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Molecular Genetic Analysis of *Saccharomyces cerevisiae* C_1-Tetrahydrofolate Synthase Mutants Reveals a Noncatalytic Function of the *ADE3* Gene Product and an Additional Folate-Dependent Enzyme

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In eucaryotes, 10-formyltetrahydrofolate (formyl-THF) synthetase, 5,10-methenyl-THF cyclohydrolase, and $NADP^+$-dependent 5,10-methylene-THF dehydrogenase activities are present on a single polypeptide termed C_1-THF synthase. This trifunctional enzyme, encoded by the *ADE3* gene in the yeast *Saccharomyces cerevisiae*, is thought to be responsible for the synthesis of the one-carbon donor 10-formyl-THF for de novo purine synthesis. Deletion of the *ADE3* gene causes adenine auxotrophy, presumably as a result of the lack of cytoplasmic 10-formyl-THF. In this report, defined point mutations that affected one or more of the catalytic activities of yeast C_1-THF synthase were generated in vitro and transferred to the chromosomal *ADE3* locus by gene replacement. In contrast to *ADE3* deletions, point mutations that inactivated all three activities of C_1-THF synthase did not result in an adenine requirement. Heterologous expression of the *Clostridium acidiurici* gene encoding a monofunctional 10-formyl-THF synthetase in an *ade3* deletion strain did not restore growth in the absence of adenine, even though the monofunctional synthetase was catalytically competent in vivo. These results indicate that adequate cytoplasmic 10-formyl-THF can be produced by an enzyme(s) other than C_1-THF synthase, but efficient utilization of that 10-formyl-THF for purine synthesis requires a nonenzymatic function of C_1-THF synthase. A monofunctional 5,10-methylene-THF dehydrogenase, dependent on NAD^+ for catalysis, has been identified and purified from yeast cells (C. K. Barlowe and D. R. Appling, Biochemistry 29:7089–7094, 1990). We propose that the characteristics of strains expressing full-length but catalytically inactive C_1-THF synthase could result from the formation of a purine-synthesizing multienzyme complex involving the structurally unchanged C_1-THF synthase and that production of the necessary one-carbon units in these strains is accomplished by an NAD^+-dependent 5,10-methylene-THF dehydrogenase.

Tetrahydrofolate (THF)-mediated one-carbon metabolism is essential in many cellular processes, including nucleic acid biosynthesis, mitochondrial and chloroplast protein biosynthesis, amino acid metabolism, and methyl group biogenesis (7). In most organisms, the major source of one-carbon units is carbon 3 of serine, derived from glycolytic intermediates (36). The one-carbon unit is transferred to THF in a reaction catalyzed by serine hydroxymethyltransferase (SHMT) (Fig. 1, reaction 4), generating 5,10-methylene-THF and glycine. This form of the coenzyme is then distributed between several pathways, depending on the needs of the cell.

In procaryotes, reactions 1 to 3 (Fig. 1) are catalyzed by three separate monofunctional enzymes, with the known exceptions of *Escherichia coli* and *Clostridium thermoaceticum*, in which the cyclohydrolase and dehydrogenase activities are catalyzed by bifunctional proteins. In eucaryotes, these three activities are present on one polypeptide in the form of a trifunctional enzyme (for a review, see reference 25). This enzyme, termed C_1-THF synthase, catalyzes the activation of formate and interconversion of the one-carbon unit attached to THF between the formate and formaldehyde oxidation levels. In the yeast *Saccharomyces cerevisiae*, two isozymes of C_1-THF synthase exist, both encoded by nuclear genes. The *ADE3* gene encodes a cytoplasmic C_1-THF synthase (20, 42), and the *MIS1* gene encodes a mitochondrial isozyme (37, 38). In both of these trifunctional enzymes, the 5,10-methylene-THF dehydrogenase activity is $NADP^+$ dependent (31, 38). Cytoplasmic C_1-THF synthase has been purified from several vertebrate sources, and cDNA clones have been isolated for the cytoplasmic enzyme from human (18) and rat (44) cells. It is not known whether higher eucaryotes also possess a trifunctional mitochondrial isozyme, but all three activities of C_1-THF synthase have been demonstrated in rat liver mitochondria (4).

We are interested in the cellular organization and role of these two isozymes in folate-mediated one-carbon metabolism. The 10-formyl-THF required in the cytoplasm for de novo purine biosynthesis can, in principle, arise from two directions, both catalyzed by activities of C_1-THF synthase. Cytoplasmic 5,10-methylene-THF (from serine) can be oxidized to 10-formyl-THF by the sequential reactions 5,10-methylene-THF dehydrogenase and 5,10-methenyl-THF cyclohydrolase (Fig. 1, reactions 3 and 2). 10-Formyl-THF can also arise from cytoplasmic formate via 10-formyl-THF synthetase (Fig. 1, reaction 1). One possible source of the formate is mitochondrial oxidation of one-carbon donors such as serine or sarcosine (4) via the mitochondrial isozyme of C_1-THF synthase (Fig. 1, reactions 3a, 2a, and 1a).

To examine the roles of these two isozymes in the synthesis of 10-formyl-THF, we have chosen a molecular genetic approach in yeast cells. Classical genetic analysis of the *ADE3* locus, encoding the cytoplasmic isozyme, has shown that deletion of the gene results in an absolute purine

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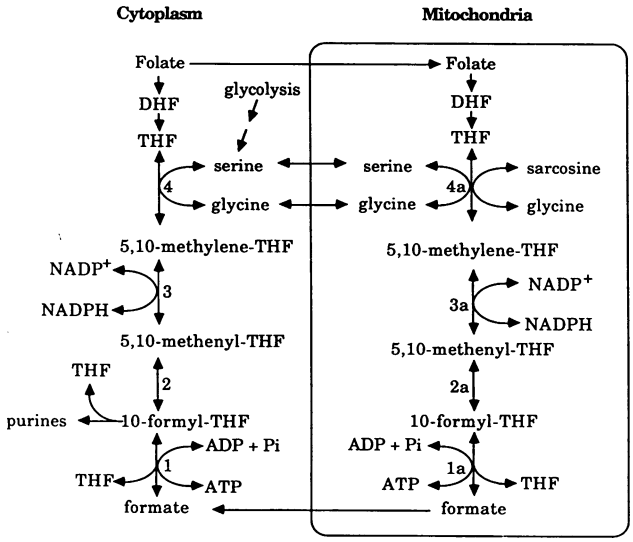

FIG. 1. Organization of THF-interconverting enzymes in eucaryotes. In reactions 1 (1a), 2 (2a), and 3 (3a), 10-formyl-THF synthetase (EC 6.3.4.3), 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9), and 5,10-methylene-THF dehydrogenase (EC 1.5.1.5), respectively, are catalyzed by C_1-THF synthase. Reaction 4 (4a), SHMT (EC 2.1.2.1).

requirement for growth (20, 21), whereas strains with decreased enzyme activities due to point mutations at the *ADE3* locus have little or no dependence on added purine for growth (28). In *ade3* deletion purine auxotrophs, the mitochondrial isozyme is obviously not able to complement the cytoplasmic deficiency. In addition, disruption of the *MIS1* gene, encoding the mitochondrial isozyme, has no apparent effect on growth compared with a wild-type strain (38). In this report, we have generated point mutations in the *ADE3* gene that affect one or more of the catalytic activities of cytoplasmic C_1-THF synthase and have replaced the wild-type *ADE3* gene with these mutant versions. The nutritional and biochemical consequences of the resulting metabolic blocks have revealed an additional folate-dependent enzyme in yeast cells and an apparent noncatalytic function of the *ADE3* gene product.

MATERIALS AND METHODS

Materials. Restriction enzymes and Moloney murine leukemia virus reverse transcriptase were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The large fragment of DNA polymerase I (Klenow fragment) and T4 DNA ligase were obtained from U.S. Biochemical Corp. (Cleveland, Ohio). [^{14}C]formate was purchased from Dupont, NEN Research Products (Boston, Mass.). Oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer, deprotected, and purified by thin-layer chromatography (1). Common reagents were commercial products of the highest grade available.

Strains and growth media. Haploid strains of *S. cerevisiae* were used in all studies and are described in Table 1. Rich medium (YPD) contained 1% yeast extract, 2% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), and 2% dextrose. Minimal medium (YMD) consisted of 0.7% yeast nitrogen base and 2% dextrose and was supplemented with the following nutrients where indicated (final concentration in milligrams per liter): L-serine, 375; L-leucine, 30; L-histidine, 20; L-tryptophan, 20; uracil, 20; adenine, 20; glycine, 20; and formate, 1,000 (pH 5.5). Yeast transformations with autonomously replicating and integrating plasmids were performed with lithium acetate (19). *E. coli* XLI-B (Stratagene, La Jolla, Calif.) was used as the cloning host. Methods for *E. coli* maintenance, plasmid isolation (26), and transformation to ampicillin resistance (15) have been described.

TABLE 1. Plasmids and *S. cerevisiae* strains used

Plasmid or strain	Relevant genotype or phenotype	Source or reference
Plasmids		
pVT-101, -102, -103U	Ap^r 2 μm *URA3*	45
pU2.1-ADE3	pVT-102U *ADE3*	5
pU3.1-ADE3	pVT-103U *ADE3*	This study
pU2-C144S	pVT-102U *ADE3*-C144S (C$^-$)	6
pU2.1-ADE3-30	pVT-102U *ADE3-30* (S$^-$)	5
pU3.1-ADE3-65	pVT-103U *ADE3-65* (D$^-$)	This study
pU2.1-ADE3-30/65	pVT-102U *ADE3-30/65* (S$^-$ D$^-$)	This study
pU2.1-ADE3-30/65/144	pVT-102U *ADE3-30/65/144* (S$^-$ D$^-$ C$^-$)	This study
pU1-CaS	pVT-101U *C. acidiurici* synthetase	5
Strains		
DAY3	a *ura3-52 ade3-130 trp1 leu2 his3 his4 ser1-171*	6
DAY4	a *ura3-52 trp1 leu2 his3 his4 ser1-171*	6
KSY8	α *ade3-130 ura3-52 ser1-171 mis1::URA3*	38
CBY1	*ADE3-30* (S$^-$)	5
CBY4	*ADE3-65* (D$^-$)	This study
CBY5	*ADE3-30/65* (S$^-$ D$^-$)	This study
CBY6	*ADE3-30/65/144* (S$^-$ D$^-$ C$^-$)	This study

Plasmid constructions. The plasmids used are described in Table 1. To facilitate subcloning after saturation mutagenesis, pU3.1-ADE3 (Fig. 2) was constructed by removing a portion of the multiple cloning site of the parent vector. This allowed utilization of unique *Pst*I and *Xho*I sites within the *ADE3* gene contained on this plasmid. Briefly, plasmid pVT-103U (45) was digested with *Hin*dIII and *Xho*I restriction endonucleases, blunt ended with the Klenow fragment (3), and ligated to create pVT-103.1U. This removes 15 bp from the multiple cloning site, including the recognition sequences for *Pst*I and *Xho*I. The *Bam*HI-*Sst*I fragment containing the entire C_1-THF synthase open reading frame was then transferred to pVT-103.1U to create pU3.1-ADE3. pU2.1-ADE3-30/65 was constructed by replacing the 1,420-bp *Bam*HI-*Pst*I fragment of pU2.1-ADE3-30 with the same fragment from pU3.1-ADE3-65. pU2.1-ADE3-30/65/144 was constructed by replacing an 802-bp *Bam*HI-*Rsr*II fragment from pU2.1-ADE3-30/65 with the same fragment from pU2-C144S (6).

In vitro mutagenesis and selection. Catalytic mutants of C_1-THF synthase were obtained by a mutagenesis and selection method previously described (5). Briefly, single-stranded pU3.1-ADE3 DNA, prepared from phagemid by using helper bacteriophage R408 (Stratagene), served as the template for saturation mutagenesis with nitrous acid. For the generation of dehydrogenase mutants, a synthetic oligonucleotide complementary to nucleotides 1648 to 1665 (38 bp

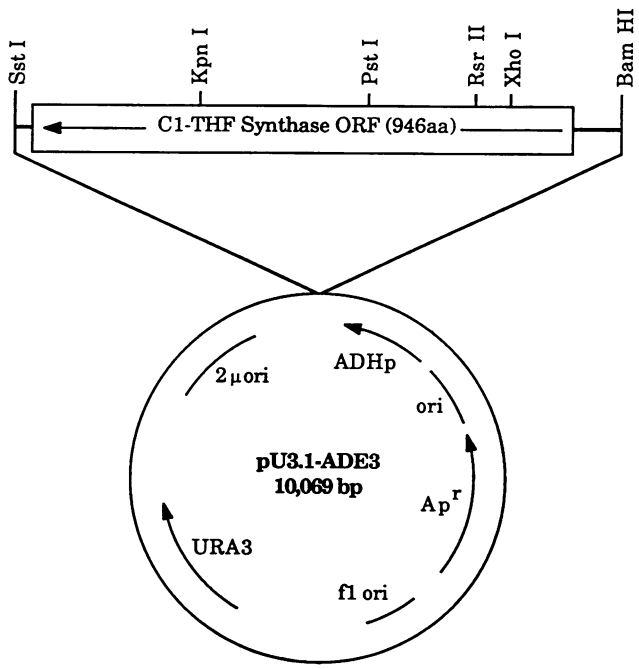

FIG. 2. Plasmid construct for expression and mutagenesis of the *ADE3* gene. Expression of the *ADE3* gene is driven by the alcohol dehydrogenase promoter (ADHp). The plasmid contains origins of replication (2μori, ori) and selectable markers (*URA3*, Ap^r) for maintenance in yeast and *E. coli* cells, respectively. The phage f1 origin allows generation of a single-stranded template for mutagenesis. Relevant restriction endonuclease sites on the *ADE3* gene are indicated. ORF, Open reading frame; aa, amino acids.

upstream of the unique *Pst*I site) (Fig. 2) was used to prime second-strand synthesis on the mutagenized template with reverse transcriptase. Primer-extended DNA was digested with *Pst*I and *Xho*I. The resulting pool of mutagenized 763-bp fragments were isolated and ligated into pU3.1-ADE3 previously cut at the unique *Pst*I and *Xho*I sites. This process generates a set of random mutations in the *ADE3* gene encoding amino acids 113 through 368, contained in the dehydrogenase-cyclohydrolase domain. This library of mutations was used to transform strain DAY3 to uracil prototrophy on plates containing serine, leucine, histidine, tryptophan, and adenine. Yeast transformants were isolated and tested for growth on control plates (same as transformation plates) or plates with glycine plus formate replacing serine. Colonies that exhibited slow growth or no growth after 4 days were selected for further study. Plasmid DNA was recovered from yeast cells (39) by *E. coli* transformation. Synthetase point mutants were obtained previously (5) by the same method.

Gene transplacement. Gene transplacement (8, 49) at the *ADE3* locus was performed as described previously (5). Mutant genes were transferred to YIP5 (43) for integrative transformation into DAY4 (to generate CBY4), CBY1 (to generate CBY5), or CBY5 (to generate CBY6). Integrations at the *ADE3* locus were confirmed by Southern analysis as previously described (5). Cells were selected for eviction of plasmid DNA by growth on 5-fluoro-orotic acid. Gene-replaced strains were examined by Southern analysis, assay of C_1-THF synthase enzyme activities, and immunoblot analysis (2) to confirm transplacement of the wild-type *ADE3* gene with intended mutations. Antisera against purified yeast C_1-THF synthase (6) were produced in rabbits by a previously described protocol (13).

Growth and metabolic labeling. Cultures were grown in 125-ml flasks at 30°C in a shaking water bath. Cells used for growth determinations were grown in YMD supplemented with serine, leucine, histidine, tryptophan, adenine, and uracil, harvested in mid-log-phase growth, and washed in YMD. Cells were then resuspended in YMD and used to inoculate flasks containing appropriate nutrients. Growth was monitored by measuring turbidity at 600 nm on a Beckman model DU40 spectrophotometer. Cells used in metabolic labeling experiments were grown in supplemented YMD media as described above and harvested in late-log-phase growth by centrifugation at room temperature. These cells were resuspended in 30 ml of YMD plus leucine, histidine, tryptophan, uracil, and reduced concentrations of serine (75 mg/liter) and adenine (4 mg/liter). After 30 min of growth in this medium, [^{14}C]formate was added to a final concentration of 1.5 mM and at a specific activity of 150 dpm/nmol. At 40, 80, and 120 min, 5-ml samples were removed and washed in cold deionized water, filtered through nitrocellulose membranes (Schleicher & Schuell no. 20330), and dried under a heat lamp. At the 120-min time point, a 10-ml sample was removed; then cells were washed with deionized water and fractionated into perchloric acid-soluble and RNA-, DNA-, and protein-containing fractions (46). Radioactivity measurements were performed in Beckman Ready Protein Plus by the H# method on a Beckman model LS1801 scintillation counter. Cell numbers were determined before filtering by measuring turbidity at 600 nm.

Subcellular fractionation. Yeast cells harboring pU1-CaS were grown in minimal medium except that 1% galactose was provided as the carbon source and uracil was omitted to maintain plasmid selection. Cells were harvested in mid-log-phase growth, converted to spheroplasts, and gently lysed. Cellular subfractions were isolated by differential centrifugation (5a). Glyceraldehyde-3-phosphate dehydrogenase (24) was used as a cytoplasmic marker activity and fumarase (33) was used as a mitochondrial marker activity to ensure the purity of cellular subfractions.

Assay of NAD^+-dependent 5,10-methylene-THF dehydrogenase. Yeast cells were grown to stationary phase in YPD, chilled, and harvested by centrifugation at 4°C. The cell pellet was suspended in 2 volumes of buffer (25 mM Tris chloride [pH 8.4], 2 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) and disrupted with glass beads. This lysate was centrifuged at 25,000 × *g* for 30 min, and the resulting supernatant fluid was assayed for NAD^+-dependent 5,10-methylene-THF dehydrogenase activity by incubation in 0.1 M KCl–0.02 M 2-mercaptoethanol–0.05 M potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (K-HEPES; pH 8.0)–0.5 mM (6*R*,*S*)-5,10-methylene-THF–2 mM NAD^+ at 37°C for 5 min (5a). Production of 5,10-methenyl-THF was determined by measuring the increase in A_{350} after adjustment to a final concentration of 0.25 M HCl, 5,10-Methylene-THF was prepared by the condensation reaction of THF with formaldehyde as previously described (2).

RESULTS

The strategy of the experiments described below is to block 10-formyl-THF synthesis from one or both directions by introducing mutations in the gene encoding cytoplasmic C_1-THF synthase. Analysis of the biochemical and nutritional phenotypes of strains harboring these mutant enzymes

TABLE 2. Phenotypes of yeast strains bearing wild-type and mutant *ADE3* loci

Strain	Enzyme activity[a] (mU/mg of protein)			S/D ratio	Growth rate[b] (doubling time, h)		
	S	D	C		+Adenine	−Adenine	Glycine/formate
DAY3	6.7 ± 0.5	1.7 ± 0.3	3.3 ± 0.4	3.9	2.2	>24	>24
DAY4[c]	151 ± 4	15 ± 1	24 ± 2	10.1	2.3	2.3	4.3
KSY8	ND	ND	ND		2.2	>24	>24
CBY1	17 ± 1	18 ± 1	25 ± 2	0.9	2.3	2.2	>24
CBY4	137 ± 5	3.8 ± 0.5	10 ± 1	36	2.1	2.1	>24
CBY5	21 ± 1	4.7 ± 0.5	10 ± 2	4.4	2.2	2.2	>24
CBY6	23 ± 1	5.4 ± 0.5	5.5 ± 0.4	4.2	2.3	3.9	>24

[a] Determined on cells grown in YPD as described in Materials and Methods. S, 10-Formyl-THF synthetase; D, 5,10-methylene-THF dehydrogenase ($NADP^+$ dependent); C, 5,10-methenyl-THF cyclohydrolase. Values represent the average of four determinations ± standard deviations. ND, Not detected.
[b] Determined in YMD supplemented as described in the legend to Fig. 5, except that uracil was included.
[c] Wild-type with respect to the *ADE3* and *MIS1* loci.

should reveal the magnitude and direction of one-carbon flux through the cytoplasmic and mitochondrial isozymes in vivo.

5,10-Methylene-THF dehydrogenase mutants do not require adenine for growth. ADE3 mutants have traditionally been characterized with respect to their ability to support purine synthesis. McKenzie and Jones (28) developed an alternate phenotypic assay for catalytic function of C_1-THF synthase not related to the purine requirement. A *ser1* mutation in yeast cells blocks the synthesis of serine from glycolytic intermediates. Serine can instead be synthesized from glycine via SHMT (reaction 4; Fig. 1), provided a source of 5,10-methylene-THF is available. Formate can satisfy this requirement via the three reactions catalyzed by C_1-THF synthase. Selection for catalytic mutants is based on the ability of *ser1 ADE3*$^+$ cells to utilize formate plus glycine to synthesize serine via C_1-THF synthase and SHMT reactions, thereby satisfying the serine requirement. Recently, we combined an in vitro random mutagenesis technique with this selection strategy to isolate catalytic mutants in any of the three C_1-THF synthase reactions (5). By using this approach, an *ade3* deletion strain (DAY3) was transformed with pU3.1-ADE3 plasmids containing random mutations in the dehydrogenase-cyclohydrolase domain. Approximately 500 DAY3 transformants were replica plated onto solid medium containing either serine or glycine plus formate instead of serine. Twenty-six colonies exhibited depressed rates of growth or no growth on glycine plus formate. After further analysis, seven of these colonies were found by immunoblot analysis to express full-length C_1-THF synthase and to possess low or undetectable levels of dehydrogenase activity when compared with the wild-type enzyme expressed from pU3.1-ADE3. Isolates that did not exhibit full-length protein by immunoblot were presumed to be a result of a mutation(s) producing a truncated or unstable protein.

One dehydrogenase mutant, designated ADE3-65, was selected for further study. Gene replacement was used to construct a strain, designated CBY4(D^-), containing the mutation in single copy at the chromosomal *ADE3* locus. This strain exhibited wild-type (e.g., DAY4) levels of synthetase activity but little dehydrogenase activity above that derived from the mitochondrial C_1-THF synthase; its cyclohydrolase activity was approximately 30% of the wild-type level when the mitochondrial background was subtracted (Table 2). As expected from the selection strategy used to isolate the mutation, CBY4(D^-) could not satisfy its serine requirement with glycine plus formate. Despite this tight block, however, CBY4(D^-) did not require adenine for growth, exhibiting a normal doubling time of 2.1 h (Table 2). This strain thus resembles the dehydrogenase *ade3* mutants isolated by McKenzie and Jones (28).

10-Formyl-THF synthetase mutants do not require adenine for growth. The results with CBY4(D^-) suggested that the dehydrogenase activity of C_1-THF synthase was not required for the production of 10-formyl-THF necessary for purine synthesis. Perhaps formate was providing the one-carbon units via the 10-formyl-THF synthetase activity of C_1-THF synthase. We had previously constructed a strain harboring a synthetase mutation in single copy at the chromosomal *ADE3* locus, designated CBY1(S^-) (5). This strain exhibits wild-type levels of dehydrogenase and cyclohydrolase activities but little synthetase activity above that derived from the mitochondrial C_1-THF synthase. Phenotypic analysis of CBY1(S^-) indicated no growth on glycine plus formate in place of serine, as expected, but normal growth in the absence of adenine (Table 2), suggesting that 10-formyl-THF was being synthesized via the dehydrogenase and cyclohydrolase activities of C_1-THF synthase.

An additional strain was generated which placed both the synthetase and dehydrogenase mutations on the same protein, expressed from a single-copy gene at the chromosomal *ADE3* locus, designated CBY5(S^-D^-). This strain exhibits greatly reduced levels of both synthetase and dehydrogenase activity and thus should be unable to synthesize 10-formyl-THF in the cytoplasm. Unexpectedly, the growth characteristics of CBY5(S^-D^-) are identical to those of CBY1(S^-) and CBY4(D^-), i.e., no growth on glycine plus formate in place of serine but normal growth in the absence of adenine (Table 2).

Since CBY5(S^-D^-) possessed some cyclohydrolase activity (Table 2), we generated one final *ade3* mutant. We previously constructed by site-directed mutagenesis a dehydrogenase-cyclohydrolase mutant enzyme, C144S, which exhibits less than 5% residual cyclohydrolase activity (6). We placed the C144S mutation into pU2.1-ADE3-30/65 (synthetase and dehydrogenase deficient [S^-D^-]). This construct (pU2.1-ADE3-30/65/144) was then used to produce by gene replacement a new strain, CBY6($S^-D^-C^-$), which expresses an S^- D^- cyclohydrolase-deficient (C^-) triple mutant enzyme. CBY6($S^-D^-C^-$) did not require adenine for growth, although its growth was slightly stimulated by the addition of adenine (Table 2).

Biochemical characterization of wild-type and mutant strains. An *ADE3* wild-type strain (DAY4) and the strains harboring deletions (DAY3 and KSY8) or point mutations (CBY1, CBY4, CBY5, and CBY6) at the *ADE3* locus were analyzed for expression of full-length C_1-THF synthase by immunoblot (Fig. 3) and for enzyme activity in crude extracts (Table 2).

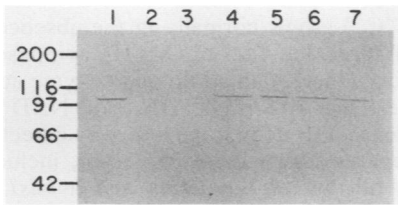

FIG. 3. Immunoblot of C_1-THF synthase in various yeast strains. Crude extracts were prepared from cells grown in YMD as described in Materials and Methods. A 30-μg sample of protein from an extract prepared from strains CBY5 and CBY6 and 20 μg from the other strains were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose for immunoassay. Lanes: 1, DAY4; 2, DAY3; 3, KSY8; 4, CBY1; 5, CBY4; 6, CBY5; 7, CBY6.

Immunoblot analysis of crude extracts prepared from these strains indicated that full-length C_1-THF synthase was expressed in all of the CBY strains (Fig. 3, lanes 4 to 7) but not in an *ade3* deletion strain (DAY3; lane 2) or an *ade3* deletion *mis1* disruption strain (KSY8; lane 3). The negative result with the DAY3 extract (lane 2), which expresses the mitochondrial isozyme, demonstrates the lack of cross-reactivity of these antisera with that isozyme. Strains CBY1(S^-) and CBY4(D^-) appeared to express C_1-THF synthase to a similar level as the wild-type DAY4; however CBY5(S^-D^-) and CBY6($S^-D^-C^-$) appeared to contain less immunoreactive protein than did the other strains, perhaps indicating an instability in the mRNA or protein when the synthetase and dehydrogenase mutations were combined.

Crude extracts prepared from these strains were analyzed for synthetase, dehydrogenase, and cyclohydrolase activities (Table 2). Enzyme assays reveal that CBY1(S^-) had greatly reduced synthetase activity and normal dehydrogenase and cyclohydrolase activities, whereas the converse was true for CBY4(D^-). CBY5(S^-D^-) exhibited greatly reduced levels of both dehydrogenase and synthetase activities and CBY6($S^-D^-C^-$) was deficient in all three activities. It is interesting that the low levels of dehydrogenase, cyclohydrolase, or synthetase activity detected in point mutant strains were approximately twofold higher than those observed in the *ade3* deletion strain (DAY3). One explanation is that the mutations isolated by phenotypic selection on glycine plus formate are slightly leaky. To investigate this possibility, the mutant proteins were overexpressed from plasmids in KSY8, which carries both an *ade3* deletion and a *mis1* disruption, thereby removing the background of mitochondrial C_1-THF synthase activity. Results of an assay of C_1-THF synthase activities in crude extracts prepared from these transformants are shown in Table 3. The wild-type enzyme was overexpressed 30-fold in this system. However, under the saturating conditions of our assay, absolutely no dehydrogenase or synthetase activity was detectable in yeast cells transformed with pU3.1-ADE3-65 or pU2.1-ADE3-30, respectively. Cyclohydrolase activity was undetectable in yeast cells transformed with pU2.1-ADE3-30/65/144. Immunoblot analysis indicated that all of these proteins were overexpressed to the same extent (data not shown). The spectrophotometric assays are capable of detecting 1.2 mU/mg of protein in crude extracts, which would correspond to 0.01% of the wild-type synthetase, 0.15% of the wild-type dehydrogenase, and 0.1% of wild-type cyclohydrolase activities when overexpressed from the wild-type *ADE3* gene. Thus, mutant strains with single copies of *ADE3-65*, *ADE3-30*, or *ADE3-30/65/144* do not appear to be leaky.

TABLE 3. Enzyme activities of overexpressed mutant and wild-type C_1-THF synthase proteins in strain KSY8

Plasmid	Enzyme Activity[a] (mU/mg of protein)		
	S	D	C
pU2.1-ADE3	3,400 ± 200	460 ± 10	900 ± 50
pU2.1-ADE3-30	ND	650 ± 20	1,400 ± 60
pU3.1-ADE3-65	3,500 ± 100	ND	230 ± 20
pU2.1-ADE3-30/65/144	ND	ND	ND

[a] S, 10-Formyl-THF synthetase; D, 5,10-methylene-THF dehydrogenase ($NADP^+$ dependent); C, 5,10-methenyl-THF cyclohydrolase. ND, Not detected.

An alternative explanation for the increased level of synthetase or dehydrogenase activity in the CBY strains compared with a deletion strain may be that expression of mitochondrial C_1-THF synthase is derepressed when the *ADE3* gene product is expressed. This alternative is supported by the fact that the ratio of synthetase to dehydrogenase activity shown in Table 2 is very similar in an *ade3* deletion strain (DAY3, 3.9) and the triple mutant (CBY6, 4.2), which is about half the ratio observed in an $ADE3^+$ strain (DAY4, 10.1). The ratio of synthetase to dehydrogenase activity for the two isozymes is significantly different (37), the lower ratio being characteristic of the mitochondrial isozyme.

Several strains were tested for their ability to utilize [^{14}C]formate as a one-carbon source (Fig. 4). Incorporation of [^{14}C]formate into cellular macromolecules should be dependent on a functional 10-formyl-THF synthetase (Fig. 1). Strains that lack cytoplasmic synthetase activity (DAY3, CBY1, and CBY5) showed decreased labeling compared with the wild-type strain (DAY4). CBY4, which lacks cytoplasmic dehydrogenase activity, exhibited less radiolabeling than did the wild-type DAY4, but greater labeling than did strains missing synthetase activity. KSY8, which lacks both the mitochondrial and cytoplasmic C_1-THF synthases, accumulated little intracellular label. At the 120-min time point, samples were taken for isolation of perchloric acid-soluble and RNA-, DNA-, and protein-containing fractions. Most of the intracellular ^{14}C label was included in the RNA and protein fractions for all strains except KSY8, in which most of the intracellular label was associated with the perchloric acid-soluble fraction. Because DAY3 and KSY8 require

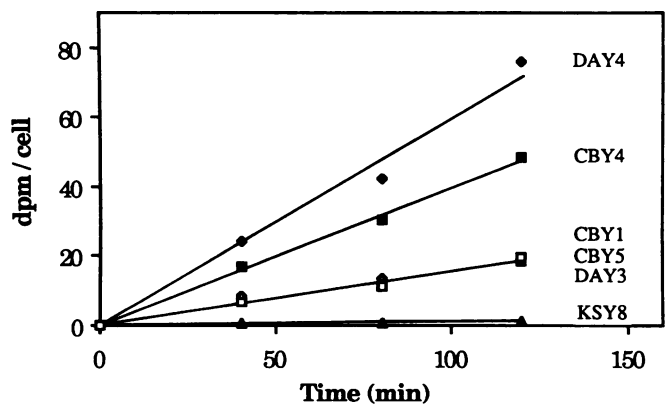

FIG. 4. Accumulation of ^{14}C label in yeast strains. [^{14}C]formate was added at zero time, and samples were taken at 40-min intervals and processed as described in Materials and Methods. Approximations of cell number were based on an optical density at 600 nm value of 0.7, corresponding to 2×10^7 cells per ml.

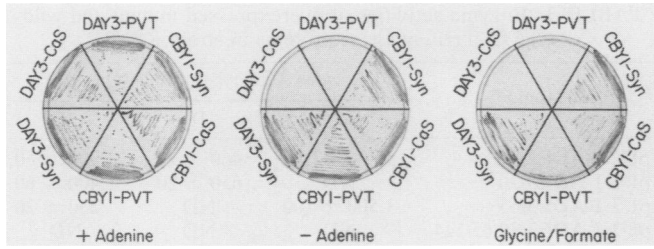

FIG. 5. Growth requirements of transformed yeast strains. +Adenine plates contained serine, leucine, histidine, tryptophan, and adenine; −Adenine plates were identical to +Adenine plates except for lacking adenine; Glycine/Formate plates were identical to +Adenine plates except that glycine and formate were added in place of serine. Uracil was omitted from all plates to maintain plasmid selection. Plasmid abbreviations: pVT, pVT-101U; CaS, pU1-CaS; Syn, pU2.1-ADE3.

adenine for growth, the labeling experiments shown were conducted in the presence of a low concentration of adenine. Reports indicate that adenine represses the level of purine enzymes and hence lowers de novo purine biosynthesis. Labeling experiments with Ade^+ strains, conducted in the absence of adenine, showed qualitatively similar results, but a threefold greater level of intracellular labeling was observed relative to the same cells grown in the presence of adenine. Fractionation of these cells indicated that the majority of this increase was associated with the RNA fraction.

In summary, none of the yeast strains that expressed full-length C_1-THF synthase required adenine for growth, regardless of enzyme activity. Furthermore, growth rates for the wild type (DAY4) and the point mutants (CBY1, CBY4, CBY5, and CBY6) were indistinguishable with respect to adenine, whereas the point mutants were unable to grow on glycine plus formate, consistent with their isolation. Thus, the ADE^+ phenotype correlates not with catalytic activity of C_1-THF synthase but rather with expression of the full-length enzyme, whether catalytically active or not.

Heterologous expression of *Clostridium acidiurici* synthetase. We next asked whether a heterologous enzyme could provide 10-formyl-THF for purine synthesis. The gene encoding a monofunctional 10-formyl-THF synthetase from *C. acidiurici* has been cloned and sequenced (47, 48). In a previous report (5), this gene was inserted into the vector pVT-101U for expression in yeast cells. Transformation of strain CBY1(S^-), which lacks cytoplasmic 10-formyl-THF synthetase activity, with pU1-CaS complements for growth on glycine plus formate as effectively as does pU2.1-ADE3 (5), thus demonstrating the catalytic competence of the clostridial synthetase in vivo. Subfractionation of this strain localized the synthetase activity to the cytoplasmic fraction, with no detectable activity in purified mitochondria above that of the untransformed strain (data not shown). Figure 5 shows the phenotypic analysis of pU1-CaS expression in an *ade3* deletion strain (DAY3) and CBY1(S^-) with respect to adenine and glycine plus formate. Surprisingly, the *ade3* deletion strain (DAY3) transformed with pU1-CaS required adenine for growth. This phenotype is in direct contrast to that of CBY4(D^-), which similarly lacks cytoplasmic dehydrogenase activity but did not require adenine for growth (Table 2).

Yeast cells possess an NAD^+-dependent 5,10-methylene-THF dehydrogenase. Clearly, strains capable of normal growth without added purines must be generating adequate 10-formyl-THF for de novo purine synthesis. Since CBY6($S^-D^-C^-$) grows normally in the absence of purines and lacks all three activities of C_1-THF synthase, we investigated the existence of an additional enzyme activity capable of generating 10-formyl-THF. An NAD^+-dependent 5,10-methylene-THF dehydrogenase was detected in crude extracts prepared from all strains tested, including KSY8, which has both an *ade3* deletion and a *mis1* disruption. NAD^+-dependent 5,10-methylene-THF dehydrogenase activity was expressed at 27 mU/mg of protein, which is similar to the level of expression of the $NADP^+$-dependent activity of $ADE3^+$ strains (e.g., DAY4; Table 2). We have recently purified the NAD^+-dependent 5,10-methylene-THF dehydrogenase activity to homogeneity from KSY8 (5a). The enzyme exists as a homodimer of 38-kDa subunits and is localized to the cytoplasm. This enzyme appears to be monofunctional, since we have been unable to detect cyclohydrolase or synthetase activity in the purified protein. The presence of a third 5,10-methylene-THF dehydrogenase in yeast cells provides an alternate pathway for the oxidation of serine-derived one-carbon units to the formyl oxidation level in an *ade3* deletion strain.

DISCUSSION

In this report, we have characterized the phenotype and metabolism of yeast strains lacking one or more activities encoded by the *ADE3* gene. Analyses of these mutant strains have revealed three unexpected results. First, we find that expression of a catalytically inactive but full-length C_1-THF synthase complements an *ade3* deletion strain for growth in the absence of adenine. Second, in vivo synthesis of cytoplasmic 10-formyl-THF by heterologous expression of the *C. acidiurici* 10-formyl-THF synthetase does not support purine synthesis in an *ade3* deletion strain. Third, a new 5,10-methylene-THF dehydrogenase has been detected in *S. cerevisiae*, one which is dependent on NAD^+ instead of $NADP^+$ for activity.

To isolate mutants that would be catalytically inactive in vivo, 10-formyl-THF synthetase, 5,10-methylene-THF dehydrogenase, and 5,10-methenyl-THF cyclohydrolase point mutants were isolated on the basis of phenotype. The selection procedure for an inactivating mutation in one or more of the trifunctional activities encoded by the *ADE3* gene was first reported by McKenzie and Jones (28). The procedure is based on the ability of cells to utilize formate plus glycine to synthesize serine via the reactions catalyzed by C_1-THF synthase and SHMT (Fig. 1, reactions 1 to 4), thereby complementing a *ser1* mutation. Using in vitro saturation mutagenesis of the *ADE3* gene, we generated several mutant proteins that lack either dehydrogenase activity, the synthetase activity, or all three activities of the trifunctional enzyme. A quite unexpected result was obtained with these full-length but catalytically compromised enzymes: none of the strains expressing the mutant enzymes requires adenine for growth. Furthermore, the growth rates of most of these strains on media lacking adenine are indistinguishable from that of the wild-type strain, DAY4 (Table 2). CBY6($S^-D^-C^-$), which expresses a C_1-THF synthase lacking all three activities, does not require adenine for growth but is slightly stimulated by its addition. Thus, the adenine prototrophy of these point mutants is in stark contrast to the phenotype of *ade3* deletion strains, which have an absolute dependence on adenine for growth.

There are several possible explanations for these observations. The simplest is that our point mutants are leaky. It is conceivable that although mutations to *ADE3* result in

undetectable levels of enzyme activity by in vitro assay, the enzyme may retain some catalytic activity in vivo. However, two results argue against this possibility. First, even at 30-fold overexpression from plasmids, the various mutants do not have detectable catalytic activity in vitro under saturating substrate conditions (Table 3). Second and more significant, the inability of these strains to grow on glycine plus formate in place of serine (Table 2) indicates that these enzymes are catalytically inactive in vivo as well.

The most striking result of these experiments is that the Ade^+ phenotype correlates not with the presence of C_1-THF synthase activities (Table 2) but with the production of a full-length *ADE3* gene product (Fig. 3), regardless of its catalytic competency. Therefore, a second interpretation of these data is that the mutant enzymes complement an *ade3* deletion strain by functioning as a structural component involved in de novo purine biosynthesis. It has been an appealing prospect for some time that the various enzyme activities of de novo purine biosynthesis (10 reactions from 5-phosphoribosyl 1-pyrophosphate to IMP) are organized as a macromolecular protein complex (9, 50). It is known that in eucaryotes several of these steps are catalyzed by multifunctional enzymes. A trifunctional enzyme catalyzing glycinamide ribonucleotide (GAR) synthetase, GAR transformylase, and aminoimidazole ribonucleotide (AIR) synthetase has been identified in chicken liver (14), *Drosophila* (17), and murine lymphoma cells (10); also identified are a bifunctional GAR synthetase-AIR synthetase in yeast cells (16), a bifunctional 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase-IMP cyclohydrolase in chicken liver (29), and a bifunctional AIR carboxylase–succino-5-aminoimidazole-4-carboxamide ribonucleotide synthetase in chicken liver (30). Furthermore, several reports support the existence of multienzyme complexes between several of the purine biosynthetic proteins. Rowe et al. found the biosynthetic enzymes of de novo purine biosynthesis partially copurified from pigeon liver (35) and human lymphocytes (27) in the presence of the nonionic polymer polyethylene glycol. Caperelli et al. (12) reported a 240-fold copurification of GAR transformylase and C_1-THF synthase from chicken liver after seven purification steps. A later report by this group provided evidence for copurification of the additional folate-requiring activities AICAR transformylase and SHMT with C_1-THF synthase and GAR transformylase (11). It was also shown that GAR transformylase and C_1-THF synthase could be cross-linked after in vitro reconstitution of the complex (40). These results all support the existence of a purine-biosynthesizing multienzyme complex in eucaryotic cells.

There is increasing support in the literature for the concept of organizing metabolic processes into multienzyme complexes (41). Recently, Kispal and co-workers used site-directed mutagenesis to engineer a structurally intact but catalytically inactive yeast citrate synthase protein (23). Analogous to our finding, they discovered that expression of this catalytically compromised tricarboxylic acid cycle enzyme complemented a strain carrying a citrate synthase deletion. They propose that the change in phenotype after transformation with an inactive citrate synthase results from the ability to form an intact tricarboxylic acid cycle complex when this protein is expressed.

The results obtained from heterologous expression of the monofunctional *C. acidiurici* 10-formyl-THF synthetase in various yeast strains are consistent with the idea of a purine-synthesizing multienzyme complex. This protein is catalytically competent in vivo, as demonstrated by its ability to complement strain $CBY1(S^-)$ for growth on glycine plus formate. However, expression of the monofunctional synthetase in an *ade3* deletion strain (DAY3) does not complement the adenine requirement even when supplemented with additional formate. Evidently, the 10-formyl-THF being produced by the monofunctional synthetase, while adequate for serine synthesis, is not efficiently utilized for purine biosynthesis. Although the amino acid identity between the synthetase domains of C_1-THF synthase and the *C. acidurici* synthetase is striking (48%) (48), the subunit structure of the *C. acidiurici* enzyme is quite different from that of the eucaryotic trifunctional enzyme. It is reported to be a homotetramer with a subunit M_r of 60,000, in contrast to a homodimer arrangement for C_1-THF synthase (25). Hence, if the *ADE3* gene product is an important structural component for purine synthesis, it is not surprising that heterologous expression of the *C. acidiurici* enzyme is unable to complement this structural function. We are now investigating the possible interaction of C_1-THF synthase with other purine biosynthetic enzymes in vitro.

Regardless of a role for C_1-THF synthase in a putative purine-synthesizing complex, the lack of catalytic activity of the enzyme in strains such as $CBY6(S^-D^-C^-)$ presents another question. $CBY6(S^-D^-C^-)$ grows quite well on media lacking adenine; it is thus capable of de novo purine synthesis, reflecting its ability to synthesize 10-formyl-THF. The question then is, Where does the 10-formyl-THF required for purine synthesis (2 mol of 10-formyl-THF per mol of purine) come from in this strain? 10-Formyl-THF could be provided by an additional cytoplasmic activity or mitochondrial C_1-THF synthase. Mitochondrial production appears unlikely because strain KSY8 (an *ade3* deletion that also lacks the mitochondrial isozyme) transformed with pU2.1-ADE3-30/65, which encodes a catalytically inactive C_1-THF synthase, does not require adenine for growth (data not shown). Furthermore, as was shown years ago (20), *ade3* deletion strains possessing a functional mitochondrial isozyme, such as DAY3, are unable to synthesize purines for growth. We have recently discovered a monofunctional NAD^+-dependent 5,10-methylene-THF dehydrogenase in the cytoplasm of *S. cerevisiae*. The level of this activity in crude extracts is comparable to the $NADP^+$-dependent 5,10-methylene-THF dehydrogenase activity in wild-type yeast. The enzyme has been purified to homogeneity and characterized with respect to kinetics and subunit structure (5a). 5,10-Methylene-THF, produced from serine via SHMT, would be readily oxidized to 5,10-methenyl-THF via the NAD^+-dependent dehydrogenase in the cytoplasm. We have been unable to detect 5,10-methenyl-THF cyclohydrolase activity in crude extracts of *ade3 mis1* mutant strains. However, at physiological pH, the equilibrium between 5,10-methenyl-THF and 10-formyl-THF lies far towards 10-formyl-THF (>90%; 22). Furthermore, the nonenzymatic hydrolysis of 5,10-methenyl-THF to 10-formyl-THF is quite rapid (32, 34). The slight decrease in growth rate exhibited by $CBY6(S^-D^-C^-)$ as compared with $CBY5(S^-D^-)$ on media lacking adenine may be an indication that this nonenzymatic step is rate limiting in this strain. In any event, the existence of a third 5,10-methylene-THF dehydrogenase in yeast cells raises several interesting questions as to its role in one-carbon metabolism and relationship with the trifunctional enzymes.

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LITERATURE CITED

1. **Alvarado-Urbana, G., G. M. Sathe, W.-C. Liu, M. F. Gillen, P. D. Duck, R. Bender, and K. K. Ogilvie.** 1981. Automated synthesis of gene fragments. Science **214**:270–274.
2. **Appling, D. R., and J. C. Rabinowitz.** 1985. Regulation of expression of the *ADE3* gene for yeast C_1-tetrahydrofolate synthase, a trifunctional enzyme involved in one-carbon metabolism. J. Biol. Chem. **260**:1248–1256.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York.
4. **Barlowe, C. K., and D. R. Appling.** 1988. In vitro evidence for the involvement of mitochondrial folate metabolism in the supply of cytoplasmic one-carbon units. Biofactors **1**:171–176.
5. **Barlowe, C. K., and D. R. Appling.** 1989. A general method for generation and analysis of defined mutations in enzymes involved in a tetrahydrofolate-interconversion pathway. Biofactors **2**:57–63.

5a. **Barlowe, C. K., and D. R. Appling.** 1990. Isolation and characterization of a novel eukaryotic monofunctional NAD^+-dependent 5,10-methylenetetrahydrofolate dehydrogenase. Biochemistry **29**:7089–7094.

6. **Barlowe, C. K., M. E. Williams, J. C. Rabinowitz, and D. R. Appling.** 1989. Site-directed mutagenesis of yeast C_1-tetrahydrofolate synthase: analysis of an overlapping active site in a multifunctional enzyme. Biochemistry **28**:2099–2106.
7. **Blakley, R. L.** 1969. The biochemistry of folic acid and related pteridines. North-Holland Publishing Co., Amsterdam.
8. **Boeke, J. D., F. LaCroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197**:345–346.
9. **Burns, V. W.** 1964. Reversible sonic inhibition of protein, purine, and pyrimidine biosynthesis in the living cell. Science **146**:1056–1058.
10. **Caperelli, C. A.** 1989. Mammalian glycinamide ribonucleotide transformylase. Kinetic mechanism and associated de novo purine biosynthetic activities. J. Biol. Chem. **264**:5053–5057.
11. **Caperelli, C. A., P. A. Benkovic, G. Chettur, and S. J. Benkovic.** 1980. Purification of a complex catalyzing folate cofactor synthesis and transformylation in de novo purine biosynthesis. J. Biol. Chem. **255**:1885–1890.
12. **Caperelli, C. A., G. Chettur, L. Y. Lin, and S. J. Benkovic.** 1978. Purification of glycinamide ribonucleotide transformylase. Biochem. Biophys. Res. Commun. **82**:403–410.
13. **Cheek, W. D., and D. R. Appling.** 1989. Purification, immunoassay, and tissue distribution of rat C_1-tetrahydrofolate synthase. Arch. Biochem. Biophys. **270**:504–512.
14. **Daubner, S. C., J. L. Schrimsher, F. J. Schendel, M. Young, S. Henikoff, D. Patterson, J. Stubbe, and S. J. Benkovic.** 1985. A multifunctional protein possessing glycinamide ribonucleotide synthetase, glycinamide ribonucleotide transformylase, and aminoimidazole ribonucleotide synthetase activities in de novo purine biosynthesis. Biochemistry **24**:7059–7062.
15. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press, Washington, D.C.
16. **Henikoff, S.** 1986. The *Saccharomyces cerevisiae ADE5,7* protein is homologous to overlapping *Drosophila melanogaster* Gart polypeptides. J. Mol. Biol. **190**:519–528.
17. **Henikoff, S., M. A. Keene, J. S. Sloan, J. Bleskan, R. Hards, and D. Patterson.** 1986. Multiple purine pathway enzyme activities are encoded at a single genetic locus in Drosophila. Proc. Natl. Acad. Sci. USA **83**:720–724.
18. **Hum, D. W., A. W. Bell, R. Rozen, and R. E. MacKenzie.** 1988. Primary structure of a human trifunctional enzyme. Isolation of a cDNA encoding methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase. J. Biol. Chem. **263**:15946–15950.
19. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
20. **Jones, E. W.** 1972. Fine structure analysis of the *ade3* locus in *Saccharomyces cerevisiae*. Genetics **70**:233–250.
21. **Jones, E. W.** 1977. Bipartite structure of the *ade3* locus of *Saccharomyces cerevisiae*. Genetics **85**:209–223.
22. **Kay, L. D., M. J. Osborn, Y. Hatefi, and F. M. Huennekens.** 1960. The enzymatic conversion of N5-formyl tetrahydrofolic acid (folinic acid) to N10-formyl tetrahydrofolic acid. J. Biol. Chem. **235**:195–201.
23. **Kispal, G., C. T. Evans, C. Malloy, and P. A. Srere.** 1989. Metabolic studies on citrate synthase mutants of yeast. A change in phenotype following transformation with an inactive enzyme. J. Biol. Chem. **264**:11204–11210.
24. **Krebs, E. G.** 1955. Glyceraldehyde-3-phosphate dehydrogenase from yeast. Methods Enzymol. **1**:407–411.
25. **MacKenzie, R. E.** 1984. Biogenesis and interconversion of substituted tetrahydrofolates, p. 255–306. *In* R. L. Blakley and S. L. Benkovic (ed.), Folates and pterins, vol. 1. John Wiley & Sons, Inc., New York.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **McCairns, E., D. Fahey, D. Sauer, and P. B. Rowe.** 1983. De novo purine synthesis in human lymphocytes. Partial co-purification of the enzymes and some properties of the pathway. J. Biol. Chem. **258**:1851–1856.
28. **McKenzie, K. Q., and E. W. Jones.** 1977. Mutants of the formyltetrahydrofolate interconversion pathway of *Saccharomyces cerevisiae*. Genetics **86**:85–102.
29. **Mueller, W. T., and S. J. Benkovic.** 1981. On the purification and mechanism of action of 5-aminoimidazole-4-carboxamide-ribonucleotide transformylase from chicken liver. Biochemistry **20**:337–344.
30. **Patey, C. A. H., and G. Shaw.** 1973. Purification and properties of an enzyme duet, phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazolesuccinocarboxamide synthetase, involved in the biosynthesis of purine nucleotides de novo. Biochem. J. **135**:543–545.
31. **Paukert, J. L., G. R. Williams, and J. C. Rabinowitz.** 1977. Formyl-methenyl-methylenetetrahydrofolate synthetase-(combined). Correlation of enzymic activities with limited proteolytic degradation of the protein from yeast. Biochem. Biophys. Res. Commun. **77**:147–154.
32. **Rabinowitz, J. C.** 1960. Folic acid, p. 185–252. *In* P. D. Boyer (ed.), The enzymes, 2nd ed., vol. 2. Academic Press, Inc., New York.
33. **Racker, E.** 1950. Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim. Biophys. Acta **4**:211–214.
34. **Robinson, D. R.** 1971. The nonenzymatic hydrolysis of N5,N10-methenyltetrahydrofolic acid and related reactions. Methods Enzymol. **18**:716–725.
35. **Rowe, P. B., E. McCairns, G. Madsen, D. Sauer, and H. Elliott.** 1978. De novo purine synthesis in avian liver. Co-purification of the enzymes and properties of the pathway. J. Biol. Chem. **253**:7711–7721.
36. **Schirch, L.** 1984. Folates in serine and glycine metabolism, p. 399–431. *In* R. L. Blakley and S. J. Benkovic (ed.), Folates and pterins, vol. 1. John Wiley & Sons, Inc., New York.
37. **Shannon, K. W., and J. C. Rabinowitz.** 1986. Purification and characterization of a mitochondrial isozyme of C_1-tetrahydrofolate synthase from *Saccharomyces cerevisiae*. J. Biol. Chem. **261**:12266–12271.
38. **Shannon, K. W., and J. C. Rabinowitz.** 1988. Isolation and characterization of the *Saccharomyces cerevisiae MIS1* gene encoding mitochondrial C_1-tetrahydrofolate synthase. J. Biol. Chem. **263**:7717–7725.
39. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

40. **Smith, G. K., W. T. Mueller, G. F. Wasserman, W. D. Taylor, and S. J. Benkovic.** 1980. Characterization of the enzyme complex involving the folate-requiring enzymes of de novo purine biosynthesis. Biochemistry **19**:4313–4321.
41. **Srere, P. A.** 1987. Complexes of sequential metabolic enzymes. Annu. Rev. Biochem. **56**:89–124.
42. **Staben, C., and J. C. Rabinowitz.** 1986. Nucleotide sequence of the *Saccharomyces cerevisiae ADE3* gene encoding C_1-tetrahydrofolate synthase. J. Biol. Chem. **261**:4629–4637.
43. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76**:1035–1039.
44. **Thigpen, A. E., M. G. West, and D. R. Appling.** 1990. Rat C_1-tetrahydrofolate synthase: cDNA isolation, tissue-specific levels of the mRNA, and expression of the protein in yeast. J. Biol. Chem. **265**:7907–7913.
45. **Vernet, T., D. Dignard, and D. Y. Thomas.** 1987. A family of yeast expression vectors containing the phage f1 intergenic region. Gene **52**:225–233.
46. **White, J. C., S. Loftfield, and I. D. Goldman.** 1975. The mechanism of action of methotrexate. III. Requirement of free intracellular methotrexate for maximal suppression of [^{14}C] formate incorporation into nucleic acids and protein. Mol. Pharmacol. **11**:287–297.
47. **Whitehead, T. R., and J. C. Rabinowitz.** 1986. Cloning and expression in *Escherichia coli* of the gene for 10-formyltetrahydrofolate synthetase from *Clostridium acidiurici* ("*Clostridium acidi-urici*"). J. Bacteriol. **167**:205–209.
48. **Whitehead, T. R., and J. C. Rabinowitz.** 1988. Nucleotide sequence of the *Clostridium acidiurici* ("*Clostridium acidiurici*") gene for 10-formyltetrahydrofolate synthetase shows extensive amino acid homology with the trifunctional C_1-tetrahydrofolate synthase from *Saccharomyces cerevisiae*. J. Bacteriol. **170**:3255–3261.
49. **Winston, F., F. Chumley, and G. R. Fink.** 1983. Eviction and transplacement of mutant genes in yeast. Methods Enzymol. **101**:211–228.
50. **Wyngaarden, J. B., S. H. Appel, and P. B. Rowe.** 1968. Control of biosynthetic pathways by regulatory enzymes, p. 415–434. *In* Exploitable molecular mechanisms and neoplasia. The Williams & Wilkins Co., Baltimore.