

ALDH1L2 Is the Mitochondrial Homolog of 10-Formyltetrahydrofolate Dehydrogenase*[§]

Received for publication, March 30, 2010, and in revised form, May 7, 2010. Published, JBC Papers in Press, May 24, 2010, DOI 10.1074/jbc.M110.128843

Natalia I. Krupenko, Marianne E. Dubard, Kyle C. Strickland, Kelly M. Moxley, Natalia V. Oleinik, and Sergey A. Krupenko¹
From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

Cytosolic 10-formyltetrahydrofolate dehydrogenase (FDH, ALDH1L1) is an abundant enzyme of folate metabolism. It converts 10-formyltetrahydrofolate to tetrahydrofolate and CO₂ in an NADP⁺-dependent reaction. We have identified a gene at chromosome locus 12q24.11 of the human genome, the product of which has 74% sequence similarity with cytosolic FDH. This protein has an extra N-terminal sequence of 22 amino acid residues, predicted to be a mitochondrial translocation signal. Transfection of COS-7 or A549 cell lines with a construct in which green fluorescent protein was introduced between the leader sequence and the rest of the putative mitochondrial FDH (mtFDH) has demonstrated mitochondrial localization of the fusion protein, suggesting that the identified gene encodes a mitochondrial enzyme. Purified pig liver mtFDH displayed dehydrogenase/hydrolase activities similar to cytosolic FDH. Real-time PCR performed on an array of human tissues has shown that although cytosolic FDH mRNA is highest in liver, kidney, and pancreas, mtFDH mRNA is most highly expressed in pancreas, heart, and brain. In contrast to the cytosolic enzyme, which is not detectable in cancer cells, the presence of mtFDH was demonstrated in several human cancer cell lines by conventional and real-time PCR and by Western blot. Analysis of genomes of different species indicates that the mitochondrial enzyme is a later evolutionary product when compared with the cytosolic enzyme. We propose that this novel mitochondrial enzyme is a likely source of CO₂ production from 10-formyltetrahydrofolate in mitochondria and plays an essential role in the distribution of one-carbon groups between the cytosolic and mitochondrial compartments of the cell.

Folate coenzymes function as one-carbon group carriers in intracellular metabolic pathways (1). Key biochemical reactions that require these coenzymes include nucleotide biosynthesis and the biosynthesis/degradation of several amino acids (1, 2). In addition, folates participate in the conversion of formate to CO₂ and the formylation of methionine-tRNA (2), a process that might be essential for initiation of translation in eukaryotic mitochondria (3). Folate pathways are compartmentalized within the cell; the glycine cleavage system, the conversion of

dimethylglycine to sarcosine and then to glycine, and the reaction of methionyl-tRNA formylation reside in mitochondria, whereas the biosynthesis of nucleotides, remethylation of homocysteine to methionine, and degradation of histidine are localized to cytosol (2, 4). Recent studies have implied that folate metabolism compartmentalization also includes the nucleus, which is a likely site for folate-dependent reactions related to thymidilate biosynthesis (5–7). It has been proposed that mitochondrial one-carbon pathways mainly serve to provide carbon groups, in the form of formate, for incorporation into the cytosolic folate pool, where they are utilized in a variety of biosynthetic reactions (4, 8).

Several folate-dependent reactions take place in both cytoplasm and mitochondria (Fig. 1) and are catalyzed by homologous enzymes, which are products of distinct genes (4). Mitochondrial serine hydroxymethyl transferase, the enzyme catalyzing the reversible conversion of serine to glycine, is structurally and functionally very similar to the cytosolic isoform (9, 10). In fact, it has been recently reported that the gene for the mitochondrial enzyme can be alternatively transcribed to produce a cytoplasm-localized enzyme, which can metabolically compensate for the lack of the true cytosolic isoform (6). In contrast, the enzymes in the pathways of 10-formyltetrahydrofolate (10-fTHF)² metabolism demonstrate significant differences in their properties between cytosolic and mitochondrial forms. In the cytoplasm, a single trifunctional enzyme, C1-synthase (MTHFD1), catalyzes both the ATP-dependent synthesis of 10-fTHF from tetrahydrofolate (THF) and formate and the reversible two-step conversion of 5,10-methylene-THF to 10-fTHF (11–13). These reactions reside in the synthetase and the bifunctional dehydrogenase/cyclohydrolase domains, correspondingly (14, 15). A similar enzyme in mitochondria, MTHFD1L, lacks the dehydrogenase/cyclohydrolase activity due to mutations in the bifunctional domain (16, 17). Furthermore, it rather catalyzes the reversal of the synthetase reaction, thus producing formate (18). Another mitochondrial enzyme, MTHFD2, is similar to the bifunctional domain of C1-synthase and catalyzes the dehydrogenase/cyclohydrolase reactions (19). An additional folate reaction, which occurs in both cytoplasm and mitochondria, also involves 10-fTHF and is the NADP⁺-dependent oxidation of the folate-bound formyl group to CO₂ (8).

The cytosolic enzyme catalyzing this reaction, 10-formyltetrahydrofolate dehydrogenase (FDH, ALDH1L1), is well charac-

* This work was supported, in whole or in part, by National Institutes of Health Grant DK54388 (to S. A. K.).

[§] The on-line version of this article (available at <http://www.jbc.org>) contains a supplemental table.

¹ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425. Tel.: 843-792-0845; Fax: 843-792-8565; E-mail: krupenko@musc.edu.

² The abbreviations used are: THF, tetrahydrofolate; 10-fTHF and CHO-THF, 10-formyltetrahydrofolate; FDH (ALDH1L1), 10-formyltetrahydrofolate dehydrogenase; mtFDH (ALDH1L2), mitochondrial FDH; cytFDH, cytosolic FDH; GFP, green fluorescent protein.

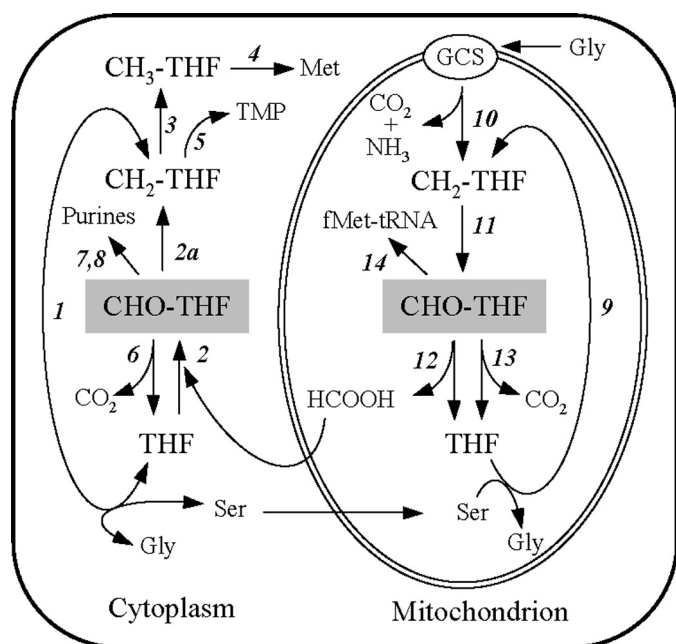


FIGURE 1. **Compartmentalization of folate metabolism in mammalian cells.** Enzymes catalyzing conversion of folate coenzymes within the cytoplasmic and mitochondrial pools are: **1**, cytosolic serine hydroxymethyltransferase; **2** and **2a**, cytosolic trifunctional C1-synthase (MTHFD1); **3**, methylenetetrahydrofolate reductase; **4**, methionine synthase; **5**, thymidylate synthase; **6**, cytosolic FDH (ALDH1L1); **7** and **8**, two enzymes of the *de novo* purine biosynthesis, glycylamide ribonucleotide formyltransferase and amidimidazole carboxamide ribonucleotide formyltransferase; **9**, mitochondrial serine hydroxymethyltransferase; **10**, glycine cleavage system (GCS); **11**, mitochondrial bifunctional 5,10-methylenetetrahydrofolate dehydrogenase-cyclohydrolase (MDHFR2); **12**, mitochondrial C1-synthase (MTHFD1L); **13**, mitochondrial FDH (ALDH1L2); **14**, methionyl-tRNA formyltransferase. Reactions catalyzed by enzymes **1**, **2**, **2a**, **9**, **11**, and **12** are reversible; the schematic shows the direction of the likely flow of the one-carbon groups within the pools in mammalian cells.

terized (reviewed in Ref. 20). It is a tetramer of four identical 902-amino acid residue subunits; each is a product of the natural fusion of three unrelated genes corresponding to three distinct functional domains (21). The N-terminal domain (residues 1–310) shares sequence homology and structural topology with other enzymes utilizing 10-fTHF as a substrate (21, 22). It carries the folate-binding site and functions as a 10-fTHF hydrolase. The C-terminal domain (residues 400–902) originates from an aldehyde dehydrogenase-related gene (21) that defines its classification as *ALDH1L1*. The two catalytic domains are connected by an intermediate linker (residues 311–399), which is a structural and functional homolog of carrier proteins with a 4'-phosphopantetheine prosthetic group (23). In the FDH mechanism, the intermediate linker domain transfers the formyl group, covalently attached to the sulfhydryl of the 4'-phosphopantetheine arm, from the N-terminal to the C-terminal domain. Thus, the overall 10-fTHF dehydrogenase reaction is a coupling of the 10-fTHF hydrolase and aldehyde dehydrogenase steps (20).

FDH is an abundant protein; its levels reach about 1.2% of the total protein in rat liver cytosol (24, 25), suggesting an important role for the reaction converting folate-bound formate to CO₂. In general, this pathway clears one-carbon groups from the folate pool and may be important to control their flux toward folate-dependent biosynthetic reactions (26, 27). The

presence of similar activity in mammalian mitochondria suggested the existence of a mitochondrial enzyme, functionally similar to cytoplasmic FDH and responsible for the production of CO₂ from 10-fTHF (8). Such an enzyme, however, has not been identified so far. Here we report the cloning of the human *ALDH1L2* gene, its mitochondrial targeting and localization, and the comparison of its tissue distribution with cytosolic FDH. We also demonstrate that the protein encoded by the *ALDH1L2* gene possesses 10-fTHF dehydrogenase/hydrolase activities.

EXPERIMENTAL PROCEDURES

Cloning *ALDH1L2*—*ALDH1L2* cDNA was amplified from the MegaMan human transcriptome library (Stratagene) using the FailSafe PCR system (Epicenter Biotechnologies) and the primers shown in the supplemental table. Conditions for the amplification were as follows: 33 cycles of 45 s at 95 °C (melting), 30 s at 58 °C (annealing), and 3 min at 72 °C (extension). The amplified fragment of 2,716 bp, which corresponds to the entire coding sequence including part of the putative mitochondrial leader, was cloned into a linearized pCR2.1 vector using a TA cloning kit (Invitrogen). The rest of the sequence corresponding to the putative mitochondrial translocation signal was reconstituted by site-directed mutagenesis. The fragment encoding *ALDH1L2* was subcloned into the pRSET-B plasmid immediately downstream of the His tag (pRSET/mtFDH expression vector) and into a pcDNA3.1 plasmid for mammalian expression (pcDNA3.1/mtFDH vector). All constructs were confirmed by DNA sequencing at the Medical University of South Carolina (MUSC) Nucleic Acid Analysis Facility.

Cell Culture, Reagents, Transient Transfection, and Cell Sorting—Cell media and reagents were from Invitrogen unless otherwise indicated. Other chemicals were from Sigma. Cell lines were obtained from the ATCC. Cells (2×10^6) were transfected with 2.0 μg of the corresponding vector using Amaxa nucleofactor protocols, which we optimized for each specific cell line. As a control, transfection with a pcDNA3.1/GFP or empty pcDNA3.1 vector was carried out. After transfection with GFP-mtFDH fusion, cell sorting was performed to select cells displaying green fluorescence. These experiments were carried out at the MUSC Flow Cytometry facility. The collected cells were used for Western blot assays.

Western Blot—*ALDH1L2* protein product was detected by SDS-PAGE followed by Western blot with a specific polyclonal antibody. The antibody was generated against a 408-amino acid-long N-terminal peptide (residues 23–429, mitochondrial leader sequence was excluded) of *ALDH1L2* protein using Harlan Laboratories, Inc. (Indianapolis, IN) services. The truncated mitochondrial FDH was expressed in *Escherichia coli* as a fusion with His₆ tag at the N terminus according to a procedure we have used in our previous studies (28, 29). The expression vector was generated from pRSET/mtFDH plasmid by deleting the mitochondrial leader sequence and introducing an in-frame stop codon immediately downstream of the codon corresponding to Asp-429. This has been done by site-directed mutagenesis using a QuikChange kit (Stratagene). The recombinant protein was purified on a metal affinity column and then on a

Mitochondrial 10-Formyltetrahydrofolate Dehydrogenase

Sephacryl S300 column (100 × 1.5 cm) as described elsewhere (28, 29). The sequence of the protein was confirmed by liquid chromatography/tandem mass spectrometry analysis of peptides after tryptic digestion at the MUSC Biomolecular Mass Spectrometry facility.

Preparation of Mitochondria—Mitochondria were isolated from cultured cells either using a mitochondria isolation kit (Thermo Scientific) or by differential centrifugation. Briefly, 20–100 × 10⁶ cells were pelleted, washed with phosphate-buffered saline, and disrupted using Dounce homogenizer in 0.25 M sucrose for 2 min on ice. Homogenates were spun down for 5 min at 700 × *g* to remove nuclei and cell debris. The supernatant was used to precipitate mitochondria by centrifugation for 20 min at 18,000 × *g*. Mitochondria from rat liver were obtained from Dr. Peter Pediaditakis.

PCR—To produce cDNA, 1 μg of the total RNA was used in a reverse-transcription reaction as described (30). The total RNA was isolated from 2 × 10⁶ cells using an RNeasy protect kit (Qiagen). Conventional PCR was carried out for 35 cycles (each cycle was run for 45 s at 95 °C, 45 s at 52 °C, and 60 s at 72 °C) using 100 ng of the total cDNA, specific primers (supplemental table), and a FailSafe PCR selection kit (Epicenter Biotechnologies). The primers were designed to amplify an 855-bp fragment of mtFDH cDNA. In the control PCR experiments, actin or glyceraldehyde-3-phosphate dehydrogenase was used as internal standard. Real-time PCR was performed using 100 ng of the total cDNA (generated as above), RT²SybrGreen/Rox qPCR master mix, and gene-specific real-time-PCR primers (both from SABioscience) with ABI 7300 Real Time PCR system as described by the manufacturer. Primers for the assay (supplemental table) were designed to generate a specific PCR product of 108 bp for FDH and 96 bp for mtFDH. For experiments with the tissue library, 1 ng of cDNA was used in the assays. All primer pairs have been tested for dimerization and the amplification of only one product. The -fold change in mRNA expression for each gene was calculated using 2^{-ΔΔC_t} (31).

Confocal Microscopy—After transfection, cells were seeded in 10-cm cell culture dishes (2 × 10⁶ cells/well). Forty-eight hours later, the plates were incubated with 50 nM MitoTracker (Molecular Bioprobes) for 15 min at 37 °C. Green (GFP) and red (mitochondria) fluorescence were visualized using a Leica TCS SP2 AOBS scanning Confocal microscope.

Purification of Mitochondrial FDH from Pig Liver—Livers were obtained from freshly slaughtered pigs from the local abattoir. Mitochondria were purified from 500 g of chopped liver homogenized in cold buffer containing 0.25 M sucrose, 2 mM HEPES, pH 7.4, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride using a powered Dounce homogenizer. Mitochondria were separated from red blood cells, unbroken cells, and cytosolic fraction using differential centrifugation as described elsewhere (32). The mitochondrial pellet was washed successively (20 ml × six times) with 2 mM HEPES buffer, pH 7.4, containing 0.25 M sucrose and 5 mM EDTA. Washed pellet was subjected to several freeze-thaw cycles at -80 and 25 °C, correspondingly. Mitochondria were then resuspended in the above buffer, sonicated, and subjected to centrifugation (9,000 × *g*, 15 min) to separate soluble (supernatant) and insol-

uble (pellet) fractions. The protein preparation extracted by freeze-thaw cycles was then loaded on a 5-formyl-THF-Sepharose affinity column (1.5 × 10 cm of packed resin) (24, 33). The column was washed with the loading buffer, 1.0 M KCl and 2.0 M KCl (50 ml of each), and bound proteins were eluted with 20 mM folic acid (25 ml). All steps were performed at a flow rate of 25 ml/h. Folic acid eluate was further subjected to size-exclusion chromatography on Sephadex S300 (1.5 × 100 cm column) as we have previously described (34, 35).

Assay of Enzyme Activity—The hydrolase and 10-fTHF dehydrogenase activities of FDH were evaluated spectrophotometrically as described previously (36). Briefly, the hydrolase and 10-fTHF dehydrogenase activities were measured using 10-formyldeazafofolate as a substrate, with the assays for dehydrogenase activity containing 100 μM NADP⁺. The monitored increase in absorbance at 295 nm (the characteristic spectrum maximum for the reaction product, deazafofolate) was used to calculate the hydrolase activity. The specific activity for the dehydrogenase reaction was measured by monitoring the increase in absorbance at 340 nm (the characteristic spectrum of produced NADPH). All assays were carried out at 30 °C in a 1-cm quartz cuvette using a Shimadzu 2401PC double-beam spectrophotometer. 10-Formyldeazafofolate was obtained from Dr. John Hynes.

RESULTS

Identification of the Mitochondrial FDH Gene and Comparison of Its Sequence with That of Cytosolic FDH—Biochemical evidence strongly suggested that an enzyme similar to cytosolic FDH is present in mammalian mitochondria (8, 37). Attempts to purify this enzyme from rat liver mitochondria using canonical protein purification techniques were previously unsuccessful.³ Since that time, annotation of the human genome (38, 39) has armed researchers with a powerful tool for exploring unknown proteins. In our studies concerning the origin of the functional domains of cytosolic FDH, we performed a homology search for rat FDH using National Center for Biotechnology Information (NCBI) BLAST (40). Predictably, this search produced human FDH as a target 92% identical to rat enzyme, with the corresponding gene located at chromosome locus 3q21.3. Unexpectedly, this search resulted in another hit, an unknown protein with about 72% sequence identity to cytosolic FDH (Fig. 2). The corresponding gene is located at chromosome locus 12q24.11 of the human genome, spans 60 kb, and is composed of 23 exons interrupted by 22 introns that range from 83 to 10,383 bases in length. The encoded protein is 923 amino acid residues in length and has a putative sequence arrangement similar to that of cytosolic FDH, with the three distinct domains easily recognizable (Fig. 2). However, when compared with cytosolic FDH, this putative protein has an additional 22 amino acid residues at the N-terminal end. The N-terminal leader sequence (residue 1–22) lacks negatively charged amino acids and is enriched with positively charged residues (five arginines and two lysines) (Fig. 3), a characteristic of a mitochondria-targeting peptide (41). The predicted cleavage site to remove the mitochondrial leader is located 19 resi-

³ C. Wagner and R. J. Cook, personal communication.

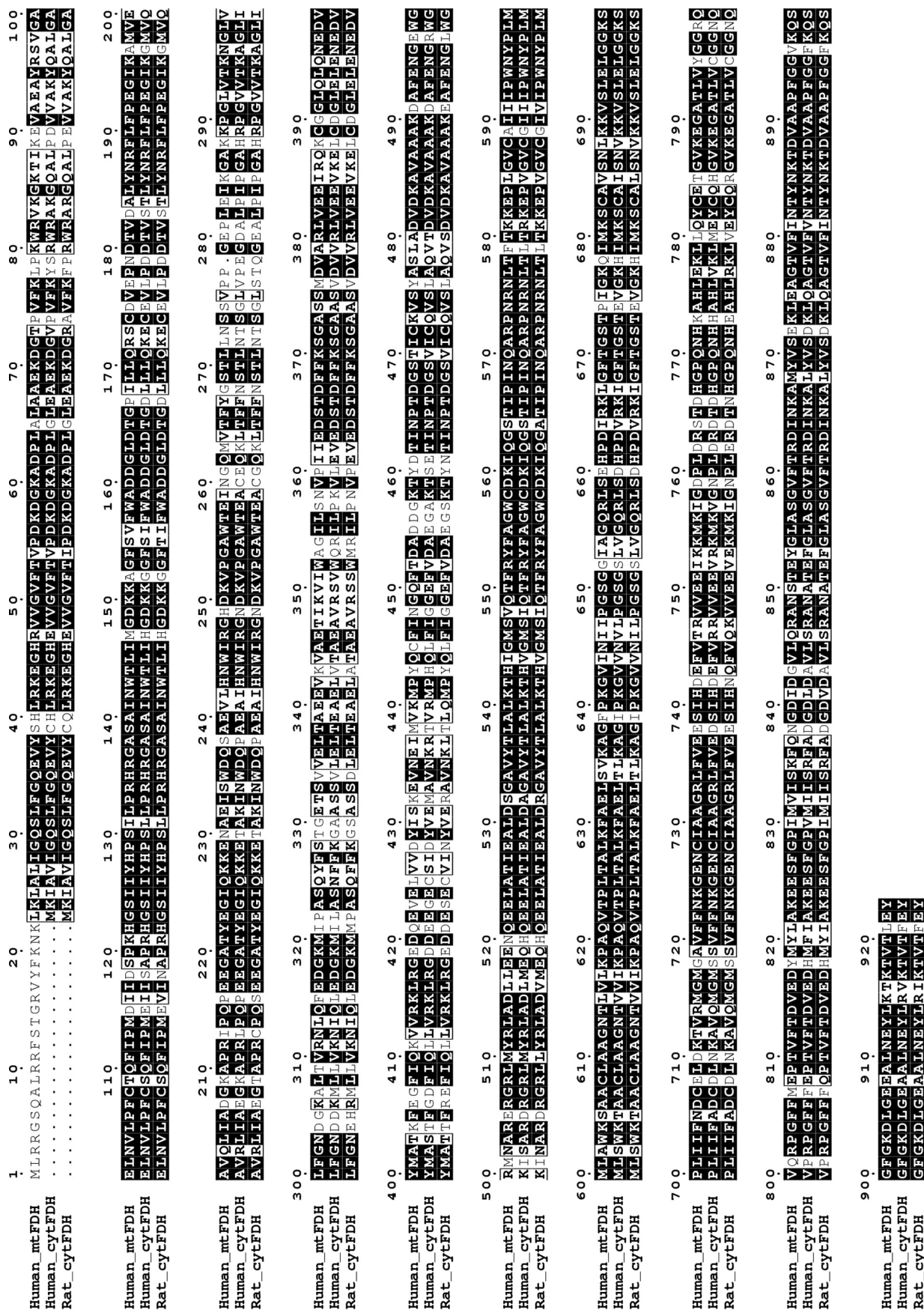


FIGURE 2. Sequence alignment of human ALDH1L1 (cytFDH) and human and rat ALDH1L2 (mtFDH). The alignment was generated using the program ClustalW2 (55), and the above image was produced using the program ESPript 2.2 (56).

Mitochondrial 10-Formyltetrahydrofolate Dehydrogenase

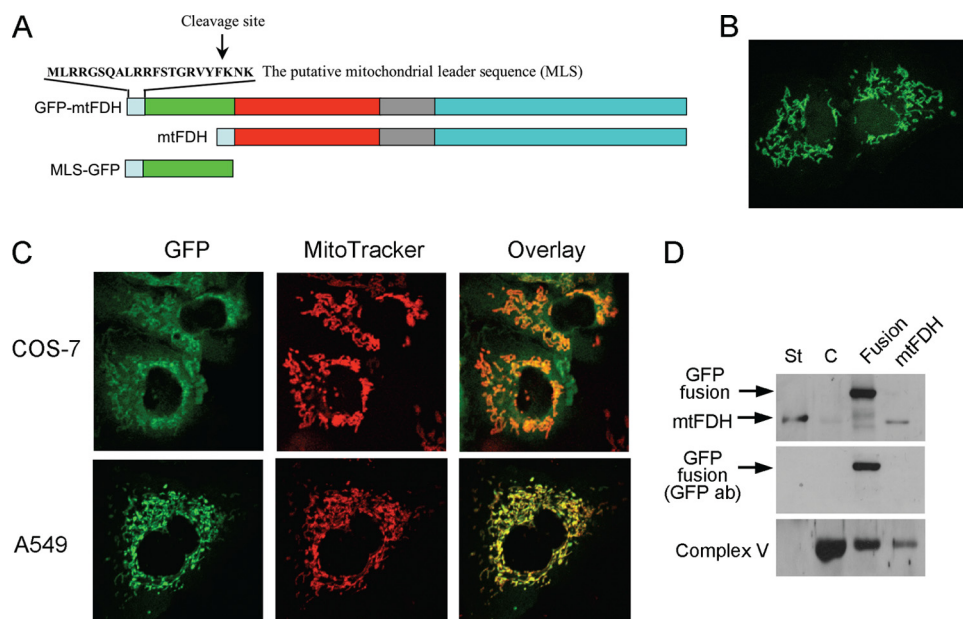


FIGURE 3. ALDH1L2 N-terminal leader targets proteins to mitochondria. *A*, schematic presentation of the domain structure of ALDH1L2 and its fusion with GFP. The sequence of the putative mitochondrial localization sequence (MLS) and the cleavage site are shown. Blue, MLS; red, N-terminal hydrolase domain; gray, intermediate acyl carrier protein-like domain; cyan, C-terminal aldehyde dehydrogenase domain; green, GFP. *B*, fluorescence confocal microscopy of COS-7 cells transfected with MLS-GFP construct. *C*, fluorescence confocal microscopy of COS-7 (top panel) and A549 (bottom panel) cells transfected with GFP-mtFDH construct. *D*, immunoblot assays of the mitochondrial fraction of COS-7 cells transfected with mtFDH or its GFP fusion construct. St., purified recombinant mtFDH; C, control (non-transfected cells). GFP ab, GFP antibody.

dues downstream of the starting methionine. Analysis of the N terminus of the putative FDH using TargetP (42) or MitoProt (43), network-based tools for prediction of peptide subcellular localization, confirmed that this protein is likely to reside in mitochondria (0.94 probability). Based upon this analysis, we suggested that this gene encodes for a mitochondrial FDH. It was later assigned the name *ALDH1L2*.

Translocation of mtFDH into Mitochondria of Mammalian Cells—To prove that the identified protein is a mitochondrial enzyme, we have generated a construct to express a fusion of mtFDH with green fluorescent protein (GFP). In this construct, GFP has been incorporated between the mitochondrial leader sequence and the N-terminal domain of mtFDH (Fig. 3A). In another experiment, we monitored the subcellular localization of GFP tagged at the N terminus with the mtFDH leader (Fig. 3A). We have observed that GFP tagged with the leader was translocated into mitochondria of transiently transfected COS-7 cells (Fig. 3B). Similar results have been observed in COS-7 cells transfected for expression of the fusion of GFP with mtFDH (Fig. 3C). The mitochondrial localization of the construct was evident as early as 24 h after transfection (data not shown), and stronger accumulation was seen 48 h after transfection (Fig. 3C). These experiments have been reproduced in another cell line, A549, a human non-small cell lung carcinoma. In these experiments, co-localization of the GFP construct and mitochondria-specific dye MitoTracker was also clearly seen (Fig. 3C). Some residual background green fluorescence in COS-7 cells could be attributed to the fact that these cells are strong overproducers of recombinant proteins, and perhaps not all fusion translocated to mitochondria in our experiments.

Immunoblot assays with GFP-specific or mtFDH-specific antibody have demonstrated the presence of a 130-kDa band in mitochondria, purified from the transfected COS-7 cells, which corresponds to the molecular mass of the full-length fusion protein. The same experiments performed with non-modified mtFDH (no GFP tag) have demonstrated the accumulation of the full-length enzyme in mitochondria. These results indicate the translocation to mitochondria of the full-length mtFDH without its truncation.

Presence of mtFDH in Cell Lines and Human Tissues—To study the abundance of mtFDH, we have evaluated its mRNA levels in an array of human tissues using real-time PCR. Similar to the cytosolic enzyme, the expression of mtFDH is tissue-specific (Fig. 4). Overall, the levels of mRNA for the cytosolic enzyme are higher than the levels of mtFDH mRNA in all evaluated tissues (Fig. 4). Of note, tissues with low levels of

cytosolic FDH mRNA also displayed low levels of mRNA for the mitochondrial enzyme. However, mtFDH mRNA levels were notably lower than those of cytosolic FDH in liver and kidney, the two major organs of the folate metabolism (44). In contrast to the cytosolic enzyme, which is typically not detectable in cancer tissues and cell lines, mtFDH mRNA and protein are clearly present in cancer cell lines (Fig. 4). These findings imply a different regulation of the two enzymes as well as potentially different functional roles in the cell.

Enzymatic Activity of Mitochondrial FDH from Pig Liver—To characterize native mitochondrial FDH, we have purified the enzyme from pig liver. We chose this source because these experiments typically require a large volume of mitochondria. High levels of cytosolic FDH in liver (24, 25) are a potential source of contamination during mtFDH purification. Therefore, the purified mitochondria were washed multiple times to remove all traces of cytosolic FDH (Fig. 5A, top panel). MtFDH was extracted from mitochondria by a series of freeze-thaw cycles (Fig. 5A, middle panel) and purified in two steps using a folate affinity column followed by size-exclusion chromatography (Fig. 5A, bottom panel, and B). Mass spectrometry analysis of tryptic peptides of the purified protein has confirmed that it is the product of the *ALDH1L2* gene. Enzyme assays of the preparation revealed the presence of both dehydrogenase and hydrolase catalytic activities (Fig. 5C, specific activity was 51.6 and 52.9 nmol/min/mg of protein for the dehydrogenase and hydrolase reactions, correspondingly). Although it is hard to make a direct comparison between the activities of the cytosolic and

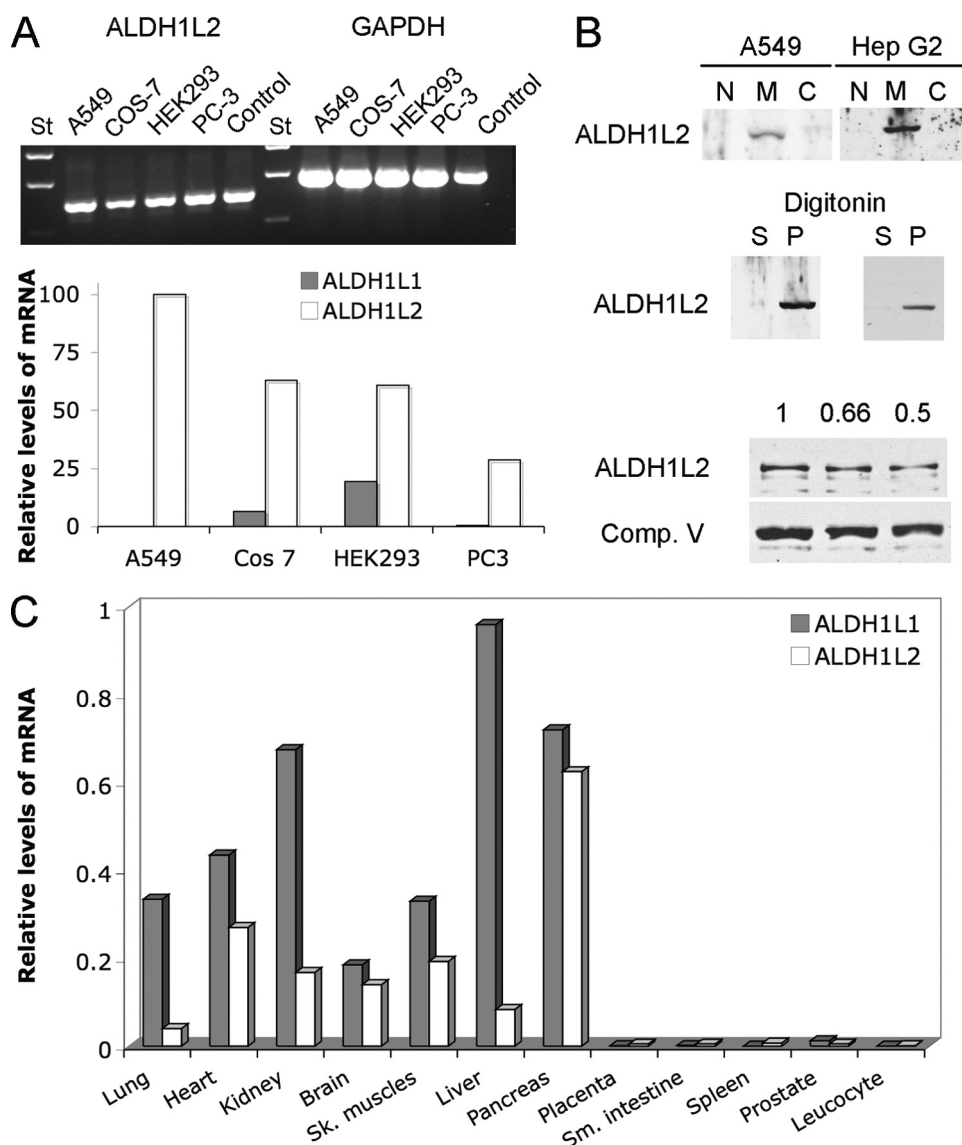


FIGURE 4. The abundance of ALDH1L2 in cell lines and tissues. *A*, levels of mtFDH (ALDH1L2) mRNA in cell lines determined by reverse transcription-PCR (top panel) and real-time PCR (bottom panel). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a control for sample normalization in top panel. Levels of cytoFDH (ALDH1L1) are shown in the bottom panel for the comparison. *St.*, purified recombinant mtFDH. *B*, levels of mtFDH proteins assessed by Western blot. Top panel, levels of mtFDH in subcellular fractions of A549 and HepG2 cells (N, M, and C indicate nuclear, mitochondrial, and cytosolic fractions, correspondingly); middle panel, levels of the enzyme in digitonin extracted mitochondria (S, digitonin-solubilized fraction; P, digitonin insoluble pellet); bottom panel, levels of mtFDH in purified rat mitochondria (different relative amounts of sample were loaded to SDS-PAGE). Complex V (β subunit of F1 complex of ATP synthase) is shown as a loading control. *C*, levels of mitochondrial and cytosolic FDH in human tissues (real-time PCR). *Sk. muscles*, skeletal muscles; *Sm. intestine*, small intestine.

mitochondrial enzymes without thorough characterization, these experiments clearly demonstrated that *ALDH1L2* encodes for mitochondrial FDH possessing both characteristic activities.

DISCUSSION

The compartmentalization of folate metabolism (Fig. 1) has resulted in the duplication of several folate enzymes as cytosolic and mitochondrial proteins (2, 4, 45). One of the folate enzymes, FDH, converts 10-fTHF to THF in an NADP⁺-dependent dehydrogenase reaction in which the formyl group is oxidized to CO₂ (20). This oxidative reaction is an active pro-

cess in the liver of animals and in humans (46–48). The enzyme itself is extremely abundant in several tissues (26) with its levels reaching up to 1.2% of the total cytosolic protein in rat liver (25). Because the generation of CO₂ from 10-fTHF was also observed in mitochondrial fractions, it was thought that a mitochondrial enzyme homologous to cytosolic FDH is present in the cell (8, 37). However, although cytosolic FDH is a well characterized enzyme (20), the enzyme responsible for the corresponding mitochondrial pathway has not been demonstrated. In the present study, we have cloned cDNA of the *ALDH1L2* gene and provide evidence that it is the mitochondrial FDH.

The product of the *ALDH1L2* gene has significant sequence similarity to cytosolic FDH. Of note, this similarity is higher for the catalytic N- and C-terminal domains (71 and 79%, correspondingly) than for the intermediate linker domain (55%). We have previously identified several amino acid residues in cytosolic FDH, which are crucial for the catalytic activity of the enzyme; replacement any of these residues completely abolished the 10-fTHF dehydrogenase activity. These residues are present in mtFDH as well, and they include His-130, Asp-165, Ser-370, and Cys-730 (corresponding to His-106, Asp-142, Ser-354, and Cys-707 of cytoFDH, Fig. 2) (28, 29, 49–51). This suggests a similar catalytic mechanism for cytosolic and mitochondrial enzymes and implies a similar metabolic role. Indeed, in our experiments, both characteristic activities of cytosolic FDH were found in mtFDH purified

from pig liver.

The reaction catalyzed by FDH irreversibly removes carbon groups from the folate pool, and as such, competes with folate-dependent biosynthetic pathways. In the cell, this pathway might fulfill several metabolic functions including: clearance of excess of folate-bound one-carbon groups (52); degradation of formate (46); regulation of *de novo* purine biosynthesis (26, 53); and the overall control of carbon group flow within the folate pool (27). These functions, however, were demonstrated for the cytosolic enzyme, but the role of mtFDH is not so clear. It could be suggested that the function of the mitochondrial and cytosolic enzymes is redundant toward the control of one-carbon

Mitochondrial 10-Formyltetrahydrofolate Dehydrogenase

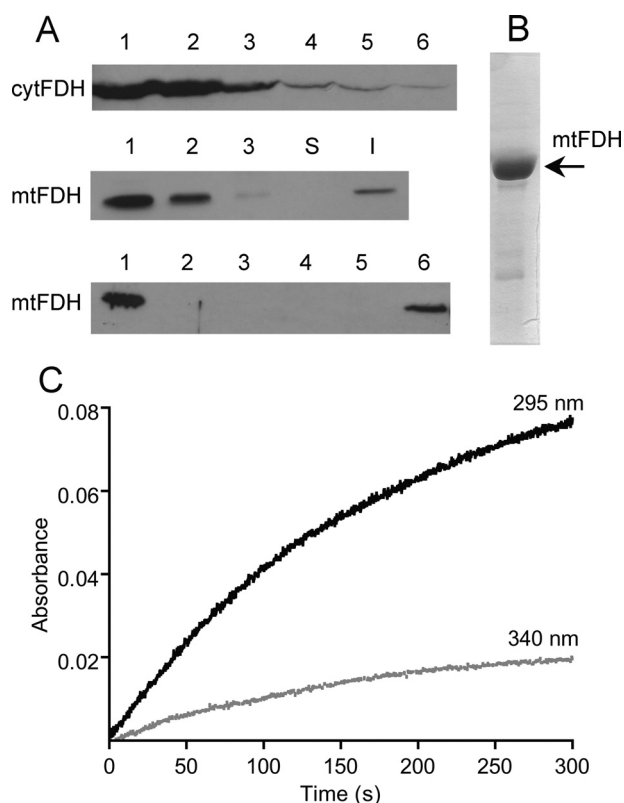


FIGURE 5. Purification of pig liver mitochondrial FDH and assays of its catalytic activities. *A*, top panel, Western blot of cytosolic FDH in mitochondrial preparation after successive washing (figures indicate the number of washings, see details under "Experimental Procedures"). *Middle panel*, Western blot showing release of mtFDH from mitochondria upon successive freeze-thaw cycles (lanes 1–3) and remaining mtFDH in the soluble (S) and insoluble (I) mitochondrial fractions. *Bottom panel*, purification of mtFDH by affinity chromatography. *Lane 1*, initial preparation of mtFDH extracted from mitochondria and loaded to the column; *lane 2*, flow-through material; *lanes 3–6*, eluates after washing the column with buffer, 1.0 M KCl, 2.0 M KCl, and 20 mM folic acid, correspondingly. *B*, purified preparation of mtFDH (Coomassie Blue staining) after affinity chromatography (*lane 1*) followed by chromatography on Sephacryl S300 (*lane 2*). *C*, time course of the hydrolase (295 nm) and dehydrogenase (340 nm) activities of purified mtFDH. Polyclonal antibodies generated against rat cytosolic FDH or human mitochondrial FDH were used in Western blot assays to detect the cytosolic or mitochondrial pig enzymes, correspondingly.

flux through the folate pool. Of note, in contrast to cytosolic FDH, mtFDH is present at detectable levels in several cancer cell lines, indicating different mechanisms of regulation of these enzymes. If, in contrast to cytosolic FDH, mtFDH is constitutively expressed, it might compensate for the lack of the cytosolic enzyme at certain conditions, thus still enabling the reaction.

In contrast to cytosolic FDH, mtFDH might play an additional functional role, which is the generation of formate, instead of carbon dioxide, from 10-fTHF. If this reaction takes place *in vivo*, it would be a part of the pathway for shuttling one-carbon groups, generated during glycine degradation or conversion of serine to glycine, from mitochondria to cytoplasm (4). Indeed, similar to cytosolic FDH (36), the mitochondrial enzyme possesses hydrolase activity *in vitro*. In both cases, this reaction requires millimolar concentrations of 2-mercaptoethanol, a compound not found in living cells. Mammalian cells, however, have high levels of glutathione, concentrations of which could reach 10–14 mM in liver mitochondria (54).

TABLE 1
Sequence similarity between ALDH1L1 and ALDH1L2 proteins

Species	ALDH1L1 (relative to human)	ALDH1L2 (relative to human)	ALDH1L1/1L2 (within species)
	%	%	%
Human	100	100	74
Mouse	92	92	75
Chicken	Not found	83	
Zebrafish	76	79/78 ^a	76/76 ^a
<i>D. melanogaster</i>	58	Not found	
<i>C. elegans</i>	59	Not found	

^a Zebrafish has two putative mitochondrial genes, which would constitute full-length mtFDH (ALDH1L2); the similarity between the product of each of these genes and the corresponding sequence of human mtFDH or zebrafish cytosolic FDH is shown.

Thus, the role of mtFDH in generating formate cannot be excluded at this time, and this avenue requires additional studies at the cellular or tissue level. Of note, the generation of formate from 10-fTHF has been demonstrated for mitochondrial C1-synthase (18), which would make such a function for mtFDH a redundant pathway.

Analysis of genomes of different species (Table 1) indicates that both cytosolic and mitochondrial enzymes are rather late evolutionary products; they are not present in bacteria, fungi, or plants. Cytosolic FDH is seen earlier than mtFDH on the phylogenetic tree; invertebrates, *Drosophila*, and *Caenorhabditis elegans*, as well as zebrafish, possess the gene for *ALDH1L1* but not for the mitochondrial isoform. This might indicate that *ALDH1L2* descended from *ALDH1L1*, although a different origin for *ALDH1L2* cannot be excluded. In zebrafish, two distinct genes on chromosomes 13 and 4 encode proteins with high similarity to the N-terminal and intermediate/C-terminal domains of mitochondrial FDH, correspondingly (GeneID: 100151288 and 572151). Both gene products have putative mitochondrial leader sequences and are predicted to reside in mitochondria, and the combination of their sequences corresponds to full-length mtFDH. The role of these genes is not known, but it is likely that they are the ancestors of mitochondrial FDH. Although the presence of two FDHs, cytosolic and mitochondrial, is perhaps evolutionarily beneficial, the function of the two forms could be just redundant. Indeed, mice lacking the cytosolic enzyme are viable (47). In further support of this possibility, birds appear to carry *ALDH1L2* but not *ALDH1L1* (Table 1).

The FDH reaction represents the final step in the course of the carbon atom oxidation, from the level of formate to the level of CO₂, and is coupled to the reduction of NADP⁺ to NADPH. Upon this reaction, one-carbon groups leave the folate pool that diverts the folate pathway from anabolic branch to the energy production. Interestingly, in uncoupled mitochondria, carbon-3 of serine is converted to CO₂, whereas in actively respiring mitochondria (state 3), it goes to formate (37), the observation supporting the importance of the energy-generating role for mtFDH at some conditions. This is in agreement with the notion that in mitochondria, one-carbon flux goes in the oxidative direction, thus generating 10-fTHF from 5,10-methylene-THF (4, 45). Of note, in mitochondria, FDH would compete with MTHFD1L for the same substrate because MTHFD1L catalyzes the reversal of the synthetase reaction of

cytosolic MTHFD1 (18), which is not the case in the cytoplasm. What direction the pathway goes, to formate or CO₂, would depend on the relative levels of the enzymes and their regulation and perhaps is tissue-specific. Further studies are required to differentiate between the role of cytosolic and mitochondrial FDH in the cell.

REFERENCES

- Wagner, C. (1995) in *Folate in Health and Disease* (Bailey, L. B., ed) pp. 23–42, Marcel Dekker, Inc., New York
- Fox, J. T., and Stover, P. J. (2008) *Vitam. Horm.* **79**, 1–44
- Spencer, A. C., and Spremulli, L. L. (2004) *Nucleic Acids Res.* **32**, 5464–5470
- Appling, D. R. (1991) *FASEB J.* **5**, 2645–2651
- Woeller, C. F., Anderson, D. D., Szebenyi, D. M., and Stover, P. J. (2007) *J. Biol. Chem.* **282**, 17623–17631
- Anderson, D. D., and Stover, P. J. (2009) *PLoS One* **4**, e5839
- Fox, J. T., and Stover, P. J. (2009) *J. Biol. Chem.* **284**, 31085–31096
- Barlowe, C. K., and Appling, D. R. (1988) *Biofactors* **1**, 171–176
- Garrow, T. A., Brenner, A. A., Whitehead, V. M., Chen, X. N., Duncan, R. G., Korenberg, J. R., and Shane, B. (1993) *J. Biol. Chem.* **268**, 11910–11916
- Masuda, T., Sakamoto, M., Nishizaki, I., Hayashi, H., Yamamoto, M., and Wada, H. (1987) *J. Biochem.* **101**, 643–652
- Paukert, J. L., Williams, G. R., and Rabinowitz, J. C. (1977) *Biochem. Biophys. Res. Commun.* **77**, 147–154
- Hum, D. W., Bell, A. W., Rozen, R., and MacKenzie, R. E. (1988) *J. Biol. Chem.* **263**, 15946–15950
- Thigpen, A. E., West, M. G., and Appling, D. R. (1990) *J. Biol. Chem.* **265**, 7907–7913
- Villar, E., Schuster, B., Peterson, D., and Schirch, V. (1985) *J. Biol. Chem.* **260**, 2245–2252
- Hum, D. W., and MacKenzie, R. E. (1991) *Protein Eng.* **4**, 493–500
- Christensen, K. E., Patel, H., Kuzmanov, U., Mejia, N. R., and MacKenzie, R. E. (2005) *J. Biol. Chem.* **280**, 7597–7602
- Walkup, A. S., and Appling, D. R. (2005) *Arch. Biochem. Biophys.* **442**, 196–205
- Pike, S. T., Rajendra, R., Artzt, K., and Appling, D. R. (2010) *J. Biol. Chem.* **285**, 4612–4620
- Patel, H., Christensen, K. E., Mejia, N., and MacKenzie, R. E. (2002) *Arch. Biochem. Biophys.* **403**, 145–148
- Krupenko, S. A. (2009) *Chem. Biol. Interact.* **178**, 84–93
- Cook, R. J., Lloyd, R. S., and Wagner, C. (1991) *J. Biol. Chem.* **266**, 4965–4973
- Chumanevich, A. A., Krupenko, S. A., and Davies, C. (2004) *J. Biol. Chem.* **279**, 14355–14364
- Donato, H., Krupenko, N. I., Tsybovsky, Y., and Krupenko, S. A. (2007) *J. Biol. Chem.* **282**, 34159–34166
- Cook, R. J., and Wagner, C. (1982) *Biochemistry* **21**, 4427–4434
- Kisliuk, R. L. (1999) in *Antifolate drugs in Cancer Therapy* (Jackman, A. L., ed) pp. 13–36, Humana Press, Totowa, NJ
- Krupenko, S. A., and Oleinik, N. V. (2002) *Cell Growth Differ.* **13**, 227–236
- Anguera, M. C., Field, M. S., Perry, C., Ghandour, H., Chiang, E. P., Selhub, J., Shane, B., and Stover, P. J. (2006) *J. Biol. Chem.* **281**, 18335–18342
- Tsybovsky, Y., Donato, H., Krupenko, N. I., Davies, C., and Krupenko, S. A. (2007) *Biochemistry* **46**, 2917–2929
- Strickland, K. C., Hoferlin, L. A., Oleinik, N. V., Krupenko, N. I., and Krupenko, S. A. (2010) *J. Biol. Chem.* **285**, 1627–1633
- Oleinik, N. V., Krupenko, N. I., and Krupenko, S. A. (2007) *Oncogene* **26**, 7222–7230
- Livak, K. J., and Schmittgen, T. D. (2001) *Methods* **25**, 402–408
- Lemasters, J. J., Grunwald, R., and Emaus, R. K. (1984) *J. Biol. Chem.* **259**, 3058–3063
- Krupenko, S. A., Horstman, D. A., Wagner, C., and Cook, R. J. (1995) *Protein Expr. Purif.* **6**, 457–464
- Reuland, S. N., Vlasov, A. P., and Krupenko, S. A. (2003) *J. Biol. Chem.* **278**, 22894–22900
- Reuland, S. N., Vlasov, A. P., and Krupenko, S. A. (2006) *Protein Sci.* **15**, 1076–1084
- Krupenko, S. A., Wagner, C., and Cook, R. J. (1995) *Biochem. J.* **306**, 651–655
- García-Martínez, L. F., and Appling, D. R. (1993) *Biochemistry* **32**, 4671–4676
- Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., et al. (2001) *Science* **291**, 1304–1351
- Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al. (2001) *Nature* **409**, 860–921
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–410
- Emanuelsson, O., and von Heijne, G. (2001) *Biochim. Biophys. Acta* **1541**, 114–119
- Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) *J. Mol. Biol.* **300**, 1005–1016
- Claros, M. G., and Vincens, P. (1996) *Eur. J. Biochem.* **241**, 779–786
- Gregory, J. F., 3rd, and Quinlivan, E. P. (2002) *Annu. Rev. Nutr.* **22**, 199–220
- Christensen, K. E., and MacKenzie, R. E. (2006) *Bioessays* **28**, 595–605
- Tephly, T. R., Green, M. D., and Gamble, J. (1992) *Toxicol. Appl. Pharmacol.* **116**, 142–145
- Champion, K. M., Cook, R. J., Tollaksen, S. L., and Giometti, C. S. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11338–11342
- Lamers, Y., Williamson, J., Theriaque, D. W., Shuster, J. J., Gilbert, L. R., Keeling, C., Stacpoole, P. W., and Gregory, J. F., 3rd. (2009) *J. Nutr.* **139**, 666–671
- Krupenko, S. A., Wagner, C., and Cook, R. J. (1995) *J. Biol. Chem.* **270**, 519–522
- Krupenko, S. A., and Wagner, C. (1999) *J. Biol. Chem.* **274**, 35777–35784
- Krupenko, S. A., Vlasov, A. P., and Wagner, C. (2001) *J. Biol. Chem.* **276**, 24030–24037
- Krebs, H. A., Hems, R., and Tyler, B. (1976) *Biochem. J.* **158**, 341–353
- Oleinik, N. V., Krupenko, N. I., Priest, D. G., and Krupenko, S. A. (2005) *Biochem. J.* **391**, 503–511
- Fernandez-Checa, J. C., and Kaplowitz, N. (2005) *Toxicol. Appl. Pharmacol.* **204**, 263–273
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) *Bioinformatics* **23**, 2947–2948
- Gouet, P., Courcelle, E., Stuart, D. I., and Métoz, F. (1999) *Bioinformatics* **15**, 305–308