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HUMAN MITOCHONDRIAL C₁-TETRAHYDROFOLATE SYNTHASE: SUBMITOCHONDRIAL LOCALIZATION OF THE FULL-LENGTH ENZYME AND CHARACTERIZATION OF A SHORT ISOFORM

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Abstract

Mammalian mitochondrial C₁-tetrahydrofolate (THF) synthase (MTHFD1L 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 C₁-THF synthase expressed in Chinese hamster ovary cells. Mitochondrial fractionation experiments show that human mitochondrial C₁-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 C₁-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 C₁-THF synthase in mitochondria from adult rat spleen and human placenta, confirming the expression of the mitochondrial isozyme in adult mammalian tissues.

INTRODUCTION

Activated one-carbon units, carried by tetrahydrofolate (THF¹), 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 (CH₂-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 C₁-tetrahydrofolate (THF) synthase. In the yeast *Saccharomyces cerevisiae*, both the cytoplasmic and mitochondrial isozymes of C₁-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

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¹Abbreviations: 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

kDa). These same characteristics are observed in all known cytoplasmic C₁-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 CO₂. All three one-carbon donors (serine, glycine, and sarcosine) produce the common intermediate CH₂-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. CH₂-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 C₁-THF synthase [17]. This mitochondrial isozyme exhibits 61% identity with cytoplasmic C₁-THF synthase, and possesses the same domain structure as the previously characterized trifunctional C₁-THF synthases. However, enzyme assays on purified recombinant enzyme revealed that human mitochondrial C₁-THF synthase is a monofunctional CHO-THF synthetase, lacking the CH₂-THF dehydrogenase and CH⁺-THF cyclohydrolase activities (Fig. 1, *mitochondrial reactions 2, 3*) found to date in all other C₁-THF synthases [18]. 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 C₁-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 C₁-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, Herndon, 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 C₁-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'-CGCCTCGAGGATCACGCGCCTGCACTCCAGCCTGGTGACAGAACGAGACTCCGTCTTGCTTTGAAGCTGGCG-3'; XhoI site underlined). SHOT3' contains the 45 extra nucleotides present only in the alternatively spliced short (1.1-kb) transcript of human mitochondrial C₁-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 [19]] using KOD Hot Start DNA polymerase and primers hmcleavedlong5' (5' TATAGGATCCAGCAGCGGCGGCGGCGGAGGC-3'; BamHI site underlined) and short isoform3' (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 hrs. This was used to inoculate a 25 ml LB/Amp/Chl culture that was grown overnight at 37°C with shaking. One liter of LB/Amp/Chl was inoculated with the overnight culture to an initial OD₆₀₀ of 0.1 and grown at 37°C with shaking until an OD₆₀₀ of 0.5. Expression of fusion protein (Maltose binding protein/Short isoform; MBP/SI) was induced using 50 µM IPTG at 15°C while shaking. The cells were harvested after overnight induction by centrifugation at 4300 × g for 20 min and washed using extraction/wash buffer [50 mM sodium phosphate, 300 mM NaCl, 5 mM 2-mercaptoethanol, 20% glycerol (pH 7.0)]. The cell pellet was resuspended in 20 ml of extraction/wash buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidinium-HCl and two complete-mini EDTA-free protease inhibitor cocktail tablets (Roche). The cells were sonicated with a Vibracell Model VC40 (Danbury, CT) on ice using a 2 mm probe for 5 × 15-sec cycles with 30 sec incubation on ice in between each cycle. The sonicated cell suspension was centrifuged at 30,600 × g for 20 min at 4°C and the supernatant was the cell extract. Purification of short isoform was done using TALON Cobalt metal affinity resin (BD Biosciences, San Jose, CA) by a batch/column method. The fusion protein, which was eluted using extraction/wash buffer containing 150 mM imidazole, was cleaved by mixing with TEV protease at a MBP/SI:TEV protease ratio of 5:1 (w/w) at room temperature while dialyzing overnight against 20 mM Tris-Cl (pH 8.0), 5 mM 2-mercaptoethanol, 100 mM KCl, 10% glycerol. The TEV-cleaved and dialyzed fusion protein was then loaded onto a fresh 5 ml TALON Cobalt column and the flow through fractions containing protein (short isoform) were collected and pooled. A Bradford assay [21] was performed to determine the protein concentration. The purified short isoform was subjected to N-terminal sequencing at the Protein Sequencing Facility at the University of Texas. 5,10-Methylene-THF dehydrogenase activity was assayed as described previously [18].

Polyclonal antibody production

Short isoform was concentrated using a Centriprep YM-10 concentrator (Millipore) by centrifuging at $1750 \times g$ at room temperature, mixed with 6X SDS sample buffer, boiled for 5 min and fractionated by SDS-PAGE [22] on a 12% polyacrylamide gel for 60 min at 180 V. The gel was stained, destained and the band containing the short isoform was cut out and sent to M.D. Anderson Cancer Center (Bastrop, TX) for polyclonal antibody production in rabbits. These antisera (anti-MTHFD1L) were stored in aliquots at -70°C until use.

Mitochondrial Isolation and Submitochondrial fractionation from CHO/pcDNA3.1-humito cells

The *glyB* line of CHO cells [23,24] was used for these experiments, since this line gave more stable transfectants than the wild-type CHO cells used previously [17]. The pcDNA3.1-humito expression construct carries the full-length human mitochondrial C_1 -THF synthase with the 14-amino acid V5 epitope fused to its C-terminus [17]. CHO cells transfected with pcDNA3.1-humito were cultured in HyQ α -minimal Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 0.25 mM glutamine, and 100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin solution in P150 dishes. The medium contained 0.8 mg/ml G-418 sulfate. Upon reaching confluency, the cells were rinsed with phosphate buffered saline (PBS) ($1 \times 5 \text{ ml}$) at room temperature and trypsinized using 0.25% trypsin in PBS containing 0.5 mM EDTA by incubating at 37°C for 5 min. Trypsin treatment was stopped by adding 6 ml of fresh medium, and the dislodged cells from all the plates were pooled into a centrifuge tube. The cells were then counted on a hemacytometer. Mitochondria were isolated essentially according to Rickwood et al [25]. A Bradford assay [21] was done on the mitochondrial fraction.

Mitoplasts were prepared by the modified swell-contrast method [26], in which mitochondria were resuspended in 20 mM potassium phosphate (pH 7.2) and incubated on ice for 20 min. ATP and MgCl_2 were then added to a final concentration of 1.0 mM each and incubated for another 5 min on ice. The suspension was centrifuged at $10,000 \times g$ for 10 min and the supernatant was saved in an ultracentrifuge tube. The pellet was resuspended in 2 ml of homogenization solution [HMS; 0.25 M sucrose, 1 mM EGTA, 0.5% BSA and 10 mM HEPES-NaOH (pH 7.4)] without BSA and centrifuged at $10,000 \times g$ for 10 min. The final pellet (mitoplasts) was saved on ice. The supernatant was combined with the first supernatant and centrifuged at $100,000 \times g$ for 1 hr. This supernatant was the intermembrane space fraction and the pellet was resuspended in HMS without BSA to yield the outer membrane fraction. Mitoplasts were also prepared by a modified digitonin method [27,28] using 0.12 mg digitonin/mg mitochondria.

Mitoplasts from either method were resuspended in HMS without BSA, mixed with an equal volume of 0.025 M HEPES pH 7.0 containing 1% Triton X-100 (0.5% final), and incubated on ice for 30 min. Triton-treated mitoplasts were centrifuged at $100,000 \times g$ for 1 hr. The supernatant was the matrix and the pellet was resuspended in HMS without BSA to yield the inner membrane fraction.

Immunoblotting

Mitochondrial subfractions (outer membrane, intermembrane space, inner membrane and matrix) were immunoblotted with mouse anti-V5 antibody (1:1000 dilution; Invitrogen) as described previously [17]. Other antibodies were used as markers, including rabbit anti-porin (Calbiochem, San Diego, CA) [29] or rabbit anti-Bclx-S/L (S-18; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) [30] for outer membrane (OM); mouse anti-COXI (Molecular Probes, Eugene, OR) for inner membrane (IM) [31]; and rabbit anti-Hsp60 (Stressgen Biotechnologies, San Diego, CA) [32] or rabbit anti-MnSOD (Stressgen Biotechnologies) [31] for inner

membrane/matrix. Secondary antibody was either goat anti-mouse or goat anti-rabbit (1:2000 dilution; Zymed laboratories Inc; San Francisco, CA).

Alkaline Carbonate Extraction of Mitochondria

Alkaline carbonate extraction of mitochondria was done essentially as described [33]. Briefly, mitochondrial suspensions were incubated on ice for 30 min with either 100 mM alkaline Na_2CO_3 (pH 11.5), 133 mM NaCl, or 0.5% Triton X-100, followed by 30 min centrifugation at $150,000 \times g$. The pellets were treated again with the same conditions. The supernatants from the two centrifugations were combined to give the soluble fraction (S) and the pellets were resuspended in an equal volume of HMS without BSA to give the membrane fraction (P). The soluble and membrane fractions were subjected to SDS-PAGE and immunoblotting as described above.

Protease Treatment of Mitochondria

Protease treatment of mitochondria was done essentially as described [34]. Briefly, mitochondria were incubated with varying concentrations of digitonin (0–1:1 mg digitonin/mg protein) in the presence or absence of proteinase K (100 $\mu\text{g}/\text{ml}$) for 20 min on ice. Mitochondria incubated with 1% Triton X-100 in the presence of proteinase K were maintained as a positive control to check for the ability of proteinase K to digest the proteins of interest. After incubation, the mitochondrial suspensions were layered on top of a sucrose cushion (HMS without BSA containing 1 M sucrose) and centrifuged at $16,600 \times g$ for 10 min. The supernatants were discarded and the pellets were resuspended in equal volumes of HMS without BSA for SDS-PAGE. In the Triton-treated controls, the pellet (P) and trichloroacetic acid (TCA)-precipitated protein from the supernatant (S) were used for SDS-PAGE. TCA precipitation was performed by adding 10% TCA to the supernatant and incubating at room temperature for 5 min. Precipitated proteins were pelleted by centrifugation at $16,600 \times g$ for 10 min, and resuspended in HMS without BSA to give the soluble (S) fraction.

Rat Spleen Submitochondrial fractionation

Two 8-week-old Sprague-Dawley male rats, housed in the Animal Resource Center at the University of Texas at Austin, were fasted overnight, euthanized, and spleens (~1 g each) removed. Isolation of mitochondria from rat spleen was performed as described [28] except for the composition of the H-medium used [0.3 M sucrose, 1 mM EGTA, 5 mM MOPS, 5 mM KH_2PO_4 , 0.1% BSA (pH 7.4)] [25]. Subfractionation of mitochondria was done in the same way as for the cultured cells, except that in the swell-contract method, outer membrane was not separated from the intermembrane space, and in the digitonin method, 0.3 mg digitonin/mg of mitochondrial protein was used.

Isolation of Mitochondria from Human Placenta

Human placenta was washed in H-medium, quick frozen in liquid nitrogen, and stored at -70°C until use. Placenta was thawed in chilled 250 mM sucrose on ice. The tissue was washed 3 times in buffer A [250 mM sucrose, 1 mM EDTA and 50 mM Tris-HCl (pH 7.5)] and diced in buffer A in a volume equal to 2.5 times the wet weight of the tissue. Homogenization was done in a Tekmar tissumizer at high speed for 5×5 -sec each, incubating on ice for 30 sec after each cycle. The homogenate was centrifuged at $960 \times g$ for 15 min, the supernatant poured through 2 layers of cheese cloth and recentrifuged at $17,200 \times g$ for 15 min. The pellet was saved on ice and the supernatant was centrifuged at $17,200 \times g$ for another 15 min. The second pellet was combined with the first using 10 ml of buffer B [250 mM sucrose, 1 mM EDTA and 10 mM Tris-HCl (pH 7.5)] and centrifuged at $17,200 \times g$ for 15 min. The supernatant was discarded and the pellet was washed twice with buffer B and resuspended in buffer B. A

Bradford assay was done to determine the protein concentration and the mitochondria were stored at -70°C .

RESULTS

cDNA Cloning and Expression of Short Isoform

Northern blot analysis of mitochondrial C₁-THF synthase in human tissues revealed a full-length 3.6 kb transcript, and an alternatively spliced shorter transcript of 1.1 kb [17]. If translated, this short transcript would encode a protein of 275 amino acids. The first 260 amino acids would be identical to the N-terminus of the full-length protein, followed by 15 residues derived from an exonized *Alu* element. To determine whether the short transcript could encode a functional enzyme, a cDNA was constructed using pMal-c2x H10TEV [19]. This vector includes a 10X His tag at the C-terminus of the MBP followed by a TEV protease recognition site and then the short isoform of human mitochondrial C₁-THF synthase (Fig. 2). This fusion protein (MBP/SI) was expressed in *E. coli* and purified on a TALON Cobalt metal affinity resin (Fig. 2; *lane 1*) and cleaved using TEV protease. A second affinity column was used to separate MBP retaining the His tag from the short isoform in the flow through. TEV protease cleavage of MBP/SI was incomplete even at a MBP/SI to TEV protease ratio of 1:5 (Fig. 2, *lane 2*). In addition, some of the uncleaved MBP/SI failed to bind to the second affinity column and eluted with the short isoform (Fig. 2, *lane 3*). Nevertheless, the predominant product was the cleaved short isoform, which remained stable and soluble. The predicted molecular weight of recombinant short isoform (lacking the 31-residue mitochondrial targeting sequence) is 26.7 kDa, but the short isoform migrated slower than expected (~ 35 kDa) on a 12% SDS-PAGE gel [The short isoform migrated closer to its predicted molecular weight on 7.5% gels (see Fig. 3A)]. 5,10-Methylene-THF dehydrogenase enzyme activity was undetectable in this purified protein.

Polyclonal antibodies raised against rat cytoplasmic C₁-THF synthase [3] cross-react with human mitochondrial C₁-THF synthase (data not shown). Since the human cytoplasmic and mitochondrial proteins share only 31% identity in the N-terminal dehydrogenase/cyclohydrolase domain [17], we predicted that polyclonal antibodies raised against the short isoform, which represents just the N-terminus of the mitochondrial isozyme, might specifically recognize the full-length mitochondrial isozyme. Polyclonal antibodies were raised in rabbits against pure short isoform, devoid of uncleaved fusion protein, as described under "Experimental Procedures". These will be referred to herein as anti-MTHFD1L antibodies.

Specificity of anti-MTHFD1L antibodies

Purified short isoform was electrophoresed and immunoblotted with either anti-MTHFD1L antibodies or the pre-immune serum. The anti-MTHFD1L antibodies gave specific signals at ~ 30 kDa (the mobility of the short isoform, SI), and at 70 kDa (corresponding to the fusion protein, MBP/SI) (Fig. 3A, *lane 1*), since the purified protein contained some uncleaved MBP/SI. A signal at 45 kDa, similar to the size of MBP, was seen in immunoblots with the anti-MTHFD1L antibodies as well as the pre-immune serum. To determine whether the polyclonal antibodies raised against the purified short isoform cross react with the cytoplasmic isozyme, 50 μg of rat liver cytosol or rat spleen cytosol were electrophoresed and immunoblotted with either anti-MTHFD1L or anti-cyto-C₁-THF synthase antibodies. While the anti-cyto-C₁-THF synthase antibodies gave a strong signal at the size of the cytoplasmic isozyme (~ 107 kDa) in both liver and spleen cytosols (Fig. 3B; *lanes 1 & 2*), the anti-MTHFD1L antibodies gave only a faint signal in rat liver cytosol (Fig. 3C; *lane 1*), and no signal in rat spleen cytosol (Fig. 3C; *lane 2*), reflecting little or no cross-reactivity with the cytosolic isozyme. Rat spleen mitochondria gave an intense signal with the anti-MTHFD1L antibodies (Fig. 3C; *lane 3*) at

the size of the mitochondrial isozyme (102 kDa), confirming the specificity of these antibodies for the mitochondrial isozyme.

CHO Cell Expression and Submitochondrial localization

We previously showed that the protein expressed from pcDNA3.1-humito in CHO cells is localized to mitochondria [17]. This expression construct carries the full-length human mitochondrial C₁-THF synthase with the 14-amino acid V5 epitope fused to its C-terminus. To determine the submitochondrial localization of this protein, mitochondria were isolated from transfected CHO cells and subfractionated as described under "Experimental Procedures". These submitochondrial fractions were subjected to SDS-PAGE and immunoblotting using antibodies against the V5 epitope to localize the human mitochondrial C₁-THF synthase, and against the mitochondrial marker proteins cytochrome oxidase subunit I (COX I; inner membrane) and Hsp60 (inner membrane/matrix). Inner membrane and matrix fractions were separated by treating the mitoplasts with 0.5% Triton X-100. After Triton X-100 treatment, most of the COX I (apparent molecular weight 35 kDa), was seen in the inner membrane fraction (Fig. 4; *lane 4*), while most of Hsp60 (60 kDa), an inner membrane/matrix marker, was released into the matrix (Fig. 4; *lane 5*). The less intense signal of Hsp60 seen in the inner membrane can be explained by the observation that Hsp60 is one of several mitochondrial DNA binding proteins that can associate with the inner membrane [32].

The mitochondrial isozyme (107 kDa; calculated size without presequence but with epitope tag) detected by the anti-V5 antibodies, was distributed between the inner membrane and matrix fractions (Fig. 4; *lanes 4 and 5*), suggesting that human mitochondrial C₁-THF synthase is at least partly associated with the mitochondrial inner membrane. Essentially the same result was obtained with anti-V5 antibodies on mitochondria fractionated using digitonin (data not shown).

Alkaline Carbonate Extraction of Mitochondria

To determine the nature of the membrane association, mitochondria were subjected to alkaline (pH 11.5) sodium carbonate treatment. Alkaline sodium carbonate is known to release peripheral membrane proteins into the supernatant while integral membrane proteins are retained in the insoluble membrane fraction [35]. The integral membrane marker protein, porin (31 kDa), was observed in the pellet after sodium carbonate treatment (membrane fraction; Fig. 5, *lane 5*), whereas almost all of the peripheral membrane marker protein, MnSOD (25 kDa), was observed in the supernatant (soluble fraction; Fig. 5, *lane 6*). In contrast to MnSOD, the mitochondrial isozyme (107 kDa) was distributed equally between the membrane and soluble fractions, detected by both anti-V5 and anti-MTHFD1L antibodies (Fig. 5; *lanes 5 and 6*). Mitochondria treated with NaCl of the same ionic strength as Na₂CO₃ failed to release the mitochondrial isozyme from the membrane fraction (Fig 5; *lane 3 vs. 4*). Triton X-100 treatment also released only about half of the mitochondrial C₁-THF synthase from the membrane (Fig. 5; *lanes 1 and 2*). MnSOD, although a matrix marker, has been reported to behave partly as an insoluble membrane protein [33], even in the presence of detergent [31], explaining its presence in the membrane fraction even after treatment with Triton X-100 (Fig. 5; *lane 1*). These data suggest that human mitochondrial C₁-THF synthase is a tightly associated peripheral membrane protein of the mitochondrial inner membrane.

Membrane topology of human mitochondrial C₁-THF synthase

To determine the topology of the mitochondrial isozyme within the mitochondrial inner membrane, mitochondria were treated with digitonin in the presence or absence of proteinase K. The digestion pattern of mitochondrial C₁-THF synthase was obviously different from the outer membrane marker (Bclx-S_L), but closely resembled the inner membrane/matrix marker Hsp60 (Fig. 6; *A and B*). Mitochondrial C₁-THF synthase behaved essentially the same in the

presence or absence of proteinase K, showing that digitonin solubilization of the outer membrane does not make it susceptible to proteolysis. Mitochondrial C₁-THF synthase was susceptible to proteinase K only when the membranes were completely solubilized by 1% Triton X-100. At the highest digitonin concentration, most of the mitochondrial isozyme was solubilized, in contrast to the matrix marker Hsp60. Anti Bclx-S/L antibodies gave two bands with different digestion patterns, the longer form being resistant to proteinase K digestion even after Triton treatment. Although the expected molecular weight of Bclx-L and Bclx-S are 30 kDa and 23 kDa respectively, anti-Bclx-S/L antibodies detected 35 kDa and 32 kDa proteins in CHO cell mitochondria. This variation in molecular weights has been observed by others at least for the longer form of Bcl-x [36]. The shorter form was digested by proteinase K at a low concentration of digitonin, as expected for an outer membrane marker. These data indicate that human mitochondrial C₁-THF synthase is associated with the matrix side of the inner membrane.

Rat Spleen Submitochondrial fractionation

We considered the possibility that the inner mitochondrial membrane localization of the recombinant human protein in the CHO cell system might be an artifact of the V5 epitope fused to the C-terminus. To examine the submitochondrial localization of the endogenous mitochondrial C₁-THF synthase in an adult mammalian tissue, rat spleen mitochondria were subjected to fractionation and immunoblotted using anti-MTHFD1L or the different marker antibodies. In both swell-contract (Fig. 7) and digitonin methods (data not shown), the rat mitochondrial isozyme (102 kDa; calculated size without presequence) partitioned between the inner membrane and matrix fractions (Fig. 7; *lanes 3 and 4*). Most of the inner membrane/matrix marker protein, Hsp60, was released into the matrix (Fig. 7; *lane 4*), and COX I was in abundance in the inner membrane fraction (Fig. 7; *lane 3*). In rat spleen mitochondria fractionated with digitonin, mitochondrial C₁-THF synthase partitions even more strongly with the inner membrane (data not shown). Thus, like in the cultured CHO cells, the mitochondrial isozyme appears to be partly associated with the inner membrane in adult rat spleen mitochondria as well.

Expression level of mitochondrial isozyme in rat and human tissues and CHO cells

Mitochondria from rat spleen (RS), human placenta (HP) and CHO/pcDNA3.1-humito cells were fractionated on a 7.5% SDS-polyacrylamide gel in duplicate lanes. One half of the gel was Coomassie-Blue stained (Fig. 8; *left panel*) and the other half of the gel was subjected to immunoblotting with anti-MTHFD1L antibodies (Fig. 8; *right panel*). Rat spleen and CHO/pcDNA3.1-humito mitochondria (*lanes 1 and 3*, respectively) gave intense signals of the size of mitochondrial C₁-THF synthase. The signal at 102 kDa (*lane 1*) represents the endogenous rat spleen mitochondrial C₁-THF synthase without the presequence, and the signal at 107 kDa (*lane 3*) represents the recombinant human mitochondrial C₁-THF synthase without the presequence but with the V5 epitope tag. Mitochondria from human placenta also gave a signal (*lane 2*; 102 kDa), confirming expression of the MTHFD1L gene at the protein level in an adult human tissue. We observed a faint cross-reacting band at about 35 kDa in human placenta (*lane 2*, marked with a *white dot*) that might represent the short isoform. Although the predicted molecular weight of the short isoform is 26 kDa (assuming the presequence is cleaved off), the recombinant protein was found to migrate anomalously at about 35 kDa in SDS-PAGE (Fig. 2). This suggests that the alternative splicing transcript may be translated *in vivo*. On the other hand, this band could be simply a proteolytic degradation product of the full-length protein. Because rodents do not possess *Alu* elements [37], and thus do not exhibit alternative splicing of the mitochondrial C₁-THF synthase transcript, the signal seen at ~ 40 kDa in lane 1 (Fig. 8) cannot be the short isoform. This immunoreactive band in rat mitochondria thus probably represents a proteolytic degradation product of the full-length protein.

DISCUSSION

The human MTHFDL1 gene encodes the mitochondrial C₁-THF synthase [17], a monofunctional CHO-THF synthetase [18]. This 975 amino acid protein is translated from a 3.6 kb transcript. Adult human tissues also express a short transcript (1.1 kb) that results from an alternative splicing event. The potential protein product (short isoform) of this short transcript would consist of 275 amino acids, with the last 15 residues being derived from exonization of an *Alu* element [17]. The short transcript, lacking exon 8 and beyond, would encode 260 of the 300 residues that make up the N-terminal dehydrogenase/cyclohydrolase domain. However, given the lack of dehydrogenase or cyclohydrolase activity in the full-length enzyme, we did not expect this short transcript to encode an enzymatically active protein. Furthermore, modeling of the short isoform onto the x-ray structure of the N-terminal dehydrogenase/cyclohydrolase domain of the human cytoplasmic C₁-THF synthase [38] revealed that it lacked amino acids that are critical for the binding of NADP⁺ and folate. Nonetheless, it was impossible to predict whether or not the 15 extra amino acids at the C-terminus of the short isoform might facilitate its folding into a stable protein with a novel function. We were able to isolate the short isoform as a soluble protein, but as expected, no CH₂-THF dehydrogenase activity could be detected.

Ignoring the 62-residue N-terminal extension, the human mitochondrial and cytoplasmic isozymes share only 31% identity in the N-terminal dehydrogenase/cyclohydrolase domain, whereas the C-terminal synthetase domains are 73% identical [17]. We predicted that since the short isoform represents just the N-terminus of mitochondrial C₁-THF synthase, polyclonal antibodies raised against it would be specific for the mitochondrial isozyme. The resulting anti-MTHFD1L antibodies do show a faint signal with a band in rat liver cytosol (Fig. 3C, lane 1), which may represent weak cross-reaction of these antibodies with the cytoplasmic isozyme. In comparison to the strong signal from mitochondrial C₁-THF synthase seen with these antibodies in rat spleen mitochondria (Fig. 3C, lane 3), this small amount of cross-reaction does not interfere with their use to specifically detect mitochondrial C₁-THF synthase.

Mitochondrial sublocalization experiments showed that human mitochondrial C₁-THF synthase partitions between the inner membrane and the matrix. Depending on the subfractionation procedure used, the membrane vs. soluble ratio varied, with the digitonin procedure typically giving more membrane-bound enzyme than the swell-contrast procedure. The membrane-associated protein behaves as a tightly associated peripheral membrane protein of the mitochondrial inner membrane, facing the matrix side. The human mitochondrial isozyme was not completely susceptible to alkaline carbonate extraction, indicating a tight association with the inner membrane. It was evident that the extraction occurs only at high pH since NaCl of the same ionic strength but pH 7 failed to solubilize the mitochondrial isozyme. Similarly, the inner membrane association of rat spleen mitochondrial C₁-THF synthase confirms that the results in CHO cells are reflected in adult mammalian tissues as well (Fig. 7).

Although mitochondrial C₁-THF synthase was found to be membrane-associated, computer analysis for transmembrane proteins (http://www.ch.embnet.org/software/TMPRED_form.html) predicted the absence of any transmembrane elements. On the other hand, the human mitochondrial isozyme has a 62 amino acid N-terminal extension, of which only the first 31 residues are cleaved upon translocation into the mitochondria [20]. The mature form of the enzyme retains the remaining 31 glycine/arginine rich amino acids that are not found in the cytoplasmic C₁-THF synthases (nor in the yeast mitochondrial C₁-THF synthase). The function of this N-terminal extension (also present in the mouse and rat mitochondrial homologs) is not known. It is not responsible for the lack of the dehydrogenase and/or cyclohydrolase activities of the mitochondrial isozyme, as a

recombinant enzyme lacking the N-terminal extension failed to regain either of these activities (unpublished data from our lab).

Northern blot analysis of mitochondrial C₁-THF synthase transcripts in adult human tissues showed high expression in spleen and placenta [17]. When immunoblots were performed on crude whole tissue extracts, rat spleen gave a faint signal while human placenta failed to give any signal of the mitochondrial isozyme (data not shown). However, immunoblots performed on mitochondria isolated from these tissues revealed the presence of mitochondrial C₁-THF synthase in both rat spleen (Fig. 8; right panel, *lane 1*) and human placental (Fig. 8; right panel, *lane 2*) mitochondria. These results confirm the expression of the mitochondrial enzyme *in vivo* in adult mammals, including humans. A faint immunoreactive signal was observed near the dye front in human placental mitochondria (Fig. 8; right panel, *white dot* in *lane 2*) that may represent either the short isoform or a proteolytic degradation product of the full-length protein. Thus, we cannot rule out the possibility that the alternatively spliced short transcript is translated *in vivo* in humans to produce low levels of the short isoform. However, given the lack of methylene-THF dehydrogenase activity in the short isoform, the physiological significance of this observation remains unknown. The short isoform is not observed in rat tissues, because the *Alu* elements responsible for the alternative splicing do not exist in rodents [37].

Adult rat liver mitochondria oxidize the 3rd carbon of serine [9,10], the 2nd carbon of glycine (Anne Tibbetts, personal communication), and N-methyl carbon of sarcosine [9,12–14] to formate or CO₂. Furthermore, Gregory *et al* [39] demonstrated in adult humans using a stable isotope method that one-carbon units flow through the mitochondria in the direction of 5,10-methylene-THF to formate. All of these results reflect the ability of adult mammalian mitochondria to oxidize 5,10-methylene-THF. The lack of dehydrogenase/cyclohydrolase activities in the mammalian mitochondrial isozyme [18] leaves a gap in the proposed pathway for one-carbon metabolism in adult mammalian mitochondria [9,10]. The bifunctional NAD-dependent 5,10-methylene-THF dehydrogenase/5,10-methenyl-THF cyclohydrolase, characterized by MacKenzie and coworkers [40,41], cannot fill this gap since it is not expressed in adult differentiated tissues [40,42–44]. An unidentified enzyme, capable of oxidizing 5,10-methylene-THF to CHO-THF, must act with the CHO-THF synthetase activity of mitochondrial C₁-THF synthase to catalyze the flow of one-carbon units through adult mammalian mitochondria. Given the membrane association of mitochondrial C₁-THF synthase reported here, it is likely that this folate-dependent one-carbon metabolism occurs at the inner mitochondrial membrane. Like mitochondrial C₁-THF synthase, several other mitochondrial folate-dependent enzymes have been reported to be associated with the inner mitochondrial membrane. The glycine cleavage system, serine hydroxymethyltransferase, dimethylglycine dehydrogenase, and sarcosine dehydrogenase (all of which produce 5,10-methylene-THF; see Fig. 1) have all been reported to be associated with the inner membrane in rat liver mitochondria [45,46]. Human mitochondrial polyglutamate synthetase (FPGS), which adds glutamate residues to the mitochondrial folate pool, is also reported to be tightly associated with the inner membrane [33]. These authors proposed that the inner membrane localization of mitochondrial FPGS would enable efficient polyglutamylation of folates by promoting substrate channeling between the inner membrane folate carrier protein and FPGS. Taking all of these data together, we hypothesize the existence of a large folate-dependent one-carbon metabolizing complex at the inner mitochondrial membrane, perhaps including the membrane carriers for substrates such as serine and formate. Experiments aimed to detect proteins that interact with mitochondrial C₁-THF synthase are currently underway.

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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 C₁-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 and 9 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.

Fig. 2. Purification of short isoform

Coomassie Blue-stained 12% SDS-PAGE of fusion protein eluted from first cobalt column (*lane 1*), products after TEV protease cleavage reaction (*lane 2*), and purified short isoform after second cobalt column (*lane 3*). MBP/SI, maltose binding protein/short isoform fusion protein; MBP, maltose binding protein; SI, short isoform; TEV, Tobacco etch virus protease. Locations and size (kDa) of molecular weight markers are shown on the left. Shown below is a schematic of the fusion protein, MBP/SI, which has MBP at its N-terminus followed by 10X His tag, TEV protease recognition sequence, followed by SI. SI is fused to the TEV protease recognition sequence beginning with amino acid 32, the first residue after the mitochondrial targeting sequence cleavage site. Amino acids 260–275 (gray shading) represent the 15 amino acids that are derived from exonization of the *Alu* element.

Fig. 3. Specificity of anti-MTHFD1L antibodies

Panel A. Immunoblot of purified short isoform (SI) with a 1:1000 dilution of anti-MTHFD1L or a 1:1000 dilution of the pre-immune serum. Each lane contains 1 μ g of purified SI (same sample shown in lane 3 of Fig. 2) resolved on a 7.5% SDS-polyacrylamide gel. Abbreviations are as in legend to Fig. 2. Panel B. Immunoblot of cytoplasmic fractions from rat liver (lane 1) and rat spleen (lane 2) with a 1:2500 dilution of anti-cyto-C₁-THF synthase antibodies. Panel C. Immunoblot of cytoplasmic fractions from rat liver (lane 1) and rat spleen (lane 2) or mitochondrial fraction from rat spleen (lane 3) with a 1:1000 dilution of anti-MTHFD1L antibodies. In panels B and C, each lane contains 50 μ g of total protein resolved on a 7.5% SDS-polyacrylamide gel.

Fig. 4. Submitochondrial localization of epitope-tagged human mitochondrial C₁-THF synthase expressed in CHO cells

Mitochondria were subfractionated by swell-contrast as described in "Experimental Procedures". Whole mitochondria, MT (50 μ g); outer membrane, OM (21 μ g); intermembrane space, IMS (6 μ g); inner membrane, IM (50 μ g); and matrix, Mat (50 μ g) were fractionated on 7.5% or 12% SDS-polyacrylamide gels, and subjected to immunoblotting with anti-V5 (1:5000 dilution), anti-Hsp60 (inner membrane/matrix marker; 1:2000 dilution), or anti-COX I (inner membrane marker; 1:1000 dilution).

Fig. 5. Alkaline carbonate extraction of CHO cell mitochondria expressing epitope-tagged human mitochondrial C₁-THF

CHO cell mitochondria were prepared and subjected to Na₂CO₃ or NaCl or Triton X-100 extraction as described in "Experimental Procedures". The insoluble integral membrane proteins (pellet; P) were separated from the soluble and peripheral membrane proteins (supernatant; S) by ultracentrifugation. Equal volumes of P and S were fractionated on a 7.5 % or a 12% SDS-polyacrylamide gel, and subjected to immunoblotting with anti-V5 (1:5000 dilution), anti-MTHFD1L (1:1000 dilution), anti-MnSOD (peripheral membrane protein marker; 1:2000 dilution), or anti-porin (integral membrane protein marker; 1:2000 dilution) antibodies.

Fig. 6. Proteinase K digestion of digitonin-treated CHO cell mitochondria expressing epitope-tagged human mitochondrial C₁-THF

Equal amounts of CHO cell mitochondria (190 μ g) were incubated with varying concentrations of digitonin (0–1:1 mg digitonin/mg protein) in the presence (panel B) or absence (panel A) of proteinase K (100 μ g/ml) as described in “Experimental Procedures”. The mitochondria were pelleted at $16,600 \times g$ and equal volumes of the pellet resuspended in HMS without BSA were fractionated on a 7.5% or a 12% SDS-polyacrylamide gel, and subjected to immunoblotting with anti-V5 (1:2000 dilution), anti-Hsp60 (matrix marker; 1:2000 dilution), or anti-Bcl-x_{S/L} (outer membrane marker; 1:1000 dilution) antibodies. Mitochondria incubated with 1% Triton X-100 in the presence or absence of proteinase K were used as positive and negative controls respectively. In the controls, the pellet (P) and TCA-precipitated proteins from the supernatant (S) were used.

Fig. 7. Localization of mitochondrial C₁-THF synthase in rat spleen

Rat spleen mitochondria were subfractionated by Swell-Contract method as described in "Experimental Procedures". The different fractions [(whole mitochondria, MT (50 μ g); outer membrane/intermembrane space, OM/IMS (18 μ g); inner membrane, IM (50 μ g); and matrix, Mat (50 μ g)] were fractionated on a 7.5% or 12% SDS-polyacrylamide gel, and subjected to immunoblotting with anti-MTHFD1L (1:1000 dilution), anti-COX I (inner membrane marker; 1:1000 dilution), or anti-Hsp60 (matrix marker; 1:2000 dilution) antibodies.

Fig. 8. Expression level of mitochondrial C₁-THF synthase

Coomassie-Blue stained 7.5% SDS-PAGE (left panel) and immunoblot (right panel) of mitochondrial fractions from rat spleen (*RS*; lane 1), human placenta (*HP*; lane 2), and CHO/pcDNA3.1-humito (*CHO*; lane 3). A 1:1000 dilution of anti-MTHFD1L antibodies was used. Each lane contains 100 μ g of total protein. Locations and size (kDa) of molecular weight markers are shown on the left. The white dot indicates a faint signal observed at approximately the size of the short isoform in lane 2 of the immunoblot.