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Human mitochondrial C_1-tetrahydrofolate synthase: Submitochondrial localization of the full-length enzyme and characterization of a short isoform

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ABSTRACT

Mammalian mitochondrial C_1-tetrahydrofolate (THF) synthase (MTHFDIL gene product) is a monofunctional 10-formyl-THF synthetase, lacking the 5,10-methylene-THF dehydrogenase and 5,10-methenyl-THF cyclohydrolase activities typically found in the trifunctional cytoplasmic proteins. Here, we report the submitochondrial localization of epitope-tagged human mitochondrial C_1-THF synthase expressed in Chinese hamster ovary cells. Mitochondrial fractionation experiments show that human mitochondrial C_1-THF synthase behaves as a peripheral membrane protein, tightly associated with the matrix side of the mitochondrial inner membrane. Inner mitochondrial membrane association was also observed for the endogenous mitochondrial C_1-THF synthase in adult rat spleen. We also purified and characterized the recombinant protein product (short isoform) of the alternatively spliced short transcript of the mitochondrial isozyme. Methylene-THF dehydrogenase assays confirmed that the short isoform is not enzymatically active. The purified short isoform was used in the production of polyclonal antibodies specific for the mitochondrial isozyme. These antibodies detected endogenous full-length mitochondrial C_1-THF synthase in mitochondria from adult rat spleen and human placenta, confirming the expression of the mitochondrial isozyme in adult mammalian tissues.

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Introduction

Activated one-carbon units, carried by tetrahydrofolate (THF)[1], are essential for cellular processes such as *de novo* purine and thymidylate biosynthesis, methionine biosynthesis, amino acid metabolism, and mitochondrial and chloroplast protein synthesis. In eukaryotes, the folate-interconverting activities of 5,10-methylene-THF (CH_2-THF) dehydrogenase, 5,10-methenyl-THF (CH^+-THF) cyclohydrolase, and 10-formyl-THF (CHO-THF) synthetase (Fig. 1, reactions 1–3) are usually present on a single trifunctional polypeptide termed C_1-tetrahydrofolate (THF) synthase. In the yeast *Saccharomyces cerevisiae*, both the cytoplasmic and mitochondrial isozymes of C_1-THF synthase have been studied extensively [1,2]. Both isozymes are trifunctional in yeast. The native proteins are homodimers of 100 kDa subunits. Each subunit has an N-terminal dehydrogenase/cyclohydrolase domain (~30 kDa) linked through a proteolytically sensitive connector region to a C-terminal synthetase domain (~70 kDa). These same characteristics are observed in all known cytoplasmic C_1-THF synthases [3–8].

Adult mammalian mitochondria can oxidize one-carbon units derived from serine [9,10], glycine [11], or sarcosine [9,12–14] to formate or CO_2. All three one-carbon donors (serine, glycine, and sarcosine) produce the common intermediate CH_2-THF, in reactions catalyzed by serine hydroxymethyltransferase (Fig. 1, mitochondrial reaction 4), glycine cleavage system (Fig. 1, reaction 5), and sarcosine dehydrogenase (Fig. 1, reaction 9), respectively. CH_2-THF can then be oxidized to formate by mitochondrial reactions 3, 2, and 1 (Fig. 1). This one-carbon metabolism pathway has been shown to be localized to the matrix in both yeast [15,16] and mammalian [9,10] mitochondria. However, the enzyme(s) catalyzing this pathway in adult mammalian mitochondria have not been identified.

Previously, we reported the identification and characterization of a gene (MTHFD1L) encoding human mitochondrial C_1-THF synthase [17]. This mitochondrial isozyme exhibits 61% identity with cytoplasmic C_1-THF synthase, and possesses the same domain structure as the previously characterized trifunctional C_1-THF synthases. However, enzyme assays on purified recombinant enzyme revealed that human mitochondrial C_1-THF synthase is a monofunctional CHO-THF synthetase, lacking the CH_2-THF dehydrogenase and CH^+-THF cyclohydrolase activities (Fig. 1, mitochondrial reactions 2, 3) found to date in all other C_1-THF synthases [18].

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[1] *Abbreviations used:* BSA, bovine serum albumin; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; FPGS, folylpolyglutamate synthetase; HMS, homogenization solution; IPTG, isopropyl-β-D-thiogalactopyranoside; MBP/SI, Maltose binding protein/short isoform; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TEV, tobacco etch virus; THF, tetrahydrofolate.

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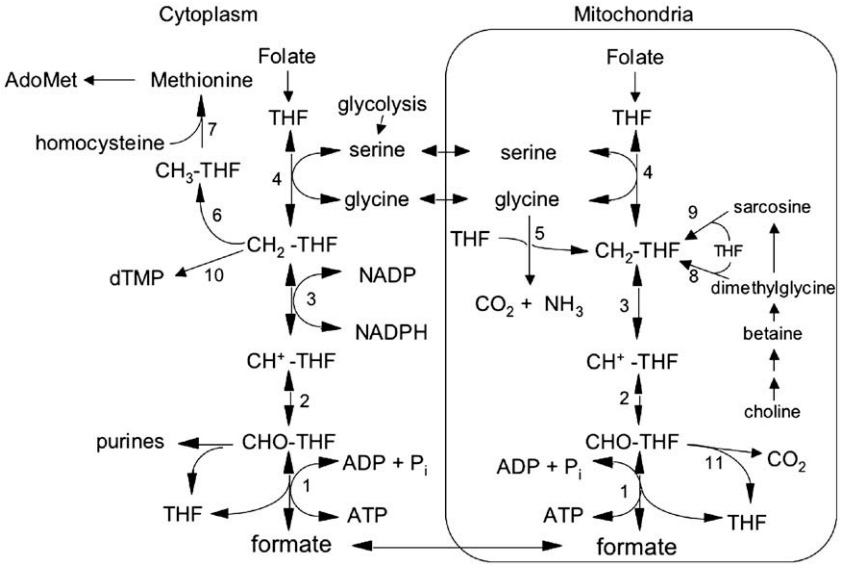

Fig. 1. Compartmentation of folate-mediated one-carbon metabolism in eukaryotes. Reactions 1, 2, and 3 are catalyzed respectively by 10-formyl-THF synthetase (EC 6.3.4.3), 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9), and 5,10-methylene-THF dehydrogenase (EC 1.5.1.5) activities of C_1-THF synthase. The other reactions are catalyzed by 4, serine hydroxymethyltransferase (EC 2.1.2.1); 5, glycine cleavage system (GCS) (EC 2.1.2.10); 6, 5,10-methylene-THF reductase (EC 1.5.1.20); 7, methionine synthase (EC 2.1.1.13); 8, dimethylglycine dehydrogenase (EC 1.5.99.2); 9, sarcosine dehydrogenase (EC 1.5.99.1); 10, thymidylate synthase (EC 2.1.1.45); 11, 10-formyl-THF dehydrogenase (EC 1.5.1.6). Coenzymes for all reactions are not shown. Reactions 8, 9 and 11 are found only in mammals. All reactions from choline to sarcosine are mitochondrial except the betaine to dimethylglycine conversion, which is cytoplasmic. AdoMet, *S*-adenosylmethionine.

The human mitochondrial isozyme includes a 62-residue N-terminal extension, with the first 31 residues predicted to function as a mitochondrial targeting sequence [17]. Expression of a full-length cDNA clone in Chinese hamster ovary (CHO) cells confirmed that the recombinant protein was indeed targeted to mitochondria. The submitochondrial localization of the protein was not determined, however. Furthermore, northern blot analysis revealed that the human MTHFD1L gene expressed two transcripts: a long transcript producing the full-length enzyme, and a shorter transcript derived from alternative splicing [17]. If translated, this short transcript would produce a truncated protein, comprised of only 275 amino acids. However, it was not known whether the protein encoded by this short transcript is translated *in vivo*, or whether it exhibits enzyme activity.

We show here that human mitochondrial C_1-THF synthase behaves as a peripheral membrane protein, tightly associated with the matrix side of the mitochondrial inner membrane. Using antibodies specific for the mitochondrial isozyme, we demonstrate that the full-length enzyme is expressed in adult tissues, including human placenta and adult rat spleen. Finally, we report the purification and characterization of the short isoform of C_1-THF synthase.

Experimental procedures

Materials

All chemicals were of the highest available commercial quality. Difco media components were obtained from VWR (West Chester, PA). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Primers for PCR and sequencing were made by IDT (Coralville, IA). TEV protease was expressed and purified essentially as described [18]. Sources of media and other supplements for cell culture were as follows: HyQ α-minimal Eagle's medium (Hyclone, Logan, UT); Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA); Glutamax and Penicillin/Streptomycin (GIBCO BRL, Gaithersburg, MD); G418 sulfate (EMD Biosciences, La Jolla, CA); Trypsin (Cellgro, Herndorn, VA). Human placenta was kindly provided by St. David's Medical Center (Austin, TX) and given to us by Dr. JoAnn Hunter Johnson (University of Texas, Austin, TX).

cDNA cloning of short isoform of mitochondrial C_1-THF synthase

cDNA for the short isoform was PCR-amplified from pcDNA3.1-humito [17] using KOD Hot Start DNA polymerase (Novagen) and primers SHOT5′ (5′-CGCCATATGGGCACGCGTCTGCCG-3′; NdeI site underlined) and SHOT3′ (5′-CGCCTCGAGGATCACGCGCCTGCACTC CAGCCTGGTGACAGAACGAGACTCCGTCTTGCTTTGAAGCTGGCG-3′; XhoI site underlined). SHOT3′ contains the 45 extra nucleotides present only in the alternatively spliced short (1.1-kb) transcript of human mitochondrial C_1-THF synthase [17]. The product (825-bp) was cloned into NdeI/XhoI-digested *E. coli* expression vector pET22b (Novagen), but attempts to express the short isoform protein from this construct in *E. coli* were not successful. So the short isoform cDNA was subcloned into the pMal-c2x H10TEV vector (obtained from Dr. John Tesmer [17]) using KOD Hot Start DNA polymerase and primers hmcleavedlong5′ (5′ TATAGGATCCAGCA GCGGCGGCGGCGGAGGC-3′; BamHI site underlined) and shortisoform3′ (5′-CGCAAGCTTTTAGATCACGCGCCTGCACTC-3′; HindIII site and stop codon following underlined). The 5′ primer, hmcleavedlong5′ was designed to amplify the cDNA from nucleotide +94 onwards only (the A of the ATG start codon is designated +1), thereby eliminating the N-terminal 31 codons representing the mitochondrial presequence [20], from the construct. The PCR product (731-bp) was cloned into BamHI/HindIII-digested *E. coli* expression vector pMal-c2x H10TEV. This construct, pMal-shortisoform was sequenced and the correct sequence confirmed.

Expression and purification of short isoform

pMal-shortisoform was transformed into chemically competent Rosetta 2(DE3)pLysS *E. coli* (Novagen) and transformants were selected on LB plates containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol (LB/Amp/Chl) at 37 °C. A single colony was used to inoculate 5 ml LB/Amp/Chl liquid media and grown at 37 °C with shaking for ~7 h. This was used to inoculate a 25 ml

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