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Identification and characterization of human mitochondrial methenyltetrahydrofolate synthetase activity

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Abstract

We present evidence for the presence of the folate metabolism enzyme methenyltetrahydrofolate synthetase (MTHFS) in mitochondria. MTHFS activity was identified in the matrix of mitochondria purified from human liver biopsies. Mitochondrial and cytoplasmic MTHFS specific activities are similar, 85% of the total cellular MTHFS activity is in the cytoplasm and both native enzymes have similar molecular weights (approximately 25 kDa). Studies using purified mitochondrial MTHFS from CA46 human Burkitt lymphoma cells reveal that mitochondrial MTHFS behaves kinetically like the cytoplasmic enzyme with K_m values of 4.7, 0.8 and 22 μM respectively for (6R,S)-5-formyltetrahydrofolate monoglutamate, (6S)-5-formyltetrahydrofolate pentaglutamate and ATP. This finding adds to previous observations that various folate-dependent enzymes reside in the mitochondria of eucaryotic cells. Intracellular tetrahydrofolate metabolism is highly compartmentalized and mitochondrial MTHFS activity is necessary for the entry of mitochondrial 5-formyltetrahydrofolate into the mitochondrial folate pool.

Keywords: Methenyltetrahydrofolate synthetase; Folate; Mitochondrion

1. Introduction

Methenyltetrahydrofolate synthetase (MTHFS; EC 6.3.3.2) catalyses the unidirectional ATP- and Mg^{2+}-dependent transformation of 5-formyltetrahydrofolate (N^5-CHO-H_4-PteGlu) to 5,10-methenyltetrahydrofolate (N^{5-10}-CH^+-H_4-PteGlu). Mammalian MTHFS activity has been purified from sheep [1], rabbit [2] and human liver [3] and the bacterial enzyme from *Lactobacillus casei* [4]. The enzyme's substrate, 5-formyltetrahydrofolate arises from the enzymatic catalysis of 5,10-methenyltetrahydrofolate by serine hydroxymethyltransferase [5]. The produced 5-formyltetrahydrofolate polyglutamates are tight-binding inhibitors of serine hydroxymethyltransferase, suggesting that their production could be involved in regulating the enzyme's activity and thus the flow of one-carbon residues necessary for de novo purine and pyrimidine syntheses [6,7]. MTHFS could thus play a role in a putative regulatory loop with serine hydroxymethyltransferase by modulating intracellular 5-formyltetrahydrofolate polyglutamate levels [7]. We have also previously shown that the inhibition of MTHFS in vivo results in increased intracellular 5-formyltetrahydrofolate polyglutamate levels which inhibit phosphoribosyl 5-aminoimidazole 4-carboxamide ribonucleotide formyltransferase, de novo purine synthesis and cell growth [8]. The inhibitory potential of 5-formyltetrahydrofolate polyglutamates against a number of other folate-dependent enzymes [7] also underlies the potential importance of MTHFS as a detoxifying enzyme.

MTHFS activity is also a potentially important enzyme in cancer chemotherapy since it is the first enzymatic step involved in the intracellular transformation of the 5-formyltetrahydrofolate (leucovorin™) administered to cancer patients either as a rescue agent in high dose methotrexate regimens [9] or more recently to enhance 5-fluorouracil's cytotoxic efficacy in the chemotherapy of colorectal cancer [10].

Mammalian cell mitochondria contain pteroylpolyglutamates and a number of folate-dependent enzymes, and tetrahydrofolate metabolism is highly compartmentalized between the cytosol and mitochondria [11]. 5-formyltetrahydrofolate accounts for 11.5% of total folates in rat liver mitochondria [12] where it is also produced enzymatically through serine hydroxymethyltransferase since Chinese hamster ovary cell mutants lacking this mitochondrial activity [13] contain no mitochondrial 5-formyltetrahydrofo-

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late (Shane, B., personal communication). A mitochondrial MTHFS activity would thus be required for the entry of mitochondrial 5-formyltetrahydrofolate into the mitochondrial folate pool and to prevent the possibly deleterious effects of its intramitochondrial accumulation.

In this paper, we present evidence supporting the presence of MTHFS activity in the matrix of human liver mitochondria and describe the characteristics of purified mitochondrial enzyme from human CA46 Burkitt lymphoma cells.

2. Experimental procedures

2.1. Chemicals

(6R,S)-N^5-CHO-H_4-PteGlu (calcium salt) and $PteGlu_5$ were purchased from B. Schircks Laboratories (Jona, Switzerland). The stereospecific reduced folate (6S)-N^5-CHO-H_4-$PteGlu_5$ was prepared from $PteGlu_5$ as previously described [8]. (6R,S)-N^5-CHO-H_4-PteGlu (potassium salt) was made from the calcium salt by exposure to potassium oxalate and the calcium oxalate formed was removed by centrifugation [3]. Aminohexyl-Sepharose-4B, Blue Sepharose CL-6B, Sephadex G-25 and Sephacryl S-200 HR were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). (6R,S)-N^5-CHO-H_4-PteGlu-aminohexyl-Sepharose-4B was prepared as previously described [3]. [2-^{14}C]Tryptamine bisuccinate (50mCi/mmol) was purchased from NEN-Dupont (Boston, MA). All other chemicals were of reagent grade and purchased either from Sigma, Fisher Scientific (Fairlawn, NJ) or Boehringer Mannheim (Penzberg, Germany).

2.2. Cell culture

Human Burkitt lymphoma CA46 cells (American Type Culture Collection, Rockville, MD) were grown in suspension culture at 37° C in the presence of 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) (Gibco, Grand Island, NY).

2.3. Subcellular fractionation

Human liver was obtained in the operating room from two patients undergoing open liver biopsies. Macroscopically normal liver was immediately dissected from any abnormal tissue and immersed in an ice-cold solution prepared from 0.25 M sucrose, 8.6 mM NaCl, 15 mM Tris-HCl and 5 mM EDTA (pH 7.5). Subcellular fractionation was initiated within the hour. For CA46 cells, approximately 1×10^{11} cells were first swelled in ice-cold deionised water for 30 min prior to homogenization. Mitochondria were isolated according to previously described protocols [14]. The swelled cells were homogenized with a Potter-Elvehjem homogenizer in 5 volumes of ice-cold deionised water while the human liver biopsies were homogenized in 5 volumes of ice-cold 0.25 M sucrose, 8.6 mM NaCl, 15 mM Tris-HCl and 5 mM EDTA (pH 7.5). The homogenate was centrifuged at $5000 \times g$ for 10 min and the supernatant centrifuged again at $12\,000 \times g$ for 20 min. The supernatant solution (cytosolic fraction) was removed from the sediment (mitochondria containing fraction) and kept at −80° C in the presence of 0.1% Tween-20 for further analysis. The mitochondria containing fraction was resuspended in 1 volume of the homogenization buffer and applied to a sucrose gradient consisting of two layers of solutions containing (from top to bottom) 2 ml of 1.0 M sucrose, 10 mM Tris, 5 mM EDTA (pH 7.5) and 2 ml of 1.5 M sucrose, 10 mM Tris, 5 mM EDTA (pH 7.5). Following centrifugation ($24\,000 \times g$ for 30 min) the purified mitochondria were collected at the interface of the 1.0 M and 1.5 M sucrose solutions. Tween-20 (0.1%) was immediately added to the purified mitochondria and the suspension was frozen at −80° C. Upon thawing, the suspension was sonicated (5 s, four times) prior to further analysis.

A portion of the purified human liver mitochondria from the second patient was further subfractionated by low concentration digitonin treatment as previously described to further localize MTHFS activity within the mitochondria [15]. Briefly, freshly prepared mitochondria were pelleted by centrifugation and resuspended in 0.3 ml of the homogenization buffer. An equal volume of digitonin solution (1.2%) was added and the solution gently stirred in a ice bath for 15 min. The lysate was then centrifuged at $10\,000 \times g$ for 10 min. The supernatant consists of outer mitochondrial membranes and intermembrane components while the mitoplasts are pelleted. The pellet was washed twice and then resuspended in 0.1 ml of the homogenization buffer prior to sonication. The extract was centrifuged again to obtain the mitochondrial matrix components in the supernatant. Tween-20 was then added to stabilize MTHFS activity.

2.4. Mitochondrial methenyltetrahydrofolate synthetase purification

Human mitochondrial MTHFS was purified from mitochondria isolated from CA46 cells. Prior to chromatography, the mitochondrial extracts were first centrifuged ($5000 \times g$, 10 min) through a 3×8 cm column of Sephadex G-25. The fraction was then purified by Blue Sepharose and (6R,S)-N^5-CHO-H_4-PteGlu-aminohexyl-Sepharose chromatography as previously described [8]. Active fractions were pooled prior to kinetic studies.

2.5. Enzyme assays

MTHFS activity was measured by monitoring the increase in absorbance at 360 nm due to the formation of

Table 1
Subcellular distribution of methenyltetrahydrofolate synthetase in human liver

Patient	Fraction	MTHFS units/mg ($\times 10^{-4}$)	LDH μmol/min/mg	CcO μmol/min/mg ($\times 10^{-2}$)	Fum	MAO
1	Cyto	4.9 (85)	1.27 (99)	0.49 (34)		
	Mito	11.0 (15)	0.06 (1)	8.55 (66)		
2	Cyto	3.6 (85)	0.77 (> 99)	1.47 (42)		
	Mito	3.0 (15)	0.01 (< 1)	11.5 (58)		
	Matrix	(66)			(72)	(12)
	Outer membrane	(34)			(28)	(88)

Subcellular fractionations were performed on fresh liver biopsy samples from two patients. MTHFS, lactate dehydrogenase (LDH) and cytochrome-*c* oxydase (CcO) activities were measured in cytoplasmic (Cyto) and mitochondrial (Mito) fractions. In patient no. 2, purified mitochondria were also treated with low concentration digitonin to remove their outer membranes and MTHFS, fumarase (Fum) and monoamine oxydase (MAO) activities measured in the outer membrane and matrix fractions. Results represent the means of duplicate assays and values in parentheses are the percentage of total enzyme activity in each fraction.

$N^{5\text{-}10}$-CH^{+}-H_4-PteGlu (E_{360}: 25.1×10^3 $M^{-1} \times cm^{-1}$). One unit of activity represent 1 μmol of $N^{5\text{-}10}$-CH^{+}-H_4-PteGlu formed per min. Reactions were carried out in a quartz cuvette of 1 cm optical path length and the temperature maintained at 30° C using a water-jacketed sample compartment. The reaction mixture contains 50 mM MES (pH 6.0), 10 mM β-mercaptoethanol, 10 mM magnesium acetate, 0.5 mM ATP and 0.2 mM (6R,S)-N^5-CHO-H_4-PteGlu [3].

Lactate dehydrogenase activity, a cytosolic marker, was measured by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADH (E_{340}: 6.22×10^3 $M^{-1} \times cm^{-1}$). Enzyme activity was expressed in μmol of NADH oxidized per min. Reactions were carried out as previously described in a reaction mixture containing 10 mM Tris-HCl (pH 7.3), 0.2 mM NADH, 1.0 mM sodium pyruvate and 0.01% Triton X-100 [16].

Cytochrome-*c* oxidase activity, a mitochondrial marker, was measured by monitoring the decrease in absorbance at 550 nm due to the oxidation of cytochrome-*c* (E_{550}: 15.3×10^3 $M^{-1} \times cm^{-1}$). Enzyme activity was expressed as μmol of cytochrome-*c* oxidized per min. Reactions were carried out as previously described in a reaction mixture containing 6.2 mM K_2HPO_4 and 33.8 mM KH_2PO_4 (pH 6.2), 0.2% Lubrol PX and 0.25 mg cytochrome-*c* [17].

Fumarase activity, a mitochondrial matrix marker, was measured by monitoring the increase in absorbance at 240 nm due to the formation of fumarate (E_{240}: 2.44×10^3 $M^{-1} \times cm^{-1}$). One unit of activity represent 1 μmol of fumarate formed per min. Reactions were carried out in a reaction mixture containing 50 mM malate in 0.1 M phosphate buffer (pH 7.6) [18].

Monoamine oxidase activity, an outer mitochondrial membrane marker, was measured by detecting the formation of [2-^{14}C]indoleacetic acid from [2-^{14}C]tryptamine. In a typical assay, 10 μl of subfractioned sample were mixed with 250 μl of [^{14}C]tryptamine] in phosphate buffer (pH 7.4) and incubated at 37° C for 20 min. Reactions were stopped by the addition of 0.2 ml 2 N HCl and the deaminated radioactive products were extracted into 6 ml toluene by shaking. After centrifugation, 4 ml aliquots of the organic material were counted in a scintillation counter. Blank values were determined by incubating [^{14}C]tryptamine with boiled samples [19].

Protein concentrations were determined using the Lowry assay with bovine serum albumin as the standard.

2.6. Gel filtration

Molecular weights of mitochondrial and cytosolic enzyme were determined by gel filtration using a Sephacryl S-200 HR column (1.0 × 30 cm) on a FPLC system (Pharmacia-LKB Biotechnology). Calibration standards and samples were run in 20 mM Pipes, 0.5 M potassium chloride, 20 mM mercaptoethanol and 0.1% (v/v) Tween 20 at pH 7.0 (flow rate: 0.25 ml/min). MTHFS was

Table 2
Purification of human mitochondrial methenyltetrahydrofolate synthetase

	Total Volume (ml)	Total protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Cytoplasm	100	800	0.903 (79)	1.12×10^{-3}		
Mitochondria	10	180	0.236 (21)	1.31×10^{-3}	1	100
Blue Sepharose	40	0.021	0.040	1.90	1,450	17
Folinate Sepharose	10	< 0.001	0.013	> 13	> 10,000	6

Human Burkitt lymphoma CA46 cells were cultured and subcellular fractions prepared. Lactate dehydrogenase and cytochrome-*c* oxydase activities were respectively distributed with 1.3 and 97% of their total activities in the mitochondrial fraction. Mitochondrial MTHFS was purified by sequential blue sepharose and folinate sepharose chromatographies. MTHFS activity and protein concentrations were determined in all fractions. Values in parentheses are the percentage of total MTHFS activity in each fraction.

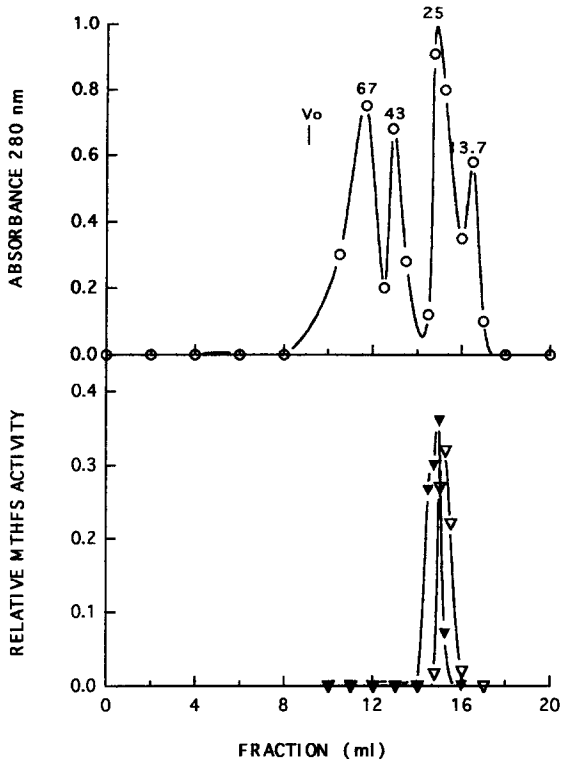

Fig. 1. FPLC elution profiles of mitochondrial and cytosolic MTHFS activity on Sephacryl 200-HR 10/30. Upper panel: the elution profiles of the molecular weight standards were monitored by UV absorbance at 280 nm. The numbers above each peak represent the molecular weight of each standard in kDa. V_0 represents the void volume. Lower panel: Mitochondrial (closed triangles) and cytoplasmic (open triangles) subcellular fractions were applied to a Sephacryl 200-HR 10/30 column on a FPLC system. MTHFS activity was detected by enzymatic activity in each 250-μl collected fraction. The sample volume was 200 μl and the elution buffer 20 mM Pipes, 0.5 M KCl, 20 mM mercaptoethanol, 0.1% (v/v) Tween 20 (pH 7.0). The flow rate was 250 μl/min.

detected in the effluent by testing for enzyme activity. The calibration standards used were bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). Column void volume was determined using blue dextran 2000.

3. Results and discussion

3.1. Subcellular localization of MTHFS

Human liver mitochondria were found to contain similar specific activity of MTHFS than cytosolic extracts with 85% of the total cellular activity in the cytoplasm (Table 1). Mitochondria preparations were free of cytoplasmic contaminants as determined by lactate dehydrogenase activity ($\leq$ 1%). Cytochrome-*c* oxidase contamination of the cytosolic fraction likely resulted from some mitochondrial breakage during homogenization. Mitochondrial MTHFS activity was further localized in the second human liver biopsy sample by subfractionating mitochondrial outer-membrane and intermembrane components from inner membrane and matrix components with low concentration digitonin treatment. Most of the mitochondrial MTHFS activity was found in the matrix components. Its distribution between the matrix and the outer membrane fraction was similar to that of fumarase activity, the matrix marker, and reflected some disruption of the mitochondrial inner membranes during digitonin treatment. The matrix preparation was relatively free of outer membrane as determined by monoamine oxidase activity. These results indicate that the mitochondrial MTHFS activity resides in the mitochondrial matrix.

Similar sublocalization of folate-dependent enzymes have been reported for serine hydroxymethyltransferase, 10-formyltetrahydrofolate synthetase, 5,10-methenyltetrahydrofolate cyclohydrolase, 5,10-methylenetetrahydrofo-

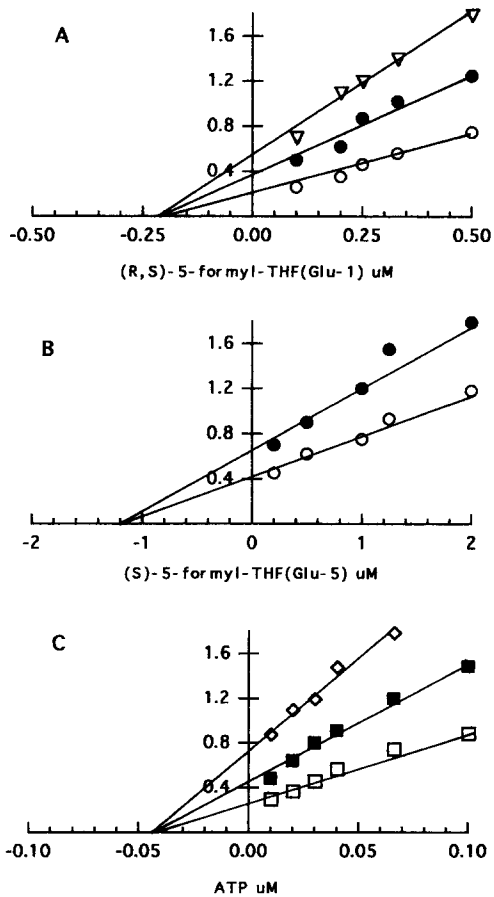

Fig. 2. Substrate affinity for purified mitochondrial MTHFS. Double reciprocal plots (1/[S] versus 1/V) with (6RS)-N^5-CHO-H_4-$PteGlu_1$ (panel A), (6S)-N^5-CHO-H_4-$PteGlu_5$ (panel B) and ATP (panel C) as substrates. Initial velocities are expressed in [μmol/min/ml$\times 10^{-5}$]$^{-1}$ (y axis) versus substrate concentration in [μM]$^{-1}$ (x axis) and represent the mean of two determinations. Velocities were measured in the presence of 15 μM (open triangles), 25 μM (closed circles) and 50 μM ATP (open circles) in panel A; 25 μM (closed circles) and 50 μM ATP (open circles) in panel B, and with 2.5 μM (open diamonds), 5 μM (closed squares) and 10 μM (6RS)-N^5-CHO-H_4-$PteGlu_1$ (open squares) in panel C.

late dehydrogenase and folylpolyglutamate synthetase [20–23]. However, we could not detect mitochondrial MTHFS activity in rabbit liver (data not shown). This interspecies difference in 5-formyltetrahydrofolate metabolism is also indicated by the observation that cytosolic rabbit liver MTHFS specific activity is approximately 4000-fold higher [2] than in our human liver biopsy samples.

3.2. Molecular weight of mitochondrial MTHFS

The molecular weight of native human liver mitochondrial enzyme was determined by gel filtration chromatography on Sephacryl-200 HR to be approximately 25 kDa and to be almost identical to the cytosolic liver enzyme (Fig. 1). These molecular weights are similar to the ones previously reported for human and rabbit liver MTHFS [2,3].

3.3. Purification and kinetic studies of mitochondrial MTHFS

MTHFS was purified > 10,000-fold from CA46 cell mitochondria. The purification protocol is shown in Table 2. We were unable to determine the protein concentration after the last purification step due to the small amount of protein left and the estimated specific activity following purification was > 13 units/mg. The K_m values of (6R,S)-N^5-CHO-H_4PteGlu, (6S)-N^5-CHO-H_4PteGlu$_5$ and ATP were found to be 4.7 μM, 0.8 μM and 22 μM respectively (Fig. 2). These values are very similar to the ones reported previously for purified human liver MTHFS (4.4 μM, 0.6 μM and 20 μM) [3]. The increased affinity for the pentaglutamate substrate is consistent with the observation that most pteroylpolyglutamate derivatives bind to folate-dependent enzymes with increased affinity compared to the monoglutamate derivatives [24]. Initial-velocity plots (Fig. 2a,c) intersected, indicating that the mitochondrial enzyme catalyzes a sequential as opposed to a ping-pong mechanism. A similar mechanism has also been reported for rabbit and human liver cytosolic MTHFS [2,3].

3.4. Significance

Mitochondrial MTHFS activity shows similar characteristics in terms of molecular weight, substrate affinity and enzyme mechanism compared to the cytoplasmic activity. We have recently reported the presence of several human cDNA isoforms coding for MTHFS [25] and are investigating to determine if one of these isoforms encodes for a mitochondrial signal sequence. The presence of MTHFS activity in the mitochondrial matrix correlates with previous observations that tetrahydrofolate metabolism is highly compartmentalized between the cytosol and mitochondria in eucaryotic cells.

Acknowledgements

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