^{13}C NMR Analysis of Intercompartmental Flow of One-Carbon Units into Choline and Purines in *Saccharomyces cerevisiae*†

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ABSTRACT: In *Saccharomyces cerevisiae*, the three-carbon of serine is normally the major one-carbon donor, although glycine and formate can substitute for serine. The second carbon of glycine enters via the glycine cleavage system in the mitochondria and can satisfy all cellular one-carbon requirements. It remains unresolved, however, as to the route by which these mitochondrial one-carbon units supply cytosolic anabolic processes. In the present work, we have used yeast mutants blocked at selected sites and ^{13}C NMR to trace the incorporation of glycine-derived mitochondrial 5,10-methylenetetrahydrofolate into nonmitochondrial synthesis of choline and purines. Label incorporation into choline traces the methylation pathway of choline synthesis from production of serine to methylation of phosphatidylethanolamine. The active one-carbon unit of *S*-adenosylmethionine involved in methylation reactions originates almost solely from C3 of serine. On the other hand, flow of mitochondrial one-carbon units to 10-formyltetrahydrofolate for purine synthesis is shown to occur via both serine and formate. Formate transport accounts for at least 25% of the total, even during growth with sufficient serine to provide for the one-carbon requirements of the cell. This work shows that the synthetase function of the cytosolic C_1-tetrahydrofolate synthase plays a critical role in the processing of mitochondrial one-carbon units to 10-formyltetrahydrofolate pools. In addition, this study provides evidence of two pools of glycine within the mitochondria and establishes a system of analyzing flux into the different folate derivatives.

Folate-mediated one-carbon metabolism is central to many metabolic activities of the cell including the synthesis of purines, choline, formylmethionine-tRNA and glycine, histidine degradation, and methyl group biogenesis. In eukaryotes, the mitochondrial and cytosolic compartments each contain a parallel set of one-carbon unit interconverting enzymes [for review, see Appling (1991)]. We have proposed that one-carbon units generated in the mitochondria, from serine (via serine hydroxymethyltransferase, SHMT)[1] or glycine (via glycine cleavage enzyme system, GCV), can be oxidized to formate for transport into the cytoplasm. In the absence of serine, glycine can support growth and presumably supply all cellular one-carbon requirements (McKenzie & Jones, 1977; Ogur et al., 1977; Zelikson & Luzzati, 1977). This process involves the mitochondrial GCV, producing 5,10-methylenetetrahydrofolate (CH_2-THF) (Ogur et al., 1977). While 5-methyl-THF (CH_3-THF) and CH_2-THF do not cross the mitochondrial membrane to any significant extent (Horne et al., 1989), two routes have been proposed for the transport of mitochondrial one-carbon units to the cytosol: as the third carbon of serine (Zelikson & Luzzati, 1977) and as formate (Barlowe & Appling, 1988). Serine, synthesized by mitochondrial SHMT from CH_2-THF and glycine, exits to the cytosol where it donates its third carbon to the cytosolic one-carbon pool as described above. Alternatively, the CH_2-THF can be converted to formate by the mitochondrial isozyme of C_1-THF synthase (mC_1-THF synthase) (Barlowe & Appling, 1988; Garcia-Martinez & Appling, 1993) (Figure 1, reactions 1–4). We have previously proposed a pathway in which mitochondrial formate is transported to the cytosol and activated by the cytoplasmic C_1-THF synthase (cC_1-THF synthase) to 10-formyl-THF (CHO-THF) for *de novo* purine synthesis or other cytosolic processes (Barlowe & Appling, 1988). This pathway is supported by *in vitro* studies demonstrating production of formate from serine by rat liver mitochondria (Barlowe & Appling, 1988; Garcia-Martinez & Appling, 1993). Formate is known to cross the mitochondrial membrane (Chappell & Haarhoff, 1967; Cybulski & Fisher, 1977; Pasternack et al., 1992) and act as a major one-carbon donor in the absence of serine (Jones & Lam, 1973; McKenzie & Jones, 1977; Ogur et al., 1977). It is unknown, however, to what degree mitochondrial formate contributes to the cytosolic CHO-THF pool *in vivo*. Indeed, can one compartment provide one-carbon units for the other compartment? By what routes do they move? How much intercompartmental flow exists?

We have begun to use *in vivo* ^{13}C NMR labeling in the yeast *Saccharomyces cerevisiae* to address these questions. The approach is to selectively direct ^{13}C to the one-carbon pool of one compartment and then monitor its use in anabolic processes in another compartment (Pasternack et al., 1992).

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[1] Abbreviations: AMP, adenosine monophosphate; AICAR, 5-aminoimidazole-4-carboxamide ribotide; cC_1-THF synthase, cytosolic C_1-THF synthase; DMSO, dimethyl sulfoxide; GAR, glycinamide ribotide; GCV, glycine cleavage enzyme system; GMP, guanine monophosphate; FAICAR, 5-formaminoimidazole-4-carboxamide ribotide; FGAR, formylglycinamide ribotide; CHO-THF, 10-formyl-THF; IMP, inosine monophosphate; mC_1-THF synthase, mitochondrial C_1-THF synthase; CH_3-THF, 5-methyl-THF; CH^+-THF, 5,10-methenyl-THF; CH_2-THF, 5,10-methylene-THF; NAD CH_2-THF dehydrogenase, 5,10-methylenetetrahydrofolate dehydrogenase; NMR, nuclear magnetic resonance; NOE, nuclear Overhouser effect; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PRPP, phosphoribosyl pyrophosphate; PS, phosphatidylserine; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate.

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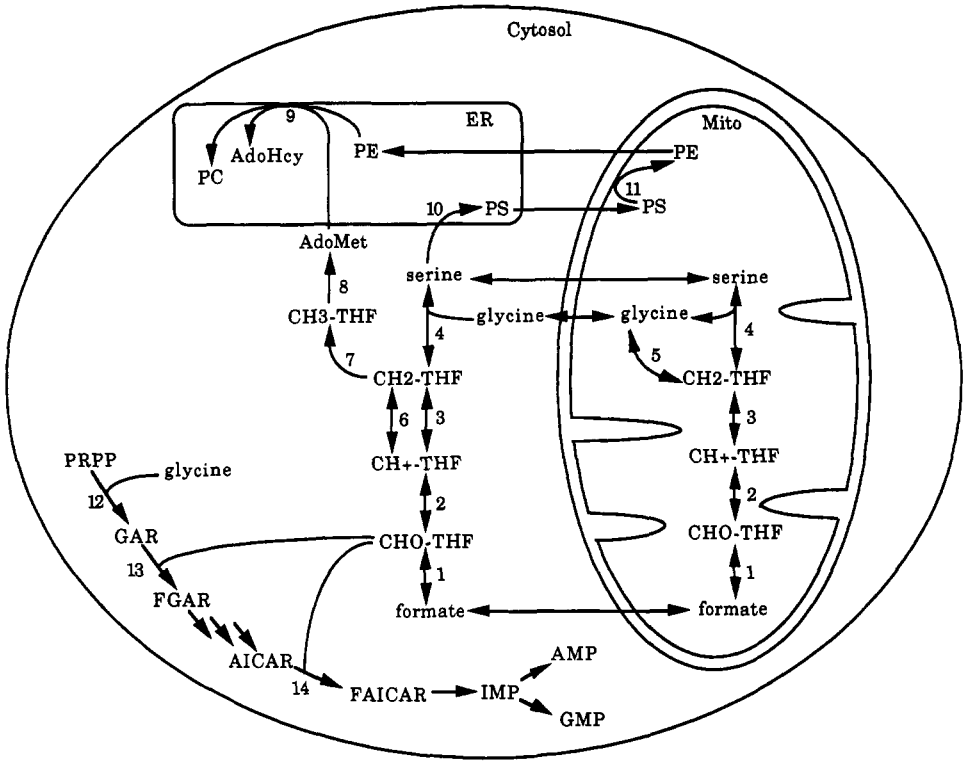

FIGURE 1: Proposed organization of the enzymes of one-carbon metabolism. Reactions 1, 2, and 3, 10-formyl-THF synthetase (EC 6.3.4.3), 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9), and 5,10-methylene-THF dehydrogenase (EC 1.5.1.5), respectively, are catalyzed by the trifunctional C_1-THF synthase. The cytoplasmic C_1-THF synthase is encoded by the *ADE3* gene (Staben & Rabinowitz, 1986). The mitochondrial C_1-THF synthase is encoded by the *MIS1* gene (Shannon & Rabinowitz, 1988). Reaction 4 is SHMT (EC 2.1.2.1), present in both compartments (Yoshida & Kikuchi, 1973; Zelikson & Luzzati, 1977). Reaction 5 is GCV (EC 2.1.2.10) and is found only in the mitochondria (Motokawa & Kikuchi, 1971; Ogur et al., 1977). 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 homocysteine methyltransferase (EC 2.1.1.14) and ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6). Reaction 9 indicates the phosphatidylethanolamine methyltransferases; reaction 10 is phosphatidylserine synthase (EC 2.7.8.8); reaction 11 is phosphatidylserine decarboxylase (EC 4.1.1.65). Localization of these enzymes is reviewed in Paltauf et al. (1992). Reactions 12, 13, and 14 are GAR synthetase (EC 6.3.4.13), GAR transformylase (EC 2.1.2.2), and AICAR transformylase (EC 2.1.2.3), respectively, and are part of the cytosolic purine synthesis system.

In order to control the point of entry of one-carbon units into the pool, strains which are *ser1*$^-$ are used. In wild-type cells, the C3 of serine synthesized from glycolytic intermediates is the major source of one-carbon units. *Ser1*$^-$ strains, however, are blocked at phosphoserine aminotransferase and require supplemental serine for growth (Jones & Fink, 1982). Glycine and formate can substitute for serine. Thus, use of *ser1*$^-$ strains allows us to introduce a one-carbon unit at the level of the synthetase of C_1-THF synthase by providing formate, SHMT by providing serine, or GCV by providing glycine.

In the present work, [2-^{13}C]glycine was chosen as the sole exogenous one-carbon source. Since glycine cleavage via GCV is strictly a mitochondrial process, [2-^{13}C]glycine provides a solely mitochondrial source of [^{13}C]CH_2-THF. Incorporation of ^{13}C-labeled one-carbon units can be detected in choline and purines, two metabolites whose synthesis depends on the one-carbon pools of compartments other than the mitochondria. Choline synthesis from glycine involves the incorporation of mitochondrial CH_2-THF during serine synthesis, conversion to phosphatidylserine (PS), decarboxylation to phosphatidylethanolamine (PE), and subsequent methylation by *S*-adenosylmethionine (AdoMet) to produce phosphatidylcholine (PC) (Figure 1). The active methyl carbon of AdoMet is derived from CH_3-THF pools and donated for choline synthesis in the endoplasmic reticulum. Purine synthesis involves 10 enzymatic steps, two of which require the incorporation of cytosolic CHO-THF. Thus comparison of the labeling pattern of these two metabolites allows direct comparison of the one-carbon pools in three structural compartments of the cell. The proposed compartmentation of the enzymes of choline and purine synthesis is summarized in Figure 1.

The experiments presented herein detect the flow of one-carbon units originating in the mitochondria to the synthesis of choline and purines in other compartments. The profile of label incorporation into choline traces the methylation pathway of choline synthesis in yeast from synthesis of serine to methylation of PE. Analysis of label incorporation at the C4 position of choline indicates that flow of mitochondrial one-carbon units for cellular methylation reactions is primarily via C3 of serine. Flow of mitochondrial one-carbon units to CHO-THF for purine synthesis is shown to occur via both serine and formate transport. Flow as formate accounts for at least 25% of cytosolic one-carbon units for purine synthesis, even during growth with sufficient serine to provide for the one-carbon requirements of the cell. In addition, we show that, as predicted by the proposed pathway, the synthetase function of the cC_1-THF synthase plays a critical role in the processing of mitochondrial one-carbon units to cytosolic CHO-THF pools for purine synthesis. This study also provides evidence of two pools of glycine within the mitochondria and establishes a method of following the flux of one-carbon units into the folate derivatives.

EXPERIMENTAL PROCEDURES

Materials and Strains. [2-^{13}C]Glycine was purchased from Cambridge Isotope Laboratories (Woburn, MA). Standards adenine, choline, glycine, and guanine were purchased from

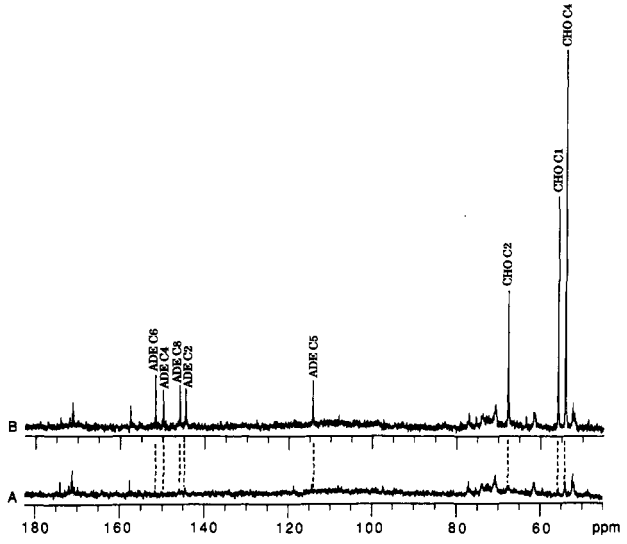

FIGURE 2: ^{13}C NMR spectra of cell extract of strain DAY4 grown in 100 mg/L unlabeled glycine and spiked with (A) nothing or (B) 16 mg/mL unlabeled choline chloride and 16 mg/mL unlabeled adenine. Chemical shifts of all relevant metabolites are listed in Table 1.

Sigma (St. Louis, MO), and L-serine was purchased from Calbiochem (La Jolla, CA). Haploid strains of *S. cerevisiae* were used in all studies. Yeast strains carrying assorted mutations in the synthases have been described and are referenced in Table 3.

Cell Growth, ^{13}C Labeling, and Extract Preparation. Yeast cultures of 1 L were grown aerobically to late log phase in yeast minimal media (Difco 0.7% yeast nitrogen base) containing 20 mg/L each of histidine, tryptophan, leucine, uracil, and 2% glucose with the addition of 100 mg/L labeled (99% [2-^{13}C]glycine) or unlabeled glycine as indicated in the figure legends. Sample preparation was by heating in 0.3 N HCl. This cleaves intracellular PC, phosphocholine, and purine nucleotides to yield choline and purine bases for analysis. Extracts were prepared as follows. The culture was centrifuged for 4 min at 4000 rpm, resuspended in 20 mL of 0.3 N HCl, warmed over a steam bath for 1 h, and centrifuged for 4 min at 4000 rpm. The pellet was reextracted in 20 mL of 0.3 N HCl, warmed over a steam bath for 1 h, and centrifuged for 4 min at 4000 rpm. The supernatant fractions were combined, evaporated in a Rotavapor-R, and dried in a Speed Vac Concentrator, and the residue was resuspended in 1.0 mL of deuterated DMSO.

NMR Analysis. NMR spectra were obtained on a Nicolet Analytical Instrument NT 360 equipped with a 5-mm probe. ^{13}C data were collected between 5-s delays at 90 MHz with a 90° flip angle pulse with continuous broad-band decoupling. A total of 2400 scans of 32K data points were acquired over a sweep width of 11 904 Hz. Data processing included line broadening of 3.0, baseline correction, and a single zero fill prior to Fourier transformation to yield the frequency domain spectrum.

Analysis of Data. Metabolites were identified by characteristic chemical shift values established from natural abundance spectra. Yeast strain DAY4 was grown with the addition of unlabeled glycine and an extract prepared as described above. The spectrum in Figure 2A serves as a control and establishes that no significant peaks exist at the resonance frequencies associated with choline, serine, adenine, or guanine. Peaks present in this spectrum result from the 1.1% naturally occurring ^{13}C of cellular metabolites normally present at high concentrations in the cell extract. Direct addition of unlabeled metabolites (1.1% natural abundance ^{13}C) allows for the identification of chemical shifts associated with the resonance of each carbon. For example, Figure 2B is the spectrum obtained from the addition of choline chloride and adenine. Table 1 lists chemical shifts of all relevant metabolites.

Table 1: ^{13}C Chemical Shifts of Metabolites[a]

metabolite	C1	C2	C3	C4	C5	C8
adenine		144.3			114.0	145.7
choline[b]	56.0	67.8		54.3		
guanine[c]		155.5			108.0	137.8
serine[b]		55.5	60.3			

[a] Chemical shifts were determined in extract of DAY4 grown in unlabeled glycine unless otherwise noted. Chemical shifts are referenced to DMSO at 39.5 ppm. [b] Chemical shifts of choline and serine may vary up to 0.8 ppm from sample to sample. [c] Chemical shifts determined in DMSO.

Percent enrichment analysis for choline C1 and C2 resonances is accomplished by deconvolution and integration using Nicolet software. The resonances for C1 and C2 of choline are a combined singlet and doublet. This indicates the presence of three labeled species with respect to the C1 and C2 position: label at the C1 position only (singlet at 56.0 ppm), label at the C2 position only (singlet at 67.8 ppm), and label at both the C1 and C2 positions (doublets surrounding each singlet). Thus each resonance is comprised of a singlet indicating the absence of a ^{13}C-labeled neighbor and a doublet indicating presence of ^{13}C label at the neighboring carbon. Percent enrichment of the neighboring carbon can be determined by dividing the integral of the doublet by the integral of the whole resonance (Malloy et al., 1990). The following equation applies:

$$FC1 = \frac{DC2}{SC2 + DC2}$$

where FC1 is the fractional enrichment of C1, SC2 is the integral of singlet at C2, and DC2 is the integral of doublet at C2.

Relative percent enrichment of adenine and choline C4 resonances is accomplished by integration with nuclear Overhouser effect (NOE) adjustment. NOE results in different signal intensity for carbons of the same compound depending on how many protons are attached and the environment of the particular carbon. Relative NOEs for the carbons within choline and adenine were determined by signal integration of natural abundance sample of the metabolite added to an extract of strain DAY4 grown in unlabeled glycine. It should be noted that the relative NOE for adenine in the extract background was found to be considerably different than the relative NOE of adenine in DMSO; thus it was critical that NOE adjustment determination be done in the actual extraction environment. A small contaminating peak on the shoulder of the methyl carbon resonance of choline introduces a potential 5% error in the calculation of relative enrichment for the C4 carbon.

RESULTS

Metabolism of Glycine to Choline. Initial studies were done with a *ser1*$^-$ yeast strain (DAY4) which is wild-type at all relevant steps of the one-carbon pathway including both isozymes of SHMT and C_1-THF synthase and GCV. Extracts prepared from DAY4 grown in the presence of [2-^{13}C]glycine (Figure 3B) reveal the presence of labeled choline. Label is apparent at all positions of choline. Figure 3C shows

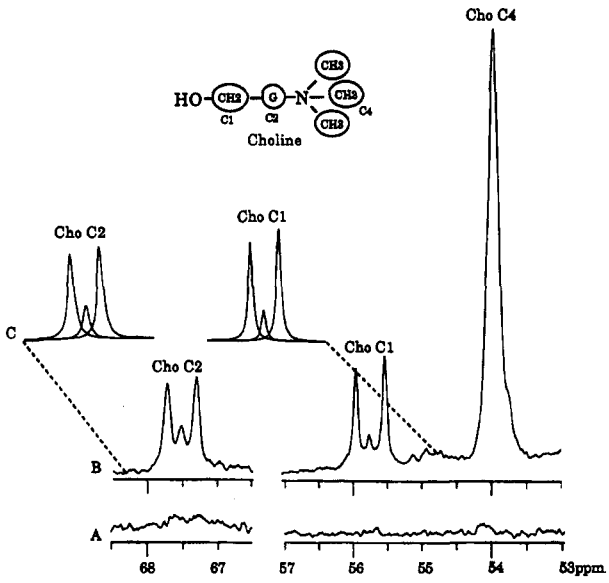

FIGURE 3: ^{13}C NMR spectra of choline resonances in cell extract of strain DAY4 grown in (A) unlabeled glycine or (B) [2-^{13}C]glycine. (C) Deconvolution and Lorenzian line fit of spectrum for integration. The choline structure indicates the source of each carbon; C1, CH_2-THF; C2, direct incorporation of glycine; C4, CH_3-THF via AdoMet.

Table 2: Effect of Exogenous Unlabeled Serine and Mutations in cC_1-THF Synthase on Enrichments in Choline and Adenine[a]

	choline			adenine
strain/grown	C1	C2	C4/C1[d]	CHO/C5[b]
DAY4	88.8 ± 0.25	89.3 ± 0.7	1.1 ± 0.08	0.92 ± 0.02
DAY4 + serine[c]	<3.0	<3.0		0.23 ± 0.07
CBY1 + serine	<3.0	<3.0		
MWY4.4	91.0 ± 4.0	87.5 ± 2.5	0.86 ± 0.08	0.88 ± 0.03

[a] Growth media is minimal media as described under Experimental Procedures with the addition of the noted nutrient. Values are means ± SEM. C1 and C2 of choline are percent enrichment, and all other values are relative enrichment. [b] Value is the average of C2/C5 and C8/C5; reflects incorporation of one-carbon unit relative to glycine backbone. [c] Average of one experiment at 178 mg/L serine and one at 375 mg/L serine. [d] Integral of C4 is divided by 3 prior to NOE adjustment in order to normalize for the presence of three methyl carbons per C1 group in each molecule.

deconvolution of the C1 and C2 resonances of choline. A percent enrichment value of 89% (Table 2) at both positions is determined from integration of the doublet and singlet associated with the resonance of a particular carbon. The doublet indicates that its neighboring carbon is ^{13}C, and the singlet indicates that its neighboring carbon is ^{12}C. Details of percent enrichment calculation are described under Experimental Procedures. That both positions are 89% enriched indicates that the mitochondrial CH_2-THF pools are enriched to the same degree as glycine pools. This suggests that glycine is the only source of CH_2-THF for serine synthesis.

Methionine and Serine Compete Glycine Metabolism to Choline. Under glycine growth conditions, SHMT uses [2-^{13}C]glycine and [^{13}C]CH_2-THF (derived from GCV cleavage of the [2-^{13}C]glycine) in the synthesis of [2,3-^{13}C]-serine. This serine goes on to PS which is decarboxylated to PE. The [2,3-^{13}C]serine also donates its C3 to the cytosolic CH_2-THF pools. Reduction of [^{13}C]CH_2-THF provides [^{13}C]-CH_3-THF for methionine synthesis and consequently the active carbon of AdoMet. AdoMet donates this labeled carbon in the methylation of PE to PC. In order to verify the origin of labeling seen in choline, label progression was competed by the provision of two metabolites along the pathway.

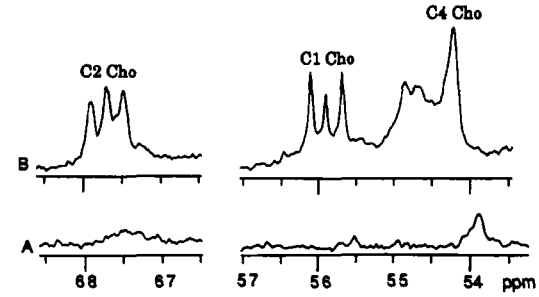

FIGURE 4: ^{13}C NMR spectra of choline resonances in cell extract of strain DAY4 grown in [2-^{13}C]glycine plus (A) 175 mg/L unlabeled serine or (B) 100 mg/L unlabeled methionine.

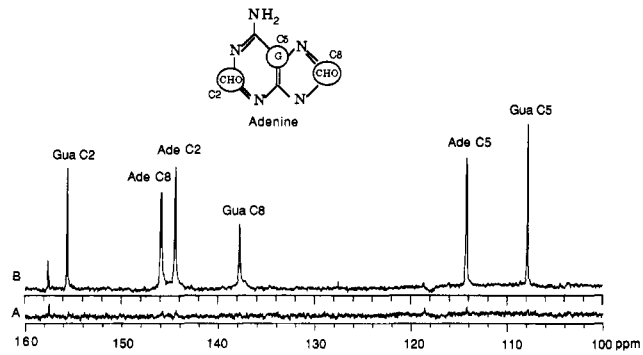

FIGURE 5: ^{13}C NMR spectra of purine resonances in cell extract of strain DAY4 grown in (A) unlabeled glycine or (B) [2-^{13}C]glycine. The purine structure indicates the source of each carbon; C2 and C8, CHO-THF; C5, direct incorporation of glycine.

DAY4 was grown in the presence of [2-^{13}C]glycine with the addition of 100 mg/L methionine (Figure 4B) or 175 mg/L serine (Figure 4A). The addition of methionine results in considerably less labeling of the methyl position C4 of choline (C4/C1 ratio = 0.25 compared with C4/C1 = 1.1 in the absence of methionine). Both the C1 and C2 positions show a slightly lower percent enrichment indicating a slight shift in preference toward use of endogenous unlabeled glycine pools instead of exogenous labeled glycine under these growth conditions. The addition of serine, however, results in loss of labeling at all positions of choline indicating either inhibition of serine synthesis or serine transport from mitochondria to cytosol. As noted in Table 1, chemical shifts of the resonances associated with choline are seen to vary up to 0.6 ppm (average chemical shift observed is 54.3) from sample to sample and were seen as low as 53.9 ppm. Thus the small resonance, approximately 3% of the C4 of choline resonance seen under growth conditions with no competing metabolite (Figure 3B), at 53.9 ppm in this spectrum is assumed to arise from labeling at the C4 position of choline. This 3% may arise from incomplete inhibition of transport of serine from the mitochondria or may indicate the degree of conversion of cytosolic CHO-THF to CH_2-THF under these growth conditions. Either way, competition by serine indicates that all positions of choline arise from carbons of serine, thus tracing the pathway from serine to PS, PE and finally PC. These observations support the methylation pathway to choline synthesis previously proposed to be the major route of synthesis in yeast (Chin & Bloch, 1988) and suggests that the flow of mitochondrial one-carbon units toward reduction to CH_3-THF occurs via the C3 of serine.

Metabolism of Glycine to Purines. Extracts prepared from DAY4 grown in the presence of [2-^{13}C]glycine (Figure 5B) also reveal the presence of labeled adenine and guanine. Label is apparent at positions C2, C8, and C5 of adenine and guanine. C2 and C8 arise from CHO-THF and C5 from direct

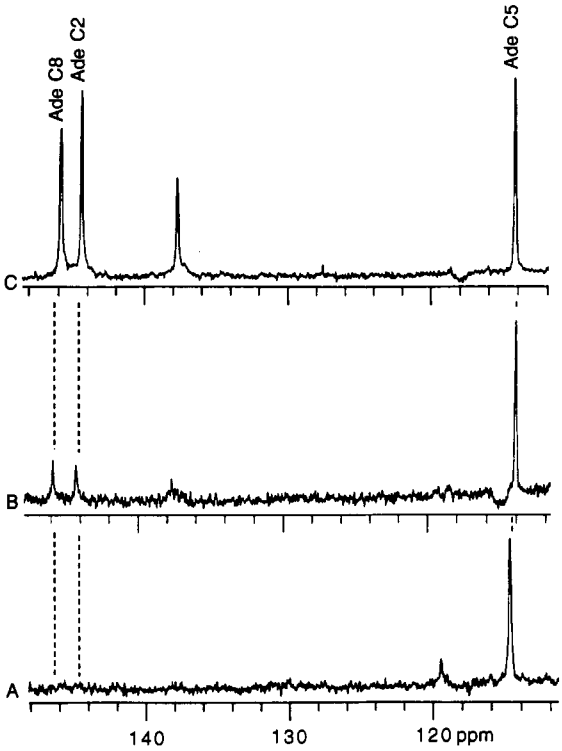

FIGURE 6: ^{13}C NMR spectra of adenine resonances in cell extracts of (A) strain CBY1 grown in 100 mg/L [2-^{13}C]glycine plus 178 mg/L serine, (B) strain DAY4 grown in 100 mg/L [2-^{13}C]glycine plus 178 mg/L serine, and (C) strain DAY4 grown in 100 mg/L [2-^{13}C]glycine alone.

incorporation of the whole glycine unit. Since synthesis of adenine and guanine diverge from a common intermediate far past the incorporation of the carbons of interest, no difference in labeling between adenine and guanine is expected. Indeed, analysis shows consistent relative enrichment patterns within adenine and guanine. Differences in the appearance of their resonances are due to differences in relative NOE of carbons within the two metabolites. Adenine was chosen for relative enrichment calculations. C2 and C8 positions show an average relative enrichment of 0.92, respectively, to the C5 position (Table 2). Note that this is not absolute percent enrichment as determined by isotopomer analysis of adjoining ^{13}C-enriched positions, but instead is strictly enrichment of the C2 and C8 (enrichment of the CHO-THF pools) relative to C5 (enrichment of C2 of glycine). Since such an internal control exists within the metabolite of interest, relative enrichment values serve our purposes as well as absolute percent enrichment.

Serine Competes Mitochondrial One-Carbon Unit Metabolism to Purines. Two routes of transport of mitochondrial one-carbon units to cytosolic CHO-THF pools for purine synthesis have been proposed, one as formate and one as the C3 of serine. To distinguish these two routes, strain DAY4 was grown on minimal media supplemented with [2-^{13}C]-glycine and 178 mg/L unlabeled serine (Figure 6B). Average relative enrichment at C2 and C8 of adenine is 0.23, which is 25% of the average 0.92 relative enrichment seen under growth conditions with no competing source of one-carbon units (Figure 6C). In order to ensure maximum competition, the serine concentration was doubled to 375 mg/L (a serine concentration that supports growth of serine auxotrophs at wild-type rates). No change in the enrichment profile of adenine was observed. Thus, at least 25% of the one-carbon units used for purine biosynthesis were derived from mitochondrial one-carbon pools, even under conditions in which

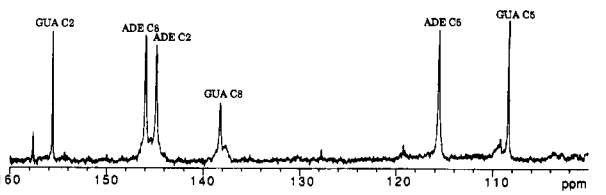

FIGURE 7: ^{13}C NMR spectra of purine resonances in cell extract of strain MWY4.4 grown in [2-^{13}C]glycine.

the cytosol had access to sufficient serine to satisfy cellular one-carbon unit requirements. Recall that, under these growth conditions, choline shows virtually no label incorporation, indicating the absence of [^{13}C]serine in the cytosol (Figure 4A). This suggests that ^{13}C at C2 and C8 of purines is derived from formate rather than serine and must be coming from the mitochondria. To confirm this pathway, strain CBY1 was grown on minimal media supplemented with [2-^{13}C]glycine and unlabeled serine (Figure 6A). Strain CBY1 is isogenic with strain DAY4 except that it lacks cC_1-THF synthase synthetase activity. Although there is good incorporation of the C2 carbon of glycine into the C5 position of adenine, C2 and C8 resonances of adenine are not labeled. In addition, strain CBY1 shows absence of labeling of choline (data not shown) as did strain DAY4 under these growth conditions (Figure 4A). This indicates either lack of serine synthesis or lack of transport of serine to the cytosol.

Two conclusions may be drawn. First, at least 25% of the CHO-THF present in the cytosol originates from mitochondrial one-carbon pools irregardless of the availability of cytosolic one-carbon units from serine. Second, the synthetase function of the cC_1-THF synthase plays a critical role in the use of these mitochondrial one-carbon units for purine synthesis.

CHO-THF for Purine Synthesis Is Derived from Mitochondrial One-Carbon Units Transported as Formate. Activation of formate by the synthetase function of the cC_1-THF synthase is one of three proposed routes to CHO-THF for purine synthesis. We have previously proposed that mitochondrial one-carbon units can be converted to formate in the mitochondria and transported to the cytosol, thus providing a route of flux for one-carbon units between compartments (Barlowe & Appling, 1988; Garcia-Martinez & Appling, 1993). In order to test this hypothesis, a strain (MWY4.4) was constructed which lacks both cytosolic CH_2-THF dehydrogenase activities. In this strain the only route to CHO-THF for purine synthesis would be formate activation by cC_1-THF synthase. Strain MWY4.4 was grown on minimal media plus [2-^{13}C]glycine and extracts were prepared as described under Experimental Procedures. The ^{13}C NMR spectrum of the extract shows incorporation into purines (Figure 7). Relative enrichment values are similar to values seen for strain DAY4, indicating that metabolism of mitochondrial one-carbon units to purines occurs independent of cytosolic dehydrogenase activity.

The absence of cytosolic dehydrogenase activity does not, however, interfere with transport of one-carbon units from mitochondria to cytosol via serine. Analysis of choline resonances in this strain (Figure 8) shows similar enrichments (88–91%) at all positions, as seen in the wild-type parent DAY4 (Table 2). Interestingly, labeled serine is also observed in this spectrum. Serine was not detected in extracts of wild-type strain DAY4 grown with [2-^{13}C]glycine. The accumulation of excess serine in this strain may be due to the absence of cytosolic dehydrogenase activity. Isotopomer analysis indicates enrichment at the C3 and C2 positions of serine is

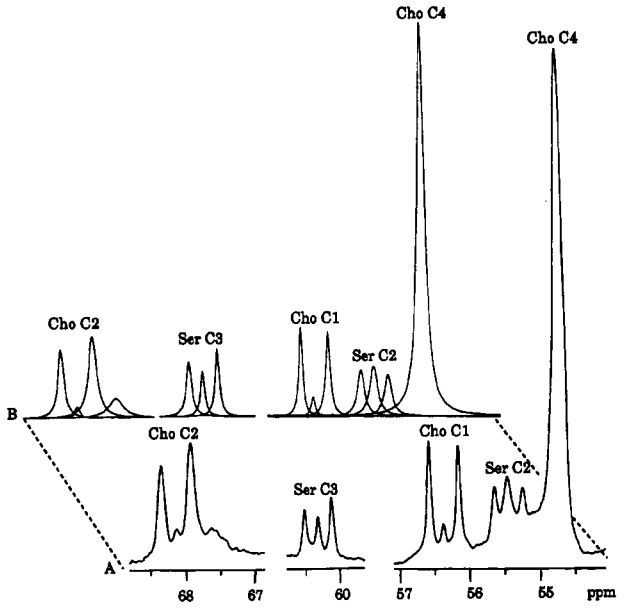

FIGURE 8: (A) ^{13}C NMR spectra of choline and serine resonances in cell extract of strain MWY4.4 grown in [2-^{13}C]glycine. (B) Deconvolution and Lorenzian line fit of spectrum for integration.

64% and 66%, respectively. The position labeled by the direct incorporation of glycine in serine versus choline indicates the presence of two pools of glycine in the mitochondria, one labeled 66% and one labeled 91%.

Glycine Growth Conditions Establish Functional Connection between Cytosolic and Mitochondrial C_1-THF Synthases. In the above experiment strain MWY4.4 is able to substitute glycine for serine as its one-carbon source and to use this glycine to synthesize purines. However, strain MWY4.6 does not grow on minimal media plus glycine, even in the presence of adenine. The two strains are isogenic except for the absence of cytosolic synthetase activity in MWY4.6. Along with being an adenine auxotroph, this strain is apparently incapable of substituting glycine for serine as its one-carbon source, even in the presence of adenine. This growth deficiency can be overcome with the addition of serine, indicating that serine synthesis is the problem. This is surprising since the serine synthesis pathway would not be expected to be disrupted by a mutation in the synthetase activity of cC_1-THF synthase. Under these conditions, one would expect serine synthesis to occur in the mitochondria, unaffected by mutation in a cytosolic protein. That this is not the case implies a more global connection between cytosolic synthetase function and utilization of mitochondrial one-carbon units. The following growth study was conducted in order to test the consistency of this phenotype.

Table 3 lists *ser1*$^-$ yeast strains tested for adenine auxotrophy and for their ability to substitute exogenous glycine for serine. The ability to substitute glycine for serine appears to be independent of (1) adenine auxotrophy (compare strains DAY3 and CBY6), (2) NAD CH_2-THF dehydrogenase activity (compare strains MWY4, MWY4.4, and MWY4.6 with their parents DAY4, CBY4, and CBY6), and (3) cC_1-THF synthase dehydrogenase activity (compare CBY4 with DAY4). Five strains are unable to grow on minimal media plus glycine and adenine (the test for ability to substitute glycine for serine independent of adenine auxotrophy). Common to these five strains is the lack of cC_1-THF synthase synthetase activity coupled with the presence of the mC_1-THF synthase. Notice that only strains carrying this genotype cannot grow on glycine. Indeed all strains we tested carrying this combination were unable to substitute glycine for serine as their one-carbon source. This indicates that mitochondrial oxidation of CH_2-THF via mC_1-THF synthase, along with the inability to activate formate in the cytosol, prevents growth, which suggests a link between these two enzymatic events.

Glycine Growth Conditions Result in Selection for Cytosolic Synthetase Function. In nonlimiting growth media, yeast typically achieve stationary growth within 48–72 h. Strains harboring mutations that render them unable to grow in the growth media provided sometimes exhibit slow but real growth during this initial 72 h. This may be due to leakiness of the mutation or to the appearance of cells harboring a reversion enabling growth. There are many types of mutations that may result in resumption of growth including: reversion of the original mutation, second site mutations, and suppressor mutations. If restoration of growth is accompanied by recovery of the deficient activity, it may be inferred that this reaction is critical to the pathway.

In this study strains CBY1 and DAY4 were grown on minimal media plus 400 mg/L glycine (Figure 9). Strain CBY1 has two point mutations in the cC_1-THF synthase gene resulting in loss of cytoplasmic CHO-THF synthetase activity (Barlowe & Appling, 1989). Initial growth of strain CBY1 is very slow, longer than the 24 h doubling time used as the criteria for growth in Table 3. However, a growth rate typical of strain DAY4 is eventually achieved. After 140 h, cells derived from this culture tested positive for ability to grow on formate and 20 mg/L glycine, which is a functional test for cytoplasmic CHO-THF synthetase activity. Enzyme assay of cell extracts from the final culture shows 86% of wild-type synthetase activity (data not shown). This compares to only 22% of wild-type activity (due to mitochondrial CHO-THF synthetase activity) in strain CBY1 grown in nonselective media. Thus the eventual growth is apparently due to selection for cells carrying a reversion rendering the CHO-THF synthetase function active again.

DISCUSSION

S. cerevisiae is an ideal model system for the study of eukaryotic compartmentation of folate-mediated one-carbon metabolism due to the ease of genetic manipulation and *in vivo* introduction of metabolic intermediates. In addition, ^{13}C NMR provides a powerful means for tracing a labeled carbon through systems such as this in which metabolite analysis is complicated by multiple labeling patterns.

The provision of glycine labeled at the C2 position as the sole one-carbon source allows us to generate labeled CH_2-THF in the mitochondria. The detection of label in choline and purines demonstrates the use of these one-carbon units outside of the mitochondria. Although ^{13}C NMR detection of purine synthesis in yeast has been previously reported (Kozluk & Spenser, 1987), we are unaware of any reports of the ^{13}C NMR detection of choline synthesis in yeast. The data presented herein support the generally accepted pathway of choline synthesis in yeast via decarboxylation and methylation of PS (Chin & Bloch, 1988).

S. cerevisiae can utilize two pathways for the biosynthesis of PC (Carman & Henry, 1989). One pathway starts with choline and leads to PC via CDP-choline. In the second pathway, PC arises by the three successive AdoMet-dependent methylations of phosphatidylethanolamine (PE). Chin and Bloch (1988) determined that this methylation pathway predominates in yeast. This pathway starts with phosphatidylserine (PS), synthesized directly from serine and CDP-diacylglycerol via PS synthase (Bae-Lee & Carman, 1984)

Table 3: Adenine and Serine Auxotrophy of Strains Harboring Mutations in Cytosolic and Mitochondrial C_1-THF Synthases[a]

strain name	genotype	relevant phenotype[b]	growth in minimal media plus[a] G	GS	GA	GAS	source or reference
CBY1	*ser1 ura3-52 trp1 leu2 his4 ade3-30*	S^-	–	+	–	+	(Barlowe & Appling, 1989)
CBY4	*ser1 ura3-52 trp1 leu2 his4 ade3-65*	D^-	+	+	+	+	(Barlowe & Appling, 1990)
CBY6	*ser1 ura3-52 trp1 leu2 his4 ade3-30/65/144*	$S^-D^-C^-$	–	+	–	+	(Barlowe & Appling, 1990)
DAY3	*ser1 ura3-52 trp1 leu2 ade3-130*	$S^-D^-C^-$	–	–	–	+	(Barlowe et al., 1989)
DAY4	*ser1 ura3-52 trp1 leu2 his4*		+	+	+	+	(Barlowe et al., 1989)
KSY7	*ser1 ade3-130 ura3-52*	$S^-D^-C^-$	–	–	–	+	(Shannon & Rabinowitz, 1988)
KSY8	*ser1 mis1::URA3 ade3-130*	$S^-D^-C^-M^-$	–	–	+	+	(Shannon & Rabinowitz, 1988)
KSY9	*ser1 mis1::URA3*	M^-	+	+	+	+	(Shannon & Rabinowitz, 1988)
MWY4	*ser1 ura3-52 mtd1::URA3 trp1 leu2 his4*	N^-	+	+	+	+	(West et al., 1993)
MWY4.4	*ser1 ura3-52 mtd1::URA3 trp1 leu2 his4 ade3-65*	D^-N^-	+	+	+	+	this study
MWY4.6	*ser1 ura3-52 mtd1::URA3 trp1 leu2 his4 ade3-30/65/144*	$S^-D^-C^-N^-$	–	–	–	+	(West et al., 1993)

[a] All minimal media includes histidine, leucine, tryptophan, and uracil. The following nutrients were added as noted in the table: G, glycine, S, serine, and A, adenine. Growth was determined in liquid media and scored (–) if no growth was apparent in 48 h. [b] Phenotypes are noted as S^-, synthetase mutant of cC_1-THF synthase, D^-, dehydrogenase mutant of cC_1-THF synthase, C^-, cyclohydrolase mutant of cC_1-THF synthase. M^-, disruption of mC_1-THF synthase. N^-, disruption of cytosolic NAD-dependent dehydrogenase.

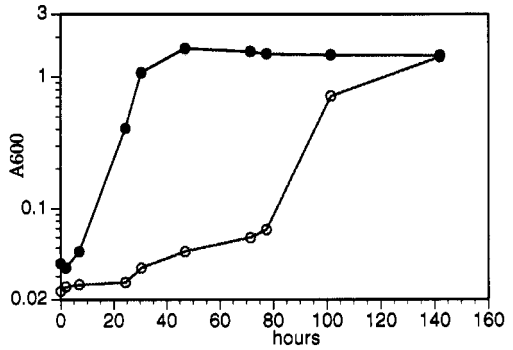

FIGURE 9: Growth curves of DAY4 and CBY1 (*ade3-30*) grown on minimal media plus glycine.

(Figure 1). We observed glycine metabolism to choline via the methylation pathway beginning with the synthesis of serine via SHMT from glycine and the CH_2-THF derived from glycine cleavage (see Figure 1). Serine then is transported to the cytosol where it is incorporated into phosphatidylserine and also provides CH_2-THF for cytosolic one-carbon requirements. CH_2-THF is reduced to CH_3-THF for methylation reactions, including methylation of PE. Thus the C2 and C3 of serine become the C1 and C2 of choline, and the C3 of serine becomes the C4 of choline.

Isotopomer analysis of C1 and C2 of choline indicates 89% enrichment at both positions. An 89% enrichment at C2 position (direct incorporation of C2 of glycine) indicates that the glycine available to SHMT for serine synthesis is 89% enriched at the C2 position and that there is an endogenous supply of unlabeled glycine of 11%. Appearance of the same enrichment at the C1 position indicates that glycine, via GCV, is the only source of one-carbon units for serine synthesis under these growth conditions.

The enzymes of choline synthesis are distributed between the cytosol, mitochondria, and endoplasmic reticulum as described in Figure 1. The route of one-carbon units from the mitochondria for use in the methylation steps appears to be via serine, as exogenous serine almost completely competes out flow of label into the C4 position of choline (Figure 4). The route of flow of one-carbon units for purine synthesis is, however, more complicated. *De novo* purine biosynthesis is cytosolic. Exogenous serine does not completely compete out label in the C2 and C8 positions of adenine, suggesting a second route of mitochondrial one-carbon units for purine synthesis. We have previously proposed that the original mitochondrial CH_2-THF (derived form GCV catalysis of glycine) may be directly oxidized to formate via mitochondrial C_1-THF synthase. Once transported to the cytosol, the formate is activated to CHO-THF for purine synthesis via the synthetase function of the cC_1-THF synthase. Several lines of evidence support this idea. Isolated mitochondria metabolize C3 of serine to formate and transport it out of the mitochondria (Barlowe & Appling, 1988; Garcia-Martinez & Appling, 1993). Exogenous formate substitutes as a source of one-carbon units in the absence of serine. We have recently demonstrated that the majority of activation of formate is via the cC_1-THF synthase (Pasternack et al., 1992). Kozluk and Spenser (1987) utilized ^{13}C NMR to confirm incorporation of CO_2, CHO-THF, and glycine into purines in yeast. These authors demonstrated that formate could compete out the labeling of C2 and C8 of purines from C2 of glycine.

In the experiments presented here, ^{13}C NMR analysis of adenine and guanine in extracts of strains grown in the presence of [2-^{13}C]glycine indicates an average enrichment of the C2 and C8 positions (incorporation of CHO-THF) of 0.92 relative to the C5 position (direct incorporation of glycine). The introduction of exogenous unlabeled serine provides an alternative one-carbon source. Under these conditions, approximately 25% of the one-carbon units used for purine synthesis are still labeled and thus are derived from mitochondrial one-carbon pools. This 25% labeling persists even with the addition of twice the concentration of serine, suggesting that there is a basal level of GCV activity and mitochondria-to-cytosol flux of one-carbon units independent of the availability of cytosolic one-carbon units. The 25% labeling, however, is lost in strain CBY1 under the same growth conditions. This strain lacks the synthetase activity of cC_1-THF synthase. Thus absence of all labeling in C2 and C8 of purines in this strain confirms the role of the synthetase activity of cC_1-THF synthase in the flow of mitochondrial one-carbon units to the cytosol for purine synthesis. Note that under these experimental conditions the direction of flow of the C3 of the exogenous serine cannot be determined. It is quite possible that a considerable portion of the competing one-carbon unit provided by serine is also passing through the mitochondria instead of the cytosol.

Genetic manipulation allows us to test the role of an enzyme in a metabolic pathway by creating metabolic blocks in alternative pathways. Genetic block of the cC_1-THF synthase synthetase function, along with all cytosolic CH_2-THF dehydrogenase functions (strain MWY4.6), renders the strain an adenine auxotroph (West et al., 1993). An isogenic strain with the synthetase activity functional (strain MWY4.4), however, grows on minimal media plus serine or glycine without the addition of adenine. Thus activation of formate via cC_1-

THF synthase is the only means available to obtain CHO-THF for purine synthesis. The appearance of label at the C2 and C8 position of adenine from mitochondrial labeled one-carbon units indicates that the formate activated in this strain originated in the mitochondria. This establishes the capacity of mitochondrial oxidation of CH_2-THF to compensate for the loss of cytosolic dehydrogenase activity in providing CHO-THF for purine synthesis. Spectral analysis shows that this strain is able to incorporate label into purines as well as produce serine and choline.

Interestingly, it was found that not all *ser1*$^-$ strains are able to grow on minimal media plus glycine. Inspection of the ability of various strains to substitute glycine for serine as their sole exogenous one-carbon source indicates a connection between the cC_1-THF synthase synthetase activity and the mC_1-THF synthase. Common to the five strains unable to use glycine is the lack of cC_1-THF synthase synthetase activity coupled with the presence of the mC_1-THF synthase. Only strains carrying this genotype cannot grow on glycine. In fact, none of the strains tested with this genotype grow on glycine. This indicates that mitochondrial conversion of one-carbon units along with a cytosolic synthetase block prevents growth, thus linking these two enzymatic events. Strain CBY1 is one of the strains that cannot substitute glycine for serine. This strain is isogenic with strain DAY4 except for the presence of two point mutations resulting in a lack of cytosolic CHO-THF synthetase activity (Barlowe & Appling, 1989). Selection for revertants of the cytosolic synthetase activity of strain CBY1 during growth in minimal media plus glycine provides additional evidence of the critical requirement for its activity in the metabolism of mitochondrial one-carbon units. Evidence that processing of one-carbon units is abnormal in this strain is provided by the ^{13}C NMR analysis of purine synthesis which shows a complete lack of labeled cytosolic CHO-THF for purine synthesis as compared to the 25% labeled CHO-THF seen in its isogenic wild-type parent under the same growth conditions. Loss of label may be a direct consequence of blocking the formate transport of mitochondrial one-carbon units pathway or may be an indirect result of the loss of GCV activity.

The data presented here support the proposed model that mitochondrial one-carbon units can be processed to CHO-THF for cytoplasmic processes by oxidation via mC_1-THF synthase, transport via formate, and activation to CHO-THF via cC_1-THF synthase. Previous work has shown the ability of mitochondria to generate and transport a one-carbon unit as formate from the C3 of serine (Barlowe & Appling, 1988; Garcia-Martinez & Appling, 1993). In the work presented here, strain MWY4.4 (unable to provide CHO-THF for purine synthesis via either cytosolic dehydrogenase activity) obtains its CHO-THF from mitochondrial one-carbon pools. Its only remaining pathway to CHO-THF is via the cC_1-THF synthase synthetase conversion of formate. In addition, strains lacking the ability to process mitochondrial one-carbon units through the cC_1-THF synthase synthetase demonstrate a more generalized improper processing of mitochondrial one-carbon units, so extreme as to prevent yeast growth.

Although the aim of this study was to trace mitochondrial one-carbon units to the one-carbon pools outside the mitochondria, as detected in the synthesis of choline and purines, several additional observations are noteworthy. Serine appears in the spectrum of strain MWY4.4 grown in [2-^{13}C]glycine, 66% enriched at both the C2 position (direct incorporation of glycine) and at the C3 position (incorporation of CH_2-THF derived from glycine cleavage) (Figure 8A). In the same spectrum, choline is enriched to 91% at the equivalent positions. The lower percent enrichment of serine is surprising since serine is the precursor of choline, and indicates the mitochondrial production of two distinct pools of serine: one pool (91% enriched at both positions) is directed to choline synthesis; the second pool (66% enriched at both positions) accumulates as serine. Since all of the doubly ^{13}C-labeled serine in these cells is derived from exogenous [2-^{13}C]glycine via GCV and SHMT, it follows that there must exist two mitochondrial pools of glycine as well, one which is diluted with 9% endogenous unlabeled glycine and one which is diluted with 34% endogenous unlabeled glycine. A second important point is that for each metabolite (serine and choline), the carbon derived from CH_2-THF is enriched to the same degree as the carbon derived from the direct incorporation of glycine. This indicates that, for each pool of glycine, the CH_2-THF produced in the GCV reaction is channeled directly to SHMT without mixing with CH_2-THF produced from the other glycine pool. One mechanism to account for these results would be a tight association of GCV and SHMT. Indeed, these enzymes have been shown to copurify from pea leaf mitochondria as a complex (Bourguignon et al., 1988). The higher percent enrichment in choline versus serine may indicate two fates for mitochondrially synthesized serine, choline synthesis and serine resynthesis. Serine resynthesis was previously demonstrated in yeast (Pasternack & Appling, 1992) in a GCV/SHMT dependent metabolism and is indicated by the 66% enrichment at the C3 position of serine.

Finally, the two compounds, choline and purines, provide detection of the incorporation of one-carbon units at three different levels of oxidation: CHO-THF at C2 and C8 of purines, CH_2-THF at C1 of choline, and CH_3-THF at C4 of choline. In addition, both choline and purine synthesis involve the direct incorporation of a glycine unit. This acts as an internal control to normalize relative enrichments between choline and purines and to directly compare the state of enrichment of the different oxidation states of the folate derivatives. This provides a metabolite pair for the evaluation of the effect of metabolic blocks or cellular conditions on the interconversion of the folate derivatives and promises to be of great value in future work in the field of folate-mediated one-carbon metabolism.

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