Purification and Characterization of a Mitochondrial Isozyme of C1-Tetrahydrofolate Synthase from *Saccharomyces cerevisiae*

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C1-Tetrahydrofolate synthase is a trifunctional polypeptide found in eukaryotic organisms that catalyzes 10-formyltetrahydrofolate synthetase (EC 6.3.4.3), 5,10-methylene tetrahydrofolate cyclohydrolase (EC 3.5.4.9), and 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) activities. In *Saccharomyces cerevisiae*, C1-tetrahydrofolate synthase is encoded by the ADE3 locus, yet ade3 mutants have low but detectable levels of these enzyme activities. Synthetase, cyclohydrolase, and dehydrogenase activities in an ade3 deletion strain co-purify 4,000-fold to yield a single protein species as seen on sodium dodecyl sulfate-polyacrylamide gels. The native molecular weight of the isozyme (Mr = 200,000 by gel exclusion chromatography) and the size of its subunits (Mr = 100,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) are similar to those of C1-tetrahydrofolate synthase. Cell fractionation experiments show that the isozyme, but not C1-tetrahydrofolate synthase, is localized in the mitochondria. Genetic studies indicate that the isozyme is encoded in the nuclear genome. Peptide mapping experiments show that C1-tetrahydrofolate synthase and the isozyme are not structurally identical. However, immunotitration experiments and amino acid sequence analysis suggest that C1-tetrahydrofolate synthase and the isozyme are structurally related. We propose to call the isozyme “mitochondrial C1-tetrahydrofolate synthase.”

EXPERIMENTAL PROCEDURES

Materials

Common reagents were commercial products of the highest grade available. Culture media were purchased from Difco. Molecular weight markers for gel electrophoresis, hydroxyapatite (Bio-Gel HTP), and protein assay dye reagent were purchased from Bio-Rad. Percol and Blue Sepharose were obtained from Pharmacia P-L Biochemicals. Proamine sulfate was purchased from Elanco Products Co. (Indianapolis, IN), Cm-cellulose from Whatman, *Staphylococcus aureus V8* protease from Miles (Naperville, IL), α-chymotrypsin from Worthington, and Zymolyase from Kirin Brewing Co. (Tokyo).

Heparin-agarose was prepared as previously described (16). (6R,S)-Tetrahydrofolate was prepared by the hydrogenation of folic acid over platinum oxide (Matheson, Coleman, and Bell, Norwood, OH) in neutral aqueous solution (17) and was purified by chromatography on DAE-cellulose (18). The stock solution contained 10 mM (6R,S)-tetrahydrofolate in 0.2 M Tris-Cl, pH 7.0, 0.5 M 2-mercaptoethanol. (6R,S)-5,10-Methylene-THF was chemically prepared from formaldehyde and (6R,S)-tetrahydrofolate (19). (6R,S)-5,10-Methenyl-THF was prepared as previously described (4).

Strains and Cell Growth

All strains used were haploid strains of *Saccharomyces*. Strain M16-14C (a serl leul) and strain 3-5281 (a serl ura1 ade3) were obtained from E. Jones (Carnegie-Mellon University, Pittsburgh, PA). Strain 3-5281 has an extensive deletion at the ADE3 locus (ade3-130) (20). Respiratory-deficient mutants were isolated from 3-5281 as described (21) except that ethidium bromide (20 μg/ml) was used as a mutagen. Respiratory-deficient mutants that failed to complement a defined ρ0 strain (a ade1 ρ0), obtained from A. Tragoloff, Columbia University,
New York) were designated \( \mu \) mutants.

Cells were grown aerobically at 30 °C. Growth was monitored by measuring turbidity at 600 nm with a Zeiss PMQ II spectrophotometer. Cells were grown in YPD medium (1% yeast extract, 2% bacto-peptone, and 1% galactose) to late log phase (OD\(_{600} \approx 10 \)) unless otherwise indicated. Extracts were prepared by disrupting washed cells with glass beads (0.45-mm diameter) in buffer containing 50 mM Tris-\( \text{SO}_4 \), pH 7.5, 10 mM KCl, 10 mM 2-mercaptoethanol, 1 mM PMSF. Homogenates were centrifuged 30 min at 16,000 × g. The supernatant fractions were used for enzyme and protein assays.

### Enzyme Assays

The purification of \( \text{C}_1 \)-THF synthase and assay of 10-formyl-THF synthetase, 5,10-methylene-THF dehydrogenase (22), and 5,10-methylene-THF cyclohydrolase (6) have been described. These activities are expressed in international units (micromoles of 5,10-methylene-THF formed per minute) based on an extinction coefficient of 24,900 M\(^{-1}\) cm\(^{-1}\) for 5,10-methylene-THF. Published procedures were used to assay fumarase (23), cytochrome \( b_2 \) (24), alkaline phosphatase (25), glyceraldehyde-3-phosphate dehydrogenase (26), phenylthiohydantoin-derivatized amino acids were identified by high performance liquid chromatography on a Beckman model 332 HPLC equipped with an Ultrasphere ODS reverse phase column.

### Gel Electrophoresis

The discontinuous buffer system described by Laemmli (30) was used for electrophoresis of polyacrylamide slab gels. Proteins and peptides were visualized by staining with Coomassie Brilliant Blue.

### Isolation of Mitochondria

Cells were grown in medium containing 1% yeast extract, 1% bacto-peptone, and 1% galactose to late log phase. Cells were harvested, converted to spheroplasts with Zymolyase, and homogenized (31 in 2–3 volumes of ice-cold “breaking” buffer (0.6 M sorbitol, 10 mM Tris-Cl, pH 7.4, 1 mM PMSF, 2 mM dithiothreitol, 8 mM ATP, 8 mM MgCl\(_2\)). All subsequent steps were carried out at 4 °C. The homogenate was centrifuged at 16,000 × g for 10 min. The supernatant fraction was then centrifuged at 9,600 × g for 10 min. The resulting pellet was resuspended in breaking buffer and centrifuged as above. The crude mitochondria (the washed pellet) were further purified on a 25% Percoll gradient formed in situ in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM PMSF, 2 mM dithiothreitol, 8 mM ATP, and 8 mM MgCl\(_2\). Centrifugation was carried out at 64,000 × g for 30 min. An orange-brown band which sedimented toward the bottom of the gradient was collected, and the Percoll was removed from the suspension by high speed centrifugation (106,000 × g for 90 min). The mitochondria were separated from the heavy pellet formed by the Percoll by rinsing with breaking buffer and were recovered by centrifuging at 9,800 × g for 10 min. Mitochondria were stored at −80 °C prior to extraction. Extracts were prepared as described above.

### Peptide Mapping Experiments

One-dimensional peptide mapping was performed as described (32). Purified enzymes (10 μg/well) were mixed with either \( S. \text{ aureus} \) V8 protease or \( \alpha \)-chymotrypsin. The mixtures were incubated at room temperature for 30 min, and the products were separated on a 12.5% SDS-polyacrylamide gel.

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wearing with 10-column volumes of the equilibration buffer containing 15 mM ATP, the column was developed with a linear gradient (10-column volumes) between 0.15 and 0.8 M KCl each in K-MES buffer. 10-Formyl-THF synthetase activity eluted in a single peak at a conductivity of 12 mmho, and fractions containing activity were pooled.

Blue Sepharose Chromatography—The pooled fraction from the heparin-agarose column was applied to a Blue Sepharose column (1.5 x 7.0 cm) equilibrated with K-MES buffer containing 0.5 M KCl. After washing with 10-column volumes of the equilibration buffer, the column was developed with a linear gradient (10-column volumes) between 0.5 and 2.5 M KCl each in K-MES buffer containing 10% glycerol. 10-Formyl-THF synthetase activity eluted in a broad peak, and fractions containing activity were pooled.

Hydroxylapatite Chromatography—The pooled fraction from the Blue Sepharose column was dialyzed against 0.1 M sodium phosphate buffer, pH 6.5, 0.05 M KCl, 10 mM 2-mercaptoethanol and applied to a hydroxylapatite column (1.0 x 3.5 cm) equilibrated with the dialysis buffer. After washing with 10-column volumes of the dialysis buffer, the column was developed with a linear gradient (20-column volumes) between 0.1 and 0.6 M sodium phosphate buffer, pH 6.5, each in 0.05 M KCl, 10 mM 2-mercaptoethanol. 10-Formyl-THF synthetase activity eluted in a single peak at a conductivity of 12 mmho, and fractions containing activity were pooled. The purified enzyme was concentrated, dialyzed against K-MES buffer containing 25 mM KCl, 50% glycerol, 1 mM PMSF, and stored at -20 °C.

RESULTS

Purification of Mitochondrial C1-THF Synthase—in S. cerevisiae, C1-THF synthase is encoded by the ADE3 locus. However, extracts from an ade3 mutant strain (3-5281) had approximately 10% of the synthetase activity, 30% of the dehydrogenase activity, and 6% of the cyclohydrolase activity as an ADE3 wild-type strain (M16-14C) (Table I). This particular mutant strain carries an extensive deletion at the ADE3 locus and lacks C1-THF synthase or any kind of derivative of C1-THF synthase (33, 37). The “residual” activities that were found in the ade3 mutant could be accounted for in two ways. First, there could exist an isozyme of C1-THF synthase catalyzing all three of the activities. Alternatively, the activities could be catalyzed by three separate monofunctional enzymes. To distinguish between these possibilities, we chose to purify the synthetase activity from the ade3 deletion strain and examine the catalytic activities of the purified protein. Extracts were treated with protamine sulfate and ammonium sulfate and then applied to Cm-cellulose, heparin-agarose, Blue Sepharose, and hydroxylapatite. We achieved a 4000-fold purification of the synthetase activity with a 16% yield (Table II). SDS-polyacrylamide gels of the purified sample reveal a single band when stained with Coomassie Brilliant Blue (Fig. 2). We performed densitometry at 560 nm of overloaded gels (10 μg protein/lane). The results demonstrate that the preparation is at least 91% pure (data not shown). The dehydrogenase and cyclohydrolase activities that were present in the crude extract co-purified with the synthetase activity (Table II). These data suggest that the purified protein is trifunctional and catalyzes the same reactions as C1-THF synthase. The purified protein is not encoded by the ADE3 gene, thus we can refer to this protein as an isozyme of C1-THF synthase.

SDS-polyacrylamide gel electrophoresis showed that the purified isozyme has a molecular weight of 100,000 (Fig. 2). Gel exclusion chromatography on Sephacryl S-300 showed that the native molecular weight of the isozyme is approximately 200,000 (data not shown). These data suggest that the isozyme is composed of two identical subunits, as is C1-THF synthase.

Subcellular Localization of Mitochondrial C1-THF Synthase—The isozyme is present in ade3 mutants, but it cannot satisfy the requirements for adenine and histidine that are characteristic of most ade3 mutants (38). This suggests that C1-THF synthase and the isozyme may have different cellular functions. Zelikson and Luzzati (15) suggested that the synthetase and dehydrogenase activities in ade3 mutants are found in the mitochondria. However, these investigators did not examine the purity of their mitochondria, and we could not distinguish between a mitochondrial localization and a vacuolar localization for these activities using their procedure for mitochondrial isolation. We isolated mitochondria from strains M16-14C and 3-5281 using a modified procedure and measured synthetase activity in the mitochondrial extracts. To test the integrity and the purity of our isolated mitochondria, we also measured the activities of a number of enzymes that mark various subcellular compartments. In both strains, the mitochondrial marker enzymes (fumarase and cytochrome b₅) were enriched in isolated mitochondria, whereas the enzymes that mark the vacuoles, the cytoplasm, the endoplasmic reticulum, and the secretory vesicles were not (Fig. 3). Synthetase was enriched in the mitochondria isolated from the

| Table I |
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| **Comparison of C1-THF synthase activity in an ade3 mutant strain and an ADE3 wild-type strain** |
| | Strain | Genotype | Synthetase | Dehydrogenase | Cyclohydrolase |
| M16-14C | ser1 leu1 | 0.14 | 0.014 | 0.007 |
| 3-5281 | ser1 ura1 ade3 | 0.015 | 0.004 | 0.0004 |

| Table II |
| --- |
| **Purification of formyl-THF synthetase from an ade3 deletion strain** |
| | Step | Protein | Total Formyl-THF synthetase activity | Specific activity | Purification fold | Yield % |
| | | mg | units | units/mg | | |
| Crude extract | 40,000 | 600 | 0.15 | 1.0 | 100 | 4 | 46 |
| Protamine sulfate | 30,000 | 600 | 0.020 | 1.8 | 100 | 3 | 14 |
| Ammonium sulfate | 9,400 | 570 | 0.080 | 4.0 | 25 | 3 | 16 |
| Cm-cellulose | 180 | 300 | 1.7 | 110 | 51 | 5 | 11 |
| Heparin-agarose | 20 | 220 | 11 | 720 | 36 | 9 | 13 |
| Blue Sepharose | 3.1 | 100 | 33 | 2,200 | 17 | 8 | 14 |
| Hydroxylapatite | 1.6 | 94 | 59 | 3,900 | 16 | 4 | 15 |

* SYN, synthetase.  
* DH, dehydrogenase.  
* CYC, cyclohydrolase.
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**FIG. 2.** Purification of mitochondrial C₁-THF synthase analyzed on a 10% SDS-polyacrylamide gel. Lanes 1 and 10, purified C₁-THF synthase (2.0 µg); lane 2, crude extract (20 µg); lane 3, protamine sulfate supernatant (20 µg); lane 4, ammonium sulfate fraction (20 µg); lane 5, dialyzed ammonium sulfate fraction (20 µg); lane 6, CN-cellulose pool (12 µg); lane 7, heparin-agarose pool (6 µg); lane 8, Blue Sepharose pool (1.2 µg); lane 9, hydroxylapatite pool (1.0 µg).

**FIG. 3.** Enrichment of enzyme activities in isolated mitochondria. Specific activities were measured in crude extracts and mitochondrial extracts from strain M16-14C (solid bar) and strain 3-5281 (hatched bar). Data are expressed as the specific activity of the mitochondrial extract to that in the crude extract. A ratio of more than one indicates enrichment of an enzyme by the isolation of mitochondria. mt., mitochondrial; ims, intermembrane space.

**TABLE III**

| Strain | Synthetase activity | Dehydrogenase activity | SYN⁺/DH⁺ |
|--------|---------------------|------------------------|----------|
|        | ρ⁻                 | ρ⁺/ρ⁻                  | ρ⁺       | ρ⁺/ρ⁻    |
|        | milliunits/mg       | ratio                  | milliunits/mg | ratio |
| p⁻     | 6.57               | 0.44                   | 1.54     | 0.35     | 4.3 |
| 2      | 12.6               | 0.84                   | 3.61     | 0.82     | 3.5 |
| 3      | 4.32               | 0.29                   | 1.28     | 0.29     | 3.4 |
| 4      | 7.37               | 0.49                   | 2.18     | 0.50     | 3.4 |
| 5      | 5.52               | 0.57                   | 1.60     | 0.37     | 3.5 |
| 6      | 8.25               | 0.55                   | 1.97     | 0.45     | 4.2 |
| 7      | 3.68               | 0.25                   | 0.82     | 0.19     | 4.5 |
| 8      | 4.53               | 0.30                   | 1.07     | 0.24     | 4.2 |
| 9      | 12.1               | 0.81                   | 4.27     | 0.97     | 2.8 |
| 10     | 3.93               | 0.26                   | 1.16     | 0.26     | 3.4 |
| p⁺     | 15.0               | 1.0                    | 4.38     | 1.0      | 3.4 |

* SYN, synthetase.
* DH, dehydrogenase.

**FIG. 4.** Peptide maps of C₁-THF synthase and mitochondrial C₁-THF synthase resulting from digestion with S. aureus V8 protease and α-chymotrypsin. Lanes 3, 5, 7, and 9 show the proteolytic degradation products of C₁-THF synthase, and lanes 4, 6, 8, and 10 show the proteolytic degradation products of mitochondrial C₁-THF synthase after digestion with 50 ng V8 protease (lanes 3 and 4), 500 ng V8 protease (lanes 5 and 6), 1.0 µg α-chymotrypsin (lanes 7 and 8), or 10 µg α-chymotrypsin (lanes 9 and 10). Lanes 1 and 2, C₁-THF synthase and mitochondrial C₁-THF synthase (1.0 µg each), respectively; lanes 11 and 12, 500 ng V8 protease and 10 µg α-chymotrypsin, respectively.

**FIG. 5.** Immunotitration of C₁-THF synthase and mitochondrial C₁-THF synthase with polyclonal antisera. Purified mitochondrial C₁-THF synthase (closed circle) or purified C₁-THF synthase (open circle) (1.5 pmol/reaction) were immunoprecipitated with various dilutions of antisera against mitochondrial C₁-THF synthase (A) or antisera against C₁-THF synthase (B). ade3 deletion strain but not in those isolated from the ADE3 wild-type strain (Fig. 3). These results indicate that the isozyme is localized in the mitochondria, whereas C₁-THF synthase is not.

Localization of Gene Encoding Mitochondrial C₁-THF Synthase—The mitochondrion has its own genome that encodes
rRNAs, tRNAs, plus a small number of mitochondrial proteins (39). Having shown that the isozyme is localized in the mitochondria, we wanted to determine whether it is encoded in the nuclear or the mitochondrial genome. The mitochondrial genome of a yeast \( p \) mutant is either absent or extensively deleted (40), thus a \( p \) mutant cannot synthesize mitochondrially encoded proteins. We isolated 10 \( p \) mutants from strain 3-5281 and found synthetase and dehydrogenase activities in extracts from each of them (Table III). Although there was significant variability in the levels of activity among the mutant strains, the ratio of the specific activity of synthetase to that of dehydrogenase remained relatively constant and was comparable to that of the parent strain. These data indicate that mitochondrial C1-THF synthase is encoded in the nucleus.

**Peptide Maps of C1-THF Synthase and Mitochondrial C1-THF Synthase**—Mitochondrial C1-THF synthase, being a nuclearly encoded mitochondrial enzyme, is presumably initially synthesized with an amino-terminal extension that directs the protein to the organelle (41). Thus, we would expect to find differences between mitochondrial C1-THF synthase and C1-THF synthase at the level of their DNA sequences. However, the two mature proteins could be structurally identical. One procedure we used to examine the structural relationship between the isoforms was peptide mapping. This method is considered to be a stringent test for protein identity. It will also reveal close structural relationships (e.g. precursor-product) among proteins. Purified mitochondrial C1-THF synthase and C1-THF synthase were partially digested with proteases, and the peptide products were separated on SDS-polyacrylamide gels. The peptide banding patterns showed no striking similarities (Fig. 4), which indicates that C1-THF synthase and mitochondrial C1-THF synthase are not structurally identical. The dissimilarities in the banding patterns probably reflect differences between the two proteins at the level of their primary sequences.

**Immunotitration of C1-THF Synthase and Mitochondrial C1-THF Synthase**—A second criteria we used to assess the structural relatedness of these isoforms was to determine whether they would cross-react immunologically. To quantitate cross-reactivity, we did immunotitration experiments by incubating the purified enzymes with polyclonal antisera directed against the purified proteins, precipitating the immune complexes and assaying the synthetase activity which remained in the supernatant fractions. Antiserum against mitochondrial C1-THF synthase reacted with both mitochondrial C1-THF synthase and C1-THF synthase; however, the immunotitration curves were distinct (Fig. 5A). The reciprocal experiment with antiserum against C1-THF synthase showed similar results (Fig. 5B). The pre-immune sera did not affect synthetase activity in these experiments (data not shown). These results are consistent with the conclusion that the two isoforms are not structurally identical, but they also suggest that there are conserved features between the two proteins probably at the level of their secondary or tertiary structures.

**Amino Acid Sequence Analysis of Mitochondrial C1-THF Synthase**—To examine the structural relatedness of the isoforms using a more stringent criteria, we compared a partial amino acid sequence of mitochondrial C1-THF synthase (residues 1-40) with the amino acid sequence deduced from the nucleotide sequence of the gene encoding C1-THF synthase (37). The sequence corresponding to the amino terminus of C1-THF synthase aligned with the amino-terminal sequence of mitochondrial C1-THF synthase (Fig. 6). In this region, there is 40% identity between the two sequences. C1-THF synthase has two functionally independent domains (5, 42). Synthetase activity is catalyzed on the carboxyl-terminal domain, and dehydrogenase and cyclodihydrolase activities are catalyzed on the amino-terminal domain. Although we have not shown that mitochondrial C1-THF synthase also has a two-domain structure, the alignment of the two sequences at their amino termini suggests that the arrangement of the activities within the protein is the same for both isoforms.

**DISCUSSION**

We have purified a mitochondrial isozyme of C1-THF synthase from *S. cerevisiae* which we call mitochondrial C1-THF synthase. Mitochondrial C1-THF synthase and C1-THF synthase are encoded by separate nuclear genes. The isoforms are not identical, but they have similar physical properties, and they are structurally related. Mitochondrial C1-THF synthase is present in *ade3* mutants that lack C1-THF synthase. The observation that the isozyme cannot supply one-carbon units for the synthesis of purines in these mutants suggests that mitochondrial C1-THF synthase and C1-THF synthase may have different cellular roles.

There are several examples of isoforms that are differentially distributed between the mitochondria and the cytoplasm. These include a number a tricarboxylic acid cycle enzymes and enzymes involved in amino acid transamination, NADPH generation, gluconeogenesis, and energy transfer (43). In most of these cases, it has been shown that the two forms of these enzymes perform different metabolic functions. For example, the mitochondrial form of malate dehydrogenase oxidizes malate in the citric acid cycle, whereas the cytoplasmic form reduces oxaloacetate to supply malate for lipogenesis and malate-aspartate shuttle reactions. The different roles of the isoforms are usually manifested by differences in their catalytic properties. The kinetic parameters of mitochondrial C1-THF synthase have yet to be determined and compared to those of C1-THF synthase (4).

Another explanation for the different roles of the isoforms is that the products of the enzymes are required in two cellular compartments but are not transported across the membrane boundaries. In fact, there is evidence that folate coenzymes are not transported across the mitochondrial membrane (44, 45), although folate derivatives (46-49) and folate-dependent enzymes (50-54) are found in mitochondria. This raises the question of the source of the folates and the one-carbon groups required for one-carbon metabolism in the mitochondria. It has been proposed that one-carbon units are shuttled between the cytoplasm and the mitochondria by cytosolic and mitochondrial isoforms of serine hydroxymethyltransferase (44). This reactions requires THF and generates 5,10-methylene-THF. In this scheme, C1-THF synthase and mitochondrial C1-THF synthase would convert the 5,10-methylene-THF generated in these reactions to other THF intermediates.
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required for various biosynthetic pathways in the cytoplasm and the mitochondria.

Reduced folates are required for mitochondrial function (55), possibly because mitochondrial protein synthesis is dependent on the formation of f-Met-tRNA^{Met} (56). Mitochondrial C_{1}-THF synthase may supply the substrate, 10-formyltetrahydrofolate, for this reaction. We are now constructing mutations in the gene encoding mitochondrial C_{1}-THF synthase to assess the physiological role of this enzyme.

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