In Vivo Analysis of Folate Coenzymes and Their Compartmentation in *Saccharomyces cerevisiae*

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ABSTRACT

In eukaryotes, enzymes responsible for the interconversion of one-carbon units exist in parallel in both mitochondria and the cytoplasm. Strains of *Saccharomyces cerevisiae* were constructed that possess combinations of gene disruptions at the *SHM1* [mitochondrial serine hydroxymethyltransferase (SHMTm)], *SHM2* [cytoplasmic SHMT (SHMTc)], *MIS1* [mitochondrial C_1-tetrahydrofolate synthase (C_1-THFSm)], *ADE3* [cytoplasmic C_1-THF synthase (C_1-THFSc)], *GCV1* [glycine cleavage system (GCV) protein T], and the *GLY1* (involved in glycine synthesis) loci. Analysis of the *in vivo* growth characteristics and phenotypes was used to determine the contribution to cytoplasmic nucleic acid and amino acid anabolism by the mitochondrial enzymes involved in the interconversion of folate coenzymes. The data indicate that mitochondria transport formate to the cytoplasmic compartment and mitochondrial synthesis of formate appears to rely primarily on SHMTm rather than the glycine cleavage system. The glycine cleavage system and SHMTm cooperate to specifically synthesize serine. With the inactivation of *SHM1*, however, the glycine cleavage system can make an observable contribution to the level of mitochondrial formate. Inactivation of *SHM1*, *SHM2* and *ADE3* is required to render yeast auxotrophic for TMP and methionine, suggesting that TMP synthesized in mitochondria may be available to the cytoplasmic compartment.

IN eukaryotes the cytoplasmic and mitochondrial compartments each possess a parallel array of enzymes catalyzing the interconversion of folate coenzymes that carry one-carbon units in different oxidation states (BLAKLEY 1969; APPLING 1991). The folate coenzyme products of one-carbon metabolism are essential participants in several metabolic processes including nucleic acid synthesis, amino acid metabolism and organelle protein synthesis. Serine is the predominant source of one-carbon units for cytoplasmic folate interconversions (SCHIRCH 1984). Serine hydroxymethyltransferase (SHMT) catalyses the transfer of a hydroxymethyl group from serine to tetrahydrofolate (THF) to yield the folate coenzyme 5,10-methylene THF (SCHIRCH 1982). The carbon group of 5,10-methylene THF is modified by C_1-THF synthase to yield two additional coenzymes, 5,10-methenyl THF and 10-formyl THF. 5,10-methylene THF is required for TMP synthesis, 5,10-methenyl THF and 10-formyl THF coenzymes are required for purine synthesis and 5-methyl THF is required for methionine biosynthesis (Figure 1).

Folylpolyglutamate synthetase is responsible for the attachment of poly-γ-glutamate tails to tetrahydrofolate (THF) (SCHIRCH and STRONG 1989). The polyglutamate tails of THF derivatives yield more efficient substrates for interconversion of folate coenzymes and are responsible for the retention of folates in eukaryotic cells and organelles (HORNE *et al.* 1989; SCHIRCH and STRONG 1989). Folylpolyglutamate synthetase activity is present in both cytoplasm and mitochondria of mammalian cells and results suggest that polyglutamate tails preclude the transport of THF derivatives across the inner mitochondrial membrane (CYBULSKI and FISHER 1981). This suggests that the products of mitochondrial one-carbon metabolism available to the cytoplasm are restricted to serine, glycine and formate, since only these compounds would be transported between the compartments (PASTERNACK *et al.* 1994). Serine and glycine donate one-carbon units to THF to yield 5,10-methylene THF via SHMT and the glycine cleavage system, respectively. Mitochondrial formate generated from serine and glycine is available for cytoplasmic metabolism (BARLOWE and APPLING 1988). Mitochondrial formate is derived from 5,10-methylene THF via C_1-THFSm encoded by the nuclear *MIS1* gene. ^{13}C-NMR analysis with various yeast strains showed that 25% of one-carbon units utilized for cytoplasmic purine synthesis are derived from mitochondrial formate (PASTERNACK *et al.* 1994). Disruption of the *MIS1* gene, however, does not yield any detectable effect on growth (SHANNON and RABINOWITZ 1988). *In vitro* studies using isolated rat mitochondria demonstrated the synthesis of formate from serine (GARCIA-MARTINEZ and APPLING 1993). Mitochondrial formate is incorporated into the cytoplasmic folate pool by the 10-formyl THF synthe-

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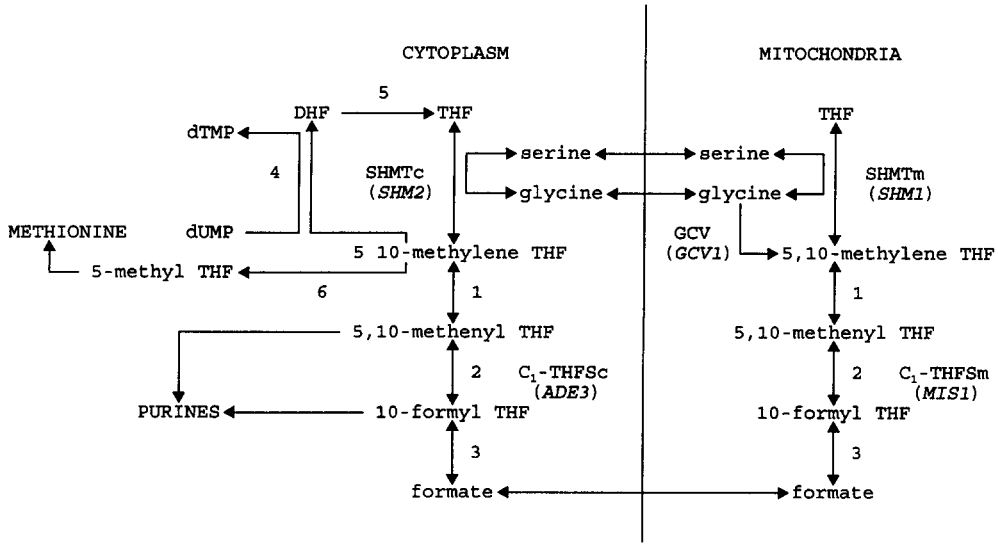

FIGURE 1.—Proposed pathway of folate-mediated one-carbon metabolism in yeast (APPLING 1991). The folate interconverting enzymes, SHMTm encoded by *SHM1*, SHMTc encoded by *SHM2*, C_1-THFSm and C_1-THFSc encoded by *MIS1* and *ADE3*, respectively, and GCV (glycine cleavage system) are indicated. The enzyme activities that comprise the trifunctional C_1-THF synthases are (1), 5,10-methylene THF dehydrogenase; (2), 5,10-methenyl THF cyclohydrolase, and (3), 10-formyl THF synthetase. Enzymatic reactions catalysed by thymidylate synthase (4), dihydrofolate reductase (5) and 5,10-methylene THF reductase (6) are also shown.

tase activity of C_1-THFSc (PASTERNACK *et al.* 1994) encoded by the nuclear *ADE3* gene (BARLOWE and APPLING 1990).

Although serine is the primary source for one-carbon units in the cytoplasm, the serine requirement of *ser1* auxotrophic yeast strains can be satisfied with formate plus glycine, or high exogenous glycine (MCKENZIE and JONES 1977). The cytoplasmic trifunctional C_1-THFSc is essential for formate assimilation into serine (MCKENZIE and JONES 1977; BARLOWE and APPLING 1990). ^{13}C-NMR studies showed that mitochondria can effect synthesis of serine and glycine from formate. The mitochondrial isozyme encoded by *MIS1*, however, does not yield serine from formate in sufficient quantities to satisfy the growth requirements of *ser1* yeast (SHANNON and RABINOWITZ 1988; BARLOWE and APPLING 1990). These results suggest that whereas the cytoplasm and mitochondria each include enzymes with identical activities, each set of enzymes of one-carbon metabolism is directed toward establishing a compartment-specific distribution of THF derivatives.

In this report, we further characterize the interactions between the cytoplasmic and mitochondrial folate-mediated one-carbon metabolism. We have recently isolated several yeast nuclear genes that encode products involved in folate metabolism (MCNEIL *et al.* 1994; J. B. MCNEIL, A. L. BOGNAR and R. E. PEARLMAN, unpublished data). These sequences were used to construct yeast strains with corresponding gene disruptions. The resultant growth characteristics and phenotypes of these strains assisted the assessment of the contribution to cytoplasmic nucleic acid and amino acid anabolism by the mitochondrial enzymes involved in the interconversion of folate coenzymes. This analysis allowed examination of both the flux of folate coenzyme interconversion within mitochondria and the functional relationship between mitochondria and the cytoplasmic compartments. Null mutations at the *SHM2* (encoding cytoplasmic SHMT) gene render yeast weak formate auxotrophs. Nearly complete formate auxotrophy occurs in *shm2 mis1* strains, indicating that formate synthesized in the mitochondria is transferred to the cytoplasm. A preferred flux for folate interconversion from 5,10-methylene THF to formate in mitochondria, is supported by the inability of mitochondria to assimilate exogenous formate in sufficient quantities to satisfy glycine auxotrophy of *shm1 shm2 gly1* yeast or serine auxotrophy of *ser1* yeast. The required mitochondrial formate contribution in *shm2* strains was used to demonstrate that, rather than the glycine cleavage system, SHMTm is responsible for most of the mitochondrial formate synthesis. When additional glycine is available, the glycine cleavage system appears to cooperate with SHMTm to yield serine. Thymidylate synthase and the coenzyme 5,10-methylene THF catalyze the synthesis of TMP from dUMP. We expected that inactivation of SHMTc and the C_1-THFSc would lead to low levels of cytoplasmic 5,10-methylene THF and concomitantly render yeast auxotrophic for TMP. However, *shm2 ade3* yeast are methionine auxotrophs but prototrophic for TMP. Null mutations in *SHM2*, *ADE3* and *SHM1* are required to yield yeast that are auxotrophic for TMP and methionine. This demonstrates that SHMTm can support cytoplasmic TMP levels and also indicates an additional interaction between the cytoplasmic and mitochondrial compartments.

MATERIALS AND METHODS

Media and growth conditions: Yeast cultures were grown as indicated in the text and figure legends, on either YEPD medium or synthetic defined (SD) medium (SHERMAN *et al.* 1986). When appropriate, the amino acids leucine (30 μg/ml), histidine (20 μg/ml), tryptophan (20 μg/ml), glycine (10 mM) and serine (400 μg/ml), the bases uracil (20 μg/ml) and adenine (20 μg/ml), formate (10 mM) and TMP (100 μg/ml) were added to SD to the final concentrations as indicated. TMP permeable derivatives of yeast strains (*tup*, TMP uptake) were isolated as described (LITTLE and HAYNES 1973). Overnight cultures were grown in YEPD, washed by centrifugation and resuspended in sterile water. For determination of growth rate, cells were inoculated with a portion of fresh overnight cultures into 10 ml of liquid SD plus appropriate supplements and monitored by measuring turbidity at 600 nm.

Yeast strains and genetic methods: The *S. cerevisiae* strains used in this study (Table 1) were constructed by either the mating of haploid strains indicated, followed by sporulation of resultant diploids to generate haploid progeny (SHERMAN *et al.* 1986) or by the disruption of gene sequences by the one-step gene replacement method (ROTHSTEIN 1983). Yeast cells were transformed by the method of CHEN *et al.* (1992). Phenotype was used to identify strains desired and genotype was verified by Southern analysis (SAMBROOK *et al.* 1989). Yeast *ade3* strains are phenotypically *His*$^-$ (JONES and MAGASANIK 1967). When appropriate, strains were transformed with yeast plasmid DNA that included the *ADE3* gene to distinguish *ade3 his3* from *ade3 HIS3* strains. All yeast strains described were derived from the haploid strains KSY8 (SHANNON and RABINOWITZ 1988), YM09 and YM13 (MCNEIL *et al.* 1994), YM50 (J. B. MCNEIL, A. L. BOGNAR and R. E. PEARLMAN, unpublished data) and SR200-12D (provided by S. ROEDER). KSY8 possesses the *ADE3* deletion allele *ade3-130* (JONES 1977) and a disrupted allele of the *MIS1* gene (SHANNON and RABINOWITZ 1988). YM09 and YM13 possess disrupted alleles of *SHM1* and *SHM2* genes. YM13 also includes a *gly1::URA3* null mutation. YM50 is a *gcv1::URA3* null mutant derivative of YM09.

Plasmids: PCR-generated fragments of the yeast *SHM1* and *SHM2* genes were each inserted at the *Eco*RI site of pEMBL10$^+$ (MCNEIL *et al.* 1994). The yeast *HIS3*, *LEU2* and *URA3* genes were inserted at the *Xho*I site of the *SHM1* gene fragment (MCNEIL *et al.* 1994) and designated pJM1153, pJM1159 and pJM1367, respectively. Insertion of the yeast *LEU2* gene into the *SHM2* gene fragment yielded pJM1162 (MCNEIL *et al.* 1994). These plasmids were used to disrupt *SHM* gene sequences in various *ade3-130* strains (Table 1) to help identify those genes whose inactivation leads to TMP auxotrophy.

Nucleic acid preparations and hybridizations: Yeast genomic DNA and total RNA were isolated as described (STRATHERN and HIGGINS 1991; MCNEIL and SMITH 1985). Northern analysis was performed as described (MCNEIL 1988) using as *LEU2* and *GLY1* probes the oligonucleotide PLEU2 (5′-GCATCGATAGCAGCACCACC-3′), and an *Eco*RI-*Sal*I restriction fragment (MCNEIL *et al.* 1994), respectively. Southern analysis was performed as described (SAMBROOK *et al.* 1989) using as probes the *GLY1 Eco*RI-*Sal*I restriction fragment (see above) and the 700-bp PCR-generated *SHM1* gene fragment (MCNEIL *et al.* 1994).

RESULTS

Mitochondrial formate is available to the cytoplasm: Formate, glycine and serine represent those metabolites of folate interconversion that when synthesized, or present in mitochondria may be available to the cytoplasm. In confirmation of the results of SHANNON and RABINOWITZ (1988), we found that the *mis1* null mutation alone had no effect on the growth rate or requirements (Table 2). Similarly, the inactivation of the SHMTm encoding gene (*SHM1*) or the inactivation of both the SHMTm and C_1-THFSm encoding genes (*SHM1 MIS1*) had no effect on growth rate or requirements. In the absence of other mutations that affect cytoplasmic folate interconversion, any serine, glycine or formate synthesized by mitochondria is not essential for folate-dependent cytoplasmic anabolic pathways. To examine the contribution made by mitochondrial one-carbon units to cytoplasmic metabolism, we compromised cytoplasmic folate interconversion through inactivation of the *SHM2* gene [encoding the cytoplasmic SHMT (SHMTc) isozyme]. Disruption of the *SHM2* gene causes a decrease in the growth rate, from a doubling time of 2 hr for the wild-type strain YM22, to 3.5 hr for the *shm2* strain YM24 (Table 2). Disruption of both *SHM1* and *SHM2* genes (YM26) increases the doubling time further to ~5 hr. In the absence of supplements to the growth medium, a severe growth defect is observed for strain YM29 (*shm2 mis1*). These results show that mitochondrial folate metabolism may make some contribution to folate-dependent cytoplasmic anabolism.

The addition of formate or adenine to the growth medium restores to wild-type, or near wild-type rates the growth of YM24 (*shm2*), YM26 (*shm1 shm2*) and YM29 (*shm2 mis1*) strains (Table 2). It appears that interruption in the synthesis of mitochondrial formate and not serine or glycine is responsible for the additional decrease in growth rate of YM26 and YM29 compared to YM24. This suggests that mitochondrial-derived formate assists cytoplasmic folate-dependent pathways in *shm2* yeast and that the *MIS1* and *SHM1* gene products assist the synthesis of mitochondrial formate. Because the double disrupted yeast strain YM29 (*shm2 mis1*) has a longer generation time than YM26 (*shm2 shm1*), SHMTm may be responsible for only a portion of mitochondrial formate available to the cytoplasm.

The glycine cleavage system and SHMTm may each contribute to the synthesis of mitochondrial formate: In addition to SHMTm, the other putative contributor to mitochondrial formate synthesis is the glycine cleavage system. Exogenous glycine can partially restore the growth rate of *shm2 shm1* yeast (YM26) and this restoration requires the *MIS1* gene product (Table 2, compare YM26 and YM27 plus glycine with the same strains plus formate). The glycine cleavage system (GCV) produces 5,10-methylene THF from glycine and THF and the 5,10-methylene THF may be oxidized to formate by the *MIS1* gene product (Figure 1). The yeast *GCV1* gene encodes the glycine cleavage T component of the GCV (J. B. MCNEIL, A. L. BOGNAR and R. E. PEARLMAN, unpublished observations). We used the

TABLE 1

***S. cerevisiae* strains**

Strain (Parent[s])	Genotype
KSY8	α *ser1 ade3-130 ura3-52 mis1::URA3*
SR200-12D	**a** *his3-11,15 leu2-3,112 ura3-52*
YM09	α *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 shm1::HIS3 shm2::LEU2 can1-100*
YM13	α *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 shm1::HIS3 shm2::LEU2 gly1::URA3 can1-100*
YM14 (SR200-12D × KSY8)	α *his3-11,15 leu2-3,112 ura3 ser1*
YM15 (SR200-12D × KSY8)	**a** *his3-11,15 leu2-3,112 ade3-130 ura3*
YM15tup (YM15)	**a** *his3-11,15 leu2-3,112 ade3-130 ura3 tup*
YM16 (SR200-12D × YM09)	α *his3-11,15 leu2-3,112 ura3 shm1::HIS3 shm2::LEU2*
YM17 (SR200-12D × KSY8)	**a** *his3-11,15 leu2-3,112 ade3-130 ura3 mis1::URA3*
YM17tup (YM17)	**a** *his3-11,15 leu2-3,112 ade3-130 ura3 mks1::URA3 tup*
YM18 (YM17 × YM09)	**a** *his3-11,15 leu2-3,112 ade3-130 ura3 mis1::URA3 tup*
YM19 (YM17 × YM09)	α *his3-11,15 leu2-3,112 ura3 shm1::HIS3 shm2::LEU2 mis1::URA3*
YM22 (YM16 × YM17)	**a** *his3-11,15 leu2-3,112 ura3*
YM23 (YM16 × YM17)	α *his3-11,15 leu2-3,112 ura3 mis1::URA3*
YM24 (YM16 × YM17)	**a** *his3-11,15 leu2-3,112 ura3 shm2::LEU2*
YM25 (YM16 × YM17)	**a** *his3-11,15 leu2-3,112 ura3 shm1::HIS3*
YM26 (YM16 × YM17)	**a** *his3-11,15 leu2-3,112 ura3 shm1::HIS3 shm2::LEU2*
YM27 (YM16 × YM17)	**a** *his3-11,15 leu2-3,112 ura3 shm1::HIS3 shm2::LEU2 mis1::URA3*
YM28 (YM16 × YM17)	**a** *his3-11,15 leu2-3,112 ura3 shm1::HIS3 mis1::URA3*
YM29 (YM16 × YM17)	**a** *his3-11,15 leu2-3,112 ura3 shm2::LEU2 mis1::URA3*
YM30 (YM14 × YM18)	α *his3-11,15 leu2-3,112 ura3 ser1*
YM34 (YM14 × YM18)	α *his3-11,15 leu2-3,112 ura3 ser1 shm1::HIS3*
YM35 (YM14 × YM18)	**a** *his3-11,15 leu2-3,112 ura3 ser1 shm2::LEU2*
YM40 (YM14 × YM18)	**a** *his3-11,15 leu2-3,112 ura3 ser1 shm1::HIS3 shm2::LEU2*
YM45 (YM14 × YM18)	**a** *his3-11,15 leu2-3,112 ura3*
YM50 (YM09 + *gcv1::URA3*)	α *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 shm1::HIS3 shm2::LEU2 gcv1::URA3 can1-100*
YM51 (YM30 × YM50)	**a** *his3-11,15 leu2-3,112 ura3 ser1 gcv1::URA3 trp1*
YM59 (YM30 × YM50)	α *his3-11,15 leu2-3,112 ura3 gcv1::URA3 trp1*
YM61 (YM30 × YM50)	**a** *his3-11,15 leu2-3,112 ura3 shm1::HIS3 gcv1::URA3 trp1*
YM62 (YM30 × YM50)	α *his3-11,15 leu2-3,112 ura3 shm2::LEU2 gcv1::URA3 trp1*
YM63 (YM30 × YM50)	α *his3-11,15 leu2-3,112 ura3 shm1::HIS3 shm2::LEU2 gcv1::URA3 trp1*
YM67 (SR200-12D × YM13)	**a** *his3-11,15 leu2-3,112 ura3 gly1::URA3 trp1*
YM69 (SR200-12D × YM13)	**a** *his3-11,15 leu2-3,112 ura3 shm1::HIS3 gly1::URA3 trp1*
YM70 (SR200-12D × YM13)	α *his3-11,15 leu2-3,112 ura3 shm2::LEU2 gly1::URA3*
YM71 (SR200-12D × YM13)	α *his3-11,15 leu2-3,112 ura3 shm1::HIS3 shm2::LEU2 gly1::URA3*
YM72 (YM16 × YM17tup)	α *his3-11,15 leu2-3,112 ura3 ade3-139 shm1::HIS3 mis1::URA3 shm2::LEU2 tup trp1*
YM74 (YM16 × YM17tup)	α *his3-11,15 leu2-3,112 ura3 ade3-130 shm1::HIS3 shm2::LEU2 tup*
YM79 (YM16 × YM17tup)	α *his3-11,15 leu2-3,112 ura3 ade3-130 mis1::URA3 shm2::LEU2 tup trp1*
YM80 (YM17tup + pJM1159)	**a** *his3-11,15 leu2-3,112 ade3-130 ura3 shm1::LEU2 mis1::URA3 tup*
YM81 (YM15tup + pJM1162)	**a** *his3-11,15 leu2-3,112 ade3-130 shm2::LEU2 ura3 tup*
YM84 (YM15tup + pJM1367)	**a** *his3-11,15 leu2-3,112 ade3-130 shm1::URA3 ura3 tup*
YM85 (YM81 + pJM1367)	**a** *his3-11,15 leu2-3,112 ade3-130 shm1::URA3 shm2::LEU2 ura3 tup*

cloned *GCV1* gene to construct *gcv1::URA3* null mutants, which we used to examine the contribution of GCV to mitochondrial formate synthesis (Figure 2). Inactivation of the *GCV1* gene yields no detectable effect on phenotype or growth rate in a wild-type (J. B. McNeil, A. L. Bognar and R. E. Pearlman, unpublished data). When cells were grown without supplement, very little difference in growth rate was observed between *shm2* (YM24) and *shm2 gcv1* (YM62) yeast, whereas a rate intermediate between that of *shm2* (YM24) and *shm2 mis1* (YM29) yeast was observed for *shm2 shm1* (YM26) yeast. The combination of the null mutations *gcv1* and *shm1* is equivalent to the *mis1* null mutation when present in a *shm2* background (compare unsupplemented growth of YM63 with YM29). Because neither serine nor glycine, but only formate could restore the growth rate of YM63 (Table 2), this demonstrates that GCV can contribute to mitochondrial formate synthesis. The inactivation of *SHM1* is required to permit detection of the contribution of GCV to the single carbon pool.

We attempted to stimulate GCV-mediated formate synthesis by adding exogenous glycine to the medium (Table 2, Figure 2). Glycine did increase the growth rate of a *shm2 shm1* strain significantly, but none was evident for *shm2* yeast, suggesting that GCV-mediated synthesis of mitochondrial formate is seen only when SHMTm is absent. One can account for these findings

TABLE 2

Growth rates of yeast strains with null mutations in genes involved in the interconversion of one-carbon units

Strain	Genotype[b]	Doubling time in SD[a] plus the supplement indicated (hr)				
		—	Serine	Glycine	Adenine	Formate
YM22	Wild type	2.0	2.1	2.2	2.0	1.9
YM25	*shm1*	2.0	2.2	2.1	2.0	2.0
YM24	*shm2*	3.5	3.4	3.6	2.0	2.1
YM26	*shm1 shm2*	5.0	5.2	3.0	2.3	2.2
YM23	*mis1*	2.0	2.1	2.5	2.0	1.9
YM28	*shm1 mis1*	2.1	2.2	3.0	2.0	2.0
YM29	*shm2 mis1*	~24	~20	~24	2.3	2.1
YM27	*shm1 shm2 mis1*	~24	~24	~24	2.6	2.5

[a] Synthetic medium with the addition of tryptophan, histidine, leucine and uracil.
[b] All relevant markers are indicated.

if exogenous glycine is normally converted via 5,10-methylene THF to serine, catalyzed by GCV and SHMTm, respectively, but will be converted via 5,10-methylene THF to formate (via GCV and mitochondrial C_1-THFS) if the route to serine is blocked by the *shm1* mutation. The formate then can be transported to the cytoplasm. Comparison of the growth of strains YM26, YM63 and YM29 (Figure 2) demonstrated that exogenous glycine assisted the growth of *shm2 shm1* but not *shm2 shm1 gcv1* or *shm2 mis1* yeasts, indicating that the *MIS1* and *GCV1* gene products are required for the glycine-mediated increase in growth rate for YM26 (*shm1 shm2*); in this instance, formate is the end product of glycine assimilation in mitochondria. In one instance (YM62), exogenous glycine decreased the growth rate. Because this effect is reversed when formate is included in the growth medium (data not shown), glycine may be repressing expression of the remaining *SHM* gene that may otherwise contribute to the synthesis of mitochondrial formate rather than that glycine or a product thereof is toxic.

Genetic analysis of the supplementation of *ser1* mutants by formate and glycine: Serine is a mitochondrial product that may be available to the cytoplasmic compartment. It has been shown previously that exogenous formate plus glycine or high levels of exogenous glycine can satisfy the serine requirement of *ser1* yeast (McKenzie and Jones 1977). Although ^{13}C-NMR studies have shown that serine can be synthesized in the mitochondria from formate (Pasternack *et al.* 1992), formate assimilation into serine in sufficient amounts to allow the growth of *ser1* yeast is independent of the *MIS1* gene product and follows a cytoplasmic route (Shannon and Rabinowitz 1988; Barlowe and Appling 1990). The *ADE3* gene product, encoding cytoplasmic C_1-THFS converts formate into 5,10-methylene THF (Jones and Magasanik 1967; Lazowska and Luzzatti 1970a,b; Lam and Jones 1973). SHMTc transfers the reactive methylene group of 5,10-methylene THF to glycine to yield serine. With the availability of *SHM* and *GCV1* null mutant strains, we were able to identify the compartment and genes required to satisfy serine auxotrophy with exogenous formate and glycine.

Figure 3 shows that high levels of exogenous glycine or formate can each fulfil the serine requirement of *ser1* yeast, however, the growth rate more closely resembles the wild-type rate when exogenous glycine is present (Figure 3). The mitochondrial SHMT isozyme, but not SHMTc, is essential for glycine-mediated satisfaction of the serine auxotrophy of *ser1* yeast (Figure 3, compare YM35 with YM34). In contrast, the *SHM2* gene product SHMTc is required to convert formate into serine (Figure 3). In agreement with other studies (Shannon and Rabinowitz 1988; Barlowe and Appling 1990), these results indicate that the assimilation of formate into serine is mediated by the cytoplasmic and not mitochondrial folate interconverting enzymes.

It has been proposed that conversion of glycine into serine involves the GCV and SHMTm enzymes (Pasternack *et al.* 1992). GCV condenses glycine and THF into 5,10-methylene THF. Serine is produced from 5,10-methylene THF by the reversal of the SHMTm isozyme. As expected, the serine requirement of *gcv1 ser1* yeast was not fulfilled with exogenous glycine (data not shown), indicating that in addition to SHMTm, the *GCV1* gene product is essential for mitochondrial conversion of glycine into serine.

The yeast *GLY1* gene is located on chromosome 5 near the *MAK10* gene (McNeil *et al.* 1994). The *GLY1* gene maps to the same region of the yeast genome as does *SUS1* (Mortimer and Hawthorne 1966), a dominant suppressor of *ser1* strains that allows growth without addition of serine or glycine (Mortimer and Hawthorne 1966). Multicopy yeast plasmids containing *GLY1* were used to transform various *ser1* strains and transformants were then tested for serine prototrophy. Table 3 shows that *ser1* yeast can be suppressed by *GLY1* on an episomal plasmid. This suppression requires both GCV and SHMTm, however, SHMTc encoded by *SHM2* is not essential. pGLY1 is present at

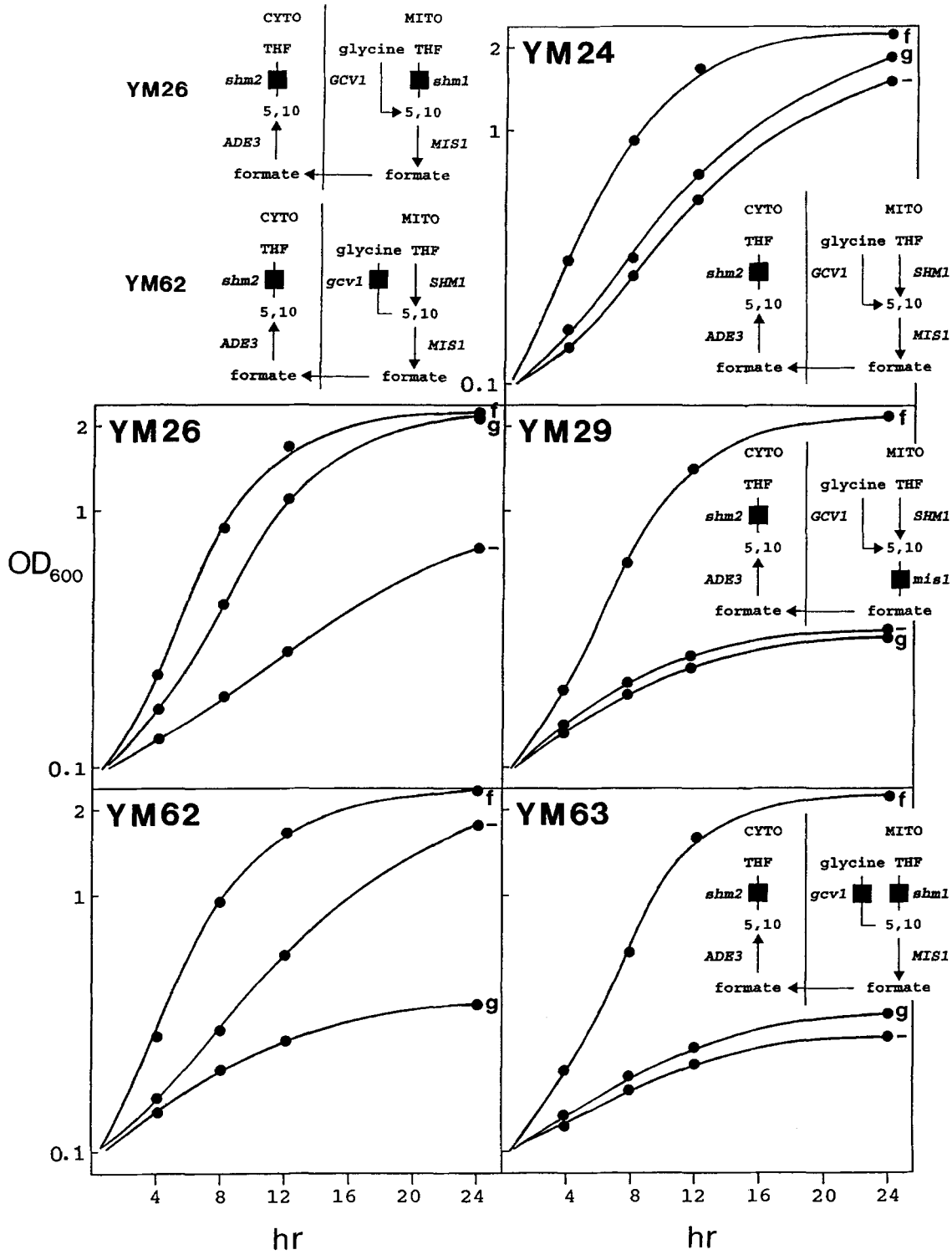

FIGURE 2.—Growth of mutant yeast strains disrupted at the *SHM2, SHM1, MIS1* and *GCV1* genes. The optical density of the liquid culture medium at 600 nm was plotted *vs.* time (hours). Yeast strains YM24 (*shm2*), YM26 (*shm1 shm2*), YM29 (*shm2 mis1*), YM62 (*shm2 gcv1*), and YM63 (*shm1 shm2 gcv1*) were grown in minimal SD medium supplemented with tryptophan, histidine, leucine, uracil and with either glycine (g), formate (f) or no additional supplements (−). In each instance a schematic representation is included that indicates the position of the genetic blocks in folate metabolism that are present for each strain.

high copy and results in overexpression of *GLY1* (Figure 4). High *GLY1* mRNA levels may yield sufficient gene product derived from the high copy plasmid to effect *ser1* suppression. Suppression of *ser1* involves a prolonged phenotypic lag of ~10–14 hr before cells reach exponential phase (data not shown). In the absence and presence of glycine, transcription of plasmid-borne *GLY1* in *ser1* yeast is approximately equivalent.

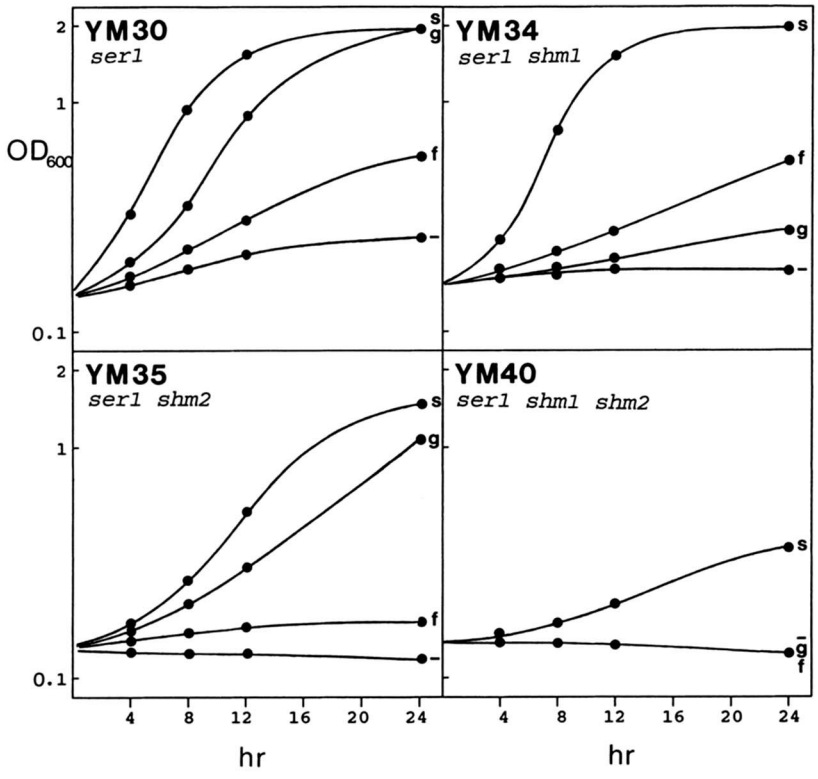

FIGURE 3.—Suppression of serine auxotrophy of *ser1* yeast by exogenous glycine and formate. Yeast strains YM30 (*ser1*), YM34 (*shm1 ser1*), YM35 (*shm2 ser1*) and YM40 (*shm1 shm2 ser1*) were grown in minimal SD medium supplemented with tryptophan, histidine, leucine, uracil and with either serine (s), glycine (g), formate (f) or no additional supplements (−).

This suggests that the lag described above does not represent an interval that is required to allow some fraction of the cells to accumulate sufficient copies of the *GLY1* gene. The effects and characteristics of *GLY1*-mediated suppression of *ser1* are identical to those of exogenous glycine-mediated supplementation of the growth requirements caused by the *ser1* mutation. Although the nature of the enzymatic activity of the *GLY1* gene product is unknown, these results suggest that *GLY1* is a significant contributor to glycine synthesis.

The flux of mitochondrial folate interconversion is toward formate: To render yeast auxotrophic for glycine, inactivation of *SHM2, SHM1* and *GLY1* genes is required. Of these genes, *GLY1* appears responsible for most of the glycine synthesized in the cell (MCNEIL *et*

TABLE 3

Suppression of *ser1* by plasmid-borne *GLY1*

Strain (plasmid)	Genotype	Phenotype[a]
YM30 (pGLY1)[b]	*ser1*	Ser$^+$
YM34 (pGLY1)	*ser1 shm1::HIS3*	Ser$^-$
YM35 (pGLY1)	*ser1 shm2::LEU2*	Ser$^+$
YM40 (pGLY1)	*ser1 shm1::HIS3 shm2::LEU2*	Ser$^-$
YM45 (pGLY1)	*SER1*	Ser$^+$
YM51 (pGLY2)	*ser1 gcv1::URA3*	Ser$^-$
YM30 (YEp24)	*ser1*	Ser$^-$
YM34 (YEp24)	*ser1 shm1::HIS3*	Ser$^-$
YM35 (YEp24)	*ser1 shm2::LEU2*	Ser$^-$
YM40 (YEp24)	*ser1 shm1::HIS3 shm2::LEU2*	Ser$^-$
YM45 (YEp24)	*SER1*	Ser$^+$
YM51 (YEp13)	*ser1 gcv1::URA3*	Ser$^-$

[a] Ser$^-$ colonies on solid medium did not grow beyond the two-cell stage. Ser$^+$ colonies reach 1 mm in size in 3–5 days.

[b] pGLY1 and pGLY2 are Yep24 (CARLSON and BOTSTEIN 1982) and YEp13 (BROACH *et al.* 1979) derivatives, respectively, which include the yeast *GLY1* gene.

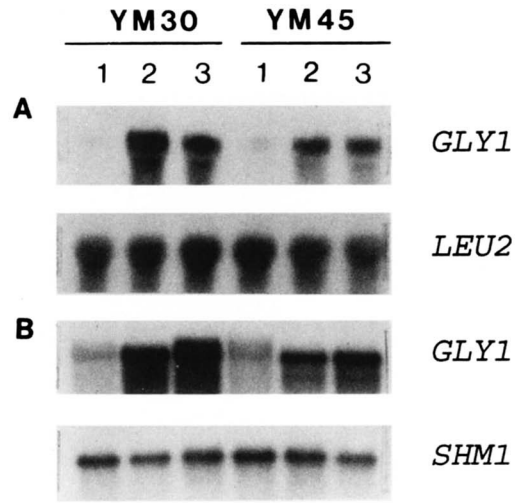

FIGURE 4.—Hybridization analysis of nucleic acids isolated from transformed yeast, demonstrating suppression of *ser1* by the over expression of the *GLY1* gene. (A) Total RNA was isolated from YM30 (*ser1*) and YM45 (*SER1*) yeast transformed with plasmids; lane 1, Yep24 (URA3); and lanes 2 and 3, pGLY1. Yeast were grown in the presence (lanes 1 and 2) or absence (lane 3) of serine and the RNA blot was hybridized with probes specific for *GLY1* and *LEU2* transcripts. (B) Genomic DNA was isolated from the same yeast strains grown under the same conditions as above and hybridized with probes specific for *GLY1* and *SHM1* (genomic single copy sequence) gene sequences.

TABLE 4

Growth rates of yeast strains disrupted at the *GLY1*, *SHM1* and *SHM2* genes

Strain	Genotype[b]	Doubling time in SD[a] plus the supplement indicated (hr)				
		—	Serine	Glycine	Adenine	Formate
YM22	Wild-type	2.2	2.3	2.2	2.3	2.1
YM67	*gly1*	6.5	2.6	2.5	6.9	14
YM69	*gly1 shm1*	12.5	10	4.0	13	>24
YM70	*gly1 shm2*	~20	~21	4.2	~20	>24
YM71	*gly1 shm1 shm2*	>24	>24	3.8	>24	>24

[a] Synthetic medium with the addition of tryptophan, histidine, leucine and uracil.
[b] All relevant markers are indicated.

al. 1994). A null mutation at *GLY1* results in a reduced growth rate that is restored to a wild-type rate when *gly1* strains are grown in the presence of exogenous glycine (Table 4). Yeast with an inactive *GLY1* allele provided us with the opportunity to determine whether some of the products of mitochondrial folate interconversion could act as substrate for glycine synthesis via mitochondrial GCV. Although serine restored the growth rate of *gly1* yeast to wild-type levels, neither formate nor adenine sufficed. This failure of formate to supplement *gly1* yeast suggests that formate is not efficiently used as substrate by C_1-THFSm to yield 5,10-methylene THF, which could be converted to glycine (via GCV) or serine (via SHMTm). Mitochondria in *ser1* yeast are unable to convert exogenous formate into sufficient quanities of serine to allow growth in the absence of serine (SHANNON and RABINOWITZ 1988; BARLOWE and APPLING 1990). These results suggest that formate is not efficiently metabolised by the *MIS1* gene product to yield 5,10-methylene THF and that the prevailing flux of folate interconversion in mitochondria is toward formate.

Inactivation of *ADE3*, *SHM2* and *SHM1* render yeast auxotrophic for TMP: TMP auxotrophy in yeast can result from a variety of different genetic lesions. For example, inactivation of the genes encoding thymidylate synthase (*TMP1*), dUTPase (*DUT1*), or dihydrofolate reductase (*DHFR*) will each yield yeast that require exogenous TMP for growth (LITTLE and HAYNES 1979; HUANG *et al.* 1992; GADSDEN *et al.* 1993). Inhibition of dihydrofolate reductase by the folate antagonist methotrexate reduces the endogenous level of THF (BARCLAY *et al.* 1982). A low level of THF causes a concomitant decrease in levels of folate coenzymes, and this effects auxotrophy for the folate-dependent metabolic end products, methionine, histidine, adenine and TMP (LITTLE and HAYNES 1979). Based on the orchestration of THF-interconverting enzymes (Figure 1) and because activated folates do not cross the mitochondrial membrane (CYBULSKI and FISHER 1981), an *ade3 shm2* strain would not be expected to convert serine or formate of cytoplasmic or mitochondrial origin into cytoplasmic 5,10-methylene THF and therefore unable to synthesize TMP or methionine for the cytoplasmic compartment.

The strains presented in Table 5 were examined for the various auxotrophic requirements indicative of stress in one-carbon metabolism. In addition to adenine and histidine, *ade3 shm2* null mutant yeast strains are also methionine auxotrophs. Such strains, however, remained TMP prototrophs. The same results were observed from strains that were constructed by mating and sporulation of diploids as they were from strains constructed by the disruption of gene sequences by gene replacement.

Only with the inactivation of *ADE3*, *SHM2* and *SHM1* genes does a methionine and TMP auxotrophy result. Because methionine synthesis is restricted to the cytoplasm (MATTHEWS 1986; GOYETTE *et al.* 1995; APPLING 1991) and must rely on cytoplasmic 5,10-methylene THF, an *ade3 shm2* strain would require exogenous methionine for growth. *SHM1* is responsible for much of the synthesis of 5,10-methylene THF in mitochondria (see above). The requirement for a *shm1* mutant to yield a TMP auxotroph may indicate that TMP synthesized in the mitochondria (via thymidylate synthase and mitochondrial 5,10-methylene THF) is available to the cytoplasmic compartment. Concomitant with inactivation of *shm1* in an *ade3 shm2* strain, depleted levels of mitochondrial 5,10-methylene THF would preclude TMP synthesis in the mitochondria. The cytoplasm could not draw upon mitochondrial TMP for nuclear DNA synthesis, rendering such a strain a TMP auxotroph. Alternatively, the cytoplasm may possess functional SHMT derived from *SHM1*, which is not transported to mitochondria.

DISCUSSION

The recent isolation of several genes encoding products that participate in one-carbon metabolism in the yeast *S. cerevisiae* has allowed the construction of an extensive series of yeast strains with various combinations of mutations in folate interconversion. We used phenotypic analysis of these mutants to examine the functional interactions between the two parallel arrays of enzymes responsible for folate metabolism. Specifically, the contributions to folate-dependent anabolic

TABLE 5

Disruption of *ADE3*, *SHM1* and *SHM2* genes renders yeast auxotrophic for TMP, adenine, methionine and histidine

Strain	Genotype	Phenotype[a]		
		Met	Tmp	Ade
YM15tup[b]	*ade3 his3 tup*	+	+	−
YM17tup[b]	*ade3 his3 mis1 tup*	+	+	−
YM81[c]	*ade3 his3 shm2::LEU2 tup*	−	+	−
YM84[c]	*ade3 his3 shm1::URA3 tup*	+	+	−
YM80[c]	*ade3 mis1 his3 shm1::LEU2 tup*	+	+	−
YM79[b]	*ade3 mis1 his3 shm2::LEU2 tup*	−	+	−
YM74[b]	*ade3 his3 shm1::HIS3 shm2::LEU2 tup*	−	−	−
YM72[b]	*ade3 mis1 his3 shm1::HIS3 shm2::LEU2 tup*	−	−	−
YM85[c]	*ade3 his3 shm1::URA3 shm2::LEU2 tup*	−	−	−

[a] Phenotypes were tested on solid medium. Neither Met$^-$ nor Ade$^-$ yeast colonies grew beyond the 2–4-cell stage. TMP auxotrophs possessed the characteristic swollen phenotype and colonies did not grow to more than 10 cells. Prototrophic colonies grew to 1 mm in diameter in 2–3 days.

[b] Yeast strain constructed through mating and sporulation of appropriate diploid.

[c] Strain constructed by transformation with null mutant derivatives of the *SHM1* and/or *SHM2* wild-type alleles

pathways in the cytosol, of folates synthesized in the mitochondrial compartment, were examined.

It has been shown that neither a *MIS1* (SHANNON and RABINOWITZ 1988), *SHM1* (see above) nor a *GCV1* (J. B. MCNEIL, A. L. BOGNAR and R. E. PEARLMAN, unpublished data) null mutation has any obvious deleterious effect on cell growth. In this study, we have shown that yeast strains deficient in both mitochondrial enzymes SHMTm and C_1-THFSm do not exhibit any growth-related stress that might indicate insufficient folate levels. Thus, while the cytoplasmic set of enzymes involved in one-carbon metabolism remains intact, the products of mitochondrial folate interconversion are not essential for folate-dependent anabolic pathways of the cytosol. A reduction in cytoplasmic synthesis of one-carbon units resulting from the disruption of the *SHM2* gene, allowed examination of the nature of the contribution by mitochondria to cytosolic folate metabolism.

It has been proposed that because of the organization of mitochondrial folate interconversion (Figure 1) and the inability of glutamyl derivatives of folates to cross the mitochondrial inner membrane (CYBULSKI and FISHER 1981; HORNE *et al.* 1989), serine, glycine or formate are the only products of mitochondrial one-carbon metabolism that may have access to the cytoplasm and that may contribute to cytosolic folate-dependent anabolic processes. However, recent studies indicate that in CHO cells that lack cytosolic folylpolyglutamate synthetase activity, the cytosolic folate pool is derived from mitochondrial efflux of folylpolyglutamates (LIN and SHANE 1994).

Because *shm2 mis1* yeast have stricter requirements for formate than do *shm2* yeast, mitochondrial-derived formate is available to the cytoplasm. NMR studies that followed the metabolism of ^{13}C one-carbon units also showed that mitochondrial 5,10-methylene THF is oxidized to formate and then transported to the cytoplasm (PASTERNACK *et al.* 1992, 1994). Mitochondrially derived formate that is made available to the cytoplasm is derived from 5,10-methylene THF, which is oxidized to formate by the *MIS1* gene product. Mitochondrial 5,10-methylene THF may be synthesized via GCV and SHMTm (Figure 1). Mutational analysis indicates that SHMTm contributes most of the 5,10-methylene THF, which is converted into mitochondrial formate and that the 5,10-methylene THF derived from GCV may be directed toward the synthesis of serine rather than toward formate. Whereas glycine could not increase the growth rate of *shm2* yeast through its conversion to formate, glycine did satisfy the serine requirement of *ser1* yeast, suggesting that serine is the predominant indirect product of the GCV. When serine is replaced with glycine during the growth of *ser1* yeast, all the folate derivatives synthesized are derived from the exogenous glycine. Glycine can stimulate vigorous growth of *ser1* yeast only if the *GCV1* and *SHM1* genes are functional. The *MIS1* gene product is not essential for this effect, therefore serine rather than formate is the predominant product of glycine assimulation. We propose that the GCV and SHMTm channel glycine through sequential enzymatic reactions to yield serine. Much of this serine may then be transported to the cytoplasm where it is a substrate for the *SHM2* gene product and is converted into the various cytoplasmic one-carbon units. Interestingly, only with the additional inactivation of SHMTm do *shm2* yeast convert exogenous glycine into significant amounts of formate. Conversion of glycine into formate is mediated by the glycine cleavage system. In the presence of glycine, *shm2 shm1* yeast possess a shorter doubling time than *shm2* yeast. We propose that in *shm1*

null mutants, the substrate channelling of glycine is uncoupled and the 5,10-methylene THF is now available to C_1-THFSm and the resultant formate is transported to the cytoplasm. NMR studies of *ser1* yeast demonstrated that 75% of the one-carbon units used for purine synthesis are derived from serine via the GCV and *SHM1* gene product. Mitochondrial formate synthesis accounts for the remaining 25% (PASTERNACK *et al.* 1994). Our work suggests that the remaining 25% alone is not sufficient to yield an observable improvement in the growth rate of shm2 yeast. This 25% may be derived in part from serine synthesized by the substrate channelling of glycine through GCV and SHMTm. Various studies support the hypothesis that gene products involved in a common metabolic process are organized into multicomponent complexes (SRERE 1987). Protein purification studies (CAPERELLI *et al.* 1978, 1980) and *in vitro* cross-linking studies (SMITH *et al.* 1980) support the putative existence of a multicomplex that includes enzymes involved in purine biosynthesis as well as C_1-THFS and SHMT.

The cytoplasmic compartment is responsible for formate mediated supplementation of *ser1* auxotrophy (MCKENZIE and JONES 1977; BARLOWE and APPLING 1990). We have shown that SHMTc and not SHMTm is essential for this effect. Because mitochondria cannot convert formate into serine or glycine in sufficient amounts to satisfy the requirements caused by *ser1* or *gly1* mutations, respectively, the prevailing flux of mitochondrial folate interconversion is from 5,10-methylene THF to formate.

We observed that *shm2 ade3* yeast are auxotrophic for methionine and histidine but not TMP. Only after inactivation of *SHM2, ADE3* and *SHM1* genes were resultant yeast auxotrophic for TMP as well as methionine and histidine. Methionine and TMP synthesis, directly or indirectly, require 5,10-methylene THF. Methionine synthesis is confined to the cytoplasm (MATTHEWS 1986; APPLING 1991; GOYETTE *et al.* 1995) and relies upon cytoplasmic folate interconversion. Therefore, *shm2 ade3* yeast are Met^- as expected. Because inactivation of *SHM1* in addition to *SHM2* and *ADE3* genes is required to yield TMP auxotrophs, the mitochondrial enzyme SHMTm contributes to the synthesis of TMP available to the cytoplasm. One can explain these results if either *SHM1* expresses a functional isozyme located in the cytoplasmic compartment, where it participates in the synthesis of cytoplasmic 5,10-methylene THF, or TMP synthesized in the mitochondria (a process dependent upon the *SHM1* gene product) is available to the cytoplasmic compartment. The growth characteristics of YM29 (*shm2 mis1*) are nearly identical to YM27 (*shm1 shm2 mis1*) indicating that SHMTm can not assist purine synthesis. In support of mitochondrial TMP, which is available to the cytoplasm, high levels of exogenous glycine can weakly suppress the TMP requirement but not the methionine auxotrophy of *shm1 shm2 ade3* yeast. We expect that the mitochondrial glycine cleavage system converts the glycine into additional 5,10-methylene THF. This folate is used to synthesize mitochondrial TMP, which is available to the cytoplasmic compartment, but mitochondrial 5,10-methylene THF is not available for methionine synthesis. In *SHM1* yeast, SHMTm is responsible for sufficient amounts of TMP that *shm2 ade3* yeast remain TMP prototrophs. It appears therefore, that there is no 5,10-methylene THF derived from a cytoplasmic SHMTm isozyme available for the interconversion of the other cytoplasmic one-carbon units required for the synthesis of purines and methionine. In addition to serine, glycine and formate, TMP may also represent a mitochondrial product that is available to the cytoplasmic compartment.

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