Regulation of de Novo Purine Biosynthesis by Methenyltetrahydrofolate Synthetase in Neuroblastoma*

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5-Formyltetrahydrofolate (5-formylTHF) is the only folate derivative that does not serve as a cofactor in folate-dependent one-carbon metabolism. Two metabolic roles have been ascribed to this folate derivative. It has been proposed to 1) serve as a storage form of folate because it is chemically stable and accumulates in seeds and spores and 2) regulate folate-dependent one-carbon metabolism by inhibiting folate-dependent enzymes, specifically targeting folate-dependent de novo purine biosynthesis. Methenyltetrahydrofolate synthetase (MTHFS) is the only enzyme that metabolizes 5-formylTHF and catalyzes its ATP-dependent conversion to 5,10-methenylTHF. This reaction determines intracellular 5-formylTHF concentrations and converts 5-formylTHF into an enzyme cofactor. The regulation and metabolic role of MTHFS in one-carbon metabolism was investigated in vitro and in human neuroblastoma cells. Steady-state kinetic studies revealed that 10-formylTHF, which exists in chemical equilibrium with 5,10-methenylTHF, acts as a tight binding inhibitor of mouse MTHFS. [6R]-10-formylTHF inhibited MTHFS with a Kᵣ of 150 nM, and [6R,S]-10-formylTHF triglutamate inhibited MTHFS with a Kᵣ of 30 nM. MTHFS is the first identified 10-formylTHF tight-binding protein. Isotope tracer studies in neuroblastoma demonstrate that MTHFS enhances de novo purine biosynthesis, indicating that MTHFS-bound 10-formylTHF facilitates de novo purine biosynthesis. Feedback metabolic regulation of MTHFS by 10-formylTHF indicates that 5-formylTHF can only accumulate in the presence of 10-formylTHF, providing the first evidence that 5-formylTHF is a storage form of excess formylated folates in mammalian cells. The sequestration of 10-formylTHF by MTHFS may explain why de novo purine biosynthesis is protected from common disruptions in the folate-dependent one-carbon network.

Tetrahydrofolates (THF) serve as a family of cofactors that carry and activate one-carbons for the synthesis of purines and thymidylate and for the remethylation of homocysteine to methionine (Figs. 1 and 2) (1). Methionine can be adenylated to form S-adenosylmethionine, which serves as a cofactor and methyl donor for numerous cellular reactions, including histone, DNA, RNA, phospholipid, and catecholamine methylation (2–4). Disruptions in the folate-mediated one-carbon metabolic network are common and result from single nucleotide polymorphisms in genes that encode folate-dependent enzymes, from low intracellular folate concentrations, and/or from other environmental factors (5, 6). De novo thymidylate synthesis and homocysteine remethylation are highly sensitive to network disruptions, which result in increased incorporation of dUTP into DNA, elevated cellular homocysteine, and impaired S-adenosylmethionine-dependent methylation reactions. De novo purine biosynthesis is less sensitive to network disruptions, although the mechanisms for this protection are unknown. Impaired folate metabolism increases risk for pathologies and developmental anomalies, including epithelial cancers, cardiovascular disease, and neural tube defects (7). Because of its role in nucleotide biosynthesis and cellular methylation reactions, this network continues to be an attractive target for the development of antiproliferative drugs (8–10).

In the cell, folates differ by the reduction state of the pteridine ring, one-carbon substitution at the N5 and/or N10 positions, and the length of the glutamate polypeptide, which can range from 1 to 9 glutamate residues linked through γ-peptide linkages (Fig. 1) (1). Folates are transported across cell membranes as monoglutamate derivatives that are converted to folate polyglutamates by the enzyme folylpolyglutamate synthetase (1, 11). The polyglutamate chain serves both to retain folates within the cell and to increase the affinity of folate derivatives for folate binding enzymes (1). Typically, folate polyglutamates bind to proteins two to three orders of magnitude tighter than corresponding monoglutamate forms (12).

There are five naturally occurring one-carbon-substituted forms of THF (Fig. 2). 5-MethylTHF carries the one-carbon unit at the oxidation level of methanol for the remethylation of homocysteine to methionine. 5,10-MethyleneTHF carries the one-carbon unit at the oxidation level of formaldehyde for thymidylate biosynthesis. Three THF derivatives, 10-formylTHF, 5-formylTHF, and 5,10-methenylTHF, carry the one-carbon unit at the oxidation level of formate. Of these forms, only 10-formylTHF is an enzyme cofactor and supplies the number 2 and number 8 carbons of the purine ring. Folate-dependent biosynthetic enzymes show absolute substrate specificity for a single folate cofactor. However, other one-carbon-substituted forms often function as potent enzyme inhibitors that regulate flux through the network (1, 13).

Although 5-formylTHF is the most stable natural folate, little is known about its physiological role and regulation in mammalian systems. 5-FormylTHF is synthesized from 5,10-methenylTHF in a reaction catalyzed by serine hydroxymethyltransferase (14–18). 5,10-Methenyltetrahydrofolate synthetase (MTHFS, EC 6.3.3.2) is the only enzyme that metabolizes 5-formylTHF and irreversibly catalyzes its ATP-dependent cyclization to 5,10-methenylTHF. In prokaryotes, there is evidence that 5-formylTHF is a storage form of folate and that its accumulation in dormant cells, including seeds and spores, is mediated by alterations in MTHFS expression (19, 20). However, 5-formylTHF is not known to account for more than 10–15% of total folate in mammalian cells (19). In mammalian systems, 5-formylTHF has been shown to inhibit several folate-dependent enzymes. 5-FormylTHF polygluta-
mates are tight binding inhibitors of serine hydroxymethyltransferase and phosphoribosylaminomimidazole carboxamide formyltransferase (21, 22). In addition to catalyzing 5-formylTHF synthesis, serine hydroxymethyltransferase also catalyzes the interconversion of serine and THF to glycine and methyleneTHF, a reaction that generates one-carbon units for purine, thymidine, and methionine biosynthesis (21). Phosphoribosylaminomimidazole carboxamide formyltransferase catalyzes the incorporation of formate into the C2 position of the purine ring. Inhibition of MTHFS in human MCF-7 cells by exposure to the MTHFS antifolate inhibitor, 5-formyltetrahydrohomofolate (5-formylTHHF), results in an accumulation of cellular folate as 5-formylTHF. This creates a purine auxotrophy because elevated levels of 5-formylTHF inhibit phosphoribosylaminomimidazole carboxamide formyltransferase (22). Therefore, it has been proposed that alterations in cellular 5-formylTHF concentrations, mediated through changes in MTHFS activity, may regulate purine biosynthesis (19). In this study, we elucidated a mechanism for feedback metabolic regulation of MTHFS by 10-formylTHF and provide evidence that this regulatory mechanism supports a role for 5-formylTHF as storage form of folate in mammalian cells. Furthermore, we discovered a novel role for MTHFS-bound 10-formylTHF in the regulation of de novo purine biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials—**MES, HEPES, and Tris were purchased from Sigma. ATP was purchased from Roche Applied Science. [6S]-5-formylTHF (the natural isomer) and [6R,S]-5-FormylTHF were a generous gift from Eprova AG. Folic acid, 10-formylfolic acid, [6R,S]-5-formyltetrahydropterate, 6-methylpterin, 5-formyltetrahydrofolate triglutamate, and folic acid polyglutamates were from Schircks Laboratories. Homofolate (NSC 79249) was a generous gift from Dr. Roy Kisluk, Tufts University.

![Figure 1. Structure of 5-formyltetrahydrofolate diglutamate (5-formylTHFGlu2). pABA, p-aminobenzoic acid.](image1.png)

**Figure 1.** The folate-dependent one-carbon metabolic network in the cytoplasm. The products of one-carbon metabolism are underlined. THF, tetrahydrofolate; MTHFS, methenyltetrahydrofolate synthetase; cSHMT, cytoplasmic serine hydroxymethyltransferase; AdoMet, S-adenosylmethionine; GARFT, glycaminide ribonucleotide formyltransferase; AICARFT, phosphoribosylaminomimidazole carboxamide formyltransferase.

![Figure 2.](image2.png)

**Figure 2.** Methylation reactions (DNA, RNA, histones, phospholipids, etc.)
TABLE 1
Substrate specificity of MTHFS

All reactions were carried out at 37 °C in 100 mM MES, pH 6.0.

| Substrate                        | $K_{cat}$ | $k_{cat}/K_{m}$ |
|----------------------------------|-----------|-----------------|
| [6R, S]-5-formylTHF pterate       | 33 ± 10   | 60 ± 20         | 1.8 |
| [6S]-5-formylTHF                  | 10 ± 3    | 64 ± 16         | 6.4 |
| [6R, S]-5-formylTHF Glu$_3$       | 0.4 ± 0.1 | 90 ± 30         | 225.0 |

All other materials were of high quality and obtained from various commercial vendors.

Synthesis of Folate and Antifolate Derivatives—[6R]-10-formylTHF (the natural isomer) was synthesized from [6S]-5-formylTHF as described previously (23). 5-Formyl, 6-methyltetrahydropterin and 5-formyltetrahydrohomofolic acid were synthesized from 6-methylpterin and homofolic acid, respectively, as described by others (11). Both homofolic acid and 6-methylpterin were reduced to tetrahydrohomofoic acid and homofolic acid, respectively, as described by others (11). Both 5-formyltetrahydrohomofolic acid were synthesized from 6-methylpterin and homofolic acid, respectively, by reduction over homofolic acid and 6-methylpterin were reduced to tetrahydrohomofoic acid and homofolic acid, respectively, as described by others (11).

TABLE 2
Inhibition of MTHFS

All reactions were carried out at 37 °C in 100 mM MES, pH 6.0 in the presence of 10 µM [6S]-5-formylTHF.

| Inhibitor         | $K_{i}$ |
|-------------------|---------|
| Folic acid        | 58 ± 5  |
| Folic acid Glu$_3$| 17 ± 3  |
| Folic acid Glu$_5$| 1.0 ± 0.3|
| 10-Formylfolic acid| 13 ± 6 |
| [6R, S]-10-formylTHF pterate| 5 ± 2 |
| [6R]-10-formylTHF | 0.15 ± 0.09|
| [6R, S]-10-formylTHF Glu$_3$ | 0.03 ± 0.01 |
| [6R, S]-5-formylTHF | 0.7 ± 0.3 |
| [6R, S]-5-formylTHF Glu$_3$ | 0.20 ± 0.02 |

RESULTS

Determinants of MTHFS Substrate Specificity—The contribution of all folate chemical moieties to substrate binding and catalysis was elucidated for recombinant murine MTHFS (Table 1 and Fig. 1). [6R, S]-5-formylTHF Glu$_4$, an endogenous form of cellular folate, is a substrate for murine MTHFS ($K_{cat}$ = 0.4 µM; $k_{cat}/K_{m}$ = 225). Previous studies have shown that the unnatural [6R]-5-formylTHF is not a substrate or inhibitor of mammalian MTHFS enzymes; therefore, the $K_{m}$ value for [6S]-5-formylTHF Glu$_4$ is likely 0.2 µM (33, 34). The $K_{cat}$ for [6S]-5-formylTHF is 10 µM ($k_{cat}/K_{m}$ = 6.0), in agreement with values determined for rabbit and human MTHFS (22, 33). Therefore, the loss of the diglutamate chain increases the $K_{m}$ by 25-fold and decreases the substrate specificity ($k_{cat}/K_{m}$) by 97%. [6R, S]-5-formyltetrahydropterate, an unnatural derivative that lacks a glutamate moiety, is also a substrate ($K_{m}$ = 33 µM; $k_{cat}/K_{m}$ = 1.8), indicating that the Glu$_1$ moiety of 5-formylTHF makes only minor contributions to the $K_{m}$ and substrate specificity ($k_{cat}/K_{m}$).

Inhibition of MTHFS—Previous studies of human MTHFS protein have demonstrated that folic acid, THF, and 5-methylTHF are weak competitive inhibitors of human MTHFS (34). Inhibition of MTHFS by 10-formylTHF has never been investigated. The inhibition of recombinant murine MTHFS by folic acid and 10-formyl-substituted folate
derivatives was examined and compared with inhibition by the synthetic antifolate and known MTHFS competitive inhibitor, 5-formylTHHF (Table 2). 5-FormylTHHF inhibited MTHFS activity with a $K_i$ of 700 nM. Folic acid, an oxidized and unnatural form of folate present in vitamin supplements, weakly inhibited MTHFS activity ($K_i$, 58 $\mu$M). The $K_i$ for folic acid triglutamate and pentaglutamate was reduced by 70 and 98%, respectively, compared with folic acid; the $K_i$ for folic acid pentaglutamate was similar to that determined for 5-formylTHHF. The tighter binding resulting from the addition of the polyglutamate binding site as a critical determinant of inhibitor affinity. N10-Formyl substitution markedly decreased the $K_i$ for folate derivatives; inhibition of all N10-substituted folates was competitive with the substrate, [6S]-5-formylTHF. N10-Formylation of folic acid decreased the $K_i$ value by nearly 80%. Similarly, [6R,S]-10-formylTHHF was a more effective inhibitor than [6R,S]-5-formylTHHF (Table 2). The $K_i$ for N10-formyl folates decreased with glutamate chain length; [6R]-10-formylTHF inhibited MTHFS with a $K_i$ of 150 nM, whereas [6R,S]-10-formylTHF triglutamate inhibited MTHFS with a $K_i$ of 30 nM. These results demonstrate that both N10-formyl substitution and the glutamate chain increase the affinity of folates for MTHFS. The effective inhibition of MTHFS by [6R]-10-formylTHF polyglutamate, a naturally occurring folate derivative, indicates that 10-formylTHF regulates MTHFS activity in vivo.

Structural Analysis of MTHFS Active Site—The murine MTHFS model shows the calculated solvent-accessible surface colored according to electrostatic potential (Fig. 3, blue for positive and red for negative). The positioning of the substrate, 5-formylTHF (in green), is iden-
tical to that observed in the active site of the *M. pneumoniae* crystal structure of the MTHFS-ADP-P;5-formylTHF complex. In this model, the MTHFS N terminus is at the lower left, the entrance to the ATP binding site is on the left side, and the view is directly into the 5-formylTHF binding site. All the inserted loops (i.e. the poorly modeled portions of the structure) are in the top half of the molecule. The positively charged region at the bottom of the structure is the putative polyglutamate binding site. In this model, the conformation of 5-formylTHF exposes the γ-carboxyl of the 5-formylTHF glutamate moiety to the solvent, but this part of the molecule was not well defined in the crystal structure (zero occupancies are given in the Protein Data Bank for all atoms past C9). Adjustment of the 5-formylTHF model using O (35) reveals that it is easily possible for hydrogen-bonding interactions to be made between polar atoms in the substrate glutamate moiety and the side chains of residues Lys-17 and Arg-147. Glu-62 could also be part of a network of H-bonds involving 5-formylTHF and the protein surface. All three of these residues are conserved in known mammalian MTHFS sequences, and Lys-17 of rabbit liver MTHFS can be cross-linked to the 5-formylTHF Glu1 (36). The lack of specific contacts for the 5-formylTHF Glu1 is consistent with its apparent weak contribution to substrate specificity (Table 1).

Residues lining the 5-formylTHF binding site include amino acid sequence regions 58–64, 97–105, 133–136, and 144–152 (corresponding to 49–55, 76–84, 104–107, and 115–123, respectively, in the *M. pneumoniae* protein) (Fig. 4a). The pterin moiety of 5-formylTHF is coordinated by the side chain of Glu-64 and the main chain O of Asp-59 and probably by Gln-58 O and Ser-97 O. The first two of these interactions are present in the *M. pneumoniae* structure and in the MTHFS of most species, whereas the latter two are not. Modeling an ATP into the MTHFS active site such that the γ-phosphate occupies the site of a phosphate in the *M. pneumoniae* structure illustrates that a simple rotation of the formyl group of 5-formylTHF places the formyl O in good approximation and orientation for nucleophilic attack on the γ-phosphorous (Fig. 4a). The importance of the highly conserved residues 144, 148, and 150–152 in defining the active site is apparent: Arg-144 occupies a critical position coordinating both the N5-formyl group of 5-formylTHF and the γ-phosphate of ATP.

10-FormylTHF can be built into the THF binding site without altering the position of side chain residues (Fig. 4b). Because the active site is not tightly constricted, several folate derivatives can be positioned to form good interactions between the pterin moiety and residues lining the binding site and between the glutamate moiety and surface hydrophilic residues. This is consistent with the kinetic data that demonstrate weak inhibition of MTHFS activity by most folate derivatives (Table 2). However, it is not possible to position the N10 formyl oxygen properly for attack on the γ-phosphorous of ATP. Approach of the formyl oxygen to the phosphorous is hindered by two of the phosphate oxygen atoms. The angle (formyl O)–P–(opposite P–O bond) is nearly linear with the 5-formyl moiety but is bent by ~40° with the 10-formyl moiety. In the case of N10 formyl species, it is possible that an H-bond could be formed between the formyl O and Lys-149 N; whether this is sufficient to explain the tighter binding of N10-formylated inhibitors is unclear (Table 2).

**Effect of MTHFS Expression on Purine Biosynthesis**—The intracellular concentration of folate derivatives is less than the binding capacity of folate-utilizing enzymes, indicating that biosynthetic pathways within the one-carbon network compete for a limiting pool of folate cofactors (6, 34). Folate-binding proteins can serve as “sinks” that sequester specific folates and thereby inhibit folate-dependent pathways or can interact with other enzymes to selectively “channel” cofactors and accelerate flux through individual biosynthetic pathways (12). 10-FormylTHF is required by GARFT and phosphoribosylaminomimidazole carboxamidase formyltransferase, two folate-dependent enzymes involved in de novo purine biosynthesis (Fig. 2) (1, 11). To determine the metabolic effects of 10-formylTHF sequestration by MTHFS on de novo purine biosynthesis, a “formate suppression” assay was developed. Mammalian cells expressing the MTHFS cDNA were cultured in the presence of [3H]hypoxanthine and [14C]formate. [3H]Hypoxanthine is converted to purines via the folate-independent salvage pathway, whereas [14C]formate is incorporated into purines via the de novo pathway. The 14C/3H dpm ratio was determined in SHSY-5Y and SHSY-SYMTHFS nuclear DNA. Nuclear DNA was isolated from SHSY-5Y (white bars) and SHSY-SYMTHFS (shaded bars) and digested to nucleosides, which were then separated by HPLC. The 14C and 3H content (dpm) in resulting fractions was quantified on a Beckman Coulter LS6500 scintillation counter. Variation is expressed as S.D. of the mean from three measurements for separated nucleosides and six experiments for nuclear DNA. dA, deoxyadenosine; dT, deoxythymidine; dG, deoxyguanosine.
be incorporated into deoxothyminidine and methylcytosine (Fig. 2) via equilibration into the folate-activated one-carbon pool, the DNA was digested to nucleosides, which were fractionated by HPLC. The deoxyguanosine and deoxycytidines 14C/3H ratio was increased by 43 and 69%, respectively, in SHSY-5Y cells compared with the parent cell line (Fig. 6). Comparison of 14C counts derived from purified deoxythymidine normalized to 3H counts from deoxyadenosine indicates that increased MTHFS expression does not affect the 14C deoxythymidine/3H deoxycytidines ratio. The enhancement of de novo purine biosynthesis by MTHFS indicates that MTHFS-bound 10-formylTHF is available for de novo purine biosynthesis.

DISCUSSION

The data presented in this study provide the first evidence that 5-formylTHF concentrations are regulated in mammalian cells. The product of the MTHFS reaction, 5, 10-methenylTHF, exists in chemical equilibrium with 10-formylTHF. Upon the hydrolysis of 5,10-methenylTHF to 10-formylTHF, the MTHFS reaction is subject to feedback inhibition by 10-formylTHF (Table 2) (Fig. 7). This discovery that MTHFS is regulated by 10-formylTHF informs the physiological function of 5-formylTHF. 5-FormylTHF can only accumulate when MTHFS is inhibited by 10-formylTHF. Therefore, 10-formylTHF accumulation both suppresses 5-formylTHF metabolism and, through its equilibrium conversion to 5,10-methenylTHF, serves as a substrate for 5-formylTHF synthesis by serine hydroxymethyltransferase. This regulatory mechanism does not support a role for physiological regulation of purine biosynthesis by 5-formylTHF. 5-FormylTHF can only accumulate when the substrate for de novo purine biosynthesis, 10-formylTHF, accumulates. Feedback regulation of MTHFS does indicate that 5-formylTHF represents a stable pool of excess formyl folates that can be mobilized by MTHFS only when 10-formylTHF levels are depleted. These studies have also identified MTHFS as a 10-formylTHF tight-binding protein. Folate cofactors are bound by enzymes that utilize one-carbon units in transfer reactions and also by folate tight-binding proteins that do not metabolize the cofactor but regulate its availability. For example, 5-methylTHF is a substrate for methionine synthase but is bound tightly to and inhibits glycine N-methyltransferase (37). THF is bound tightly by and inhibits 10-formyltetrahydrofolate dehydrogenase (38), and 5-formylTHF and 5-methylTHF are bound tightly by and inhibit serine hydroxymethyltransferase (39). MTHFS is the first 10-formylTHF tight-binding protein to be identified. Previously, we have shown that increased MTHFS expression in SHSY-5Y cells increased relative concentrations of 10-formylTHF at the expense of 5-methylTHF levels; 10-formylTHF levels constituted as much as 90% of total cellular folate in MTHFS-expressing cells (21). These results are consistent with MTHFS serving as a reservoir for 10-formylTHF that permits its accumulation.

The results from this study indicate that MTHFS may determine the fate of cellular 10-formylTHF (Fig. 7). 10-FormylTHF can be used by three pathways. It is a cofactor for phosphoribosylaminomimidazole carboxamide formyltransferase and GARFT during de novo purine biosynthesis. Alternatively, the 10-formyl group can be reduced and the cofactor converted to other one-carbon forms of folate, a pathway initiated by the enzyme methylene tetrahydrofolate cyclohydrolase. MTHFS binds 10-formylTHF tightly and facilitates its use for purine biosynthesis.
