Metabolic Role of Cytoplasmic Isozymes of 5,10-Methylenetetrahydrofolate Dehydrogenase in *Saccharomyces cerevisiae*†

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ABSTRACT: *Saccharomyces cerevisiae* possesses two cytosolic 5,10-methylenetetrahydrofolate (CH_2-THF) dehydrogenases that differ in their redox cofactor specificity: an NAD-dependent dehydrogenase encoded by the *MTD1* gene and an NADP-dependent activity as part of the trifunctional C_1-THF synthase encoded by the *ADE3* gene. The experiments described here were designed to define the metabolic roles of the NAD- and NADP-dependent CH_2-THF dehydrogenases in one-carbon interconversions and *de novo* purine biosynthesis. Growth studies showed that the NAD-dependent CH_2-THF dehydrogenase is interchangeable with the NADP-dependent CH_2-THF dehydrogenase when flow of one-carbon units is in the oxidative direction but that it does not participate significantly when flux is in the reductive direction. ^{13}C NMR experiments with [2-^{13}C]glycine and unlabeled formate confirmed the latter conclusion. Direct measurements of cellular folate coenzyme levels revealed substantial levels of 10-formyl-THF (CHO-THF), the one-carbon donor used in purine synthesis, in the purine-requiring *ade3* deletion strain. Thus, CHO-THF is necessary but not sufficient for *de novo* purine synthesis in yeast. Disruption of the *MTD1* gene in this strain resulted in undetectable CHO-THF, indicating that the NAD-dependent CH_2-THF dehydrogenase was responsible for CHO-THF production in the *ade3* deletion strain. Finally, we examined the ability of wild-type and catalytically-inactive domains of the cytoplasmic C_1-THF synthase to complement the adenine auxotrophy of the *ade3* deletion strain. Both the dehydrogenase/cyclohydrolase (D/C) domain and the synthetase domain could functionally replace the full-length protein, but, at least for the D/C domain, complementation was not dependent on catalytic activity. These results reveal a catalytic role for the NAD-dependent CH_2-THF dehydrogenase in the oxidation of cytoplasmic one-carbon units and indicate that the cytoplasmic C_1-THF synthase plays both catalytic and noncatalytic roles in *de novo* purine biosynthesis in yeast.

Serine, derived from glycolytic intermediates, is the major source of one-carbon units in most organisms (Schirch, 1984). Its 3-carbon is transferred to tetrahydrofolate (THF)[1] in a reaction catalyzed by serine hydroxymethyltransferase (SHMT) (Figure 1, reaction 4) to generate 5,10-methylene-THF (CH_2-THF) and glycine. This form of the coenzyme is used for thymidylate synthesis or can be reduced to 5-methyl-THF for methyl group biogenesis. In rapidly growing cells, the synthesis of purines is a critical folate-dependent pathway, requiring 2 mol of 10-formyl-THF (CHO-THF) per mole of purine ring. CH_2-THF is converted to CHO-THF via the sequential enzymes CH_2-THF dehydrogenase and 5,10-methenyl-THF (CH^+-THF) cyclohydrolase (reactions 3 and 2, respectively, in Figure 1).

CH_2-THF dehydrogenase is typically found as part of a multifunctional protein in eukaryotes. NADP-dependent CH_2-THF dehydrogenases in mammals, birds, and yeast are components of a trifunctional enzyme called C_1-THF synthase that also contains CH^+-THF cyclohydrolase and CHO-THF synthetase activities (reactions 1−3). In the yeast *Saccharomyces cerevisiae*, both the cytoplasmic and mitochondrial isozymes are trifunctional, encoded by the *ADE3* and *MIS1* genes, respectively (Staben & Rabinowitz, 1986; Shannon & Rabinowitz, 1988). In these eukaryotic enzymes (subunit M_r = 100 000), the dehydrogenase and cyclohydrolase activities are contained in a 30−35 kDa N-terminal domain and the synthetase activity is located in a 70 kDa C-terminal domain.

S. cerevisiae also expresses an NAD-dependent CH_2-THF dehydrogenase activity in the cytoplasm (reaction 6) (Barlowe & Appling, 1990a), encoded by the *MTD1* gene (West et al., 1993). In contrast to all other eukaryotic CH_2-THF dehydrogenases characterized to date, this enzyme appears to be monofunctional. The enzyme is a homodimer with a subunit molecular mass of 36 kDa, reminiscent of the dehydrogenase/cyclohydrolase (D/C) domain of the trifunctional C_1-THF synthase. However, the deduced amino acid sequence shows only slight (20%) similarity to other CH_2-THF dehydrogenase sequences (West et al., 1993).

Why do yeast possess two cytoplasmic enzymes catalyzing the same reaction, but using different redox cofactors (NAD vs NADP)? Most cytoplasmic NADP-linked dehydrogena-

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[1] Abbreviations: THF, tetrahydrofolate; CH_3-THF, 5-methyl-THF; CHO-THF, 10-formyl-THF; CH_2-THF, 5,10-methylene-THF; CH^+-THF, 5,10-methenyl-THF; D/C, dehydrogenase/cyclohydrolase; SHMT, serine hydroxymethyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; CHES, 2-[*N*-cyclohexylamino]ethanesulfonic acid; DMSO, dimethyl sulfoxide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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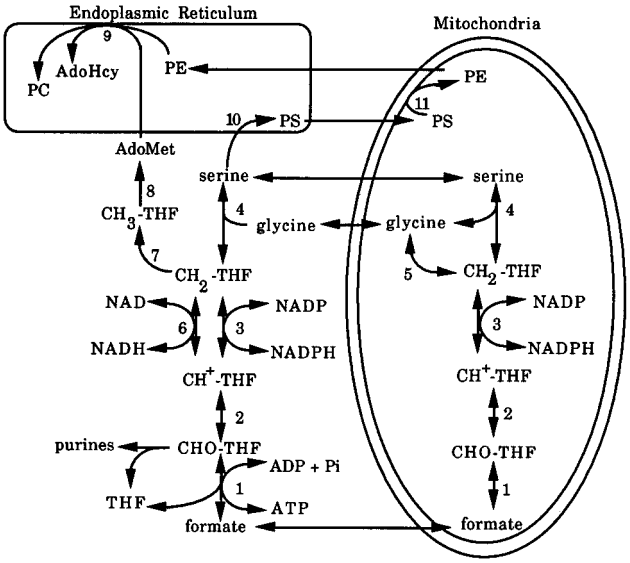


FIGURE 1: Proposed organization of the enzymes of one-carbon metablism in *S. cerevisiae*. Reactions 1−3: CHO-THF synthetase (EC 6.3.4.3), CH^+-THF cyclohydrolase (EC 3.5.4.9), and NADP-dependent CH_2-THF dehydrogenase (EC 1.5.1.5), respectively, are catalyzed by cytoplasmic or mitochondrial C_1-THF synthase. Reaction 4 is cytoplasmic or mitochondrial serine hydroxymethyltransferase (EC 2.1.2.1). Reaction 5 is the glycine cleavage system (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 homocysteine methyltransferase (EC 2.1.1.14) and ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6); reaction 9 is the phosphatidylethanolamine methyltransferases; reaction 10 is phosphatidylserine synthase (EC 2.7.8.8); and reaction 11 is phosphatidylserine decarboxylase (EC 4.1.1.65).

ses operate in the reductive direction (*i.e.*, reduction of substrate) (Atkinson, 1977). The NAD/NADH redox ratio typically observed in the cytoplasm of eukaryotic cells (Sies, 1982) would favor the oxidation of CH_2-THF by a NAD-linked dehydrogenase such as the *MTD1* gene product. Both dehydrogenases appear to be expressed at all stages of cell growth and in rich or minimal media (Appling & Rabinowitz, 1985b; Barlowe & Appling, 1990a). One possibility is that the NADP-dependent enzyme supports reductive flux whereas the NAD-dependent enzyme is responsible for the oxidative direction.

Deletion of the *ADE3* gene encoding the cytoplasmic trifunctional C_1-THF synthase leads to a strict adenine requirement (Jones, 1972, 1977). This observation suggested that C_1-THF synthase was solely responsible for the production of CHO-THF for purine biosynthesis, either from CH_2-THF via its dehydrogenase and cyclohydrolase activities (reactions 2 and 3) or from formate via its synthetase activity (reaction 1). We reported the construction of a yeast strain (CBY6) expressing a catalytically-inactive cytoplasmic C_1-THF synthase, which retained the ability to synthesize purines (Barlowe & Appling, 1990b). We reasoned that the required CHO-THF in this strain was being produced from CH_2-THF by an additional cytoplasmic activity, leading to the discovery of the monofunctional NAD-dependent CH_2-THF dehydrogenase (Barlowe & Appling, 1990a). Although this enzyme lacks CH^+-THF cyclohydrolase activity, at physiological pH, the equilibrium between CH^+-THF and CHO-THF lies far toward CHO-THF (Kay et al., 1960) and the nonenzymatic hydrolysis of CH^+-THF to CHO-THF is quite rapid (Rabinowitz, 1960; Robinson, 1971). In support of this hypothesis, disruption of the *MTD1* gene leads to a purine requirement in CBY6 but not in strains expressing catalytically-active C_1-THF synthase (West et al., 1993).

In order to rationalize all of these observations, we proposed that the C_1-THF synthase has both catalytic and noncatalytic functions in *de novo* purine synthesis (Barlowe & Appling, 1990b). The catalytic function can be replaced by the NAD-dependent CH_2-THF dehydrogenase, as we subsequently demonstrated (West et al., 1993). We envision the noncatalytic function as involving the formation of a purine-synthesizing multienzyme complex, or metabolon (Srere, 1987), in which C_1-THF synthase is a required structural component. We propose that this metabolon is required *in vivo* for the high rates of purine synthesis necessary to support the rapid growth of yeast cells. This model predicts that in the absence of C_1-THF synthase protein, regardless of whether CHO-THF is available, an active complex is unable to form, resulting in purine auxotrophy.

Song and Rabinowitz (1993) have questioned this model on the basis of their observation that expression in yeast of either a heterologous monofunctional synthetase or the D/C domain of yeast C_1-THF synthase is sufficient to complement the adenine requirement of *ade3* deletion strains. They argued that the catalytic activity of C_1-THF synthase is required for purine biosynthesis. However, Song and Rabinowitz (1993) used yeast strains that are wild-type at the *MTD1* locus. The presence of the monofunctional NAD-dependent CH_2-THF dehydrogenase in their strains makes it impossible to discern the contribution of each activity to the production of CHO-THF required in purine synthesis.

Here we report experiments designed to investigate the role(s) of the *ADE3* and *MTD1* gene products, and their respective NADP- and NAD-dependent CH_2-THF dehydrogenase activities. Three specific questions are addressed. First, is one of the CH_2-THF dehydrogenases responsible for the oxidative direction and the other the reductive direction, or are both enzymes fully reversible *in vivo*? Second, can cellular CHO-THF levels be correlated with purine prototrophy, or are other factors involved? Finally, can the results of Song and Rabinowitz (1993) be reconciled with our own?

EXPERIMENTAL PROCEDURES

Materials. Common reagents and media components were of high-grade commercial quality. DIFCO growth media were purchased from Baxter (McGraw Park, IL). [2-^{13}C]-Glycine was purchased from Cambridge Isotope Laboratories (Woburn, MA), and dimethyl-d_6 sulfoxide was purchased from Isotec Incorporated (Miamisburg, OH).

Growth Conditions and Yeast Strains. Rich media (YPD) contained 1% yeast extract, 2% Bactopeptone, and 2% glucose. Synthetic minimal media consisted of 0.7% yeast nitrogen base without amino acids, 2% glucose, and the following supplemental nutrients when indicated (final concentration): L-serine (375 mg/L), L-leucine (30 mg/L), L-histidine (20 mg/L), L-tryptophan (20 mg/L), uracil (20 mg/L), adenine (20 mg/L), glycine (20 mg/L), formate (1000 mg/L). Yeast were grown at 30 °C with ample aeration in a 250 rpm rotary shaker. Supplemental nutrients used in the ^{13}C NMR labeling experiment are described in a later section.

The yeast strains used in the following experiments are summarized in Table 1. Disruption of the *MTD1* gene was

Table 1: *S.* cerevisiae Strains and Plasmids

strains and plasmids	genotype	relevant phenotype[a]	ref
	Strains		
DAY3	*ser1 ura3−52 trp1 leu2 ade3−130*	$S^-D^-C^-$	Barlowe et al. (1989)
DAY4	*ser1 ura3−52 trp1 leu2 his4*	wild-type	Barlowe et al. (1989)
CBY1	*ser1 ura3−52 trp1 leu2 his4 ade3−30*	S^-	Barlowe and Appling (1989)
CBY4	*ser1 ura3−52 trp1 leu2 his4 ade3−65*	D^-	Barlowe and Appling (1990b)
CBY5	*ser1 ura3−52 trp1 leu2 his4 ade3−30/65*	S^-D^-	Barlowe and Appling (1990b)
CBY6	*ser1 ura3−52 trp1 leu2 his4 ade3−30/65/144*	$S^-D^-C^-$	Barlowe and Appling (1990b)
MWY3	*ser1 ura3−52 trp1 leu2 ade3−130* Δ*mtd1*	$S^-D^-C^-$	West et al. (1993)
MWY4	*ser1 ura3−52 trp1 leu2 his4* Δ*mtd1*	N^-	West et al. (1993)
MWY4.1	*ser1 ura3−52 trp1 leu2 his4 ade3−30* Δ*mtd1*	S^-N^-	this study
MWY4.4	*ser1 trp1 leu2 his4 ade3−65* Δ*mtd1*	D^-N^-	Pasternack et al. (1994)
MWY4.5	*ser1 ura3−52 trp1 leu2 his4 ade3−30/65* Δ*mtd1*	$S^-D^-N^-$	this study
MWY4.6	*ser1 ura3−52 trp1 leu2 his4 ade3−30/65/144* Δ*mtd1*	$S^-D^-C^-N^-$	West et al. (1993)
	Plasmids		
pVT103U	pVT103U		Vernet et al. (1987)
pADE3	pU3.3-*ADE3*	wild-type	Barlowe (1990)
pD/C-WT	pU3.3-*ADE3*-Δ*Hin*dIII	ΔS	this study
pD/C-65/144	pU2.1-*ADE3−30/65/144*-Δ*Hin*dIII	D^-C^-DS	this study
pSynthetase-WT	pVT101U-*ADE3-Apa*I/*Sph*I	DD/C	this study
pSynthetase-30	pVT101U-*ADE3−30/65/144-Apa*I/*Sph*I	S^-DD/C	this study

[a] Phenotypes are noted as follows: S^-, lacks synthetase activity of C_1-THF synthase; D^-, lacks dehydrogenase activity of C_1-THF synthase; C^-, lacks cyclohydrolase activity of C_1-THF synthase; N^-, disruption of cytosolic NAD-dependent CH_2-THF dehydrogenase; ΔD/C, deletion of dehydrogenase/cyclohydrolase domain of C_1-THF synthase; ΔS, deletion of synthetase domain of C_1-THF synthase.

reported previously (West et al., 1993); however, a new generation of *MTD1*-disrupted strains was made to take advantage of an excisable *URA3* cassette (Roca et al., 1992). A 3.3-kbp *Bam*HI−*Cla*I fragment containing the *MTD1* gene (West et al., 1993) was subcloned into a pUC19 vector lacking both *Hin*dIII and *Kpn*I sites in its multiple cloning region. A 0.15-kbp *Bgl*II−*Hin*dIII fragment of the *MTD1* open reading frame (nucleotides 712−861) was replaced by a 1.4-kbp *Sac*I−*Hin*dIII fragment containing the "hit-and-run" *URA3* gene cassette. The sequences flanking the *URA3* gene are recognition sequences for a site-specific yeast recombinase. A 5.0-kbp *Pvu*II linear fragment containing the *mtd1::URA3* disruption construct was transformed into haploid *ura3*$^-$ yeast strains in a one-step gene disruption (Rothstein, 1983) of the *MTD1* gene. Yeast transformations with linearized DNA or autonomously replicating plasmid were performed with lithium acetate (Ito et al., 1983). Gene disruption was detected by polymerase chain reaction of yeast colonies using primers with sequences complementary to the 5′ and 3′ ends of the *MTD1* gene. Disruptants were verified by loss of NAD-dependent CH_2-THF dehydrogenase activity in crude yeast extracts. The *URA3* marker was then evicted by transformation with pHM53, a vector carrying a *LEU2* marker and the recombinase gene expressed from the *S.* cerevisiae *GAL1* promoter (Roca et al., 1992). Thus, the disrupted *MTD1* lacks approximately 0.15 kbp near the 3′ end and is denoted as Δ*mtd1* in Table 1.

Growth Studies. Appropriate synthetic minimal medium was inoculated with 0.03% (v/v) of an overnight saturated culture grown in YPD. Growth was monitored by turbidity at 600 nm until saturation, and doubling time was determined from the exponential region of the growth curve.

[2-^{13}C]Glycine Labeling, Extract Preparation, and NMR Analysis. Yeast cultures of 500 mL were grown aerobically to saturation in synthetic minimal media containing leucine, histidine, tryptophan, and uracil at the previously detailed concentrations, 100 mg of labeled 99% [2-^{13}C]glycine/L, and 1.3 mM unlabeled formate.

Sample preparation was as described earlier (Pasternack et al., 1994) but with the following modifications. Yeast were harvested and washed once with deionized water. The washed pellet was resuspended in 30 mL of 0.3 N HCl and steamed over a boiling water bath until the cell suspension was reduced to approximately 15 mL. This converts all intracellular phosphatidylcholine and phosphocholine to choline. The acid suspension was centrifuged at 10 000*g* for 30 min, and the supernatant was transferred to a round-bottomed flask for drying of the sample by Rotovap. The resulting residue was resuspended in 1.0 mL of deuterated DMSO and transferred to NMR sample tubes.

The ^{13}C NMR spectra were obtained on a Bruker AMX500 using a 5 mm probe. ^{13}C data were collected during a 0.5 s acquisition time with a 5 s delay at 125 MHz with full proton decoupling. 1200 scans of 32K data points were acquired over a sweep width of 31 250 Hz. For purposes of integration, the spectra were processed with an exponential multiplier window with a line-broadening expansion of 1 Hz. The spectra were base-line corrected with a fifth-order polynomial function.

The carbons in choline were identified by characteristic chemical shift values established from natural abundance spectra, and enrichments were determined as described previously (Pasternack et al., 1994). The relative percent enrichment of choline C4 (RE_{C4}) is the ratio of the integral of the C4 peak to the integral of the C2 peak (direct incorporation of the labeled glycine) along with an empirically determined normalization correction constant for the C4 position.

CHO-THF Determination. The method used for cellular extraction was modified from that used for rat liver extracts (Wilson & Horne, 1984). Yeast were grown in synthetic minimal media until late log, harvested, and washed once with deionized water. The cell pellet was resuspended in 0.5 mL of deionized water, transferred into a screw-capped tube, and recentrifuged. Once the excess water was removed the "wet weight" was determined. The samples were resuspended in twice their wet weight with extraction

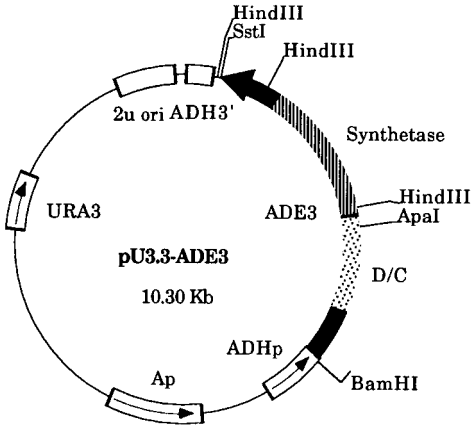


FIGURE 2: Plasmid construct for expression of the *ADE3* gene. Plasmid pU3.3-*ADE3* was used to construct [pD/C-WT], [pSynthetase-WT], and [pSynthetase-30]. [pD/C-65/144] is a similar construct based on pVT-102U (see Experimental Procedures). The *ADE3* gene is contained within a *Bam*HI−*Sst*I fragment, depicted as an arrow showing the direction of transcription. The regions encoding the dehydrogenase/cyclohydrolase (D/C) (stippled) and synthetase (cross-hatched) domains of the C_1-THF synthase are shown within the *ADE3* open reading frame. Expression of all gene constructs is driven by the alcohol dehydrogenase promoter (ADHp). Relevant restriction endonuclease sites that are referred to in Experimental Procedures are indicated.

buffer: 50 mM HEPES, 50 mM CHES, 0.2 M 2-mercaptoethanol, and 2% (w/v) ascorbic acid, final pH of 7.85. Acid-etched glass beads were added at 1.5 times the wet weight of the pellet. The samples were boiled in a 100 °C sand bath for 10 min and vortexed for 4 min at the highest setting for complete disruption of the yeast cells. Cellular debris was separated by centrifugation at 20 000*g* for 30 min. Finally, the supernatants were transferred to new tubes, their volumes measured, and stored at −70 °C until analysis.

Cellular extracts were analyzed for total folate by using the *Lactobacillus casei* microbiological assay (Horne & Patterson, 1988). CHO-THF pools were determined by HPLC and converted from nmol of CHO-THF/mL of extract to nmol of CHO-THF/g of wet weight of cells. Typically, the recovery (the sum of HPLC detectable folates divided by total folate) ranged from 70% to 90%.

C_1-THF Synthase Domain Constructs. A. [pD/C-WT] and [pD/C-65/144]. All of the following constructs were made within a family of yeast expression vectors called pVT-U (Vernet et al., 1987). Each of the pVT-U vectors (pVT-100U, -101U, -102U, -103U) (6.9 kbp) differs from each other only in the orientation of the intergenic region of the phage f1 (f1 *ori*) and the multiple cloning site. Cloned inserts are transcribed from the *ADH* promoter and expression is essentially identical in the different orientations. The pVT-103U-derived vector pU3.3-*ADE3* (Figure 2) lacks the *Pst*I, *Xho*I, and *Pvu*II sites in the multiple cloning region but contains the entire C_1-THF synthase open reading frame between the *Bam*HI and *Sst*I sites (Barlowe et al., 1989). This plasmid was digested with *Hin*dIII and religated to excise the synthetase domain that resides in the 3′ two-thirds of the open reading frame. The 5′ *Hin*dIII site within the *ADE3* gene is located just 3′ of the putative domain linker region, and the 3′-most *Hin*dIII site is in the multiple cloning site of the pVT vector. This construct, lacking the synthetase domain, was named [pD/C-WT]. The same *Hin*dIII digestion/religation was performed with the plasmid pU2.1-*ADE3−30/65/144* (a pVT-102U-derived vector), and the new construct was named [pD/C-65/144]. pU2.1-*ADE3−30/65/144* expresses a mutant *ADE3* gene lacking all three activities of the C_1-THF synthase and was used previously to construct strain CBY6 (see Table 1) (Barlowe & Appling, 1990b).

B. [pSynthetase-WT] and [pSynthetase-30]. Deletion analysis defines the interdomain region of the human C_1-THF synthase to residues 292−310 (Hum & MacKenzie, 1991). This corresponds to residues 297−315 of the yeast enzyme. For the construction of an active synthetase domain, we isolated a 2.1-kbp *Apa*I−*Sst*I fragment from pU3.3-*ADE3*, which includes the sequence for the domain linker region and the entire synthetase domain (see Figure 2). The fragment was blunt-ended and ligated into the *Pvu*II site of pVT101U to construct the plasmid [pSynthetase-WT], which lacks the D/C domain. The analagous *Apa*I−*Sst*1 fragment of pU2.1-*ADE3−30/65/144* was ligated into pVT101U to produce [pSynthetase-30].

Immunoassays. Yeast strains were grown in yeast minimal media containing serine and adenine. Crude extracts of yeast strains were prepared as described previously (West et al., 1993). A 40 μg sample of protein from each extract was resolved by electrophoresis and transferred electrophoretically to nitrocellulose for immunoassay using antisera against purified yeast C_1-THF synthase as described by Barlowe et al. 1989.

RESULTS

Growth Studies of C_1-THF Synthase and NAD-Dependent CH_2-THF Dehydrogenase Mutants. Barlowe and Appling (1990b) previously constructed a set of yeast mutants (CBY strains) that lacked various combinations of the dehydrogenase, cyclohydrolase, and synthetase activities of the *ADE3*-encoded C_1-THF synthase. However, all the CBY strains are wild-type at the *MTD1* locus. In the present work, we have disrupted the *MTD1* gene in the various CBY backgrounds to examine the role of the NAD-dependent CH_2-THF dehydrogenase in one-carbon metabolism.

The following growth experiments were designed to examine the contributions of the cytoplasmic NAD- vs NADP-dependent dehydrogenases in the flow of one-carbon units in the oxidative and reductive directions. All of the strains used are *ser1*$^-$. The *ser1* mutation blocks the synthesis of serine from glycolytic intermediates. Serine can be added exogenously or instead synthesized from glycine via SHMT (reaction 4, Figure 1), provided that a source of CH_2-THF is available. Formate can satisfy the CH_2-THF requirement by being reduced to CH_2-THF via the three reactions catalyzed by C_1-THF synthase (reactions 1−3, Figure 1).

Thus, to study one-carbon flux in the oxidative direction, cells were given serine as the one-carbon donor in media lacking purines. To supply one-carbon units for purine biosynthesis, CH_2-THF produced from the 3-carbon of serine must be oxidized to CHO-THF by one of the three CH_2-THF dehydrogenases located in the cytoplasm or mitochondria (see Figure 1). Growth of *ser1*$^-$ strains on glycine plus formate in place of serine was used to investigate the ability of the cytosolic dehydrogenases to support flux of one-carbon units in the reductive direction. Serine is in high demand by these *ser1*$^-$ strains, not only as a source of one-carbon units but also for protein synthesis. Formate-derived one-carbon units must be reduced by a CH_2-THF dehydrogenase to support serine synthesis under these conditions.

Table 2: Effect of Mutations in the NAD-Dependent CH_2-THF Dehydrogenase and C_1-THF Synthase on Growth

strain and phenotype[a]	DAY4 wild-type	MWY4 N^-	CBY4 D^-	MWY4.4 D^-N^-	CBY1 S^-	MWY4.1 S^-N^-	CBY5 S^-D^-	MWY4.5 $S^-D^-N^-$	CBY6 $S^-D^-C^-$	MWY4.6 $S^-D^-C^-N^-$
YPD[b]	1.5[c]	1.5	1.5	1.4	2.0	1.9	1.7	1.8	1.8	1.8
YMD+S	2.1	2.1	1.9	6.5	2.0	2.1	2.2	>100	4.0	>100
YMD+S,A	2.5	2.2	2.3	2.0	2.2	2.2	2.2	2.2	2.0	2.3
YMD+G,F	5.0	5.7	13	12	25	18	23	>100	19	>100
YMD+G,F,A	4.0	4.1	9.5	9.0	18	12	17	18	9.5	12
YMD+G,F,S	1.9	1.8	1.7	3.9	2.0	1.9	2.1	>100	4.1	>100
YMD+G,F,A,S	2.2	2.1	2.2	2.0	2.0	2.2	2.0	2.3	1.8	2.2

[a] Phenotypes are noted as follows: S^-, lacks synthetase activity of C_1-THF synthase; D^-, lacks dehydrogenase activity of C_1-THF synthase; C^-, lacks cyclohydrolase activity of C_1-THF synthase; N^-, disruption of cytosolic NAD-dependent CH_2-THF dehydrogenase. [b] Growth media was YPD or synthetic minimal media with (mg/L) leucine (20), histidine (20), tryptophan (20), uracil (20), and the designated additions: serine (S) (20), adenine (A) (20), glycine (G) (20), formate (F) (1000). [c] Doubling times are expressed in hours.

Growth rates on various media are shown in Table 2, where the strains are organized in three groups (depicted as three panels): those with an active CHO-THF synthetase domain of C_1-THF synthase, those lacking an active synthetase, and those strains expressing a completely inactive C_1-THF synthase. All the strains are isogenic except at the *ADE3* and/or *MTD1* loci. In rich media, all strains had growth rates similar to that of the wild-type *ser1*$^-$ strain DAY4. When the cells are grown in minimal media, the strains require supplementation with serine or with nutrients that lead to serine (glycine and formate). In minimal media containing serine but no adenine, strains with at least one active cytoplasmic CH_2-THF dehydrogenase (MWY4 or CBY4) had doubling times similar to those of DAY4. Thus, when one CH_2-THF dehydrogenase is inactive, the other appears capable of supporting the oxidative flux without a change in growth rate.

What contribution does the mitochondrial pathway make? We have shown previously that serine can be transported into the mitochondria and metabolized to formate by the mitochondrial isozymes of SHMT and C_1-THF synthase (Pasternack et al., 1994). The formate can then exit the mitochondria and be converted to CHO-THF by the synthetase activity of cytoplasmic C_1-THF synthase. The reduced growth rate of MWY4.4 in minimal media containing serine (6.5 h) illustrates the contribution of the mitochondrial pathway. This strain lacks both cytoplasmic CH_2-THF dehydrogenases; CHO-THF can be produced only from mitochondrially-generated formate. Addition of adenine to the media allowed MWY4.4 to grow at wild-type rates.

Blocking the cytoplasmic synthetase activity allows us to eliminate any contribution of the mitochondrial pathway. Comparing the strains in the middle panel of Table 2, it can be seen that either cytoplasmic CH_2-THF dehydrogenase alone could supply oxidized one-carbon units for purine synthesis. MWY4.1, which lacks the NAD-dependent activity, and CBY5, which lacks the NADP-dependent activity, both grew at normal rates. Only when both cytoplasmic dehydrogenases were missing, in a synthetase-minus background, did the cells require purines for growth (MWY4.5 and MWY4.6).

The last panel in Table 2 illustrates the role of the cyclohydrolase activity. CBY6, which has an active NAD-dependent CH_2-THF dehydrogenase but no cyclohydrolase or synthetase activity, grew at half the rate of CBY5, which has retained 40% of its cyclohydrolase activity (Barlowe & Appling, 1990b). Thus, in CBY6, the non-enzymatic hydrolysis of CH^+-THF to CHO-THF becomes growth-limiting in the absence of purines.

The bottom part of Table 2 shows growth rates on glycine plus formate media to investigate flux in the reductive direction. Wild-type strain DAY4 grew with a 5 h doubling time in minimal media with glycine and formate in place of serine. This slower growth was due mainly to the serine requirement, since addition of adenine did not restore normal growth rates. If only the NADP-dependent CH_2-THF dehydrogenase was active, as in MWY4, growth was essentially identical to DAY4. On the other hand, if only the NAD-dependent CH_2-THF dehydrogenase was active (CBY4), or if neither dehydrogenase was active (MWY4.4), the doubling time increased to 12−13 h.[2] These results suggest that the NAD-dependent CH_2-THF dehydrogenase does not contribute to flux in the reductive direction.

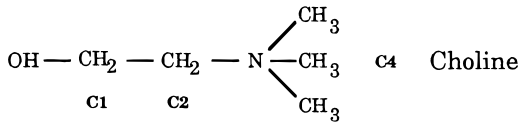
Absence of cytoplasmic CHO-THF synthetase activity (Table 2, last 2 panels) profoundly affected growth on glycine and formate, whether or not any CH_2-THF dehydrogenase was present. For example, CBY1, which expresses both dehydrogenases, exhibited a 25 h doubling time compared to the 5 h doubling time of DAY4. Supplementation of these strains with adenine gave slightly faster growth, but normal growth rates could only be achieved in these strains if serine was supplied in the media. Since strains lacking cytoplasmic synthetase activity must produce serine from formate via the mitochondrial pathway, this route must be growth-limiting.

Strains with both cytoplasmic dehydrogenases inactive in addition to an inactive synthetase (MWY4.5 and MWY4.6) showed no growth on glycine plus formate, since these cells are unable to produce a cytoplasmic pool of CHO-THF for purine synthesis. Addition of adenine to the glycine/formate media supported doubling times of 18 h (MWY4.5) and 12 h (MWY4.6). Serine could not replace adenine for these two strains. Supplementation with both adenine and serine enabled these strains to grow similarly to DAY4.

^{13}C NMR Analysis of Reductive One-Carbon Flux through the NAD- and NADP-Dependent CH_2-THF Dehydrogenases in Vivo. The similar growth rates of strains CBY4 and MWY4.4 in minimal media with glycine plus formate suggested that the NAD-dependent CH_2-THF dehydrogenase does not participate in the reduction of formate to CH_2-THF.

[2] We previously reported (Barlowe & Appling, 1990b) that CBY4 grows with a doubling time of >24 h in this media. Repeated experiments revealed that CBY4 typically grows with a 12−13 h doubling time, as shown in Table 2.

Table 3: Effect of Exogenous Unlabeled Formate on NAD- and NADP-Dependent CH_2-THF Dehydrogenase Mutant Strains Grown in [2-^{13}C]Glycine[a]



strain	RE_{C4}[b]	$\frac{RE_{C4} + \text{formate}}{RE_{C4} - \text{formate}}$
DAY4		
−formate	0.64	0.23
+formate	0.15	
MWY4		
−formate	0.57	0.32
+formate	0.18	
CBY4		
−formate	0.60	0.68
+formate	0.41	
MWY4.4		
−formate	0.74	0.69
+formate	0.51	

[a] Growth was in minimal media as described under Experimental Procedures with the addition of 100 mg of [2-^{13}C]glycine/L, with or without 1.3 mM unlabeled formate as noted. [b] RE_{C4} is the relative enrichment, reflecting incorporation of labeled one-carbon units at C4 relative to labeled glycine incorporation into choline at C2.

To investigate this possibility more directly, we used ^{13}C NMR to monitor the incorporation of one-carbon units derived from [2-^{13}C]glycine into phosphatidylcholine (PC), a metabolite whose synthesis depends on cytosolic CH_2-THF.

Four strains were studied: DAY4 (expressing both cytoplasmic CH_2-THF dehydrogenases), MWY4 (expressing only the NADP-dependent dehydrogenase), CBY4 (expressing only the NAD-dependent dehydrogenase), and MWY4.4 (expressing neither cytoplasmic CH_2-THF dehydrogenase). The cells were grown to saturation in minimal media containing [2-^{13}C]glycine, either with or without unlabeled formate, and extracts containing cellular metabolites were prepared for ^{13}C NMR analysis. In the extraction procedure (see Experimental Procedures), cellular PC and phosphocholine were converted to choline by heating the cells in acid.

All five carbons of choline (structure depicted with Table 3) can become labeled when the cells are grown in [2-^{13}C]-glycine. The glycine is metabolized to PC beginning with the mitochondrial synthesis of serine (Figure 1, reaction 4) from glycine and the CH_2-THF derived from glycine cleavage (reaction 5). Serine, labeled in both C2 and C3, is then transported to the cytosol where it is incorporated into phosphatidylserine (PS) and phosphatidylethanolamine (PE) (Figure 1, reactions 10 and 11) (Bae-Lee & Carman, 1984; Chin & Bloch, 1988); serine C3 also provides CH_2-THF for cytosolic one-carbon requirements. CH_2-THF is reduced to CH_3-THF (reaction 7) for AdoMet synthesis (reactions 8 and 9) (Banerjee & Matthews, 1990). CH_2-THF is the sole one-carbon donor, via CH_3-THF and AdoMet, for all three methylations of PE. Thus, the C2 and C3 of serine become the C1 and C2 of the choline moiety of PC, and the C3 of serine is incorporated into the C4 of the choline moiety of PC (Pasternack et al., 1994).

Cytoplasmic CH_2-THF can also be produced by reduction of cytoplasmic CHO-THF pools, which can be synthesized from exogenously added formate (Figure 1, reactions 1−3 and 6). If cells are grown with both [2-^{13}C]glycine and unlabeled formate, dilution of ^{13}C labeling in choline by the formate-derived one-carbon units can be used to estimate reductive flux through the CH_2-THF dehydrogenase reaction (Pasternack et al., 1996). Thus, metabolism of unlabeled formate to CH_2-THF will decrease the ^{13}C enrichment of that pool, detected by a decreased enrichment of the choline C4. Figure 3 shows representative spectra for several strains. All three positions are enriched with ^{13}C in each sample, but the relative enrichment of the C4 varies considerably. For example, when DAY4 is grown in the presence of formate, the C4 resonance is reduced relative to the C2 (*cf.* the first two panels). MWY4, CBY4, and MWY4.4 show varying degrees of dilution by formate. The minus-formate spectra for each of these (not shown) are similar to the minus-formate spectrum for DAY4.

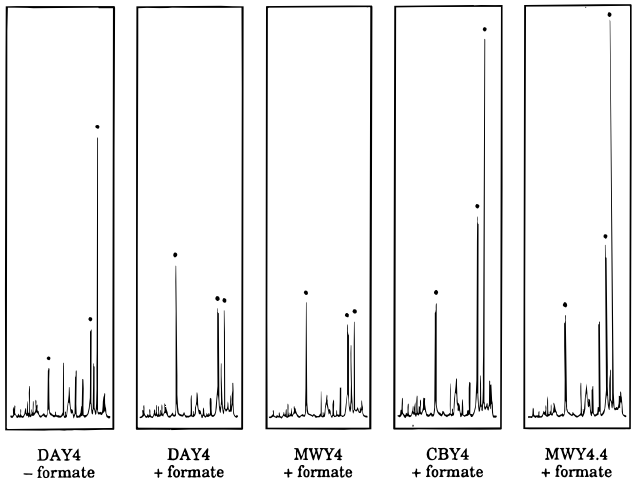


FIGURE 3: ^{13}C NMR spectra. Choline resonances of cell extracts from different strains grown in [2-^{13}C]glycine, with and without 1.3 mM unlabeled formate as indicated. Three dots in each panel indicate the labeled carbons of choline; the left-most dot is C2 (68 ppm), the middle is C1 (56 ppm), and the right-most is C4 (54 ppm). The ^{13}C NMR profile shown in each panel spans from 50 to 80 ppm, referenced to DMSO at 39.5 ppm.

Analysis of the spectra was done as follows. First, the C4 relative enrichment (RE_{C4}) was determined, representing the amount of ^{13}C label present at C4 of choline compared to the amount of label present at C2 (which is not dependent on the CH_2-THF pool). Next, we compared the RE_{C4} from cells grown with formate to that obtained from cells grown without formate to determine the flux through the CH_2-THF dehydrogenase step. This ratio of relative enrichment in C4 (with vs without formate) for a particular strain indicates the extent of dilution of label at C4 of choline and thus the ability of the CH_2-THF dehydrogenase to reduce unlabeled CH^+-THF to CH_2-THF. Growing the cells in labeled glycine but no formate established the maximal RE_{C4} for each strain. The results are summarized in Table 3. The relative enrichments at C4 for DAY4, MWY4, CBY4, and MWY4.4 grown without formate were roughly the same (57%−74%). When unlabeled formate was included, some dilution of ^{13}C in choline C4 was observed for all strains. However, the ratio of relative enrichment in C4 (with vs without formate) was much lower for DAY4 and MWY4 than for CBY4 and MWY4.4, indicating greater dilution of label in the former strains. DAY4 and MWY4 both express the NADP-dependent CH_2-THF dehydrogenase, whereas CBY4 and MWY4.4 express only the NAD-dependent enzyme (CBY4) or neither (MWY4.4). Thus, significant reductive flux

Table 4: Analysis of CHO-THF Levels in Selected Strains[a]

strain	adenine phenotype[b]	nmol of CHO-THF: g of wet weight of cells	nmol of total folate: g of wet weight of cells
DAY3	−	16.0 ± 0.8 (2)	123.5 ± 0.8
MWY3	−	<1.0 (2)	84 ± 8
CBY6	+	9.31 ± 0.06 (2)	61.2 ± 0.5
MWY4.6	−	<1.0 (2)	71 ± 6
DAY4	+	<1.0 (3)	68.5 ± 0.2

[a] Yeast were grown to late-log phase in minimal media with the addition of adenine when required for growth: DAY3, MWY3, MWY4.6. Cell extracts were prepared as described in Experimental Procedures. Values are means ± SEM. SEMs for <1.0 were not determined since this value represents the lower limit of the detection method used. The number in parentheses is the sample size. [b] Adenine phenotypes are designated as + for strains that grow without adenine added to minimal media and − for strains which do not grow without added adenine.

through the CH_2-THF dehydrogenase reaction correlates only with an active NADP-dependent enzyme. In fact, CBY4 and MWY4.4 exhibited essentially identical ratios, indicating no contribution by the NAD-dependent dehydrogenase under these conditions.

CHO-THF Levels in Wild-Type and Mutant Strains. The phenotype of an *ade3* deletion strain such as DAY3 is a strict requirement for adenine, whereas a strain (CBY6) expressing a stable triple point mutant of *ADE3* (*ADE3−30/65/144*) does not require adenine (Barlowe & Appling, 1990b). This surprising result led us to propose the existence of a purine-synthesizing multienzyme complex in which C_1-THF synthase is a required structural participant. This model predicts that CHO-THF, the one-carbon donor used in purine synthesis, is necessary but not sufficient for purine biosynthesis. To test this prediction, we measured the CHO-THF levels in cytosolic extracts of selected strains grown in minimal media. The results are presented in Table 4. There was a significant accumulation of CHO-THF in DAY3 and CBY6 cytosolic extracts. Since neither of these strains expresses synthetase or NADP-dependent dehydrogenase activity, the CHO-THF is being produced by the *MTD1*-encoded NAD-dependent CH_2-THF dehydrogenase. CHO-THF was undetectable when these two strains were additionally disrupted at the *MTD1* locus (MWY3 and MWY4.6, respectively). CHO-THF was also undetectable in the wild-type DAY4. Clearly, cytoplasmic CHO-THF levels do not correlate with the ability to synthesize purines *in vivo*.

Complementation of the ade3 Deletion Phenotype by Individual Domains of C_1-THF Synthase. The C_1-THF synthase is modeled as two functionally independent domains with the D/C activities residing in the 33 kDa N-terminal region and the synthetase activity residing in the remaining C-terminal 70 kDa (Appling & Rabinowitz, 1985a). It has been reported that the D/C domain of yeast C_1-THF synthase (Song & Liebman, 1989) or a monofunctional CHO-THF synthetase from spinach or *Clostridium acidiurici* (Song & Rabinowitz, 1993) can complement the adenine requirement of an *ade3* deletion strain. However, the host strain used in those studies was wild-type at the *MTD1* locus, and thus expressed the NAD-dependent CH_2-THF dehydrogenase, which enables the cell to make CHO-THF (Table 4). Thus, it is impossible to determine whether complementation was due to catalytic activity of the expressed domains, to some structural role of the protein, or to both. The domain

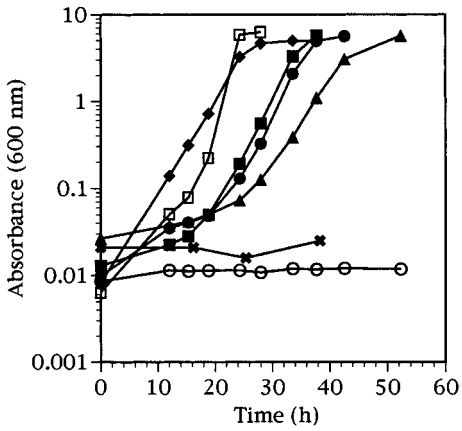


FIGURE 4: D/C domain of yeast C_1-THF synthase complements *ade3* deletion phenotype. Growth profiles of DAY4 (◆), DAY3-[pVT-103U] (×), DAY3[pADE3] (■), DAY3[pD/C-WT] (●), DAY3[pD/C-65/144] (▲), MWY3[pADE3] (□), and MWY3[pD/C-WT] (○) all grown in minimal media with serine (no adenine).

expression experiments described below utilize strain MWY3, which carries both an *ade3* deletion and a disruption of the *MTD1* locus. This strain requires adenine but, in contrast to DAY3, has undetectable levels of cytoplasmic CHO-THF (Table 4).

The first experiment utilized the multicopy expression vector pVT-U with the following two gene constructs: (i) the D/C domain of *ADE3* (named [pD/C-WT]) and (ii) a catalytically inactive D/C domain of *ADE3* (named [pD/C-65/144]). These plasmids were constructed from either the wild-type *ADE3* gene, or *ADE3−30/65/144* (Barlowe & Appling, 1990b) as described in Experimental Procedures. These plasmids, as well as one containing the wild-type *ADE3* ([pADE3]), were introduced into strains DAY3 and MWY3. The growth profiles of the transformed strains in minimal media with serine, but lacking adenine, are shown in Figure 4. DAY3 transformed with vector alone (×) did not grow without adenine. DAY3[pD/C-WT] (●) growth was similar to DAY3[pADE3] (■) and DAY4 (◆). DAY3 expressing the inactive D/C domain (▲) grew at a slightly slower rate. In contrast, [pD/C-WT] did not support growth of strain MWY3 without purines (○). This last result implies that the plasmid-encoded D/C domain is not catalytically active *in vivo*. In fact, we were unable to detect NADP-dependent CH_2-THF dehydrogenase activity in the strains containing either [pD/C-WT] or [pD/C-65/144]. It should be noted that we were also unable to detect the D/C domain by immunoblot of cellular extracts of strains containing [pD/C-WT] or [pD/C-65/144] using polyclonal antisera to yeast C_1-THF synthase (data not shown).

The parallel experiment using the CHO-THF synthetase domain from yeast C_1-THF synthase yielded somewhat different results (Figure 5A). These plasmids were also constructed from either the wild-type *ADE3* gene, or the *ADE3−30/65/144* mutant (Barlowe & Appling, 1990b). Both DAY3[pSynthetase-WT] (●) and MWY3[pSynthetase-WT] (○) grew without adenine but at slower doubling times than DAY3[pADE3] (■) or MWY3[pADE3] (□) and to only one-sixth of the final cell density. The [pSynthetase-30] plasmid was unable to support growth of either DAY3 (▲) or MWY3 (△) without adenine. The [pSynthetase-WT] plasmid over-expressed synthetase activity to about half the level obtained with [pADE3], whereas no synthetase activity was detected

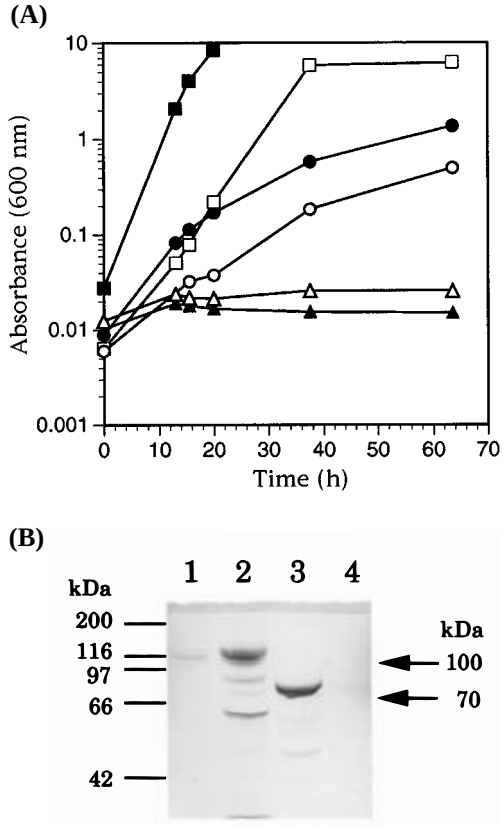


FIGURE 5: Synthetase domain of yeast C_1-THF synthase complements *ade3* deletion phenotype. (A) Growth profiles of DAY3[pADE3] (■), DAY3[pSynthetase-WT] (●), DAY3[pSynthetase-30] (▲), MWY3[pADE3] (□), MWY3[pSynthetase-WT] (○), and MWY3[pSynthetase-30] (△), all grown in minimal media with serine (no adenine). (B) Immunoblot of yeast extracts using polyclonal C_1-THF synthase antisera. Relative molecular masses of standard proteins are shown along the left side of the immunoblot and the approximate mass of the C_1-THF synthase and synthetase domain proteins are shown along the right side. Lane 1, DAY4; lane 2, DAY3[pADE3]; lane 3, DAY3[pSynthetase-WT]; and lane 4, DAY3[pSynthetase-30].

in strains transformed with [pSynthetase-30] (data not shown). An immunoblot using polyclonal antisera to the yeast C_1-THF synthase revealed high levels of immunoreactive species in cellular extracts of DAY3[pADE3] and DAY3[pSynthetase-WT] (Figure 5B, lanes 2 and 3) but little or no staining in the DAY3[pSynthetase-30] extract (lane 4).

DISCUSSION

The experiments described here revealed four important results concerning the functions of the *ADE3* and *MTD1* gene products in *S. cerevisiae*. First, either cytoplasmic CH_2-THF dehydrogenase can support one-carbon flux in the oxidative direction, whereas only the NADP-dependent enzyme appears to be involved in the reductive direction. Second, when neither cytoplasmic dehydrogenase is active, the cell must rely on the mitochondrial pathway for one-carbon interconversions, and this pathway is limiting for growth. Third, cytoplasmic CHO-THF is necessary, but not sufficient, for purine biosynthesis, and can be provided from CH_2-THF via either CH_2-THF dehydrogenase, or directly from formate via the synthetase activity of the cytoplasmic C_1-THF synthase. Finally, while the individual dehydrogenase/cyclohydrolase or synthetase domains can complement the adenine requirement of an *ade3* deletion strain, at least in the case of the D/C domain, complementation does not require catalytic activity as long as an alternate source of CHO-THF is available.

The interchangeability of the NADP- and NAD-dependent CH_2-THF dehydrogenases in supporting *oxidative* one-carbon flux is apparent from the growth studies. The presence of either enzyme alone supported identical, wild-type growth rates in minimal media containing serine but no adenine, which requires the oxidation of serine-derived one-carbon units to CHO-THF. Only when both cytoplasmic dehydrogenases were missing, in a synthetase-minus background, did the cells require purines for growth.

Growth of *ser1*$^-$ strains on glycine plus formate in place of serine was used to investigate the ability of the cytosolic dehydrogenases to support flux of one-carbon units in the *reductive* direction. The growth studies suggested that the NAD-dependent CH_2-THF dehydrogenase does not play a role in the reductive direction. Thus, strains lacking only the *MTD1* gene product exhibited identical growth rates to the wild-type strain on glycine plus formate. When the NADP-dependent enzyme was missing, growth was significantly slower than normal; additional loss of the NAD-dependent activity had no further effect. This interpretation was confirmed by the ^{13}C-NMR experiments. When wild-type cells were grown with [2-^{13}C]glycine to label the one-carbon pools, unlabeled formate effectively diluted the reduced CH_3-THF pools used in PC biosynthesis. The ability of formate to dilute the C4 labeling of choline was unaffected in the strain lacking only the NAD-dependent CH_2-THF dehydrogenase (MWY4), whereas it was greatly reduced in strains lacking the NADP-dependent enzyme (CBY4 and MWY4.4). Thus, the NAD-dependent enzyme seems to operate only in the oxidative direction *in vivo*.

These results are consistent with what is known about the redox states of the cytoplasmic NAD/NADH and NADP/NADPH redox couples in eukaryotic cells. The free concentration ratios reported for the cytosolic NAD/NADH redox system in various growth conditions consistently indicate an oxidized state in yeast (Gancedo & Gancedo, 1973), as in other systems (Sies, 1982; Veech, 1987). Where both have been measured, the cytosolic NADP/NADPH redox state is always more reduced than the NAD/NADH couple (Sies, 1982). These observations support the generalization that most cytoplasmic NADP-linked dehydrogenases function in biosynthetic reductions whereas most cytoplasmic NAD-dependent dehydrogenases catalyze substrate oxidations (Atkinson, 1977). Thus, we would predict that the NAD-linked CH_2-THF dehydrogenase would operate efficiently in the oxidative direction, but not in the reductive direction. This is precisely what we observed. Likewise, we would predict that the NADP-linked enzyme would support flux in the reductive direction more efficiently than in the oxidative direction. However, we found that the NADP-dependent dehydrogenase supported normal one-carbon flux in both directions. The relative oxidative flux through the two dehydrogenases in wild-type cells expressing both enzymes is not known. Certainly the NAD-dependent CH_2-THF dehydrogenase is more likely to oxidize CH_2-THF on the basis of redox states. However, in many organisms where the physiological direction of the CH_2-THF dehydrogenase reaction is thought to be in the oxidative direction, the reaction is catalyzed by an NADP-dependent enzyme

(Ragsdale & Ljungdahl, 1984). Atkinson (1977) has argued that this exception to the rule stems from the fact that the equilibrium between CH_2-THF and CHO-THF lies far toward CHO-THF; if this oxidation relied solely on NAD, the cell would find it difficult to maintain adequate steady state levels of CH_2-THF. The use of NADP instead may have evolved to keep this reaction from going too far *in vivo*.

S. cerevisiae is the only organism reported to have two distinct extramitochondrial NAD- and NADP-dependent CH_2-THF dehydrogenases. Redundant systems are commonly seen in metabolic pathways in yeast, and, rather than being truly redundant, they generally reflect the ability of the cell to respond to differing anabolic needs of the cell as oxygen or nutrient levels vary (Wills, 1990). In the case of the folate metabolic pathway, the existence of both an NAD- and NADP-requiring CH_2-THF dehydrogenase would allow shifting of the interconversion of one-carbon units toward either the more oxidized form for *de novo* purine synthesis or the more reduced form for methyl group generation or thymidylate biosynthesis as conditions change. Further experiments will be required to fully define the physiological roles for the NAD-dependent CH_2-THF dehydrogenase.

The second important result obtained concerns the role of the mitochondrial pathway of folate-mediated one-carbon metabolism. In a wild-type strain in which both pathways are functional, it appears that at least 25% of one-carbon units for purine synthesis are metabolized through the mitochondrial pathway (Pasternack et al., 1994). In a strain in which neither cytoplasmic dehydrogenase is active, cells must rely on the mitochondrial pathway for one-carbon interconversions. Studies with MWY4.4 and CBY1 indicate that metabolism of one-carbon units exclusively by the mitochondrial pathway is growth-limiting. Pasternack et al. (1994) previously showed using ^{13}C NMR that MWY4.4 is capable of supplying virtually all of its cytoplasmic one-carbon units from mitochondrially-derived formate. The slower growth of MWY4.4 (lacking both dehydrogenases) compared to CBY1 (lacks cytoplasmic synthetase activity) in minimal media containing serine, but no adenine, reveals that when the mitochondrial pathway is the *only* way to generate one-carbon units for purine synthesis, there is not sufficient flux through it to maintain wild-type growth rates. On the other hand, CBY1 grown in glycine and formate is forced to synthesize serine from glycine and CH_2-THF, produced by the reduction of formate by the mitochondrial C_1-THF synthase. CBY1 grew quite slowly, again reflecting dependence on the limiting mitochondrial pathway.

There are several curious results in the bottom part of Table 2 for which there is no clear explanation. For example, CBY1 growth on glycine and formate was not improved significantly by addition of adenine, suggesting that inactivation of the synthetase affects other reactions as well. It is also surprising that glycine, formate, and serine do not support normal growth rates for MWY4.4, since formate should be providing ample CHO-THF for purine synthesis. In fact, adenine is required to restore normal growth. This is reminiscent of growth of this strain in minimal media and serine. Apparently, the inability of MWY4.4 to utilize the one-carbon units from serine through the cytosolic dehydrogenases is still limiting growth.

The third important conclusion from these studies is that CHO-THF is necessary, but not sufficient for purine synthesis. Before our discovery of the NAD-dependent CH_2-THF dehydrogenase, the strict adenine requirement of *ade3* deletion strains had been assumed to result from lack of cytoplasmic CHO-THF due to loss of C_1-THF synthase catalytic activities (Jones, 1972, 1977; Song & Rabinowitz, 1993). However, CHO-THF levels in yeast containing the *ade3−130* deletion mutation had not previously been determined. Both CBY6 and DAY3 showed large accumulations of CHO-THF, while in MWY3 and MWY4.6 (sister strains lacking NAD-dependent CH_2-THF dehydrogenase), CHO-THF was below detection. Therefore, the NAD-dependent CH_2-THF dehydrogenase provides CHO-THF in both CBY6 and DAY3, as previously suggested (West et al., 1993). Clearly, the strict adenine requirement of the *ade3* deletion strain is not due to loss of C_1-THF synthase's catalytic activity, since the *MTD1* gene product can supply the necessary CHO-THF. The question that remains is why the CHO-THF produced in CBY6 can be utilized in purine synthesis, while the CHO-THF pool in DAY3 cannot. A possible resolution to this dilemma is our proposal that, in addition to its catalytic function, the *ADE3* gene product also plays a noncatalytic, structural role in purine synthesis (Barlowe & Appling, 1990b).

CHO-THF was also below detection in DAY4, even though this strain synthesizes purines at normal rates. Lor and Cossins (1972) reported a measurable CHO-THF pool in *S. cerevisiae* strain ATCC 9763. Strain 9763 is apparently a serine prototroph, whereas DAY4 carries a *ser1* mutation. In both studies, the yeast were grown in minimal media containing serine. It is possible that in serine-requiring strains, flux through the folate-mediated pathway may be such that no CHO-THF accumulates under conditions where both the cytosolic and mitochondrial routes for one-carbon interconversion to CHO-THF are unimpeded.

The final result of this work concerns the role of the C_1-THF synthase protein in purine synthesis. Expression of the D/C domain of the yeast C_1-THF synthase in *ade3* deletion strains was previously shown to enable growth without adenine (Song & Liebman, 1989; Thigpen et al., 1993). However, those experiments used strains that are wild-type at the *MTD1* locus. Thus, differentiating between a catalytic versus a structural function of the D/C domain in supporting purine synthesis was not possible because there existed an alternative way for the cell to make CHO-THF. Performing the same experiment in strains lacking the NAD-dependent CH_2-THF dehydrogenase sheds new light on this result. Both DAY3[pD/C-WT] and DAY3[pD/C-65/144] grew in minimal media lacking adenine, suggesting that production of the necessary CHO-THF for purine synthesis was catalyzed by the *MTD1* gene product present in these cells. This was confirmed by the inability of MWY3[pD/C-WT] to grow in media lacking adenine. This last result suggests that neither the wild-type nor mutant forms of the plasmid-borne NADP-dependent D/C domain were catalytically active *in vivo*.

In support of this, NADP-dependent CH_2-THF dehydrogenase activity was undetectable by enzyme assay of extracts from cells transformed with either DAY3[pD/C-WT] or DAY3[pD/C-65/144]. Although *in vitro* proteolysis of purified mammalian C_1-THF synthase can yield an active D/C domain (Tan & MacKenzie, 1977; Villar et al., 1985), genetic or biochemical manipulation has yet to successfully produce a catalytically active yeast D/C domain (Paukert et al., 1977). Curiously, our polyclonal antibodies raised against the full-length yeast C_1-THF synthase did not react

with any protein in immunoblots of crude extracts of any of the strains containing either [pD/C-WT] or [pD/C-65/144] (data not shown). Immunoprecipitation experiments done previously with these antibodies showed that the synthetase activity was much more sensitive to inhibition than either dehydrogenase or cyclohydrolase activity (Appling & Rabinowitz, 1985a). It is possible then, that the D/C domain is expressed but is recognized by such a small fraction of the antibody population that it remains below the detection limits of our immunoblot.

Despite our inability to detect expression of the D/C domain, the wild-type and mutant constructs were clearly able to complement the adenine-requiring phenotype of the *ade3* deletion strain (DAY3), whereas vector alone did not. Since neither D/C domain construct exhibited catalytic activity, complementation in DAY3 by this domain is clearly not related to its catalytic function—CHO-THF is being produced by the NAD-dependent CH_2-THF dehydrogenase in DAY3. If there is no alternate supply of CHO-THF, as in MWY3[pD/C-WT], the strain remains auxotrophic for adenine.

In contrast to the D/C domain constructs, the overexpressed wild-type synthetase domain was both active and easily visualized by immunoblot of cell extracts. Both DAY3[pSynthetase-WT] and MWY3[pSynthetase-WT] grew well without adenine, indicating that the wild-type synthetase domain complemented the adenine-requiring phenotype, and was able to synthesize CHO-THF from mitochondrially-derived formate for purines with or without participation of the NAD-dependent CH_2-THF dehydrogenase. On the other hand, the mutant synthetase domain ([pSynthetase-30]) did not complement the *ade3−130* deletion phenotype of DAY3 or MWY3. In this case, however, it was clear from immunoblots that the mutant synthetase domain was not stably expressed from the [pSynthetase-30] construct. Thus, we are unable to determine whether complementation by the wild-type synthetase domain was due to its catalytic activity, or a noncatalytic function. However, CBY6, which expresses a stable full-length, but catalytically-inactive C_1-THF synthase (contains the same synthetase point mutations as our [pSynthetase-30] construct), does not require purines [Table 4 and Barlowe and Appling (1990b)]. Clearly, CHO-THF synthetase catalytic activity is not necessary for production of CHO-THF and purine synthesis in *MTD1*$^+$ cells.

Song and Rabinowitz (1993) demonstrated that a partial deletion of *ADE3*, lacking residues 115−170 in the D/C domain, supported growth in the absence of purines at rates only slightly slower that the wild-type allele. In addition, they reported that a monofunctional CHO-THF synthetase from spinach complemented the adenine requirement of the *ade3−130* deletion strain; the monofunctional synthetase from *C. acidiurici* allowed very slow growth (23 h doubling time) on minus-adenine media only if supplemented with formate.[3] However, the levels of activity of the three constructs described by Song and Rabinowitz did not correlate with growth rates in media lacking adenine. In any event, the presence of the *MTD1* gene product in their host strain brings into question their conclusion that complementation was due to synthetase catalytic activity rather than some structural function.

Our domain expression experiments, coupled with direct measurement of CHO-THF levels, shed further light on the proposed structural role of C_1-THF synthase in purine biosynthesis. The results show that, while CHO-THF is necessary for purine biosynthesis, it is not sufficient. Cell growth in the absence of purines requires, in addition, the *ADE3* gene product or a stable form of one of its domains, or certain homologs of one of the domains. However, that gene product or domain does not have to be catalytically active, as long as an alternate source of CHO-THF is available (*e.g.*, from the NAD-dependent CH_2-THF dehydrogenase). In normal cells, the necessary CHO-THF is synthesized by the activities of C_1-THF synthase, by the *MTD1* gene product, or by both. In any event, its utilization in *de novo* purine synthesis depends on some structural function of C_1-THF synthase.

The differential complementation efficiency of the various constructs appear to correlate with differences in sequence or quaternary structure of the proteins and how they interact with other proteins in the cell. For example, the spinach synthetase, which supported a 5 h doubling time in the absence of purines, was much more effective than the bacterial enzyme (23 h doubling time) (Song & Rabinowitz, 1993). The synthetase domain of the *ADE3*-encoded C_1-THF synthase complements the adenine requirement slightly more effectively (Figure 5, ∼4.0 h doubling time), but less so than the full-length homologous protein. Both C_1-THF synthase (Paukert et al., 1977) and the spinach enzyme (Nour & Rabinowitz, 1991) are homodimers, whereas the bacterial enzyme exists as a tetramer (Scott & Rabinowitz, 1967).

How can individual domains complement the proposed structural function of C_1-THF synthase? Multienzyme complexes are most likely stabilized by multiple protein-protein interactions (Srere, 1987). While each individual interaction is weak, cooperativity results in a stable complex, analogous to the hydrogen bond networks that stabilize secondary structures of proteins. This concept is seen in recent studies on RNA polymerase II transcription complexes (Chi et al., 1995; Koleske & Young, 1995). Thus, we envision that both domains of C_1-THF synthase are involved in multiple protein−protein interactions within the putative purine-synthesizing complex. While the complex might withstand loss of one set of contacts or another, loss of both sets of contacts destabilizes the complex to the point at which it can no longer catalyze purine synthesis at the high rates required *in vivo*.

In summary, these results further define the metabolic capabilities of the two cytoplasmic CH_2-THF dehydrogenases in both the reductive and oxidative directions, and reveal the growth-limiting nature of one-carbon flux through the mitochondrial pathway. Finally, the experiments confirm that a supply of CHO-THF is necessary, but not sufficient, for purine biosynthesis in growing yeast, and the *ADE3* gene product has, in addition, a required noncatalytic role, perhaps as an integral component of a multienzyme complex involved in *de novo* purine synthesis as previously proposed.

[3] We previously reported (Barlowe & Appling, 1990b) that the *C. acidiurici* monofunctional synthetase did not complement the *ade3−130* mutation on the basis of plate assays, which would not have detected colonies with a 23 h doubling time.

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