Compartmentalization of Mammalian Folate-Mediated One-Carbon Metabolism

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Abstract

The recognition that mitochondria participate in folate-mediated one-carbon metabolism grew out of pioneering work beginning in the 1950s from the laboratories of D.M. Greenberg, C.G. Mackenzie, and G. Kikuchi. These studies revealed mitochondria as the site of oxidation of one-carbon donors such as serine, glycine, sarcosine, and dimethylglycine. Subsequent work from these laboratories and others demonstrated the participation of folate coenzymes and folate-dependent enzymes in these mitochondrial processes. Biochemical and molecular genetic approaches in the 1980s and 1990s identified many of the enzymes involved and revealed an interdependence of cytoplasmic and mitochondrial one-carbon metabolism. These studies led to the development of a model of eukaryotic one-carbon metabolism that comprises parallel cytosolic and mitochondrial pathways, connected by one-carbon donors such as serine, glycine, and formate. Sequencing of the human and other mammalian genomes has facilitated identification of the enzymes that participate in this intercompartmental one-carbon metabolism, and animal models are beginning to clarify the roles of the cytoplasmic and mitochondrial isozymes of these enzymes. Identifying the mitochondrial transporters for the one-carbon donors and elucidating how flux through these pathways is controlled are two areas ripe for exploration.

Contents

INTRODUCTION

Folate coenzymes (or the related archaeal pterins) are present in virtually every known organism and cell type. One-carbon (1C) transfers mediated by folate coenzymes play essential roles in many major cellular processes, including nucleic acid biosynthesis, mitochondrial and chloroplast protein biosynthesis, amino acid metabolism, methyl group biogenesis, and vitamin metabolism. The diversity of reactions that involve these 1C units results from the ability of the coenzyme tetrahydrofolate to carry activated 1C units at several different oxidation states and from the ability of the cell to readily interconvert these forms. The activated one-carbon units are carried on the N5, N10, or both positions of tetrahydrofolate (**Figure 1**). The major cellular forms of folate coenzymes contain poly-γ-glutamate tails attached to the *p*-aminobenzoic acid moiety in a reaction catalyzed by folylpolyglutamate synthetase (FPGS). The polyglutamate chain lengths of the folates differ from one cell type to another and even within different organelles of a given cell, but in most eukaryotic cells, the penta- and hexaglutamate forms predominate. Polyglutamylation is essential for the cellular retention of folates in eukaryotic cells and results in more efficient substrates for many folate-dependent enzymes.

Figure 2 summarizes the major pathways of folate-mediated 1C metabolism in eukaryotic cells. Folic acid is reduced to 7,8-dihydrofolate (DHF) and then to 5,6,7,8-tetrahydrofolate (THF, the active form of the coenzyme) by dihydrofolate reductase (DHFR). In most organisms, the major source of 1C units is carbon 3 of serine, derived from glycolytic intermediates. The 1C unit is transferred to THF in a reaction catalyzed by serine hydroxymethyltransferase (SHMT; reaction 4), generating 5,10-methylene-THF (CH_2-THF) and glycine. This form of the coenzyme is required for de novo thymidylate synthesis in the reaction catalyzed by thymidylate synthase (reaction 10). CH_2-THF may also be reduced to 5-methyl-THF (for methyl group biogenesis) (reaction 6) or oxidized to 10-formyl-THF depending on the needs of the cell. In rapidly growing cells, the synthesis of purines is a critical folate-dependent pathway, requiring two moles of 10-formyl-THF per mole of purine ring. CH_2-THF is converted to 10-formyl-THF via the sequential enzymes CH_2-THF dehydrogenase and 5,10-methenyl-THF (CH^+-THF) cyclohydrolase (reactions 3 and 2, respectively).

One of the hallmarks of eukaryotic cells is the existence of distinct intracellular compartments, and we now recognize that folate-mediated 1C metabolism is highly compartmentalized between the cytoplasm, mitochondria, and nucleus (**Figure 2**). This topic has previously been discussed in several excellent reviews (5, 18, 19, 23, 38). This review focuses on compartmentalization of mammalian 1C metabolism, with special emphasis on the role of mitochondria in these interdependent pathways.

INTRACELLULAR FOLATE POOLS

The major pools of folate coenzymes are distributed primarily in the cytosol and

1C: one-carbon

FPGS: folylpolyglutamate synthetase

DHF: dihydrofolate

THF: tetrahydrofolate

DHFR: dihydrofolate reductase

SHMT: serine hydroxymethyltransferase

CH_2-THF: 5,10-methylene-THF

CH^+-THF: 5,10-methenyl-THF

mitochondria of liver cells [see (23) and references therein], with smaller amounts associated with the nucleus (128, 136). Cook & Blair (24) reported an equal distribution of rat liver folates between the cytoplasmic and mitochondrial fractions, whereas mitochondria from leukemia cells contain only about 20% of the total cellular folates (142). Mitochondrial and cytosolic pools differ in the distribution of the specific forms of the folate cofactors and in the extent of the polyglutamylation of these compounds. For example, rat liver mitochondria contain predominantly THF and 10-formyl-THF, with 11% as 5-formyl-THF and less than 7% as 5-methyl-THF (56). In the cytoplasm, THF cofactors are approximately 45% 5-methyl-THF, 30% 10-formyl- and 5-formyl-THF, and 25% unsubstituted THF. The distribution is quite different in leukemia cells but still shows distinct mitochondrial and cytoplasmic pools (142). Studies of polyglutamate chain lengths indicate that Chinese hamster ovary (CHO) cells have longer glutamate chain lengths in mitochondrial folates as compared to the cytosolic pools, with heptaglutamates predominating in the mitochondria and hexaglutamates in the cytoplasm (77). Rat liver mitochondria also contain penta-, hexa-, and heptaglutamates, although the average chain length was reported to be slightly shorter than in the cytoplasmic folate pools (13). Several studies indicate that limited flux occurs between these two pools of cellular folates. Following an 18-hour exposure of rats to nitrous oxide and subcellular fractionation of harvested liver, the cytosolic folate distribution pattern was markedly changed (56). However, the distribution of mitochondrial folates was not affected by nitrous oxide treatment. Similarly, leukemia cells treated with the DHFR inhibitor, trimetrexate, showed decreased levels of THF and increased levels of DHF in the cytosol, but no change in THF or DHF levels in the mitochondria (142). These results clearly suggest that only a limited exchange of free folates occurs between the two compartments.

MITOCHONDRIAL ONE-CARBON METABOLISM

CHO: Chinese hamster ovary cells

Based primarily on studies with the yeast *Saccharomyces cerevisiae*, a model of eukaryotic 1C metabolism was proposed that comprises parallel cytosolic and mitochondrial pathways (5, 6) (**Figure 2**). Although there is limited exchange between the cytoplasmic and mitochondrial pools of THF, these compartments are metabolically connected by transport of the 1C donors (serine, glycine, and formate) across the mitochondrial membranes, supporting a mostly unidirectional flow (clockwise in **Figure 2**) of 1C units from serine to formate, and onto methionine.

Transport Processes

The presence of folate coenzymes in mammalian mitochondria was first established decades ago, and subsequent studies have demonstrated carrier-mediated transport of folate into the mitochondria (28, 54, 141). However, the capacity of this transport system is not great enough to supply the one-carbon needs of the mitochondrial compartment (56). Rather, the transport of 1C units between mitochondria and cytoplasm occurs via 1C donors such as serine, glycine, and formate (14, 26, 27, 104). When considering the movement of folate coenzymes and 1C units between the cytoplasm and mitochondria, it is important to distinguish between the 1C units, which are required in *stoichiometric* amounts, and the carrier (THF), required only in *catalytic* amounts. The THF coenzyme is regenerated when 1C units are released by the enzymes 10-formyl-THF synthetase (**Figure 2**, reaction 1m), 10-formyl-THF dehydrogenase (reaction 11), methionyl-tRNA transformylase (reaction 12), or mitochondrial SHMT (mSHMT) (in the serine direction; reaction 4m). Thus, mitochondria have evolved transport systems with different capacities for the folates and for the various 1C donors. In addition to serine,

MFT: mitochondrial folate transporter

glycine, and formate, dimethylglycine and sarcosine can serve as 1C donors in mitochondria via the dimethylglycine and sarcosine dehydrogenase reactions (reactions 8 and 9), thus suggesting transport mechanisms for these compounds as well. *S*-adenosylmethionine, the 1C donor for mitochondrial methylation reactions, is also transported to the mitochondria in a carrier-mediated process.

Mitochondrial folate transport. Fractionation of rat liver following intraperitoneal injection (155) or oral administration (24) of radiolabeled folic acid revealed the rapid appearance of label in mitochondrial fractions, suggesting a specific transport system for folates into mitochondria. The form of the folate coenzyme transported through the mitochondrial inner membrane has been investigated on several levels. The presence of polyglutamylated forms of the folate coenzyme are essential for mitochondrial function, and mammalian cells possess both mitochondrial and cytoplasmic FPGS isozymes (77), resulting from differential splicing of a single nuclear gene (17, 39). Mitochondrial FPGS activity is required for mitochondrial folate accumulation (17), suggesting that the monoglutamate forms of the coenzyme are transported into mitochondria, followed by conversion to polyglutamates. In experiments with cultured CHO cells, Lin & Shane (78) showed that folylpolyglutamates could not enter the mitochondria, but mitochondrial folylpolyglutamates could exit without prior cleavage of the polyglutamate chain.

With respect to the oxidation state of the pteroyl moiety of the coenzyme, Cybulski & Fisher (28) reported that tetrahydrofolates are not transported into isolated rat liver mitochondria, whereas folic acid, dihydrofolate, and methotrexate are rapidly transported into the matrix by a carrier-mediated mechanism. In contrast, using more physiological conditions, Horne et al. (54) demonstrated a carrier-mediated uptake of reduced folylmonoglutamates (5-methyl-THF and 5-formyl-THF) in isolated rat liver mitochondria. Uptake of the oxidized form, folic acid, was significantly slower. Inhibitors of the dicarboxylate or tricarboxylate carriers had no effect on 5-formyl-THF uptake into mitochondria, suggesting the existence of a distinct carrier for reduced folates. Taken together, these data suggest that mitochondria receive folates from the cytoplasm in a reduced, monoglutamate form. This pool of THF coenzyme would then be polyglutamylated and charged with 1C units in situ. This view is also consistent with lack of DHFR activity in mitochondria (147).

It has now been shown that folate coenzyme entry in mammalian mitochondria is mediated by the mitochondrial folate transporter (MFT) (141). Isolation of the human MFT was facilitated by the availability of the *glyB* CHO cell line, which is auxotrophic for glycine and lacks measurable mitochondrial folates. This cell line was isolated in an early mutagenesis study selecting for glycine-requiring mutants, and it represents one of four separate complementation groups (along with *glyA*, *glyC*, and *glyD*) (61). Glycine auxotrophy is a common phenotype resulting from dysfunctional mitochondrial 1C metabolism, presumably due to the dependence of glycine synthesis on mitochondrial SHMT activity, as discussed below (see Cultured Cell Models section). When *glyB* cells were transduced with a retroviral human cDNA library, the expression of a previously uncharacterized gene, now identified as the human mitochondrial folate transporter, was found to complement the glycine auxotrophy (141). Further studies confirmed a G192E mutation in the hamster MFT gene of *glyB* CHO cells that inactivates mitochondrial folate transport (83). Transfection of the wild-type hamster MFT cDNA into *glyB* cells resulted in glycine prototrophy and restored accumulation of mitochondrial folates, verifying the mitochondrial folate transport role of this protein. The MFT-encoded mitochondrial folate transporters are members of the mitochondrial carrier family of inner mitochondrial membrane transport carriers (101), which includes the well-studied ADP/ATP (adenosine triphosphate/adenosine diphosphate) exchanger (108). Homology modeling, based on the bovine

ADP/ATP carrier, and site-directed mutagenesis suggest that the G192E mutation most likely affects substrate binding (110). An R249A MFT mutant was unable to complement the *glyB* phenotype, and both the R249A mutant and the original *glyB* mutant (G192E) demonstrated a severe deficiency in uptake of [^{3}H]-(*6S*)-5-formyl-THF into mitochondrial fractions as compared to wild-type cells. Because *glyB* cells lack measureable mitochondrial folates, it is likely that the MFT carrier represents the only folate transporter in the mitochondria of these cells. However, the distribution of the MFT in other cell types or tissues has not been reported. At least one report suggests the localization of the reduced folate carrier, a major plasma membrane folate transporter, to the mitochondrial membrane in human leukemia cell lines (143), but to date there are no other reports of mitochondrial localization of this transporter, particularly in tissues or nonleukemia cells.

The physiological form of folate that is transported into mitochondria remains undefined. Studies showing uptake of 5-formyl-THF and 5-methyl-THF used these species because they are the most stable forms of reduced folates. The 1C unit would have to be removed from either of these forms once in the mitochondria in order to provide a functional pool of THF for the organelle. The only enzyme known to utilize 5-formyl-THF is methenyltetrahydrofolate synthetase, which catalyzes the irreversible conversion of 5-formyl-THF to CH^+-THF in an ATP-dependent reaction (59). This CH^+-THF could then be processed by reactions 2m plus 1m, 11, or 12 (**Figure 2**) to generate free mitochondrial THF. Methenyltetrahydrofolate synthetase is found predominately in the cytoplasm in mammals (4, 81), although there is one report of mitochondrial localization (10). 5-Methyl-THF is less likely to be a physiologically important substrate for mitochondrial transport. The only enzyme known to utilize 5-methyl-THF is methionine synthase (**Figure 2**, reaction 7). However, this enzyme appears to be exclusively localized to the cytoplasm (117), and without a mitochondrial 5-methyl-THF methyltransferase activity, there would be no way to generate free THF from transported 5-methyl-THF. Taken together, the available data suggest that the physiological form of folate transported into mitochondria is a reduced monoglutamate, probably the monoglutamate of tetrahydrofolate or 5-formyl-THF. However, this remains an open question.

Mitochondrial transport of one-carbon donors. Transport of 1C units between mitochondria and cytoplasm occurs via 1C donors such as serine, glycine, and formate. It was previously demonstrated that isolated rat liver mitochondria could take up serine, oxidize its 3-carbon to formate, and export the formate out of the mitochondria (6) in a process dependent on the respiratory state of the mitochondria (44). The same pathway could be shown to occur in vivo in yeast using ^{13}C-NMR methods (104, 105). Similar experiments demonstrated the uptake and conversion of [1-^{14}C] and [2-^{14}C]glycine to $^{14}CO_2$ in intact rat liver mitochondria (51). The glycine cleavage system (**Figure 2**, reaction 5) is strictly mitochondrial in mammalian cells, and the conversion of glycine to CO_2 in isolated organelles indicates facile transport of glycine into mitochondria. Clearly, these transport processes are active enough to satisfy the 1C needs of each compartment; however, little is known about the mechanisms of serine, glycine, or formate transport.

Early work with mammalian mitochondria suggested that serine and glycine utilize the same inner membrane neutral amino acid carrier (26, 27). In addition to transporting glycine, the carrier shows stereospecific transport of the L-isomers of serine, alanine, valine, methionine, and leucine, and it is inactivated by protein-modifying reagents. These are characteristics of a facilitated diffusion process involving a membrane transport carrier protein. Benavides et al. (8) observed saturation kinetics for glycine uptake by mitochondria from rat brain and liver. It is not clear whether this is the same carrier studied by Cybulski & Fisher (26, 27), since alanine did not inhibit glycine

BHMT: betaine hydroxymethyltransferase

DMG: *N,N*-dimethylglycine

DMGDH: dimethylglycine dehydrogenase

AdoMet: *S*-adenosylmethionine

uptake, and serine was not tested. However, the presence of multiple carriers for serine and/or glycine was not conclusively eliminated in any of these studies with isolated mitochondria.

Even less is known about formate transport across the mitochondrial inner membrane. Formate is known to enter intact mitochondria, as evidenced by swelling assays (14, 27). Formate effluxes rapidly from mitochondria, both in vivo in yeast (105) and in vitro (6, 44). Given the toxicity of formate to mitochondrial respiration via inhibition of cytochrome *c* oxidase (97), it seems likely that its transport is carrier mediated.

In mammals, choline represents a major source of dietary methyl groups (156). It is obtained in the diet primarily as phosphatidylcholine and is released during phospholipid turnover. Free choline is preferentially reincorporated into phospholipids; however, excess choline is oxidized to betaine (*N,N,N*-trimethylglycine), which in the liver donates a methyl group to homocysteine via the cytoplasmic betaine hydroxymethyltransferase (BHMT) reaction, forming methionine (**Figure 2**, reaction 14). *N,N*-dimethylglycine (DMG), the other product of the BHMT reaction, can serve as a mitochondrial 1C donor via the mitochondrial dimethylglycine dehydrogenase (DMGDH) reaction, forming CH_2-THF (reaction 8). Sarcosine (*N*-methylglycine) formed either from the DMGDH reaction or via cytosolic glycine *N*-methyl transferase can also serve as a 1C donor in the mitochondria via sarcosine dehydrogenase (reaction 9). These pathways of 1C flux require the transport of choline, betaine, dimethylglycine, and sarcosine across the inner mitochondrial membrane. Mitochondrial choline transport has been characterized in rat liver and kidney (98, 112) and was shown to be the rate-limiting step of choline oxidation in isolated mitochondria (98, 113). Choline uptake is saturable, inhibited by hemicholinium-3, and sensitive to mitochondrial uncoupling, all indicative of a carrier-mediated transport process. One candidate is the solute carrier 44A1 (SLC44A1), which mediates hemicholinium-3-sensitive choline transport at the plasma membrane (43). SLC44A1 has recently been detected in mitochondria from mouse and human tissue culture cells and mouse tissues (87) and therefore could be responsible for mitochondrial choline transport. However, immunoblot analysis indicated significant loss of SLC44A1 signal in mitoplasts as compared to whole mitochondria, suggesting possible outer membrane localization, thus leaving the role of this protein in choline transport into the mitochondrial matrix in question. Efflux of radiolabeled betaine from isolated rat liver mitochondria has also been studied, with results arguing against a specific carrier-mediated mechanism (113). Betaine efflux was not saturable within physiologically relevant concentrations, and betaine analogs and known mitochondrial transport inhibitors did not affect betaine transport.

Few reports have addressed the mitochondrial transport of dimethylglycine and sarcosine. However, Cybulski & Fisher (27) showed that neither sarcosine nor dimethylglycine was able to prevent *p*-mercuribenzoate inhibition of neutral amino acid transport. Therefore, it is likely that a separate mitochondrial carrier(s), other than that described for neutral amino acids, is responsible for dimethylglycine and sarcosine transport. Using isolated rat liver mitochondria, Porter et al. (113) measured the mitochondrial swelling rate in response to various concentrations of betaine, dimethylglycine, sarcosine, and glycine. This experiment provided virtually no evidence for facilitated diffusion of betaine (as mentioned above), but increased mitochondrial swelling was observed as the level of substitution on the α-amino group decreased. Currently there is no molecular information about the mitochondrial transporters for serine, glycine, dimethylglycine, sarcosine, or formate. Elucidation of the proteins involved in these transport processes is essential for complete understanding of the compartmentalization of 1C metabolism.

Mitochondrial transport of *S*-adenosylmethionine/*S*-adenosylhomocysteine. *S*-adenosylmethionine (AdoMet) is required for mitochondrial methylation reactions, including

the methylation of DNA, RNA, and proteins (1 and references therein). However, due to lack of methionine adenosyltransferase activity (55, 127), AdoMet cannot be synthesized in the mitochondria, suggesting the presence of a mitochondrial AdoMet carrier/transporter. The mechanism of AdoMet uptake in rat liver mitochondria was first investigated by Horne et al. (55), who demonstrated counter-transport of AdoMet and inhibition by *S*-adenosylhomocysteine (AdoHcy) and thus provided clear evidence of a carrier-mediated process. The human mitochondrial AdoMet transporter, *S*-adenosylmethionine (SAMC), was recently identified (1) based on sequence identity between the yeast AdoMet carrier and human expressed sequence tags (ESTs). This carrier, a 274 amino acid member of the mitochondrial carrier family, was overexpressed in *E. coli*, reconstituted in liposomes, and shown to catalyze the uptake of AdoMet using a counter-exchange mechanism. This carrier presumably exchanges cytosolic AdoMet for mitochondrial AdoHcy in vivo. Mitochondrial localization of human SAMC was verified by expression of the green fluorescent protein-tagged protein in CHO cells, and real-time PCR indicated SAMC mRNA expression in many human tissues, with highest expression in testis (1).

Mitochondrial Pathways

Folate-mediated 1C metabolism in mammalian mitochondria can be divided into three processes: transfer of 1C units from donors to the mitochondrial pool of THF; interconversion of the activated 1C unit carried by THF; and donation or release of 1C units from THF and their export to cytoplasm. Mitochondrial 1C flux is predominately in the oxidative direction, and mammalian mitochondria can oxidize 1C units derived from serine (6, 44), glycine (51), or sarcosine (6, 40, 75, 89) to formate or CO_2.

CH_2-THF production. At least four mitochondrial 1C donors (serine, glycine, dimethylglycine, and sarcosine) are known to generate activated 1C units at the level of CH_2-THF. The 3-carbon of serine is the major 1C donor in most organisms, including humans (31), and extensive studies by Kikuchi and coworkers demonstrated that the major pathway for serine and glycine catabolism in vertebrates is in mitochondria (65, 153). Serine is cleaved to CH_2-THF and glycine by the mitochondrial isozyme of SHMT (**Figure 2**, reaction 4m). The other product of the SHMT reaction, glycine, can also serve as a source of 1C units (72). It is broken down by the mitochondrially localized glycine cleavage system (GCS) (reaction 5), producing CH_2-THF from the 2-carbon of glycine. Glycine is also an important source of 1C units in yeast (99, 104). Dimethylglycine and sarcosine, products of choline oxidation, also produce the common intermediate CH_2-THF, in reactions catalyzed by dimethylglycine dehydrogenase (reaction 8) and sarcosine dehydrogenase (reaction 9), respectively.

mSHMT, encoded by the *SHMT2* gene, is expressed in most mammalian tissues (42, 47, 91, 94, 111) and is often the predominate SHMT activity (47, 79, 120). mSHMT is also expressed in embryos (140). The mitochondrial and cytoplasmic isozymes share >60% identity, existing as homotetramers of 52 kDa subunits (46). SHMT catalyzes the reversible transfer of C3 of serine to THF to form glycine plus CH_2-THF, as well as the THF-independent cleavage of 3-hydroxy-amino acids such as L-threonine and *allo*-threonine (122). The enzyme uses pyridoxal 5′-phosphate (PLP) to bind the amino acid substrates as aldimines (Schiff bases) and is proposed to catalyze a retro-aldol reaction involving a transient formaldehyde intermediate (122). mSHMT activity has been shown to be sensitive to dietary vitamin B-6 (pyridoxine) levels in rats (120), but marginal vitamin B-6 nutritional status appears to have little effect on flux through the mitochondrial SHMT reaction in humans (30). SHMT also catalyzes the irreversible hydrolysis of CH^+-THF to 5-formyl-THF, in a reaction that depends on bound glycine (132). Notably, the mSHMT from rabbit liver has significantly higher affinity for glycine than the

AdoHcy: *S*-adenosylhomocysteine

SAMC: *S*-adenosylmethionine mitochondrial carrier

ESTs: expressed sequence tags

GCS: glycine cleavage system

mSHMT: mitochondrial serine hydroxymethyltransferase

PLP: pyridoxal 5′-phosphate

NKH: nonketotic hyperglycinemia

SDH: sarcosine dehydrogenase

cytoplasmic isozyme (121); thus, mSHMT may be an important in vivo source of 5-formyl-THF. 5-Formyl-THF has been proposed to play a regulatory role in 1C metabolism, as it is an inhibitor of several folate-dependent enzymes, including SHMT (48, 133). Like many folate-dependent enzymes, mSHMT is sensitive to the polyglutamate chain length, exhibiting 200-fold tighter binding of the hexaglutamate form of THF relative to the monoglutamate form (K_d = 70 nM versus 14 μM, respectively) (134). The high affinity for polyglutamylated folate coenzymes is consistent with the long chain lengths (6–9 residues) typically found in mitochondria and suggests that mSHMT competes very well for limited mitochondrial THF.

Glycine is the second important mitochondrial 1C donor. Using [1,2-$^{13}C_2$]glycine, Lamers et al. (70, 72) showed that glycine cleavage is a major source of 1C units in humans. The metabolic fate of glycine-derived 1C units may be cell or tissue specific. Whole-body flux measurements in humans suggest that nearly all GCS-derived CH_2-THF ends up in serine via reversal of the mSHMT reaction (71, 72). On the other hand, the 2-carbon of glycine is readily oxidized to formate or CO_2 in mitochondria from rat liver (51, 52, 154), avian liver (154), and mouse embryos (111a).

The strictly mitochondrial glycine cleavage system (GCS) catalyzes the THF-, PLP-, and NAD-dependent oxidation of glycine to CO_2, NH_3, NADH, and CH_2-THF (66). GCS is a multienzyme complex composed of four subunits: P-protein, which catalyzes the PLP-dependent decarboxylation of glycine; T-protein, which catalyzes a THF-dependent aminomethyl transfer; L-protein, an FAD-dependent lipoamide dehydrogenase; and H-protein, a hydrogen carrier protein containing lipoic acid. The P-, T-, and H-protein subunits are unique to GCS, whereas the L-protein is shared with other mitochondrial α-ketoacid dehydrogenases including the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes. The reaction catalyzed by GCS is fully reversible and involves an aminomethyl moiety covalently bound to the lipoic acid cofactor of H-protein as a reaction intermediate. In the glycine cleavage direction, following release of glycine C1 as CO_2, the aminomethyl group (α-amino and C2 of glycine) is transferred from the lipoate thiol to THF to give CH_2-THF and NH_3. The dihydrolipoamide on the H-protein is then reoxidized by the L-protein, producing NADH. These electrons are delivered to Complex I of the mitochondrial respiratory chain, and flux through GCS is very sensitive to the state of the NAD^+/NADH redox couple (51, 52). The T-protein of human GCS exhibits a 10 μM K_m for the tetraglutamate of THF, 15-fold lower than for the monoglutamate (100). Like the mSHMT reaction, flux through GCS is resilient to the effects of marginal vitamin B-6 deficiency in humans (71). B-6 deficiency did cause increased tissue glycine levels, and it was suggested that this might counteract the effect of PLP depletion on GCS activity, thereby maintaining adequate glycine cleavage flux even during marginal vitamin B-6 status.

GCS function is probably required throughout life. Nonketotic hyperglycinemia (NKH) is an autosomal recessive brain disease caused by defects in subunits of the GCS, resulting in elevated glycine levels in cerebrospinal fluid, plasma, and urine (50). NKH typically occurs as a severe neonatal disorder, but some patients present during infancy or childhood (late onset). GCS is expressed during embryogenesis (60, 140), and the severe neurological symptoms exhibited within the first few days of life in NKH neonates suggest this activity is essential for normal brain development.

DMGDH and sarcosine dehydrogenase (SDH) are the other two enzymes that catalyze the production of mitochondrial CH_2-THF. Dimethylglycine and sarcosine are the final intermediates in the choline oxidation pathway (**Figure 2**, reactions 8 and 9). This pathway yields glycine, one methyl group for methionine synthesis, four pairs of electrons for the mitochondrial respiratory chain, and two 1C units in the form of CH_2-THF (45). DMGDH and SDH both catalyze the transfer of a methyl

group from the glycine α-amino group to THF to form CH_2-THF. In the process, the 1C unit is oxidized from the methyl to the methylene oxidation state, and the electrons are transferred to covalently bound FAD (9, 25, 74). DMGDH and SDH are linked to the mitochondrial respiratory chain via electron-transferring flavoprotein (ETF) and the ETF:ubiquinone reductase (57, 130). DMGDH and SDH were initially discovered as major mitochondrial folate-binding proteins, and both proteins are isolated with THF pentaglutamate tightly bound (150, 151). Both DMGDH and SDH can catalyze oxidative demethylation of their substrates in the absence of THF, producing free formaldehyde (130, 151). However, when polyglutamylated THF is present on the enzyme, the formaldehyde is trapped by the THF, and the 1C unit is released as the condensation product, CH_2-THF (134, 151). SDH and DMGDH are both expressed most highly in liver but are also present at lower levels in adult lung, kidney, pancreas, and brain (9, 73). DMGDH mRNA is also detectable in 14-day rat embryos (73). Thus, while choline oxidation takes place primarily in liver and kidney (45), the pathway may be more widely distributed than first thought.

All four of these enzymes (mSHMT, GCS, SDH, and DMGDH) have been shown to be partly or wholly associated with the inner membrane in liver mitochondria (9, 29, 91). As we discuss below, several other folate-dependent enzymes are also localized to the inner membrane, suggesting the existence of a large folate-dependent 1C metabolizing complex at the inner mitochondrial membrane.

CH_2-THF oxidation. Sarcosine and DMG (from choline), glycine, and serine are important sources of 1C units in mammals. A role for mitochondria in the metabolism of these 1C donors was first recognized nearly 60 years ago, when Mitoma & Greenberg (89) showed that rat liver mitochondria incorporated the *N*-methyl carbon of sarcosine into formate, formaldehyde, and serine. The 2-carbon of glycine was shown to produce CO_2 in mitochondria isolated from rat liver (51, 52, 154) and avian liver (154). The 3-carbon of serine was reported to be metabolized to formate in vivo (148) as well as in liver slices (129), but isolated mitochondria were not investigated in that work. Yoshida & Kikuchi (153) demonstrated that rat liver mitochondria could catalyze the oxidation of the 3-carbon of serine to CO_2, but they did not attempt to measure formate production. However, avian liver mitochondria could produce a 1C product from the 2-carbon of glycine that was incorporated into purines when a cytoplasmic extract was added to the mitochondria (154). Yoshida & Kikuchi proposed that in uricotelic animals, 1C units produced by mSHMT or GCS might emerge from the mitochondria to the cytoplasm to provide 1C units for purine synthesis, and they considered formate as a possible intermediate in this pathway (154).

As described in the previous sections, all of these mitochondrial 1C donors produce CH_2-THF, and this 1C unit can be oxidized by mitochondria to formate or CO_2. However, the enzyme(s) catalyzing this pathway in adult mammalian mitochondria remained unidentified until very recently. A trifunctional enzyme catalyzing CH_2-THF dehydrogenase, CH^+-THF cyclohydrolase, and 10-formyl-THF synthetase (termed C_1-THF synthase) was known to exist in the cytoplasm of eukaryotic cells [see (18) and references therein]. These three activities catalyze the reversible interconversion of CH_2-THF to THF + formate (**Figure 2**, reactions 1–3). CH_2-THF dehydrogenase and 10-formyl-THF synthetase activities had been reported in yeast mitochondria (157), but no mammalian mitochondrial enzyme was identified until MacKenzie and coworkers discovered a bifunctional NAD-dependent CH_2-THF dehydrogenase/CH^+-THF cyclohydrolase in ascites tumor cells (84, 86). This enzyme was shown to be a nuclear-encoded mitochondrial protein (7, 85) and might thus account for reactions 3m and 2m (**Figure 2**) in mammalian mitochondria. However, this enzyme is detectable only in transformed mammalian cells and embryonic

MEFs: mouse embryonic fibroblasts

or nondifferentiated tissues, but not adult differentiated tissues (19). Furthermore, because this enzyme lacks 10-formyl-THF synthetase activity (**Figure 2**, reaction 1m), it would not explain the reports of formate production in adult mammalian mitochondria (89, 154).

In 1988, Barlowe & Appling (6) showed that intact mitochondria isolated from adult rat liver readily oxidize the 3-carbon of serine or the *N*-methyl carbon of sarcosine to formate and CO_2 in a process that requires no additional cofactors. This indicated that all the enzymes and cofactors necessary for oxidation of the 1C unit carried by THF are present in mitochondria. The labeled formate exits the mitochondria rapidly and can be incorporated into purines by a cytoplasmic purine synthesizing system, confirming the earlier prediction of Yoshida & Kikuchi (154). When these mitochondria were subfractionated using digitonin, CH_2-THF dehydrogenase, CH^+-THF cyclohydrolase, 10-formyl-THF synthetase, and 10-formyl-THF dehydrogenase activities were detected in the matrix fraction (**Figure 2**, reactions 3m, 2m, 1m, and 11, respectively) (6). During this same period, Shannon & Rabinowitz (125) showed that the yeast *Saccharomyces cerevisiae* possesses a mitochondrial version of the trifunctional enzyme, encoded by the *MIS1* gene. Given the existence of a trifunctional C_1-THF synthase in yeast mitochondria, it seemed likely that mammalian mitochondria also possessed a trifunctional enzyme capable of catalyzing reactions 1m–3m (**Figure 2**). Based on all of these reports, Barlowe & Appling (6) proposed the model of eukaryotic one-carbon metabolism illustrated in **Figure 2**. Although many details of this model were confirmed in yeast (63, 105, 149) and adult rat liver (44), ultimate identification of the enzymes in adult mammalian mitochondria had to await sequencing of the human genome. **Figure 3** summarizes our current understanding of the mammalian family of enzymes that participate in intercompartmental 1C metabolism.

The cytosolic isozyme of the trifunctional C_1-THF synthase is encoded by the *MTHFD1* gene in mammals (20, 58, 80). C_1-THF synthase exists as a homodimer of ~100 kDa subunits. Each subunit consists of a ~30 kDa N-terminal domain that contains NADP-dependent CH_2-THF dehydrogenase and CH^+-THF cyclohydrolase activities, connected to a ~70 kDa C-terminal domain that contains the 10-formyl-THF synthetase activity (**Figure 3**) [see (19) and references therein]. The yeast mitochondrial trifunctional C_1-THF synthase (*MIS1* gene product) has this same domain structure (126). Cytosolic C_1-THF synthase is ubiquitously expressed in embryos (34) and adult mammals, but expression is highest in kidney and liver (16, 80, 139). *MTHFD1* is an essential gene, as homozygous disruption in mice results in embryonic lethality (80). *MTHFD1*$^{+/-}$ heterozygotes are viable but exhibit lower hepatic AdoMet levels and decreased uracil misincorporation into liver DNA. Mouse embryonic fibroblasts (MEFs) derived from *mthfd1*-null embryonic stem cells are purine auxotrophs (20), and MacFarlane et al. (80) propose that the embryonic lethality of *mthfd1*-null mice is due to inadequate de novo purine synthesis.

The mitochondrial, bifunctional NAD-dependent CH_2-THF dehydrogenase/CH^+-THF cyclohydrolase discovered by MacKenzie and coworkers is encoded by the *MTHFD2* gene in mammals (18). The human gene is located on chromosome 2 at 2p13.1. This protein is homologous to the N-terminal dehydrogenase/cyclohydrolase domain of the trifunctional C_1-THF synthases, existing as a ~34 kDa homodimer (**Figure 3**). The bifunctional MTHFD2 enzyme is proposed to have evolved from the trifunctional MTHFD1 through gene duplication and subsequent loss of the synthetase domain and change of the redox cofactor specificity from $NADP^+$ to NAD^+ (106). As described above, *MTHFD2* expression is restricted to embryonic and transformed cells and has been shown to be essential during embryonic development (33, 34). Embryonic lethality was proposed to be due to a defect in hematopoiesis in the embryonic liver.

MTHFD2 can account for reactions 3m and 2m (**Figure 2**) in embryos but not in adult

mitochondria. In 2003, Prasannan et al. (115) identified a human cDNA from the German Genome Project that encoded an open reading frame with high similarity to, but distinct from, human cytoplasmic C_1-THF synthase. The homology extended the length of the proteins, suggesting that this cDNA encoded another trifunctional C_1-THF synthase. The corresponding gene, *MTHFD1L*, spans 236 kbp on chromosome 6 and consists of 28 exons plus one alternative exon. The gene encodes a protein of 978 amino acids, including an N-terminal mitochondrial targeting sequence, and was shown to localize exclusively to mitochondria (**Figure 3**). The N-terminus is cleaved after residue 31 upon import (135) to produce a mature protein of 957 amino acids that resides in the matrix and inner mitochondrial membrane (114). The human mitochondrial isozyme is 61% identical to the human cytoplasmic isozyme (115) and shares the same domain structure (146). However, the sequence identity is considerably lower in the N-terminal domain (33%) than in the C-terminal synthetase domain (77%) (135). Christensen et al. (20) noted that several residues in the N-terminal domain involved in pyridine dinucleotide binding or critical for cyclohydrolase function are substituted in the human mitochondrial C_1-THF synthase, and they proposed that the MTHFD1L gene encodes a monofunctional 10-formyl-THF synthetase. This was subsequently confirmed when recombinant human MTHFD1L protein was purified and characterized (146). The enzyme exhibits a typical 10-formyl-THF synthetase activity but lacks CH_2-THF dehydrogenase and CH^+-THF cyclohydrolase activities. Like that of other C_1-THF synthases, the human mitochondrial 10-formyl-THF synthetase activity is very sensitive to polyglutamate chain length of the THF substrate. The K_m for THF decreases at least 100-fold in going from the mono- to the pentaglutamate. The K_m values for formate and ATP also decrease dramatically, and the k_{cat}/K_m values increase with increasing polyglutamate chain length (146). This increased catalytic efficiency with increasing polyglutamate chain length nicely matches the long chain lengths typically found in the folate pools of mammalian mitochondria (13, 77, 128) and may explain why formate production from serine in sonicated mitochondria shows a complete dependence on polyglutamylated THF (44).

The lack of CH_2-THF dehydrogenase and CH^+-THF cyclohydrolase activities in MTHFD1L leaves a gap in the pathway proposed for mitochondrial 1C metabolism in adult mammals. Recently, a fourth C_1-THF synthase homolog has been identified in the human genome (S. Bolusani, B. Young, N. Cole, A.S. Tibbetts, and D.R. Appling, manuscript in preparation). This gene, designated *MTHFD2L* (**Figure 3**), is present in all sequenced vertebrate genomes, including mouse and rat. The human gene is located on chromosome 4 at 4q13.3. The mammalian *MTHFD2L* gene is predicted to be composed of 8 exons, encoding a protein of about 338 amino acids homologous to the mitochondrial bifunctional dehydrogenase/cyclohydrolase (*MTHFD2* gene) and the N-terminal dehydrogenase/cyclohydrolase domain of C_1-THF synthase (**Figure 3**). The predicted MTHFD2L proteins possess the four critical dehydrogenase/cyclohydrolase residues (20) that are substituted in the monofunctional mitochondrial MTHFD1L, and the rat enzyme exhibits robust NAD^+-dependent CH_2-THF dehydrogenase activity (S. Bolusani, B. Young, N. Cole, A.S. Tibbetts, and D.R. Appling, manuscript in preparation). The rat *MTHFD2L* cDNA was expressed in CHO cells, and the protein localized exclusively to mitochondria. Very little information on *MTHFD2L* expression is available to date, but the gene appears to be expressed during embryogenesis and adult stages. The rat cDNA was cloned from adult brain RNA (S. Bolusani, B. Young, N. Cole, A.S. Tibbetts, and D.R. Appling, manuscript in preparation), and human *MTHFD2L* ESTs are detected in both fetal and adult tissues. These data suggest that the *MTHFD2L* gene encodes the missing CH_2-THF dehydrogenase/CH^+-THF cyclohydrolase activities in adult mitochondria.

Why do mammals possess two mitochondrial CH_2-THF dehydrogenases (MTHFD2 and MTHFD2L)? One possibility is that these two genes follow different developmental programs. *MTHFD2* expression is turned on early in embryogenesis, at least by embryonic day 4.5 (E4.5) in mice (111a), but then declines as the fetus approaches birth (34). We hypothesize that the *MTHFD2L* gene is turned on late in embryogenesis, replacing *MTHFD2* expression, thereby maintaining mitochondrial CH_2-THF dehydrogenase/CH^+-THF cyclohydrolase activities (**Figure 2**, reactions 3m and 2m) throughout development and adulthood. *MTHFD1L* is expressed continuously from E4.5 throughout fetal development (111a) and adulthood (115), supplying the essential 10-formyl-THF synthetase activity (**Figure 2**, reaction 1m) for mitochondrial formate production.

10-formyl-THF metabolism. As shown in **Figure 2**, 10-formyl-THF represents a branch point in mitochondria where the 1C unit can be converted to formate or CO_2. The formate branch, catalyzed by the 10-formyl-THF synthetase activity of MTHFD1L (reaction 1m), is discussed above. The CO_2 branch was proposed to be catalyzed by NADP-dependent 10-formyl-THF dehydrogenase (reaction 11), based on *in organello* oxidation experiments with rat liver mitochondria (44). Oxidation of the third carbon of serine to formate and CO_2 is dependent on the respiratory state of the mitochondria. CO_2 production is greatest in uncoupled mitochondria and lowest in respiratory-inhibited mitochondria. In soluble mitochondrial extracts, CO_2 production depends on $NADP^+$ and THF (44). 10-Formyl-THF dehydrogenase was initially characterized as an abundant cytoplasmic protein (69) and in fact represents a major cytosolic folate-binding protein (88). 10-Formyl-THF dehydrogenase activity was subsequently detected in mitochondria from rat liver (6), retina and brain (96). Barlowe & Appling further localized the mitochondrial activity to the matrix (6). Completion of the human genome sequence revealed a gene on chromosome 12 (*ALDH1L2*) homologous to, but distinct from, the gene encoding the cytoplasmic 10-formyl-THF dehydrogenase (*ALDH1L1*). This gene has now been shown to encode a mitochondrial isozyme of 10-formyl-THF dehydrogenase (S. Krupenko, personal communication). Like the cytoplasmic isozyme, the mitochondrial enzyme is composed of an N-terminal hydrolase domain and a C-terminal aldehyde dehydrogenase domain connected by an intermediate domain. The catalytic mechanism is a two-step process in which the N-terminal domain hydrolyzes the formyl group from THF, followed by NADP-dependent oxidation of the formyl group to CO_2 in the C-terminal domain (67). A 4′-phosphopantetheine "swinging arm" bound to the intermediate domain transfers the formyl group between the hydrolase and dehydrogenase catalytic domains (35). In the presence of exogenous thiols such as 2-mercaptoethanol or dithiothreitol (but not glutathione), 10-formyl-THF dehydrogenase can catalyze the hydrolysis of 10-formyl-THF to formate and THF (68, 88, 118, 124). If the mitochondrial enzyme exhibits this hydrolase activity in vivo, it would represent another mechanism to synthesize formate from 10-formyl-THF. However, given the high levels of exogenous thiols required for this activity (10–100 mM), it is not likely that this enzyme contributes significantly to mitochondrial formate production in vivo.

There is one other important fate of mitochondrial 10-formyl-THF: formylation of the initiator tRNA for mitochondrial protein synthesis. Initiation of protein synthesis in mitochondria normally uses a formylated initiator methionyl-tRNA (fMet-$tRNA_f^{Met}$) [see (76) and references therein]. 10-Formyl-THF serves as formyl donor in this reaction catalyzed by methionyl-tRNA formyltransferase (**Figure 2**, reaction 12), which is localized to mitochondria in all eukaryotes. Given the small number of mitochondrially synthesized proteins (13 in mammalian mitochondria), initiator tRNA formylation probably uses only a few percent of the total 1C units in mitochondria.

Enzyme Organization

As mentioned above, several of the mitochondrial folate-dependent enzymes have been shown to be partly or wholly associated with the inner mitochondrial membrane. Mitochondrial SHMT, GCS, SDH, and DMGDH (all of which produce CH_2-THF) have all been reported to be associated with the inner membrane in rat liver mitochondria (9, 29, 91). MTHFD2L (S. Bolusani, B. Young, N. Cole, A.S. Tibbetts, and D.R. Appling, manuscript in preparation) and MTHFD1L (114), enzymes required to convert CH_2-THF to formate, have also been found to tightly associate with the inner mitochondrial membrane. Finally, human mitochondrial FPGS, which adds glutamate residues to the mitochondrial folate pool, is also reported to be tightly associated with the inner membrane (93). 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. Considering all of these observations, it is tempting to hypothesize the existence of a large folate-dependent one-carbon metabolizing complex at the inner mitochondrial membrane, perhaps including membrane carriers for substrates (e.g., serine, formate, folate) and/or components of the respiratory chain where the redox cofactors are reoxidized (**Figure 4**).

NUCLEAR ONE-CARBON METABOLISM

Thymidylate synthase (TS) catalyzes the methylation of deoxyuridylate (dUMP) to form thymidylate (dTMP) using CH_2-THF as the 1C donor (**Figure 2**, reaction 10 and 10n). This reaction is unique in folate 1C transfers in that the THF carrier is oxidized to DHF, with the electrons being used to reduce the 1C unit to the methyl level. To reenter the active pool, DHF must be reduced back to THF, in a reaction catalyzed by DHFR (**Figure 2**, reaction 13). Stover and coworkers have recently presented evidence of folate-mediated dTMP synthesis in the nucleus (3, 152). They observed in HeLa and MCF-7 cells that cytoplasmic serine hydroxymethyltransferase (cSHMT), TS, and DHFR are all translocated into the nucleus during S and G2/M phases following modification by the small ubiquitin-like modifier (SUMO). These three enzymes constitute a dTMP synthesis cycle in which serine serves as 1C donor through the cSHMT reaction (**Figure 2**). Purified intact liver nuclei are able to catalyze the formation of tritiated dTMP from dUMP, NADPH, and [2,3-^{3}H]serine (2). TS and DHFR had previously been observed in nuclei as components of a putative replitase complex, perhaps as a mechanism to produce dTTP directly at the replication fork during S-phase (116). Inclusion of cSHMT in this complex provides the necessary source of 1C units and may explain previous results showing that cSHMT is rate-limiting for dTMP synthesis and preferentially supplies 1C units for dTMP synthesis at the expense of methyl group biogenesis in MCF-7 cells (53).

Because the tissue-specific expression of cSHMT is much more restricted than that of TS and DHFR, nuclear dTMP synthesis may not occur in all cells, at least not with serine as direct 1C donor. Indeed, *shmt1* knockout mice lacking cSHMT are viable and fertile, indicating that cSHMT is not an essential source of 1C units. However, liver nuclei from these *shmt1*-null animals exhibit residual SHMT-dependent dTMP synthesis, suggesting the existence of an alternative SHMT activity (2). It was shown that the *SHMT2* gene, encoding the mitochondrial SHMT isozyme, produces two transcripts. The shorter transcript lacks exon 1 and encodes an isoform of SHMT2 (SHMT2α) that localizes to cytoplasm and nuclei as well as mitochondria (2). Thus, SHMT2α may support nuclear dTMP synthesis in those tissues that lack SHMT1 expression and probably accounts for the viability of *shmt1*-null animals.

PHYSIOLOGY

Mitochondrial oxidation of the C3 of serine to formate and CO_2 is dependent on the respiratory state (6, 44), but the mechanisms

TS: thymidylate synthase

dUMP: deoxyuridylate

dTMP: thymidylate

cSHMT: cytoplasmic serine hydroxymethyltransferase

that underlie this dependence are not known. Redox and equilibrium considerations certainly favor the clockwise direction illustrated in **Figure 2**. In rat liver mitochondria, the redox potential of the NAD^+/NADH couple (approximately −300 mV) is considerably more positive (oxidizing) than the $NADP^+$/NADPH couple (approximately −400 mV) (145). Thus, as pointed out by Pelletier & MacKenzie (109), use of NAD^+ rather than $NADP^+$ by the CH_2-THF dehydrogenase activity of MTHFD2 and MTHFD2L (**Figure 2**, reaction 3m) shifts the equilibrium 60- to 200-fold in favor of 10-formyl-THF production in mitochondria. [This estimation includes the CH^+-THF cyclohydrolase reaction (**Figure 2**, reaction 2m) since at physiological pH, the equilibrium between CH^+-THF and 10-formyl-THF lies farther toward 10-formyl-THF (>90%; Reference 64)]. Release of formate from 10-formyl-THF at the ADP-dependent 10-formyl-THF synthetase step (**Figure 2**, reaction 1m) would be favored by the high ADP/ATP ratio maintained in mitochondria (12). Conversely, once formate exits the mitochondria, its activation back to 10-formyl-THF (**Figure 2**, reaction 1) would be favored by the high ATP/ADP ratio in the cytoplasm (145). The cytoplasmic $NADP^+$/NADPH redox couple is maintained in a reduced state (145), greatly favoring the reductive direction of NADP-linked dehydrogenases such as the CH_2-THF dehydrogenase of MTHFD1. The cytoplasmic and mitochondrial redox states exert considerable control over metabolic processes (145), but we do not yet understand how 1C flux responds to changes in redox state.

Cultured Cell Models

Glycine auxotrophy is a common phenotype of mammalian cell lines with defects in mitochondrial 1C metabolism (61, 62, 82), suggesting that glycine biosynthesis is primarily a mitochondrial process. For example, although SHMT is found in both the cytoplasm and mitochondria, loss of the mitochondrial isozyme alone results in glycine auxotrophy in *glyA* CHO cells (15, 138). Flux studies in wild-type and *glyA* CHO cells support the hypothesis that mSHMT is the primary pathway for glycine synthesis in mammals (95, 111). Another glycine-requiring CHO cell line, AUXB1 (*glyC*), is deficient in FPGS activity (137) and thus contains only monoglutamate forms of folate coenzymes (37). Even though these cells possess normal levels of cytoplasmic and mitochondrial SHMT, they are unable to synthesize adequate glycine. Shane's group (78) showed that expression of a bacterial FPGS in the mitochondria of these mutants restored mitochondrial folylpolyglutamate pools and overcame the glycine requirement. Likewise, *mthfd2*-null MEFs derived from *mthfd2*-null embryos (E9.5-E11.5) are auxotrophic for glycine (107). The authors speculated that lack of MTHFD2 CH_2-THF dehydrogenase and CH^+-THF cyclohydrolase activities prevents regeneration of THF in mitochondria of these cells, blocking production of glycine from serine by mSHMT. Radiolabeling experiments indicated that the *mthfd2*-null MEFs produce much less mitochondrial formate for incorporation into DNA than do wild-type MEFs, confirming the participation of the MTHFD2 CH_2-THF dehydrogenase/CH^+-THF cyclohydrolase activities (**Figure 2**, reactions 3m and 2m) in mitochondrial 1C production (107). These results suggest that the *mthfd2*-null MEFs do not express another mitochondrial CH_2-THF dehydrogenase (e.g., *MTHFD2L*). Stable isotope studies with a human breast cancer cell line (MCF-7) grown on ^{13}C- or deuterium-labeled serine showed that more than 90% of the 1C units incorporated into purines, dTMP, and methyl groups are derived from mitochondrial formate (41). Finally, as described above, the *glyB* CHO cell line was shown to be missing the mitochondrial folate transporter (83, 141). The fact that glycine is the only nutrient required to rescue growth of these cells suggests that glycine biosynthesis (via mSHMT) is the only folate-dependent process that absolutely requires mitochondrial 1C metabolism. De novo purine and dTMP biosynthesis, while preferring mitochondrial formate as 1C donor, can

apparently utilize cSHMT-derived 1C units, at least in this rodent cell line.

Animal Models

Further support for the mostly unidirectional flow of 1C units as shown in **Figure 2** comes from knockout mouse studies. For example, MacFarlane et al. (79) showed that mice lacking the cytoplasmic SHMT isozyme (*SHMT1* gene product) are viable and fertile, suggesting that cSHMT is not an essential source of 1C units. Instead, it is proposed that in these animals serine donates its 1C unit in mitochondria via mSHMT, producing CH_2-THF, which is then oxidized to formate via reactions 3m, 2m, and 1m (**Figure 2**). This formate would then exit the mitochondria and flow into the cytoplasmic THF pool via the 10-formyl-THF synthetase activity of C_1-THF synthase (MTHFD1) for use in cytoplasmic processes such as de novo purine and thymidylate biosynthesis. However, as noted above, closer evaluation of these *shmt1*-null mice revealed that the *SHMT2* gene, encoding the mitochondrial SHMT isozyme, encodes an isoform of SHMT2 (SHMT2α) that localizes to cytoplasm and nuclei as well as mitochondria (2). The contribution that this cytoplasmically localized mSHMT makes to the cytoplasmic 1C pool remains to be determined.

In contrast to *SHMT1*, the *MTHFD1* gene is essential in mice (80). Loss of all three activities of this enzyme would prevent production of 10-formyl-THF in the cytoplasm, and the embryonic lethality of *mthfd1*-deficient mice is attributed to inadequate purine synthesis (80). The gene trap insertion in the *MTHFD1* gene disrupts the C-terminal 10-formyl-THF synthetase domain of the enzyme and was shown to eliminate the synthetase activity, whereas the CH_2-THF dehydrogenase activity was essentially normal in the fusion protein. Homozygous *mthfd1* gene trapped mice would be expected to retain the CH_2-THF dehydrogenase and CH^+-THF cyclohydrolase activities of the N-terminal domain and should thus be able to oxidize cytoplasmic CH_2-THF, if it were available, to 10-formyl-THF to support purine synthesis. The embryonic lethality of these animals is consistent with a critical role for mitochondrial formate as 1C donor in purine synthesis. However, it was noted that the gene trapped fusion protein is present at very low levels (<10% of wild type) in heterozygotes (80), so this gene trapped allele might behave essentially as a null allele, with all three cytoplasmic C_1-THF synthase activities deficient.

The *MTHFD2* gene is also essential in mice (33). Homozygous *mthfd2*-null mice exhibit a failure of erythropoiesis in the fetal liver as early as E12.5 and begin to die by E13.5. Hypoxanthine stimulated the proliferation of *mthfd2*-null MEFs derived from *mthfd2*-null embryos (107), suggesting that inefficient de novo purine synthesis underlies the defect in erythropoiesis (34). Inefficient purine synthesis is presumably caused by a block in mitochondrial formate production in the *mthfd2*-null mice due to loss of the MTHFD2 CH_2-THF dehydrogenase/CH^+-THF cyclohydrolase activities (**Figure 2**, reactions 3m and 2m). As mentioned above, these results imply that the *mthfd2*-null MEFs do not express another mitochondrial CH_2-THF dehydrogenase (e.g., *MTHFD2L*) and are consistent with the proposed developmental switch from *MTHFD2* to *MTHFD2L* expression during late embryogenesis (i.e., around E13) (111a).

Human Studies

Stable isotope flux studies in humans also support the model shown in **Figure 2**. Gregory et al. (49) showed in adult humans that serine-derived 1C units can flow through the mitochondria, in the direction of CH_2-THF to 10-formyl-THF to formate, and can be incorporated into methionine. Whole-body flux studies with glycine reveal that the mitochondrial GCS produces CH_2-THF at a rate that is ~20 times greater than the methylation demand in healthy adults (71, 72). Most of these 1C units are used to synthesize serine or are oxidized to CO_2, with the remainder presumably going into purines, dTMP, and homocysteine remethylation. Although the contribution of

NTDs: neural tube defects

SNP: single nucleotide polymorphism

serine and glycine to mitochondrial 1C pools is certainly cell and tissue specific, these studies indicate that the flux is considerable in humans.

Although serine and glycine are quantitatively the most important 1C donors, there is considerable flux through DMGDH and SDH from choline oxidation. Inborn errors in SDH and DMGDH have shed some light on the contribution of choline oxidation to the 1C pool. Deficiency of SDH in humans is characterized by increased levels of sarcosine in plasma and urine (123). In a clinical study on patients with SDH deficiency (sarcosinemia), Mudd et al. (92) estimated that basal flux is approximately 2 mmol sarcosine produced from choline every 24 h. To date, only one case of DMGDH deficiency has been confirmed (11, 90). This patient had an unusual fish-like body odor and accumulated DMG in plasma and urine, again consistent with a significant flux of 1C units from choline through this enzyme in normal animals.

The importance of the mitochondrial-cytoplasmic 1C cycle in humans is also revealed by recent genetic analyses. Common polymorphisms in the *MTHFD1L* gene are strongly associated with coronary artery disease (22, 119) and neural tube defects (NTDs) (103). Surprisingly, all of these polymorphisms are located in introns in the middle of the *MTHFD1L* gene (introns 7–13). The lead single nucleotide polymorphism (SNP) in the coronary artery disease study [rs6922269 (G/A) in intron 11] exhibited an increased odds ratio of 1.24 for the risk allele (A) (119). Although the individual risk attributed to the disease-associated allele is relatively modest, the A allele has a prevalence of about 25% and thus could contribute substantially to overall coronary artery disease in the population. These intronic SNPs may not be directly causal, but rather simply inherited in the same haploblock that carries an unidentified disease-causing mutation/polymorphism. Nonetheless, all of the positive risk markers fall within introns, not exons. Intronic variation can affect splicing efficiency or accuracy by altering the binding of splicing factors that enhance or silence splicing. One can further imagine effects on transcript stability or on alternative splicing. Indeed, Parle-McDermott et al. (103) identified a common deletion/insertion polymorphism (rs3832406) in *MTHFD1L* that influences alternative splicing and is strongly associated with NTD risk in an Irish population. This deletion/insertion polymorphism is located at the 3′ end of intron 7, in the polypyrimidine tract adjacent to an alternatively spliced exon. Human *MTHFD1L* produces two transcripts, 3.6 kb and 1.1 kb. The long transcript encodes the full-length active enzyme; the short transcript results from an alternative splicing event, where exon 7 is spliced to exon 8a instead of exon 8. Exon 8a is derived from an exonized *Alu* element and contains a premature stop codon and polyadenylation signal (115). The short transcript thus encodes a truncated protein lacking the 10-formyl-THF synthetase domain (114). Both transcripts are detected in most human tissues, but the ratio differs from tissue to tissue (115). Three deletion/insertion alleles were detected with varying numbers of ATT repeats. In lymphoblast cell lines homozygous for the most common alleles, the ATT_7 allele was associated with a higher ratio of long-to-short transcript relative to the ATT_8 allele, suggesting a functional effect on alternative splicing of exon 8a (103). Surprisingly, the allele associated with the highest expression of the long transcript (ATT_7), and therefore potentially more functional mitochondrial 10-formyl-THF synthetase activity, is the risk allele for NTDs. It is not obvious why increased expression of the full-length transcript would increase NTD risk.

A common SNP in the *MTHFD1* gene encoding the cytoplasmic C_1-THF synthase has been found to be a maternal risk factor for NTDs in several populations (32, 102), but not all (144). This polymorphism, which causes an Arg to Gln substitution at codon 653 (R653Q), is also associated with increased risk of bipolar disorder, schizophrenia, and migraine [see (21) and references therein] and may also be involved in the pathogenesis of Alzheimer's and Parkinson's diseases (36). Christensen et al. (21) have recently shown that the R/Q polymorphism at position 653 affects the stability of

the purified human protein, with the Q variant being more thermolabile than the wild-type variant. Furthermore, cells expressing the Q variant exhibited reduced flux of formate into DNA. They also found that the homozygous 653QQ genotype in children was associated with increased risk for congenital heart defects. A second nonsynonymous SNP of the cytoplasmic C_1-THF synthase, R134K, has been associated with a significant increase in risk for postmenopausal breast cancer (131).

OPEN QUESTIONS

These disease associations clearly point out the importance of the mitochondrial-cytoplasmic one-carbon cycle in mammals, including humans. But many fundamental questions remain to be answered. How is flux through the mitochondrial-cytoplasmic one-carbon cycle controlled? Can rate-limiting steps be defined (transport, oxidation, etc.)? There is some evidence that expression of the enzymes in this pathway is coordinated during embryogenesis, but the details remain to be worked out. Likewise, we know very little about the regulation of these genes in response to changes in nutritional status of the animal. Why do mammals use separate enzymes [MTHFD2 (or MTHFD2L) + MTHFD1L] to catalyze the mitochondrial pathway whereas yeast use a single trifunctional enzyme?

With the exception of the mitochondrial folate carrier, none of the mitochondrial transporters that support this intercompartmental 1C cycle have been identified yet. Carriers for serine, glycine, formate, dimethylglycine, and sarcosine need to be identified and characterized. What kind of substrate specificity do they exhibit? What is the actual form of folate transported by mitochondria? How are these carriers regulated? Is their expression coordinated with that of the interconverting enzymes?

Although in vitro studies have provided many insights into the mitochondrial-cytoplasmic one-carbon cycle, to fully test this model and determine the role of this family of enzymes in health and disease (both during embryonic development and adult stages), studies with knockout mice are warranted. Knockout mouse models for the cytoplasmic enzymes SHMT (79) and MTHFD1 (80) have shed considerable light on the operation of this cycle in vivo. Generation and analysis of knockout mouse models for the mitochondrial MTHFD1L and MTHFD2L enzymes will likewise contribute to better understanding of mammalian folate-mediated 1C metabolism.

Finally, we have discussed the possibility of a large folate-dependent one-carbon metabolizing complex localized to the matrix side of the inner mitochondrial membrane (**Figure 4**). An organized multienzyme complex might facilitate channeling of substrates or coordination of interconversion of the 1C units with transport of the donors. Do any of these enzymes physically interact with each other? Because none of these enzymes are integral membrane proteins, it is likely that they interact with other membrane-associated proteins, such as inner membrane transporters or respiratory chain components. These binding partners remain to be identified.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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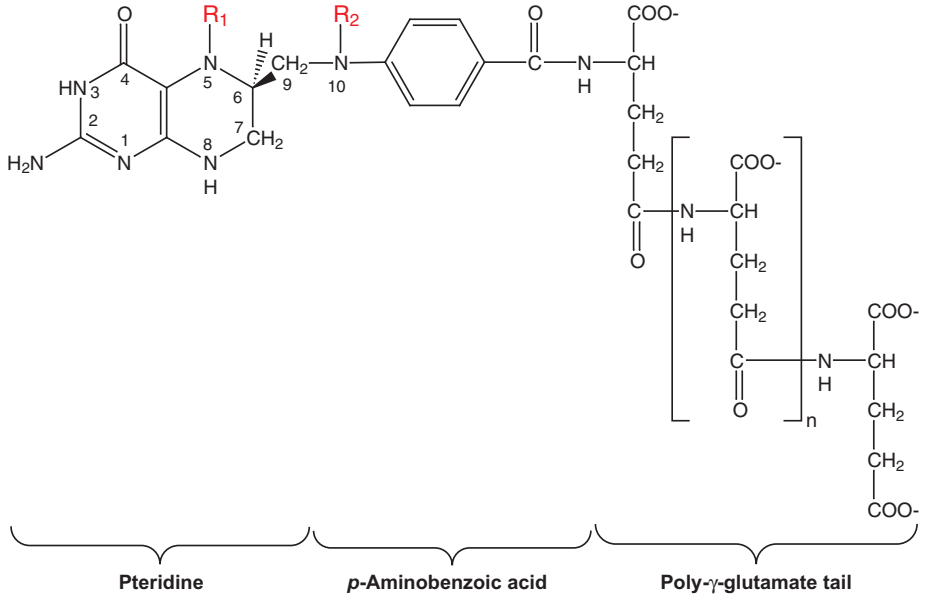

Figure 1

Structure of tetrahydrofolate. Activated one-carbon units are carried at N5 and/or N10 (R_1 and R_2). The 6*S*-isomer (shown here) is the naturally occurring form used by enzymes.

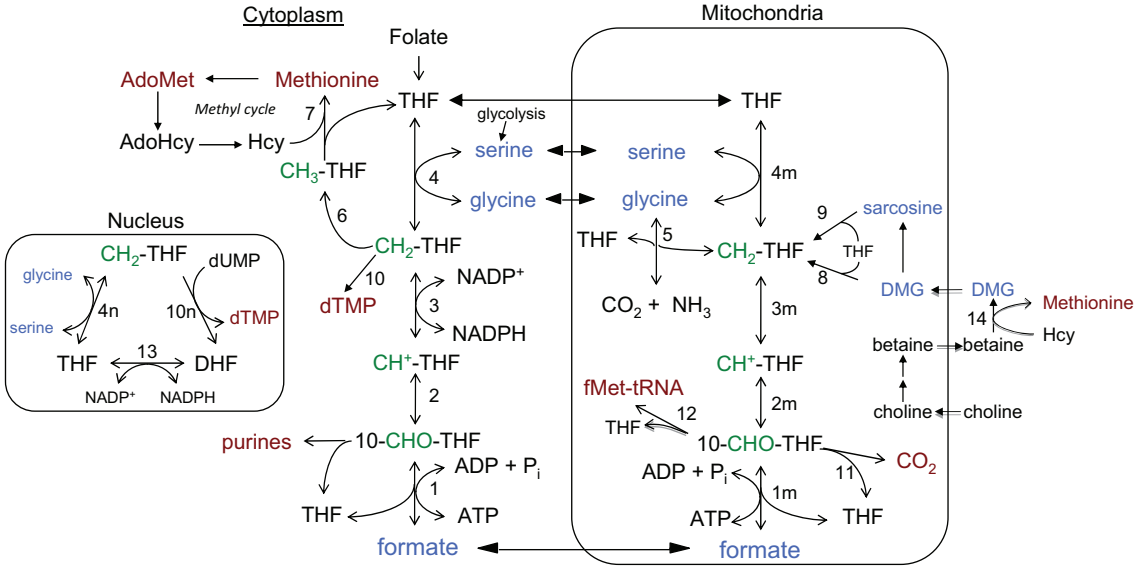

Figure 2

Compartmentalization of mammalian one-carbon metabolism. End products of one-carbon metabolism are in red. One-carbon donors are in blue. Activated one-carbon units carried by tetrahydrofolate (THF) are in green. Reactions 1–4 are in both the cytoplasmic and mitochondrial (m) compartments. Reactions 4 and 10 are also present in the nucleus (n). Reactions 1, 2, and 3: 10-formyl-THF synthetase, 5,10-methenyl-THF (CH^+-THF) cyclohydrolase, and 5,10-methylene-THF (CH_2-THF) dehydrogenase, respectively, are catalyzed by trifunctional C_1-THF synthase in the cytoplasm (MTHFD1). In mammalian mitochondria, reaction 1m is catalyzed by monofunctional MTHFD1L and reactions 2m and 3m by bifunctional MTHFD2 or MTHFD2L. The other reactions are catalyzed by the following: 4, 4n, and 4m, serine hydroxymethyltransferase; 5, glycine cleavage system; 6, 5,10-methylene-THF reductase; 7, methionine synthase; 8, dimethylglycine (DMG) dehydrogenase; 9, sarcosine dehydrogenase; 10 and 10n, thymidylate synthase; 11, 10-formyl-THF dehydrogenase (only the mitochondrial activity of this enzyme is shown, but it has been reported in both compartments in mammals); 12, methionyl-tRNA formyltransferase; 13, dihydrofolate (DHF) reductase; 14, betaine-homocysteine methyltransferase. AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; Hcy, homocysteine.

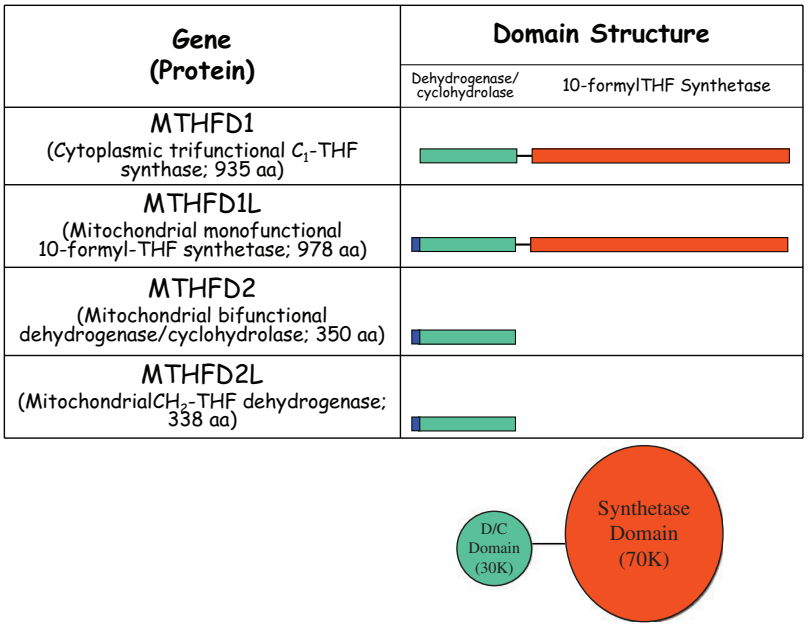

Figure 3

Mammalian gene family of one-carbon interconverting enzymes. *MTHFD1* encodes a cytoplasmic trifunctional enzyme composed of two domains, a ~30 kDa N-terminal D/C domain (*green*), which contains NADP-dependent CH_2-THF dehydrogenase and CH^+-THF cyclohydrolase activities, connected to a ~70 kDa C-terminal domain (*orange*), which contains the 10-formyl-THF synthetase activity. *MTHFD1L* encodes a mitochondrial protein with the same two-domain structure, but with an N-terminal mitochondrial targeting sequence (*blue*). The D/C domain of *MTHFD1L* is catalytically inactive in this monofunctional 10-formyl-THF synthetase. *MTHFD2* and *MTHFD2L* both encode bifunctional NAD-dependent CH_2-THF dehydrogenase/CH^+-THF cyclohydrolases that are localized to mitochondria. These proteins are homologous to the D/C domain of *MTHFD1*.

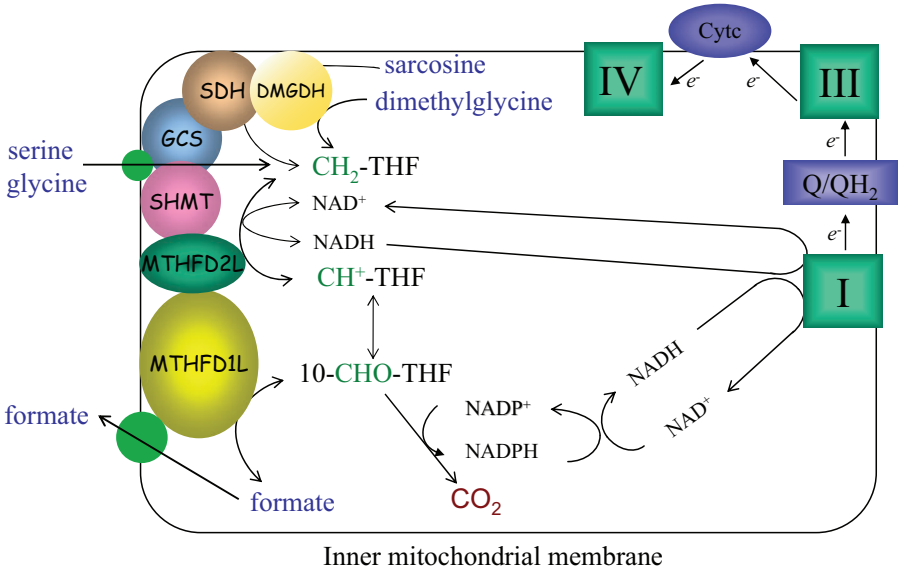

Figure 4

Proposed organization of folate-dependent one-carbon metabolism in adult mammalian mitochondria. Mitochondrial SHMT (serine hydroxymethyltransferase), GCS (glycine cleavage system), SDH (sarcosine dehydrogenase), DMGDH (dimethylglycine dehydrogenase), MTHFD2L, and MTHFD1L have all been shown to be partly or wholly associated with the matrix side of the inner mitochondrial membrane. SHMT, GCS, SDH, and DMGDH each produce CH_2-THF from serine, glycine, sarcosine, and dimethylglycine, respectively. MTHFD2L catalyzes the oxidization of CH_2-THF to 10-formyl-THF, and MTHFD1L catalyzes production of formate. Alternatively, 10-formyl-THF dehydrogenase catalyzes oxidization of 10-formyl-THF to CO_2. The CH_2-THF and 10-formyl-THF oxidation reactions are linked to electron transport through Complex I of the respiratory chain. The FAD (flavin adenine dinucleotide)-dependent SDH and DMGDH reactions are linked to electron transport through the coenzyme Q pool. Predicted inner membrane carriers for serine, glycine, and formate are hypothesized as components of the multiprotein complex.

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Errata

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