G-H8 resonances are indicated. The central A-H8 resonance at 8.66 ppm represents adenine molecules having ^{12}C at both the 8- and 5-positions. The doublet at 8.65 and 8.67 ppm represents molecules with ^{13}C at the 5-position only (adenine C5-H8 coupling constant 9.0 Hz). The doublet at 8.876 and 8.438 ppm represents molecules with ^{13}C at the 8-position only (C8-H8 coupling constant 219 Hz). The doublet of doublets at 8.882, 8.864, 8.448, and 8.430 ppm represents molecules with ^{13}C at both the 5- and 8-positions. The same pattern is seen for guanine but with slightly different coupling constants (guanine C8-H8 222 Hz; C5-H8 6.4 Hz). For the proton on the adenine C2 (A-H2), only coupling to C2 can be resolved (217 Hz) as the H2-C5 coupling constant is too small (*ca.* 1.3 Hz). Each multiplet (or singlet) in a spectrum can be separately integrated providing complete information about the isotopomer composition of the purine pool. Adenine and guanine, which are both derived from the same compound (IMP) in purine biosynthesis, showed the same pattern of labeling, further validating the quantitation method. Although the A-H2 resonance cannot be used to discriminate C5-labeled from unlabeled molecules (due to the small H2-C5 coupling

Table 4: Incorporation of ^{13}C into Purines from [2-^{13}C]Glycine[a]

	fractional isotopomer distributions				absolute enrichments	
strain/addition	A8A5	A8A5*	A8*A5	A8*A5*	A8*	A5*
DAY4 (m+ c+)[b]						
no serine	0.11 (0.09)	0.41 (0.43)	0.06 (0.08)	0.42 (0.40)	0.48	0.83
1/4× serine	0.41 (0.39)	0.41 (0.43)	0.06 (0.08)	0.12 (0.10)	0.18	0.53
1/2× serine	0.74 (0.74)	0.26 (0.26)	0.00 (0.00)	0.00 (0.00)	0.00	0.26
EKY1 (m− c+)						
no serine	0.19 (0.17)	0.48 (0.50)	0.07 (0.09)	0.26 (0.24)	0.33	0.74
1/4× serine	0.57 (0.57)	0.29 (0.30)	0.07 (0.08)	0.06 (0.05)	0.13	0.35
1/2× serine	0.71 (0.71)	0.22 (0.22)	0.05 (0.05)	0.02 (0.02)	0.07	0.24
EKY2 (m+ c−)						
no serine	0.26 (**0.18**)	0.35 (**0.43**)	0.04 (**0.12**)	0.35 (**0.27**)	0.39	0.70
1/4× serine	0.38 (0.37)	0.46 (0.48)	0.04 (0.06)	0.11 (0.09)	0.15	0.57
1/2× serine	0.59 (0.58)	0.35 (0.36)	0.03 (0.04)	0.03 (0.02)	0.06	0.38
EKY3 (m− c−)						
1/4× serine	0.21 (**0.15**)	0.34 (**0.41**)	0.04 (**0.11**)	0.40 (**0.33**)	0.44	0.74
1/2× serine	0.45 (**0.36**)	0.25 (**0.34**)	0.07 (**0.16**)	0.23 (**0.14**)	0.30	0.48

[a] Cells were grown in minimal medium containing 100 mg/L [2-^{13}C]glycine plus either no unlabeled serine, 1/4× serine (94 mg/L), or 1/2× serine (187 mg/L). The asterisk (*) indicates ^{13}C at that position. Values in parentheses are the theoretical distributions calculated using the model described in the Discussion. [b] m± c± represents presence or absence of mitochondrial (m) or cytoplasmic (c) SHMT.

constant), it could be used to measure the absolute enrichment of C2. In all cases, the C2 enrichment was in close agreement to the C8 enrichment, indicating that both carbons were derived from the same pool of CHO-THF.

The absolute enrichment values determined from the ^1H NMR spectra for all the strains grown in [2-^{13}C]glycine as a one-carbon source are shown in Table 4. In the wild-type strain (DAY4), nearly 85% of the C5 atoms (A8A5* + A8*A5*) and about 50% of the C8 atoms (A8*A5 + A8*A5*) were labeled when grown in glycine alone. EKY1 and EKY2 showed 70−75% enrichments at C5 and 30−40% enrichments at C8. EKY3 (lacking both SHMT isozymes) did not grow on glycine as the sole source of one-carbon units (Table 3).

Competition by Unlabeled Serine. Unlabeled serine was added as a competing source of one-carbon units. Two different amounts of serine were used, representing $^1/_4$ the normal level of serine (94 mg/L) and $^1/_2$ the normal level of serine (187 mg/L). The degree of dilution of label at the C8 and C5 positions should reflect the contribution of each SHMT isozyme in providing one-carbon units and glycine for purine biosynthesis. The effect of serine competition on the purine labeling pattern is illustrated for EKY2 in Figure 4. The A-H8, A-H2, and G-H8 signals reveal a sharp decrease in ^{13}C incorporation at C8 and C2 (compare the outer satellites in panel A vs B), whereas the enrichment at C5 (compare the central doublets) hardly changed.

The results from these competition experiments are summarized in Table 4, which shows the fractional distribution of each isotopomer as well as the absolute enrichments of C8 and C5 (last two columns). In DAY4, serine diluted label incorporation at C5 substantially. This was expected since glycine can be produced from unlabeled serine and thus compete with the exogenous labeled glycine for incorporation into purines. Label incorporation at C8 was diluted even greater (completely by 1/2× serine) due to the production of unlabeled one-carbon units from serine. In strain EKY1 (lacking mSHMT), dilution of label at C5 was at least as great as in the wild-type strain, indicating the contribution of cSHMT in glycine production for purines. Dilution of label at C8 was almost complete with 1/2× serine. Strain EKY2, on the other hand, showed much less dilution of label

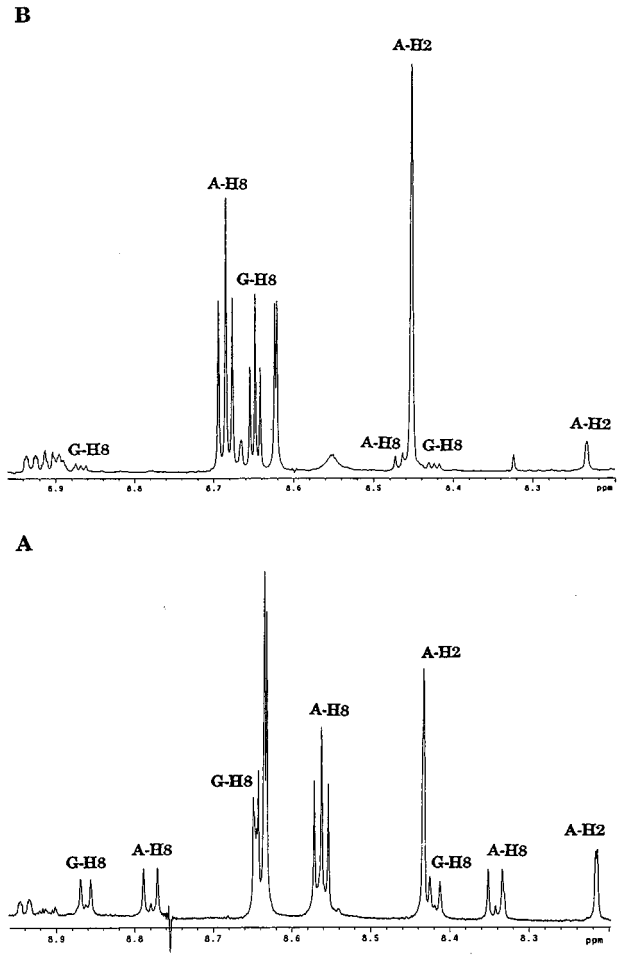

FIGURE 4: ^1H NMR spectra of extracts from strain EKY2 (lacking cSHMT). The cells were grown on 100 mg/L [2-^{13}C]glycine with the addition of (A) no serine and (B) 94 mg/L serine. The A-H2, A-H8, and G-H8 resonances are indicated.

at C5 than did EKY1, whereas dilution of label at C8 was similar to that seen for EKY1. EKY3 grown with 1/4× serine exhibited essentially the same enrichment of C5 and C8 as the other three strains grown with no serine; 1/2× serine resulted in a small, but significant dilution at both positions. Because EKY3 cannot be grown without serine in the medium, dilution effects in this strain must be

compared against the 1/4× and 1/2× serine treatments of the other strains. However, it is obvious that serine was a very poor competitor of glycine and one-carbon units in the double mutant EKY3.

DISCUSSION

The goal of this work was to further define the roles of the two serine hydroxymethyltransferase isozymes in folate-mediated one-carbon metabolism in yeast. Toward this end, the *SHM1* and *SHM2* genes encoding the cytoplasmic and mitochondrial enzymes were disrupted singly and in combination and the resulting strains were characterized by growth and NMR studies.

The contributions of the cytoplasmic and the mitochondrial SHMT isozymes in supplying serine and one-carbon units were deduced from the growth characteristics of the four strains on different one-carbon sources. *S. cerevisiae* can utilize serine, glycine, or formate as one-carbon donors (*6*, *7*). SHMT is absolutely required for utilization of serine as a one-carbon donor, whereas glycine and formate provide one-carbon units independent of SHMT. In addition, these strains all depend on SHMT activity to synthesize serine from glycine since they all harbor the *ser1* mutation. Both compartments (mitochondria and cytoplasm) require adequate serine and one-carbon (C1) units to support normal growth rates. Glycine is probably not limiting in any of these strains due to their ability to synthesize glycine from threonine via the *GLY1*-encoded threonine aldolase (*17*, *18*). Thus, with serine as the sole exogenous one-carbon source, the normal growth of EKY1 (lacking mSHMT) (Table 3) can be explained by the ability of the active cSHMT to produce cytoplasmic one-carbon units. Glycine (from *GLY1* or cSHMT) can then move into the mitochondria where it supplies mitochondrial one-carbon units via GCV (Figure 1, reaction 5), thereby satisfying the needs of both compartments. In the double disruptant (EKY3), both compartments are deficient in C1 units, resulting in very slow growth on serine. EKY2 lacks only the cSHMT; its intermediate growth rate on serine is due to deficiency of cytoplasmic C1 units. These explanations were confirmed by the ability of formate to rescue the growth of EKY2 and EKY3 to normal rates (Table 3; serine + formate).

In minimal media supplemented with 100 mg/L glycine as sole one-carbon source, growth of even the wild-type strain DAY4 was slow (Table 3, 5× glycine). Under these conditions, where no exogenous serine is supplied, all one-carbon units must be synthesized from glycine in the mitochondria, via GCV, and used to produce mitochondrial serine (Figure 1, reactions 5 + 4), some of which must be transported out to the cytoplasm. This serine is required for protein synthesis as well as cytoplasmic one-carbon units. Thus, the cytoplasm would be expected to be deficient in serine and/or C1 units, limiting growth on 5× glycine. Loss of the mSHMT (EKY1) led to a dramatic decrease in growth rate. EKY1 cannot synthesize serine in the mitochondria and must convert glycine-derived one-carbon units to formate for transport to the cytoplasm. Although the cytoplasmic pathway of C_1-THF synthase plus cSHMT can produce serine (*33*) (Figure 1, reactions 1−4), this pathway is growth limiting (*5*, *34*, *35*) and the cytoplasm is deficient in both C1 units and serine. EKY2 (lacks cSHMT) grew at about the same rate as the parental strain, suggesting that the mSHMT plus GCV can satisfy all the mitochondrial requirements, but serine and/or C1 units remain limiting in the cytoplasm. The double mutant (EKY3) showed no growth at all in 5× glycine, indicating that this strain has no other way of producing serine from glycine.

Formate rescued fully the 5× glycine growth of DAY4 and EKY2 (Table 3), indicating that these strains were deficient in cytoplasmic C1 units but not serine. Thus, it appears that mitochondrial serine synthesis via mSHMT can satisfy cytoplasmic serine needs in the absence of cSHMT activity. In contrast, formate only partially rescued the growth of EKY1, indicating that cSHMT is not able to fully satisfy the cell's serine needs in the absence of mSHMT activity. As expected, formate had no effect on the growth of EKY3. The slow growth of cells on 5× glycine could also be rescued in certain cases by adenine. Thus, adenine can spare the bulk of the cytoplasmic one-carbon requirements but cannot satisfy either a cytoplasmic or mitochondrial serine requirement.

The poor growth on 5× glycine of the strains lacking mSHMT (EKY1 and EKY3) suggests that the mitochondrial isozyme is the main source of serine under these conditions (*ser1* mutation; glycine as sole one-carbon source). This conclusion is further supported by the fact that formate fully rescued the growth of cells expressing mSHMT (DAY4, and EKY2), but only partially rescued the growth of cells reliant on cSHMT (EKY1). The cSHMT can produce serine when the mSHMT is absent, but requires a cytoplasmic source of one-carbon units such as formate.

The situation is more complicated when the metabolic flux is in the serine-to-glycine direction. Although serine and glycine are readily transported between cytoplasm and mitochondria, transport of one-carbon folate derivatives is negligible. Previous work from our laboratory has shown that the mitochondrial pathway, including mSHMT, accounts for at least 25% of the total serine-derived one-carbon units destined for purine biosynthesis (*32*). Yet EKY1, lacking mSHMT activity, grew normally on serine (Table 3), probably due to the existence of the mitochondrial glycine cleavage system, which can produce one-carbon units even when mSHMT is absent. On the other hand, in cells lacking cSHMT, regardless of the rate of serine cleavage by the mSHMT, the growth rate of the cell will be limited by transport of one-carbon units to cytoplasm. Thus, experiments measuring growth on serine cannot accurately reflect the contribution of the mSHMT to serine catabolism.

The relative contribution of each isozyme, however, could be deduced from NMR analysis of competition between ^{13}C-glycine and unlabeled serine. Table 4 shows that serine diluted ^{13}C incorporation at C5 of the purine ring more in EKY1 (cSHMT only) than in EKY2 (mSHMT only). This reveals the prevailing role of cSHMT as a source of glycine for purine biosynthesis. Absolute enrichment at C8 shows near the same level of serine competition for both EKY1 and EKY2, indicating that when serine is available, the mSHMT can operate in the direction of serine catabolism, providing one-carbon units that exit the mitochondria as formate for cytoplasmic purine synthesis. However, the glycine produced by the mitochondrial isozyme is apparently not used efficiently for purine synthesis. One explanation is that this glycine is channeled directly from mSHMT to GCV for further oxidation. These two mitochondrial activities may be closely coupled in yeast (*19*) and other organisms

(*36*, *37*), and McNeil *et al.* (*38*) showed that the *GCV1* gene encoding the yeast GCV T-protein is strongly induced during growth with glycine.

The proton NMR data also reveal the possible existence of at least two sites of *de novo* purine biosynthesis, each being fed by distinct pools of precursors. The ability to directly measure the absolute enrichment in each position of the purine pool (by ^{1}H NMR) allows us to calculate the theoretical (statistical) distribution of each of the four possible isotopomers using a simple model. This model assumes that (i) label is incorporated independently into the C8 (C2) and C5 positions of the purine ring and (ii) that each precursor (CHO-THF and glycine) is drawn from a single cellular pool with a characteristic ^{13}C enrichment value. Thus, the absolute enrichment of adenine C5 (A5* in Table 4), equal to the sum of A8A5* + A8*A5*, represents the enrichment of the pool of glycine that labels C5. Likewise, the absolute enrichment of adenine C8 (A8* in Table 4), equal to the sum of A8*A5 + A8*A5*, represents the enrichment of the pool of CHO-THF that labels C8. From these *experimentally determined* values, we can calculate the expected fractional distribution of all four isotopomers:

$$(\text{A8*A5*} + \text{A8A5} + \text{A8*A5} + \text{A8A5*} = 1.0)$$

$$\text{A8*A5*} = (\text{A8*})(\text{A5*})$$

$$\text{A8A5} = (\text{1-A8*})(\text{1-A5*})$$

$$\text{A8*A5} = (\text{A8*})(\text{1-A5*})$$

$$\text{A8A5*} = (\text{1-A8*})(\text{A5*})$$

These calculations were done for each experiment in Table 4, and the calculated results are shown in parentheses next to the experimentally observed results. The statistically expected distribution of isotopomers corresponds closely to the experimentally observed distribution in almost every case, indicating the validity of the model. Three exceptions stand out, however: EKY2, grown with no serine competition, and the two EKY3 experiments. In these three cases, the observed distribution is quite different from the expected distribution. For example, the experimentally observed fraction of molecules labeled at C8 only (A8*A5) was one-half to one-third the expected value. It is unlikely that the experimental data are artifactual as similar results were obtained from repeated experiments and the total enrichments of C5 and C8 were always within the same range seen in the other strains. Thus, we must conclude that one of the assumptions of the model used in calculating the statistical distribution is invalid for these three cases. The first assumption is that labeling at the two positions is independent. Given the complexity of the *de novo* purine biosynthetic pathway and the fact that the two carbons are incorporated at distinct steps, it is difficult to imagine a situation where incorporation of a ^{13}C at one position would depend on incorporation of ^{13}C at the other position. The second assumption, on the other hand, is more tenuous. It states that all purine molecules are synthesized from a single pool of glycine, of some characteristic enrichment, and a single pool of CHO-THF, of some characteristic enrichment. If, however, two or more pools of either of these precursors exist which feed separate purine synthesis systems and the pools differ in their ^{13}C enrichment, then the second assumption is invalid and the calculated isotopomer distribution should no longer correspond with the observed distribution.

There is some evidence for multiple precursor pools in yeast, at least in the case of glycine. We have previously observed labeling patterns of serine (*6*) and choline (*32*) that are consistent with multiple glycine pools. There are at least three sources of glycine in our experiments, depending on the strain and growth conditions. All the cells receive [2-^{13}C]-glycine (99% enriched) in the media. This is diluted to varying degrees by endogenous unlabeled glycine produced by the SHMT reactions and by the *GLY1*-dependent pathway from threonine (*17*, *18*). Strain differences or growth conditions could easily affect the contributions of each of these to the total glycine content of the cell. For example, in EKY3 where neither SHMT is functional, the contribution from unlabeled serine is negligible and only the *GLY1*-dependent pathway can dilute the ^{13}C-glycine provided in the media.

Assuming multiple precursor pools exist, if all purines are synthesized by a single collection of enzymes, the enrichment of the product would simply reflect the average enrichment of the combined precursor pools, and the calculated distribution of isotopomers should still correspond to the observed distribution. The fact that they do not, at least in the three cases here, suggests that there may be at least two sites of purine biosynthesis, each being fed by distinct pools of precursors (glycine, CHO-THF, etc.).

While there may be other explanations for the discrepancy between observed and expected isotopomer distributions, we favor the hypothesis that *Saccharomyces* somehow compartmentalizes its *de novo* purine biosynthesis, possessing both a major system that produces most of the purines and a minor system that is revealed only when the major system is somehow restricted. How might these two systems be compartmentalized? When EKY2 and EKY3 are grown with glycine as the sole one-carbon source, the cytoplasmic one-carbon unit supply is limiting (due to lack of cSHMT), thereby restricting the major, cytoplasmic, purine biosynthesis system. On the other hand, mitochondrial one-carbon units should be abundant in these strains through the activity of GCV. If the minor purine biosynthesis system is associated with mitochondrial one-carbon units, then its relative contribution would be increased in these strains. This could also explain why addition of serine eliminates the difference between the observed and calculated isotopomer distributions in EKY2, but not in EKY3 (Table 4). Unlabeled serine could be converted into one-carbon units and glycine in EKY2 mitochondria, thereby decreasing the enrichments of the mitochondrial pools of these precursors relative to the enrichments of the cytoplasmic pools. As the differences in absolute enrichment decrease, our ability to detect two pools of purines diminishes. Unlabeled serine does not eliminate the isotopomer distribution discrepancy in EKY3, as it lacks mSHMT and cannot convert serine into unlabeled glycine and one-carbon units in either compartment. In fact, compartmentation of *de novo* purine biosynthesis has recently been reported in the root nodules of cowpea (*39*). Both plastids and mitochondria were shown to have a full complement of enzymes for *de novo* purine synthesis, whereas the cytoplasm was devoid of these activities. Similar biochemical studies have not been carried out in yeast, but it is curious that *Saccharomyces* expresses two

isozymes for the last two steps of the *de novo* pathway (*40*). The functions and cellular localization of these two isozymes is currently under investigation.

In summary, these studies suggest that, in yeast, the roles of the SHMT isozymes change as the nutritional requirements of the cell changes. Under conditions where serine is provided in the media (and presumably also when serine can be synthesized from glycolytic intermediates), the cytoplasmic isozyme is the major contributor of one-carbon units and glycine via the *breakdown* of serine. Although the mitochondrial SHMT also catabolizes serine, as shown by our previous ^{13}C NMR studies (*7*, *32*), its contribution is clearly not as important under theses conditions. However, when serine is limiting, for example during growth on glycine, the mitochondrial SHMT functions preferentially in the direction of serine *synthesis*. This observation is consistent with recent work (*19*), which suggests that mitochondrial, but not cytoplasmic, SHMT is required for glycine-mediated satisfaction of the serine auxotrophy of *ser1* yeast. It is interesting to compare these role assignments to those for the SHMT isozymes other systems. For example, based on the glycine requirement of chinese hamster ovary (CHO) cells lacking the mitochondrial SHMT, it has been proposed that the mSHMT functions mainly in the direction of serine catabolism (*41*, *42*). The role of the cSHMT in CHO cells is less clear. Narkewicz *et al.* (*42*) suggest that the cSHMT functions mainly in the direction of serine synthesis, whereas earlier data indicated that the cSHMT was more active in serine catabolism (*41*). Of course, yeast and CHO cells differ in their nutritional requirements and the activities of other reactions such as threonine aldolase and glycine cleavage. Other factors such as transport of metabolites and cofactors between compartments may also differ, making it difficult to predict which factors might contribute to differences in SHMT function from one cell type to another. However, the ability to make defined alterations in these pathways in yeast coupled with NMR analysis of the metabolism should allow us to determine the contribution of some of these factors to the regulation of SHMT function.

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