The folate derivative 5-formyltetrahydrofolate (folinic acid; 5-CHO-THF) was discovered over 40 years ago, but its role in metabolism remains poorly understood. Only one enzyme is known that utilizes 5-CHO-THF as a substrate: 5,10-methenyltetrahydrofolate synthetase (MTHFS). A BLAST search of the yeast genome using the human MTHFS sequence revealed a 211-amino acid open reading frame (YER183c) with significant homology. The yeast enzyme was expressed in Escherichia coli, and the purified recombinant enzyme exhibited kinetics similar to previously purified MTHFS. No new phenotype was observed in strains disrupted at MTHFS or in strains additionally disrupted at the genes encoding one or both serine hydroxymethyltransferases (SHMT) or at the genes encoding one or both methyltetrahydrofolate reductases. However, when the MTHFS gene was disrupted in a strain lacking the de novo folate biosynthesis pathway, folinic acid (5-CHO-THF) could no longer support the folate requirement. We have thus named the yeast gene encoding methenyltetrahydrofolate synthetase FAU1 (folinic acid utilization). Disruption of the FAU1 gene in a strain lacking both 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase isoenzymes (ADE16 and ADE17) resulted in a growth deficiency that was alleviated by methionine. Genetic analysis suggested that intracellular accumulation of the purine intermediate AICAR interferes with a step in methionine biosynthesis. Intracellular levels of 5-CHO-THF were determined in yeast disrupted at FAU1 and other genes encoding folate-dependent enzymes. In fau1 disruptants, 5-CHO-THF was elevated 4-fold over wild-type yeast. In yeast lacking MTHFS along with both AICAR transformylases, 5-CHO-THF was elevated 12-fold over wild type. 5-CHO-THF was undetectable in strains lacking SHMT activity, confirming SHMT as the in vivo source of 5-CHO-THF. Taken together, these results indicate that S. cerevisiae harbors a single, nonessential, MTHFS activity. Growth phenotypes of multiply disrupted strains are consistent with a regulatory role for 5-CHO-THF in one-carbon metabolism and additionally suggest a metabolic interaction between the purine and methionine pathways.

Although 5-formyl-tetrahydrofolate (5-CHO-THF); folinic acid or leucovorin) is the most stable derivative of the reduced folates (1), it does not have a known direct role as a one-carbon donor. Leucovorin has been employed extensively as a rescue agent in chemotherapeutic protocols, but the physiological role of this folate derivative remains poorly understood. 5-CHO-THF can arise nonenzymatically from the hydrolysis of 5,10-methylene-THF (5,10-CH-THF) under mild conditions (2). For this reason, it was considered likely that the 5-CHO-THF detected in cell extracts was an artifact of preparation rather than a normal component of folate metabolism. More recent studies designed to prevent the possibility of artifactual conversion concluded that 5-CHO-THF is indeed a naturally occurring metabolite (3). The biological source of 5-CHO-THF, however, remains in question. Stover and Schirch (4) showed that in the presence of glycine, serine hydroxymethyltransferase (SHMT) catalyzes the hydrolysis of 5,10-CH-THF to 5-CHO-THF and proposed that this reaction is the source of cellular 5-CHO-THF. Supporting evidence was provided by the observation that cells deficient in SHMT activity do not accumulate 5-CHO-THF (3, 5). SHMT is an abundant enzyme that normally catalyzes the reversible conversion of serine to glycine coupled to the production of 5,10-methylene-THF (5,10-CH-THF); this reaction is the major source of one-carbon units in most cells (6). The physiological significance of the SHMT-catalyzed hydrolysis of 5,10-CH-THF to 5-CHO-THF has recently been called into question. Baggott (7) studied the chemical hydrolysis of 5,10-CH-THF under mildly acidic conditions and concluded that the intermediate proposed by Stover and Schirch does not accumulate and that the likely source of 5-CHO-THF is chemical hydrolysis of 5,10-CH-THF within subcellular organelles rather than the SHMT-catalyzed reaction.

Whereas the biological source of 5-CHO-THF remains controversial, an enzyme that catalyzes its conversion back to 5,10-CH-THF has been identified and characterized from several prokaryotic and eukaryotic sources (8–11). Methenyltetrahydrofolate synthetase (MTHFS; EC 6.3.3.2) catalyzes the irreversible (12) ATP-dependent reaction,

\[
5\text{-CHO-THF} + \text{MgATP} \rightarrow 5\text{-CH-THF} + \text{MgADP} + \text{P}.
\]

REACTION 1

This is the only enzyme known that utilizes 5-CHO-THF as a

*This work was supported by National Institutes of Health Grant RR09276. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: 5-CHO-THF, 5-formyltetrahydrofolate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; DHFR, dihydrofolate reductase; DHFS, dihydrofolate synthase; THF, (6R,6S)-tetrahydrofolate; 10-CHO-THF, 10-formyltetrahydrofolate; 5,10-CH-THF, 5,10-methenyltetrahydrofolate; SHMT, serine hydroxymethyltransferase(s); MTHFS, 5,10-methenyltetrahydrofolate synthetase; ORF, open reading frame; ZTP, 5-aminoimidazole carboxamide riboside 5′-triphosphate.
substrate. The existence of this enzyme from bacteria to humans strongly suggests that 5-CHO-THF plays a universally important role in folate-mediated one-carbon metabolism. 5-CHO-THF has been proposed to function as a stable storage form of one-carbon units (3) and as a regulator of the flow of one-carbon units (13), based on its ability to inhibit several folate-utilizing enzymes, including SHMT. Indeed, Girgis et al. (14) have presented evidence from cultured cells that 5-CHO-THF regulates homocysteine methylation via inhibition of SHMT.

In an effort to better understand the metabolic role of 5-CHO-THF and MTHFS, we have cloned and disrupted the gene encoding MTHFS (YER183c) from the yeast Saccharomyces cerevisiae. We have purified and characterized the yeast enzyme. Genetic experiments revealed that this enzyme is required for folinic acid utilization in yeast, and we have named the gene FAU1. We have also characterized yeast strains lacking MTHFS in combination with other enzymes of folate acid metabolism and measured cellular 5-CHO-THF levels in these strains. These studies confirm the role of MTHFS in the metabolism of 5-CHO-THF in yeast and reveal several interesting metabolic interactions between methionine and purine biosynthesis.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of the highest available commercial quality. Unless otherwise noted, chemicals were obtained from Sigma. Difco media components were obtained from VWR (West Chester, PA). The pentagalacturate derivative of (6R,6S)-5-formyl-THF was purchased from Schircks Laboratories (Jona, Switzerland). Restriction enzymes, shrimp alkaline phosphatase, calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from Invitrogen. Purified rabbit SHMT was purchased from Schircks Laboratories (Jona, Switzerland). Restriction enzymes, monoclonal anti-GST antibodies, and monoclonal antibodies against GST were purchased from Sigma.

Isolation of the Yeast Gene Encoding MTHFS—The ORF encoding the putative yeast MTHFS (YER183c; designated FAU1) was PCR-amplified from yeast genomic DNA using the primers 5′-GTTGCCCATATGAGCACTAAGCA-3′ and 5′-CTGTGCTCAGAGTAAATCCGAAAAACGACAG-3′, which include the restriction sites for NdeI and XhoI, respectively. The restriction sites were placed at the first and last codons of the yeast ORF in frame. Following restriction digestion and gel purification, the PCR product was cloned into the NdeI and XhoI sites of pET101U and transformed into XL-1 Blue. The resulting plasmid, pET-MTHFS, carries the 211-residue yeast MTHFS (no N- or C-terminal fusions) under transcriptional control of the ADH1 promoter.

Enzyme Assay of MTHFS—MTHFS activity was determined by following production of 5,10-CH2-THF at 360 nm (ε280 = 25.1 × 103 M−1 cm−1) using a modification of a previously published method (8). The standard reaction mixture contained 1 mm MgATP, 500 µM (6R,6S)-5-CHO-THF (calcium salt of monoglutamate derivative), 0.5% Triton-X-100, 14 mm 2-mercaptoethanol, and 50 mm K-HEPES, pH 7.0, in a final volume of 300 µl. Reactions were performed at 30 °C and monitored with a SpectraMax Plus spectrophotometer. One unit of activity represents 1 µmol of 5,10-CH2-THF formed/min. Assay of MTHFS activity in crude cell extracts required a gentle disruption method. Yeast spheroplasts, prepared from midlog cultures as described (21), were lysed by mild sonication. Cellular debris was removed by centrifugation, and aliquots of the supernatant were used to assay for folate-utilizing enzymes. Steady state kinetic parameters for purified MTHFS were determined using 0.5 µg of purified His-tagged enzyme in each reaction. The concentration of (6R,6S)-5-CHO-THF monoglutamate was varied from 1 to 500 µM to saturating MgATP concentration (1 mm). Similarly, the concentration of MgATP was varied from 0.025 to 5 mm at saturating (6R,6S)-5-CHO-THF concentration (500 µM). Both substrate series were performed in triplicate. Substrate-velocity data were plotted and fit to the Michaelis-Menten equation using nonlinear regression with Deltagraph Pro3 software on a Macintosh computer.

Production of Recombinant Yeast MTHFS—The yeast MTHFS construct and the pREP4-GroESL plasmid (22) were co-transformed into E. coli BL21(DE3) cells. Transforms containing both plasmids were used to inoculate a 3-ml 37 °C starter culture in 2YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) containing 50 µg/ml ampicillin and 50 µg/ml kanamycin. When the A600 of the culture reached 0.6, an aliquot was used to inoculate a 25-ml 2YT/ampicillin/kanamycin culture at 37 °C. When the A600 was 0.6, the culture was cooled in a water bath to 21 °C, and protein production was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mm. The induced culture was incubated at room temperature for 8–9 h on an orbital shaker. The cell-free extract was then centrifuged at 8,000 rpm for 15 min in a GSA rotor (Sorvall). The medium was discarded, and the cell pellet was frozen at −20 °C until use.

The frozen cells were resuspended in an equal volume of column buffer (0.1 M KPO4, 10% glycerol (v/v), 1.0 mm NaATP, 0.05% Triton X-100, 2.0 mm imidazole, pH 7.0) and disrupted by two passes through a French pressure cell at 12,000 p.s.i. Debris was removed by centrifugation at 12,000 rpm in an SS-34 rotor (Sorvall) for 15 min. The supernatant was passed through a 0.22-µm cellulose acetate syringe filter (Whatman) to yield the cell-free extract. Expression of MTHFS was confirmed by SDS-PAGE through a 12% acrylamide gel. The cell-free extract was loaded onto a column immobilized with affinity ligands (Talon; CLONTECH) equilibrated with column buffer. The column was washed with 10 volumes of column buffer containing 20 mm imidazole. Protein was eluted with a 50–500 mm imidazole linear gradient in column buffer and collected in 5-ml fractions. Protein purity was demonstrated by a single band after SDS-PAGE, and fractions containing pure protein were pooled for study. Concentration of the pooled protein was determined by densi-
Yeast Methylenetetrahydrofolate Synthetase

TABLE I

| Strain     | Genotype                        | Reference or source |
|------------|---------------------------------|---------------------|
| DAY4       | ura3–52 trp1 leu2 hisd ser1     | Ref. 58             |
| DAY4a/     | ura3–52 trp1 leu2 hisd ser1     | Ref. 44             |
| ATY3       | ura3–52 trp1 leu2 hisd ser1 Δade16 Δade17 | Ref. 45           |
| ATY3.1     | ura3–52 trp1 leu2 ser1 Δade16 Δade17 HIS4 | Ref. 45          |
| TR3        | ura3–52 trp1 leu2 ser1 Δade16 HIS4 | C. Guthrie         |
| WHY3       | ura3–52 trp1 leu2 hisd ser1 Δmet12 Δmet13 | This study       |
| RRY3       | ura3–52 trp1 leu2 hisd ser1 Δmhm1 Δmhm2 | Ref. 44          |
| EKY3       | ura3–52 trp1 leu2 hisd ser1 Δmhm1 Δmhm2 | Ref. 35          |
| CD208–2B   | his3 leu2 ura3 trp1 fol3 : HIPS3 | Ref. 36             |
| WHY1a      | ura3–52 trp1 leu2 hisd ser1 Δufu1 | This study          |
| WHY1a/     | ura3–52 trp1 leu2 hisd ser1 Δufu1 | This study          |
| WHY1.1     | ura3–52 trp1 leu2 ser1 Δufu1 Δade16 | This study         |
| WHY1.12    | ura3–52 trp1 leu2 ser1 Δufu1 Δade17 | This study         |
| WHY1.13    | ura3–52 trp1 leu2 ser1 Δufu1 Δade17 | This study         |
| WHY1.4     | ura3–52 trp1 leu2 hisd ser1 Δufu1 Δmhm1 | This study      |
| WHY1.5     | ura3–52 trp1 leu2 hisd ser1 Δufu1 Δmhm2 | This study      |
| WHY1.6     | ura3–52 trp1 leu2 hisd ser1 Δufu1 Δmhm1 Δmhm2 | This study       |
| WHY1.7     | ura3–52 trp1 leu2 hisd ser1 Δufu1 Δmhm2 | This study       |
| WHY1.8     | ura3–52 trp1 leu2 hisd ser1 Δufu1 Δmet12 | This study     |
| WHY1.9     | ura3–52 trp1 leu2 hisd ser1 Δufu1 Δmet13 | This study     |
| WHY1.3.1   | ura3–52 trp1 ser1 Δufu1 Δade16 Δade17 ade2 HIS4 | This study |
| WHY3.1     | ura3–52 trp1 ser1 Δufu1 Δade16 Δade17 HIS4 ade2 | This study |
| YCY3SB     | ade2 lys2–80 ura3–52 trp1       | This study          |

The upstream primer (5′-CAAGGGAATCCA-3′) and the downstream primer (5′-AATCACAGTCAGTCAGCTATGACCATGATTACGCC-3′) were designed to target the URA3 locus and are underlined. Subsequent excision of the URA3 cassette was confirmed by PCR.

Construction of Mutant Yeast Strains—Disruptions in yeast genes were effectuated using a PCR-based gene targeting method (23). The URA3 cassette of pJR-URA3 (24) was co-transformed with primers that contain URA3 sequences in their 5′-ends and FAU1 sequences in their 5′-ends. The upstream primer (5′-GCTTGCGAGCCCGGTAAAACGACGGCCAGT-3′) includes 39 nucleotides of sequence 300 bp upstream of the FAU1 locus. The downstream primer (5′-CCATCAAAGGCCTGTTTGCT-3′) includes 39 nucleotides of sequence 300 bp upstream of the FAU1 locus.

The amplified fragment was gel-purified and used to transform diploid strain DAY4a to uracil prototrophy. PCR using Tag polymerase was performed directly on positive colonies (25) with FAU1-specific primers 5′-CCATCAAAGGCCCTTGGTGCT-3′ and 5′-CCATCGGCAACAGGGAATCCA-3′ to confirm the FAU1 disruption. FAU1-disrupted diploids were plated on solid medium (26) for 3–4 days. Tetrad were prepared for dissection using lyticase (Sigma) and dissected using a Zeiss microscope. The resulting spores were replica-plated onto selective and rich media. Several spores were tested for mating type by mating with an α and a test strain. The URA3 cassette was excised as described by Roca et al. (24). Strains that had excised the URA3 cartridge were identified by plating onto medium containing uracil (2 mg/ml) and 5-fluoroorotic acid (1 mg/ml). These strains were screened by colony PCR to demonstrate excision of the URA3 cartridge. The resulting Δufu1Δmet12Δmhm1Δmhm2 strain was designated WHY1 (Table I). Haploid strains disrupted at the FAU1 locus in combination with disruptions at other loci involved in folate metabolism were obtained through crosses of WHY1 with strains carrying the disruption(s) of interest. Following mating, the strains were sporulated, and tetrads were dissected onto YPD plates. Progeny were screened for disrupted genes by colony PCR in some cases by growth phenotype. The new haploid strains (Table I) were characterized by their ability to grow on selective medium and in some cases by growth rates in selective medium.

RESULTS

Identification and Cloning of Yeast MTHFS—An ORF (YER183c) with homology to human MTHFS was identified in the Saccharomyces Genome Database using a BLAST search against the human MTHFS sequence (30). When aligned with human and rabbit MTHFS using the ClustalW algorithm (31) (Fig. 1), the three proteins were found to share 23% identities and 25% conservative substitutions. The yeast ORF encodes a 211-amino acid protein with a predicted molecular mass of 24.1 kDa. The amino acid sequence was analyzed using PSORTII (humangen.med.un.es/tools/PSort2_form.html) and is pre-

ometry of Coomassie Blue-stained SDS-PAGE gels, using bovine serum albumin as a standard.

This study
dicted to localize to the cytoplasm. No other ORF in the yeast genome with significant similarity to human MTHFS was identified by the BLAST search.

Purification and Kinetic Analysis of Recombinant Yeast MTHFS—The His-tagged recombinant protein was expressed from the pET-16b vector in *E. coli* BL21(DE3) following induction by isopropyl-β-D-thiogalactopyranoside. Expression of soluble MTHFS required coexpression of the *E. coli* GroEL and GroES proteins from the pREP4-GroESL plasmid (22) along with a shift in induction temperature from 37 to 21 °C. The pREP4-GroESL plasmid, a gift from Dr. Martin Stieger (Hoffman-La Roche), contains the *E. coli* genes encoding GroEL and GroES behind the Lac promoter/operator element. This construct allowed for production of these chaperonins in conjunction with expression of MTHFS. Without coexpression of the chaperone proteins, all MTHFS protein was found to be in the particulate fraction at both temperatures. However, when the co-transformed cells were induced at 21 °C, an adequate portion of the expressed protein was found in the soluble fraction.

Enzyme activity was found to be unstable when the bacterial cells were disrupted by sonication; therefore, cells were disrupted with a French pressure cell. Chromatography on a cobalt metal affinity column yielded a pure protein that migrated at 28 kDa on SDS-PAGE (Fig. 2), consistent with the predicted molecular weight of the recombinant protein with its N- and C-terminal extensions. The purified protein precipitated rapidly during concentration by ultrafiltration or during dialysis. The addition of 10% glycerol and 1 mM ATP and storage at 4 °C stabilized the protein for up to 1 week.

Purified His-tagged recombinant protein was used to demonstrate that the cloned ORF encodes an active MTHFS. Preliminary experiments revealed that enzyme activity was inhibited by salt; thus, MgATP was used instead of MgCl₂ and NaATP. Activity was followed spectrophotometrically by monitoring the formation of 5,10-CH₂-THF at 360 nm. The purified protein exhibited a specific activity of 7 units/mg of protein.

The steady state kinetic parameters for ATP and (6R,6S)-5-CHO-THF monoglutamate were determined by nonlinear regression analysis of the kinetic data (Fig. 3). The *Kₘ* values obtained for ATP and (6R,6S)-5-CHO-THF were 43 µM and 33 µM, respectively. These values are similar to those obtained for the human enzyme (Table II), confirming the identity of this protein as a functional MTHFS.

Disruption of Yeast Gene Encoding MTHFS—The gene encoding the yeast MTHFS was disrupted in the diploid strain DAY4α/α using a PCR-based gene targeting method (23). DAY4α/α was transformed to uracil prototrophy with a PCR fragment containing the *URA3* cartridge flanked by sequences from the MTHFS ORF at both ends. Recombination at the correct locus resulted in the replacement of about 900 bp of the MTHFS gene, including the entire ORF, with the 1.2-kbp *URA3* cartridge. Uracl prototrophs were screened for proper insertion of the *URA3* cartridge by colony PCR using a primer pair designed to amplify from 150 bp upstream to 150 bp downstream of the expected insertion site. A heterozygous diploid gave the expected 1043-bp band for the wild type locus and a 1481-bp band for the disrupted locus (Fig. 4, lane 2). A
The yeast enzyme (this study) was assayed at pH 7.0. The folate $K_m$ for (6-R,S)-5-CHO-THF monoglutamate of 33 $\mu$M and a $K_m$ for MgATP of 43 $\mu$M.

**Table II**

| Organism       | (6R,S)-5-CHO-THF $K_m$ | Mg-ATP $K_m$ | Specific activity | Reference |
|----------------|------------------------|--------------|-------------------|-----------|
| Human          | 4.4                    | 20           | 37                | Ref. 40   |
| Rabbit         | 8                      | 330          | 13                | Ref. 41   |
| Yeast          | 33                     | 43           | 7                 | This study|

The kinetic parameters for all three species were determined at 30 °C. The human and rabbit enzymes were assayed at pH 6.0 (40, 41). The yeast enzyme (this study) was assayed at pH 7.0. The folate $K_m$ values were determined using the RS mixture in each case.

**Fig. 3.** Dependence of MTHFS activity on (6-R,S)-5-CHO-THF concentration (A) and MgATP concentration (B) of purified Histagged yeast MTHFS. Nonlinear regression analysis yielded a $K_m$ for (6-R,S)-5-CHO-THF monoglutamate of 33 $\mu$M and a $K_m$ for MgATP of 43 $\mu$M.

**Fig. 4.** Disruption of ORF YER183c in yeast. PCR primers amplify a 1043-bp product from the wild-type gene and a 1481-bp product from the disrupted gene. Lane 1, wild-type haploid (DAY4); lane 2, heterozygous diploid; lanes 3–6, haploid progeny of a dissected tetrad. MW, 1-kbp ladder molecular weight markers.

haploid strain carrying the disrupted gene (DAY4 mthfs::URA3) was obtained by sporulation and tetrad dissection onto YPD plates. The spore progeny were screened for uracil prototrophy and by PCR (Fig. 4, lanes 3–6) to identify those carrying the disrupted gene. Finally, the URA3 cassette was evicted, and the resulting haploid disruptant strain was designated WHY1 (Table I).

**MTHFS Activity in Yeast**—Extracts from the disrupted and wild type strains were assayed for MTHFS enzyme activity. No activity was detected in extracts prepared by glass bead disruption. Instead, cells were treated with lyticase, and the resulting spheroplasts were lysed by brief sonication. Extract was cleared of debris by centrifugation, and the supernatant was used for the enzyme assay. MTHFS activity in the wild type strain, DAY4, was readily detectable (1.46 milliunits/mg of protein), whereas no MTHFS activity could be detected in the disrupted strain WHY1 (data not shown).

Phenotype of Yeast Lacking MTHFS—5-CHO-THF has been proposed as a storage form of readily available one-carbon units and of reduced folate in fungal spores (3) and as a regulator of SHMT (13). Cells lacking MTHFS activity might be expected to accumulate 5-CHO-THF. If *S. cerevisiae* spores normally accumulate 5-CHO-THF, a possible phenotype of mthfs mutants might be poor germination of spores incapable of converting the stored cofactor to its active form. To determine the effect on spore germination, a homozygous disrupted diploid strain, WHY1a/a (Table I) was created through mating. Sporulation was induced by nitrogen starvation, and tetrad were dissected onto rich medium. No difference in germination rate was observed between the mutant and the wild type diploid strain DAY4a/a (data not shown).

Accumulation of 5-CHO-THF in growing cells may also lead to inhibition of SHMT activity, producing a phenotype similar to that of strains lacking one or both SHMTs. In yeast cells carrying a ser1 mutation, which blocks the synthesis of serine from glycolytic intermediates (32), SHMT activity is required to satisfy serine requirements. High levels of glycine (100 mg/liter) can provide both the two-carbon unit and the 5,10-methylenethenyl-THF (via the glycine cleavage system) required for serine synthesis via SHMT (33, 34). ser1 mutants lacking mitochondrial SHMT have longer doubling times than the parental strain when grown on high glycine (35). The addition of adenosine to the high glycine medium completely rescues the growth of SHMT− cells, but mitochondrial SHMT mutants grow very slowly (9-h doubling time) (35). We used this medium to test whether deletion of MTHFS affected SHMT activity in vivo. The mthfs deletion strain WHY1 was grown in high glycine minimal medium supplemented with adenosine, and the growth rate was directly compared with the parental strain DAY4. Doubling rates for the strains were identical (2.9 h), suggesting no impairment in the conversion of glycine to serine by SHMT in the MTHFS mutant.

We next took a genetic approach to detecting a phenotype resulting from deletion of the MTHFS gene. *S. cerevisiae* possesses a de novo pathway for the synthesis of folate acid that begins with GTP and requires the activities of dihydrofolate synthase (DHFS; FOL3 gene) and dihydrofolate reductase (DHFR; DFR1 gene). DHFR catalyzes the reduction of folate acid to dihydrofolate and dihydrofolate to tetrahydrofolate. Folic acid (5-CHO-THF) can also satisfy the tetrahydrofolate requirement in a pathway that requires the activities of methenyl-THF synthetase (MTHFS; FAU1 gene) and methenyl-THF cyclohydrolase (ADE3 gene) to produce 5-formyl-THF, which yields tetrahydrofolate after donation of the formyl group in the transformylase reactions of purine synthesis.

**Fig. 5.** Two metabolic routes to tetrahydrofolate. The de novo pathway begins with GTP and requires the activities of dihydrofolate synthase (DHFS; FOL3 gene) and dihydrofolate reductase (DHFR; DFR1 gene). DHFR catalyzes the reduction of folate acid to dihydrofolate and dihydrofolate to tetrahydrofolate. Folic acid (5-CHO-THF) can also satisfy the tetrahydrofolate requirement in a pathway that requires the activities of methenyl-THF synthetase (MTHFS; FAU1 gene) and methenyl-THF cyclohydrolase (ADE3 gene) to produce 5-formyl-THF, which yields tetrahydrofolate after donation of the formyl group in the transformylase reactions of purine synthesis.
tetrad dissection, spore germination was examined on medium supplemented with either 5-CHO-THF or folic acid versus medium with no supplementation (Table III). Tetrads dissected onto unsupplemented rich medium gave two viable and two nonviable progeny, consistent with the folate requirement of fol3 mutants. On medium with 5-CHO-THF added, three of four spores were observed to germinate and produce colonies. Folic acid supplementation was found to support complete germination of tetrads, demonstrating that the failure to germinate on other media is due to folate starvation. If the diploid was first transformed with a multicopy plasmid containing the MTHFS gene, germination was nearly 100% when tetrads were dissected onto plates containing 5-CHO-THF (data not shown). These results confirm the function of MTHFS in vivo, and we have named the gene FAU1 (for folic acid utilization).

The fau1 deletion strain was mated with strains lacking other enzymes involved in folate utilization to generate double or triple disruptants (Table I). The resulting strains were examined for growth requirements on plates. Strains that carried fau1 disruptions in combination with disruptions of the genes encoding mitochondrial and/or cytosolic SHMTs (WHY1.4, WHY1.5, WHY1.6) and either one or both MTHFRs (WHY1.7, WHY1.8, WHY1.9) demonstrated no growth differences from the parental strains. Strains that carried the fau1 disruption in combination with disruptions of the genes encoding either of the bifunctional AICAR transformylase/IMP cyclohydrolases (WHY1.1, WHY1.2) also showed no new phenotype. However, when FAU1 was disrupted in combination with both ade16 and ade17, a new methionine deficiency was detected in the triple mutant strain (WHY1.3). The methionine-deficient strain demonstrated a normal growth rate in minimal medium supplemented with methionine, but growth was reduced in medium lacking methionine (Table IV). Neither parental strain (ATY3.1, WHY1) exhibited a methionine deficiency. S-adenosylmethionine, but not homocysteine, could rescue the methionine deficiency (data not shown), suggesting a homocysteine remethylation defect.

ade16 ade17 double mutants require adenine (37) and might be expected to accumulate the substrate of AICAR transformylase, AICAR (Fig. 6). AICAR has been reported to inhibit S-adenosylhomocysteine hydrolase and adenosine deaminase (38), two enzymes in the homocysteine remethylation pathway, and thus might interfere with methionine synthesis. AICAR is actually produced in two pathways: purine and histidine biosynthesis (Fig. 6). To test whether AICAR might be involved in the methionine deficiency of the triple mutant, a new strain was generated (WHY3.1; Δfau1 Δade16 Δade17 Δhis4 ade2) (Table I). ADE2 and HIS4 encode enzymes that catalyse steps preceding AICAR in the purine and histidine biosynthetic pathways, respectively (Fig 6). WHY3.1 is thus unable to make AICAR from either pathway. When grown in minimal media, WHY3.1 grew at the same rate with or without methionine (Table IV). This suggests that the combination of the fau1 mutation with the ade16/ade17 mutations leads to excessive accumulation of AICAR, which affects some aspect of homocysteine remethylation, leading to the methionine deficiency.

![Diagram](image)

**Intracellular 5-CHO-THF Levels**—The new methionine deficiency observed in the Δfau1 Δade16 Δade17 strain (WHY1.3) suggested that 5-CHO-THF might also be elevated. Intracellular 5-CHO-THF levels were determined by spectrophotometrically measuring the formation of the ternary complex SHMT-glycine-5-CHO-THF. The ternary complex has an extinction coefficient of 40,000 M⁻¹ cm⁻¹ at 502 nm (29); thus, 5-CHO-THF concentrations as low as 0.25 μM (A₅₀₂ = 0.01) may be reliably determined. Because SHMT forms a light-absorbing ternary complex with all reduced folates except 10-CHO-THF (39), samples were prepared in the absence of reducing agents and boiled; under these conditions, only the 5-CHO-THF derivative is stable (27, 28). The accuracy of the ternary complex formation method of determining 5-CHO-THF was demonstrated by testing a known concentration of the pentaglutamate derivative of 5-CHO-THF. The standard was found to be stable under the conditions of preparation, and the concentration determined by the binding assay was the same as the concentration given by spectrophotometric determination (data not shown).

Cellular 5-CHO-THF levels in various yeast strains are reported per mg of soluble protein and per g, wet weight, of cells in Table V. Intracellular 5-CHO-THF concentration (Table V, last column) was calculated assuming that a typical haploid yeast cell contains 3 pg of soluble protein and has a volume of 70 μm³ (26). 5-CHO-THF was approximately 3 times higher in WHY1 (Δfau1) than in the wild type DAY4. WHY1.3 (Δfau1 Δade16 Δade17) exhibited a 5-CHO-THF level ~12 times higher than the wild type. In the two strains lacking SHMT activity, EKY3 (Δshm1 Δshm2) and WHY1.6 (Δshm1 Δshm2 Δfau1), 5-CHO-THF levels were below detection.

To obtain an estimate of the total cellular folate pool, one set of extracts was prepared using 2-mercaptoethanol and ascorbate to protect reduced folates other than 5-CHO-THF. Using the values from these samples, a ratio of 5-CHO-THF to total

**TABLE III**  
| Media supplementation | Germination ratio (viable/nonviable spore progeny) |
|------------------------|-----------------------------------------------|
| No addition            | 2:2                                           |
| 5-CHO-THF (100 μg/ml)  | 3:1                                           |
| Folic acid (1.0 mg/ml)  | 4:0                                           |

**TABLE IV**  
| Strain        | Relevant genotype | Doubling time (+ Met − Met) |
|---------------|-------------------|-----------------------------|
| DAY4 Wild type|                   | 2.6 − 2.4                   |
| ATY3.1        | Δade16 Δade17 ADE2 HIS4 | 3.1 − 2.8                   |
| WHY1          | Δfau1             | 2.5 − 2.4                   |
| WHY1.3        | Δade16 Δade17 Δfau1 ADE2 HIS4 | 3.8 − 6.8                   |
| WHY3.1        | Δade16 Δade17 Δfau1 Δade2 Δhis4 | 2.3 − 2.3                   |
folate can be estimated (Table V, percentage of total folate). The results indicate that a higher proportion of folates were in the form of 5-CHO-THF in the Δfau1 strain WHY1 (30%) when compared with the wild type strain DAY4 (10%). In the strains with no SHMT activity, (EKY3 and WHY1.6), 5-CHO-THF levels were below detection, and this ratio could not be calculated. In the methionine-deficient strain (WHY1.3), 5-CHO-THF represented 80% of the total cellular folate.

**DISCUSSION**

The experiments described here confirm that the yeast gene, YER183c, encodes a functional MTHFS, with properties very similar to the mammalian homologs. The ORF predicts a protein of 211 residues with 23% identity to the human and rabbit enzymes. An N-terminally His-tagged version of the yeast protein was expressed in *E. coli* and purified to homogeneity by metal affinity chromatography on a cobalt column. Like the rabbit (8) and *Lactobacillus casei* (9) enzymes, the yeast enzyme was inhibited by high salt. The removal of salt and inclusion of glycerol and the nonionic detergent, Triton-X-100, were found to increase the stability and activity of the enzyme. The *K*$_m$ values for (6R,8S)-5-CHO-THF monoglutamate (Table II) were found to be similar to those obtained for the human (40) and rabbit enzymes (41), confirming the identity of this protein as a functional MTHFS. The rabbit enzyme has a *K*$_m$ of 0.2 µM for the pentaglutamate form of 5-CHO-THF (10), indicative of a polyglutamate binding site on the enzyme. Although we have not examined the yeast enzyme with polyglutamate substrates, the high sequence homology suggests the yeast enzyme will also exhibit a polyglutamate binding site.

The gene encoding MTHFS is not essential in yeast. The gene was first disrupted in a diploid yeast strain. This heterozygous diploid was then sporulated, and tetrads were dissected to obtain haploid disruptants. Loss of one MTHFS locus in the diploid did not affect sporulation or subsequent spore germination. Haploid disruptants grew normally under all conditions tested, and tetrads were dissected to obtain haploid disruptants. Loss of one MTHFS locus in the diploid did not affect sporulation or subsequent spore germination.

Although the folate status of yeast spores was not measured, the inability to metabolize 5-CHO-THF via MTHFS clearly did not affect the processes of sporulation or spore germination under the conditions tested.

**In vivo** function of the yeast MTHFS was demonstrated by a genetic approach. Yeasts are capable of synthesizing folic acid by a pathway that begins with GTP (Fig. 5). Disruption of the folate biosynthetic pathway results in yeast auxotrophic for folate; this requirement can be satisfied by the addition of 5-CHO-THF (folinic acid) to the media (36, 42). As illustrated in Fig. 5, utilization of 5-CHO-THF should require MTHFS activity. Folic acid, on the other hand, can bypass both the DHFs and MTHFS steps, requiring only a functional DHFR for its conversion to THF. Haploid strains WHY1 (MTHFS disruptant) and CCD208B (fo13 disruptant) were crossed to produce an *mthfs/MTHFS fo13/FOL3* heterozygous diploid. Upon sporulation of this diploid, one of four spores in each tetrad should harbor both mutations, since the two loci are on separate chromosomes. As expected, when tetrads were dissected onto media containing 5-CHO-THF, on average only three of four spores germinated (Table III). Folic acid supplementation was found to support complete germination of tetrads. If the diploid was first transformed with a multicopy plasmid containing the yeast MTHFS gene, germination was nearly 100% on plates containing 5-CHO-THF. These data confirm the role of MTHFS in the utilization of folic acid in vivo. We have thus designated the YER183c gene *FAU1* for folic acid utilization.

The metabolic role of MTHFS was further investigated by studying strains carrying the *faul* disruption in combination with mutations in other folate enzyme genes. No new growth phenotypes were observed when the *faul* disruption was combined with disruption of the two SHMT genes (*SHM1* and *SHM2*; Refs. 30 and 38) or the two methylenetetrahydrofolate reductase genes (*MET12* and *MET13*; Ref. 39). However, when the *faul* disruption was combined with disruptions of two genes in purine biosynthesis, a striking methionine deficiency was observed, and it appeared to be related to the purine intermediate, AICAR.

AICAR is produced in one of the latter steps of the *de novo* purine biosynthetic pathway. It is converted to IMP by the two activities of the bifunctional AICAR transformylase/IMP cyclohydrolase (Fig. 6). Yeast have two isozymes of this bifunctional enzyme, encoded by the *ADE16* and *ADE17* genes (37). Either of these enzymes is sufficient to support purine biosynthesis in yeast, whereas the double disruptant (Δade16Δade17) is an adenine auxotroph. In addition to a purine requirement, an Δade16Δade17 strain is also auxotrophic for histidine (45). The connection between purine and histidine metabolism is not well understood, but AICAR is also generated as a by-product of histidine biosynthesis (32), and this AICAR can presumably be utilized in purine biosynthesis (Fig. 6). Yeast that are *ade3* mutants also exhibit a secondary histidine requirement (46).

The *ADE3* gene encodes the trifunctional enzyme C$_7$-THF syn-

### Table V

| Strain     | Relevant genotype     | Intracellular 5-CHO-THF levels | Intracellular 5-CHO-THF levels | Intracellular 5-CHO-THF levels |
|------------|-----------------------|-------------------------------|-------------------------------|-------------------------------|
|            |                       | nmol/mg protein | Percentage of total folate | nmol/g, wet weight | µg/mg |
| DAY4       | Wild type             | 0.13             | 10                      | 1.5               | 5.4  |
| WHY1       | Δfau1                 | 0.36             | 30                      | 4.5               | 15.6 |
| EKY3       | Δshm1 Δshm2           | BD               | BD                      | BD                | BD   |
| WHY1.6     | Δshm1 Δshm2 Δfau1     | BD               | BD                      | BD                | BD   |
| WHY1.3     | Δfau1 Δade16          | 1.44             | 80                      | 18.0              | 62.2 |

*Calculated assuming that a typical haploid yeast cell contains 3 pg of soluble protein and has a volume of 70 µm$^3$ (26).

BD, below detection.

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*S. cerevisiae* spores accumulate 5-CHO-THF in a similar manner, then spores from a homozygous diploid disruptant might display reduced germination rates or impeded growth. However, a homozygous diploid MTHFS disruptant (WHY1a/a) sporulated efficiently, and the germination and growth of its haploid progeny was the same as that of the wild type diploid DAY4a/a.
to inhibit methionine metabolism. AICAR was found to inhibit histidine biosynthesis by feedback regulation, explaining the histidine requirement of both ade16ade17 and ade3 mutants. This hypothesis is strengthened by the observation that blocks in the de novo purine pathway that precede AICAR formation do not result in histidine auxotrophy.

The fau1 disruption was introduced into an ade16 ade17 background by crossing and tetrad dissection to create the haploid strain WHY1.3 (Δfau1Δade16Δade17), which lacks all AICAR transformylase and MTHFS activities. In addition to the adenine and histidine requirements caused by the ade16ade17 disruptions, this strain exhibited a new methionine-dependent growth defect (Table IV). The parental strains WHY1 (Δfau1) and ATY3.1 (Δade16Δade17) were expected to accumulate 5-CHO-THF and AICAR, respectively. Since neither parental strain had a methionine deficiency, we speculated that the effect required the simultaneous accumulation of both metabolites. This was tested by introducing additional mutations that block the synthesis of AICAR. Mutation of the HIS4 gene blocks AICAR production from the histidine biosynthetic pathway, and mutation of ADE2 blocks its production from the de novo purine pathway (32) (Fig. 6). If just one of the pathways was blocked, the resulting strains remained methionine-deficient (data not shown). When both pathways were blocked, however, the strain WHY3.1 (Δfau1Δade16Δade17 Δade2his4) regained normal growth on media lacking methionine (Table IV). Therefore, it appears that the accumulation of AICAR, combined with elevated 5-CHO-THF, leads to a defect in methionine synthesis. There is some precedent for an interaction between AICAR and methionine metabolism. AICAR was found to inhibit S-adenosylhomocysteine hydrolase in extracts of blood cells from rabbit and human, although significant inhibition required 1 mM AICAR (38). S-Adenosylhomocysteine hydrolase is involved in the remethylation cycle, and its inhibition would lead to decreased homocysteine levels, thereby slowing methionine synthesis. However, homocysteine deple tion is probably not the mechanism in this case, since the methionine deficiency was not alleviated by the addition of homocysteine in the medium (data not shown).

Whatever the mechanism, it appears to require elevated 5-CHO-THF as well as AICAR. Intracellular 5-CHO-THF was indeed elevated in fau1 mutants and was highest in WHY1.3 (Δfau1Δade16Δade17). The intracellular 5-CHO-THF concentration was estimated to be 5 μM in wild-type cells (Table V). This increased to 16 μM in the Δfau1 mutant (WHY1) and to 62 μM in the Δfau1Δade16Δade17 mutant. The polyglutamate forms of 5-CHO-THF are known inhibitors of several mammalian folate-dependent enzymes, including AICAR transformylase (48) and SHMT (29), with Kₜ values in the low micromolar range. If the yeast enzymes have similar kinetic properties, the 5-CHO-THF levels observed in the MTHFS mutants are clearly sufficient to inhibit AICAR transformylase and SHMT activity in vivo. In yeast grown with serine, where the cytoplasmic SHMT operates in the direction of glycine and 5,10-CH₂-THF production (35), inhibition of SHMT would lead to a deficiency of cytoplasmic 5,10-CH₂-THF and thus the 5-methyl-THF required for methionine synthesis. The extreme elevation of 5-CHO-THF in the Δfau1Δade16Δade17 strain (12-fold) may explain why the methionine deficiency is seen only when both MTHFS and AICAR transformylase activities are missing in the same strain.

Additional evidence that 5-CHO-THF can inhibit SHMT activity in vivo comes from the recent work of Piper et al. (49) on the regulation of one-carbon metabolism in yeast. Based on experiments with various mutant strains, they concluded that yeast respond to a deficiency of 5,10-CH₂-THF by increasing expression of the mitochondrial glycine cleavage system, which produces 5,10-CH₂-THF from glycine. They showed that strain WHY1 (Δfau1) has altered control of the glycine cleavage system gene expression, indicative of low cytoplasmic 5,10-CH₂-THF levels. This effect is consistent with the elevated 5-CHO-THF in that strain inhibiting SHMT, causing the deficiency of 5,10-CH₂-THF.

We can only speculate as to why the loss of AICAR transformylase activity is associated with additional accumulation of 5-CHO-THF in the fau1 mutant. Based on studies with Salmonella typhimurium grown under folate starvation conditions, Bochner and Ames (50) proposed that 5-aminomimidazole carboxamide riboside 5’-triphosphate (ZTP; the 5’-triphosphate derivative of AICAR) is an “alarmone” that signals a cellular deficiency of 10-CHO-THF. AICAR (ZMP) accumulates in the folate-starved cells and is subsequently phosphorylated to the triphosphate level (ZTP). The authors proposed that this ZTP then triggers alterations in cellular metabolism to remedy the one-carbon unit deficiency (50). If AICAR does influence the cellular content of one-carbon units, these might be stored as 5-CHO-THF. In the absence of MTHFS activity, the effect would be the trapping of the one-carbon unit and perhaps more importantly the coenzyme as 5-CHO-THF. ZTP can be detected in eukaryotic cells (51, 52), but there are no reports on its existence in yeast. AICAR itself, however, has been shown to be an activator of the mammalian AMP-activated protein kinase (53, 54). Members of the AMP-activated protein kinase subfamily are found in most eukaryotes, where they are the central component of a protein kinase cascade that acts as a metabolic sensor for the AMP/ATP ratio (55). S. cerevisiae also has an AMP-activated protein kinase homologue, the SNF1 protein kinase. In yeast, the SNF1 protein kinase plays a central role in the glucose repression response but is also involved in the regulation of sporulation, glycogen storage, peroxisome biogenesis, and lipid metabolism as well (56). Although the intracellular signal in yeast is not known, changes in the AMP/ATP levels correlate reasonably well with SNF1 kinase activity under a variety of nutritional conditions (57). Attempts to demonstrate a direct effect of AMP on the activity of the purified yeast kinase in vitro have been unsuccessful, but AICAR was not tested (57). Thus, it remains a possibility that the elevated AICAR in the ade16ade17 mutants, acting through the SNF1 kinase cascade, alters folate-mediated one-carbon metabolism, and in cells lacking the ability to move 5-CHO-THF into the active one-carbon pool, this form accumulates to the extreme levels observed in the Δfau1Δade16Δade17 strain.

Finally, our data also shed new light on the source of 5-CHO-THF in vivo. Stover and Schirch (4) showed that SHMT catalyzes the conversion of 5,10-CH⁻THF to 5-CHO-THF in vitro, and proposed that this is the primary source of 5-CHO-THF in vivo. Consistent with this proposal is the observation that E. coli and N. crassa cells deficient in SHMT activity do not accumulate 5-CHO-THF (3, 5). On the other hand, Baggott (7) has argued that all cellular 5-CHO-THF is due to the nonenzymatic hydrolysis of 5,10-CH⁻-THF in mildly acidic subcellular organelles, based on the in vitro observation that the equilibrium between 5,10-CH⁻-THF and 5-CHO-THF favors 5-CHO-THF at pH 4.5. In the present study, 5-CHO-THF was undetectable in yeast strains entirely lacking SHMT activity (EKY3, WHY1.6). Deletion of fau1 was shown to cause accu-
mulation of 5-CHO-THF but only in strains with active SHMT (compare WHY1 and WHY1.6; Table V), confirming that SHMT is the only significant source of 5-CHO-THF in vivo. Thus, results in S. cerevisiae, E. coli, and N. crassa strains with reduced or absent SHMT activity all support the hypothesis that the cellular source of 5-CHO-THF is the SHMT-catalyzed hydrolysis of 5,10-CH2-THF.

Acknowledgments—We thank Dr. Yolande Surdin-Kerjan (CNRS, Gif-sur-Yvette, France) for providing the foU3 yeast strain and Drs. Verne Schirch (Virginia Commonwealth University) and Patrick Stover (Cornell University) for gifts of purified SHMT and helpful discussion. We thank Carol Chang for help with tetra dissections and strain construction.

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