Methylenetetrahydrofolate Reductase: Biochemical Characterization and Medical Significance

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Running title: Characterization and Medical Significance of MTHFR

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**ABSTRACT:** Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5,10-methylenetetrahydrofolate (CH₂-H₄folate) to 5-methyltetrahydrofolate (CH₃-H₄folate). The enzyme employs a noncovalently-bound flavin adenine dinucleotide (FAD), which accepts reducing equivalents from NAD(P)H and transfers them to CH₂-H₄folate. The reaction provides the sole source of CH₃-H₄folate, which is utilized by methionine synthase in the synthesis of methionine from homocysteine. MTHFR plays a key role in folate metabolism and in the homeostasis of homocysteine; mutations in the enzyme lead to hyperhomocyst(e)inemia. A common C677T polymorphism in MTHFR has been associated with an increased risk for the development of cardiovascular disease, Alzheimer’s disease, and depression in adults, and of neural tube defects in the fetus. The mutation also confers protection for certain types of cancers. This review presents the current knowledge of the enzyme, its biochemical characterization, and medical significance.

**KEY WORDS:** flavin, FAD, folate, MTHFR, methylenetetrahydrofolate, methyltetrahydrofolate, homocysteine, C677T polymorphism

**1. INTRODUCTION**

The *de novo* synthesis of methyl groups is one of the more incredible feats achieved by biological organisms. This biosynthesis requires the action of several enzymes, including methylenetetrahydrofolate reductase (MTHFR), and five coenzymes, folic acid, riboflavin, niacin, cobalamin, and pyridoxine. As shown in Fig. (1) depicting mammalian, cytoplasmic one-carbon metabolism (reviewed in [1]), 5,10-methylenetetrahydrofolate (CH₂-H₄folate), the substrate for MTHFR, derives its methylene group from the β-carbon of serine or from formate. CH₂-H₄folate has three possible fates. MTHFR catalyzes the direct reduction of CH₂-H₄folate to 5-methyltetrahydrofolate (CH₃-H₄folate). In the thymidylate synthase reaction, the methylene group is transferred from CH₂-H₄folate to dUMP and then reduced to form dTMP. Lastly, CH₂-H₄folate can be converted in two steps to 10-formyltetrahydrofolate (10-CHO-H₄folate) for purine biosynthesis.

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**Fig. (1).** Overview of mammalian one-carbon metabolism in the cytoplasm. The enzymes involved are: CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; DHFR, dihydrofolate reductase; FDH, 10-formyltetrahydrofolate dehydrogenase; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFC, 5,10-methylenetetrahydrofolate cyclohydrolase; MTHFD, 5,10-methylenetetrahydrofolate dehydrogenase; MTHFR, 5,10-methylenetetrahydrofolate reductase; SAHH, S-adenosylhomocysteine hydrolase; SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase.
MTHFR is a flavoprotein that catalyzes the flavin adenine dinucleotide (FAD)-dependent reduction of CH$_2$-H$_4$folate by NAD(P)H (Fig (2)). The methyl group of the product CH$_3$-H$_4$folate is then transferred to homocysteine (Hcy) to generate methionine and H$_4$folate in a reaction catalyzed by methionine synthase. In prokaryotes, this reaction is the terminal step in methionine biosynthesis; methionine is then converted to S-adenosylmethionine (AdoMet), the major methyl donor for biosynthetic reactions in the cell. In mammals, which do not synthesize methionine de novo, methylation of homocysteine allows regeneration of the methyl group of methionine in support of AdoMet-dependent methylation reactions. Adenosylhomocysteine (AdoHcy), formed as a product in these methylation reactions, is then hydrolyzed to regenerate homocysteine (Fig. (1)).

**Fig. (2).** Reaction catalyzed by MTHFR. The noncovalently bound flavin adenine dinucleotide (FAD) accepts reducing equivalents from NAD(P)H and transfers them to CH$_2$-H$_4$folate to form the products NAD(P)$^+$ and CH$_3$-H$_4$folate. R is benzoyl-L-glutamate.

Homocysteine is a critical branch point in one-carbon metabolism; it can be methylated to form methionine or be converted to cystathionine during times of excess dietary methionine (Fig. (1)). Elevation of blood homocysteine, hyperhomocyst(e)inemia, has been associated with an increased risk of cardiovascular disease [2] and Alzheimer’s disease [3] in adults, and of neural tube defects in the fetus [4]. This condition can result from defects in MTHFR. Patients with severe MTHFR deficiency exhibit very elevated homocysteine levels and show a wide range of clinical symptoms, such as developmental delay, vascular complications, and neurodegenerative disorders [5]. A milder form of MTHFR deficiency arising from a common C677T polymorphism has also been described, in which Ala222 in the human enzyme is replaced by a valine [6]. The polymorphism is associated with mild hyperhomocyst(e)inemia and is considered a risk factor for cardiovascular disease, neural tube defects, Alzheimer’s disease, and depression. Recent studies suggest that the C677T mutation is also correlated with a lower risk of colon cancer [7] and acute lymphoblastic leukemia [8, 9].

This review will summarize our current knowledge of the enzyme methylenetetrahydrofolate reductase, its biochemical characterization and medical significance. The prior reviews on MTHFR, written by Rowena G. Matthews, were published in 2005 [10], 2002 [11], 1992 [12], and 1990 [13]. MTHFR was first characterized from porcine liver and shown to be allosterically regulated by AdoMet. The availability of peptide sequences from the porcine enzyme led to the cloning of the human MTHFR cDNA. Although attempts at production of human MTHFR in E. coli were not successful, the enzyme was eventually overexpressed in insect cells and its properties determined. Eukaryotic MTHFRs from yeast, Arabidopsis, and Leishmania have also been characterized. The E. coli enzyme, initially chosen for construction of the homologous C677T mutation, has been fully characterized and due to the available crystal structures, this enzyme is being used for extensive mechanistic studies. In a recent, exciting development, the properties and crystal structure of MTHFR from the bacterium Thermus thermophilus HB8 have been determined. MTHFRs from Peptostreptococcus productus and Clostridium formicoacetium have also been investigated. Following discussion of the biochemical characterization and regulation of MTHFR, a summary of the studies connecting MTHFR to medicine will be presented. Finally, the review will describe the biochemical phenotypes of the common C677T polymorphism and a second polymorphism A1298C.

2. BIOCHEMICAL CHARACTERIZATION OF EUKARYOTIC MTHFR

2.1. Porcine MTHFR

2.1.a. Purification and Physical Properties

MTHFR activity was first observed in pig liver by Donaldson and Keresztesy in 1959 [14]. The enzyme was purified 20-fold and shown to require the addition of FAD for maximal activity. It catalyzed the conversion of a natural folate prefolic A (later identified as CH$_3$-H$_4$folate) to H$_4$folate and formaldehyde in the presence of menadione as electron acceptor [15]. The reaction observed can be shown as Eq (1). CH$_3$-H$_4$folate is oxidized to CH$_2$-H$_4$folate, which dissociates nonenzymatically into H$_4$folate and formaldehyde. Menadione is needed to oxidize the enzyme-bound FAD.
\[
\text{CH}_3\text{-H}_4\text{folate} + \text{menadione} \longrightarrow \text{CH}_2\text{-H}_4\text{folate} + \text{menadiol} \quad (1)
\]

These studies indicated that MTHFR could also catalyze the reverse direction, the reduction of \(\text{CH}_2\text{-H}_4\text{folate}\) to \(\text{CH}_3\text{-H}_4\text{folate}\), as shown in Eq (2). \(\text{CH}_2\text{-H}_4\text{folate}\) (added directly or made \textit{in situ} from \(\text{H}_4\text{folate}\) and formaldehyde) is converted to \(\text{CH}_3\text{-H}_4\text{folate}\) in the presence of reduced diphosphopyridine nucleotide (NADH) [15].

\[
\text{CH}_2\text{-H}_4\text{folate} + \text{NADH} + \text{H}^+ \longrightarrow \text{CH}_3\text{-H}_4\text{folate} + \text{NAD}^+ \quad (2)
\]

This early work was followed upon by Kutzbach and Stokstad [16], who purified MTHFR almost 400-fold from porcine liver and shown it to be a flavoprotein specific for FAD. They confirmed catalysis of Eq (1), but demonstrated that the enzyme greatly preferred NADPH over NADH as the electron donor in the physiological NADPH-\(\text{CH}_2\text{-H}_4\text{folate}\) oxidoreductase reaction, shown as Eq (3). An additional MTHFR activity, the NADPH-\text{menadione} oxidoreductase reaction, was also described (Eq (4)) [16].

\[
\text{CH}_2\text{-H}_4\text{folate} + \text{NADPH} + \text{H}^+ \longrightarrow \text{CH}_3\text{-H}_4\text{folate} + \text{NADP}^+ \quad (3)
\]

\[
\text{NADPH} + \text{menadione} \longrightarrow \text{NADP}^+ + \text{menadiol} \quad (4)
\]

Kutzbach and Stokstad reported for the first time the inhibition of MTHFR by AdoMet [16]. All three reactions of the enzyme (Eq (1), (3), and (4)) were strongly inhibited by AdoMet, although the onset of inhibition was slow. AdoHcy could partially relieve this inhibition, but it was not an activator of the enzyme. AdoMet was hypothesized to be an allosteric inhibitor of MTHFR based on it having no structural resemblance to any of the substrates, the competition of AdoMet and AdoHcy for the same enzyme site, and AdoMet being an intermediate in the pathway [16].

In 1982, Matthews and coworkers purified MTHFR to homogeneity in five steps, 32,000-fold, from pig liver [17]. The flavin cofactor, noncovalently bound, was identified as FAD, and the purified enzyme exhibited a visible absorbance spectrum typical for a flavoprotein with a maximum at 450 nm. The protein migrated as a single band of 77.3 kDa on SDS/PAGE, similar to the 74.5 kDa per mole of flavin determined by amino acid analysis (excluding tryptophan and cysteine residues) [17]. Sephacryl S-300 chromatography, however, yielded an apparent molecular mass of the holoenzyme of 210 ± 20 kDa, not a clear multiple of 77 kDa, suggesting that the protein could exist as a trimer of identical subunits or an ellipsoidal homodimer [17]. To solve this conundrum, Matthews collaborated with Joseph Wall to subject the MTHFR protein to scanning transmission electron microscopy (STEM). The STEM analysis generated a mass distribution of protein particles with a mean particle size of 136 ± 29 kDa, consistent with the native enzyme being a dimer of identical subunits, each containing a noncovalently-bound FAD [18].

### 2.1.b. Kinetic and Thermodynamic Characteristics of the Reaction

Homogeneous MTHFR catalyzes the \(\text{CH}_3\text{-H}_4\text{folate}-\text{menadione}\) (Eq (1)), NADPH-menadione (Eq (4)), and NADPH-\(\text{CH}_2\text{-H}_4\text{folate}\) (Eq (3)) oxidoreductase reactions with the kinetic parameters \(k_{\text{cat}}\) and \(K_m\) shown in Table (1) [17, 19, 20]. These assays were performed at pH 7.2 and 25 °C and utilized the monoglutamate form of \(\text{CH}_2\text{-H}_4\text{folate}\) or \(\text{CH}_3\text{-H}_4\text{folate}\). Intracellular forms of folate are found as polyglutamyl derivatives containing two to seven glutamates linked by their \(\gamma\)-carboxyl groups. To examine the specificity of MTHFR, the NADPH-\(\text{CH}_2\text{-H}_4\text{folate}\) oxidoreductase assay was run with \(\text{CH}_2\text{-H}_4\text{folate}\) polyglutamyl substrates containing one to seven glutamates [21]. The \(k_{\text{cat}}\) values increased 1.76-fold in going from the mono- to the diglutamyl substrate, and \(K_m\) values decreased as chain length increased from one to six glutamyl residues. Optimum catalytic efficiency (\(k_{\text{cat}}/K_m\)) occurred with the hexaglutamate form of \(\text{CH}_2\text{-H}_4\text{folate}\). These results are consistent with inhibition studies of polyglutamyl derivatives of dihydrofolate. The dihydrofolates were competitive inhibitors with respect to folate and those with one to six glutamyl residues showed decreasing \(K_i\) values, binding increasingly more tightly to the enzyme, but the addition of a seventh glutamate weakened the affinity [21].

<table>
<thead>
<tr>
<th>Table 1. Steady-state kinetic constants*</th>
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<tbody>
<tr>
<td>NAD(P)H-(\text{CH}_2\text{-H}_4\text{folate}) Oxidoreductase Assay</td>
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<tr>
<td>(K_m) for NAD(P)H (µM)</td>
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<tr>
<td>(K_i) for NAD(P)H (µM)</td>
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<tr>
<td>(K_m) for (\text{CH}_2\text{-H}_4\text{folate}) (µM)</td>
</tr>
<tr>
<td>(K_i) for (\text{CH}_2\text{-H}_4\text{folate}) (µM)</td>
</tr>
<tr>
<td>(k_{\text{cat}}) (s(^{-1}))</td>
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<tr>
<td>Calculated (k_{\text{cat}}) (s(^{-1}))</td>
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<tr>
<td>NAD(P)H-Menadione Oxidoreductase Assay</td>
</tr>
<tr>
<td>(K_m) for NAD(P)H (µM)</td>
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<tr>
<td>(K_i) for NAD(P)H (µM)</td>
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Spectrophotometric titrations of MTHFR with substrate were performed to directly observe reduction and oxidation of the enzyme-bound FAD. Under anaerobic conditions, titration of enzyme with NADPH yielded a stoichiometry of 1.09 mol NADPH oxidized/mol FAD reduced [17]. Thus, the pyridine nucleotide can react with the enzyme-bound flavin in the absence of the folate substrate in the reductive half-reaction. When the enzyme is photoreduced in the presence of deazaflavin, EDTA, and light and then reacted with CH₂-H₄folate, near stoichiometric reoxidation of the enzyme-bound FAD occurs, demonstrating the ability of the enzyme and folate to react in the absence of the NADPH substrate in the oxidative half-reaction [17]. The anaerobic titration of oxidized MTHFR with CH₃-H₄folate was also performed to determine the midpoint potential (Eₘ) of the enzyme-bound FAD [22]. The potential of the CH₂-H₄folate/CH₃-H₄folate couple at pH 7.2 is -200 mV at pH 7.0 [23], and the Eₘ is calculated to be -212 mV at pH 7.2. Using this potential, the titration data yielded an Eₘ of -223 mV for the MTHFR-bound FAD/hydroquinone couple [22]. The Eₘ of MTHFR allows calculation of the free energy values for both half-reactions. At pH 7.0, the Eₘ for the NADPH/NADP⁺ couple is -324 mV [24], and at pH 7.2, the Eₘ is calculated to be -330 mV. Thus, the reduction of MTHFR by NADPH occurs with a free energy decrease of -4.9 kcal mol⁻¹, indicating that reduction of enzyme by NADPH is essentially irreversible. In combination with the potential of -212 mV for the CH₂-H₄folate/CH₃-H₄folate couple at pH 7.2 [23], it can be calculated that the oxidation of MTHFR by CH₂-H₄folate occurs with a free energy decrease of -0.51 kcal mol⁻¹, indicating that the oxidative half-reaction is reversible for MTHFR. Thus, of the two half-reactions, the reductive half-reaction with NADPH is the irreversible part of the overall enzyme reaction.

Homogeneous MTHFR demonstrates ping-pong Bi-Bi kinetics in catalyzing the oxidoreductase reactions given as Eq (1), (3), and (4) [17, 20], consistent with the direct spectrophotometric titrations described above [17, 22]. Parallel lines were first observed in the double reciprocal plots of the NADPH-CH₂-H₄folate oxidoreductase reaction (Eq 3) using less purified enzyme [25]. Marked inhibition in the assay at high CH₂-H₄folate concentrations further supported the ping-pong kinetic mechanism [25].

In order to establish that the enzyme could catalyze the individual half-reactions comprising the oxidoreductase reactions (1), (3), and (4) in a kinetically competent manner, each half-reaction was examined under anaerobic conditions in a stopped-flow spectrophotometer. Fig. (3) shows the kinetic constants assigned to the

![Kinetic mechanisms and assigned rate constants for MTHFR half-reactions.](image)
reductive and oxidative half-reactions. Only monophasic reactions were observed. The maximum first-order rate constants associated with reduction of enzyme-bound FAD by NADPH or CH$_2$-H$_4$folate in addition to oxidation of FAD by menadione or CH$_2$-H$_4$folate are shown in Table (2) [20]. From the rate constants of the individual half-reactions, the turnover number for each of the oxidoreductase reactions can be calculated, assuming a ping-pong Bi-Bi mechanism [26]. As shown in Table (1), the calculated turnover numbers are in reasonable agreement with the $k_{cat}$ values observed for the steady-state oxidoreductase assays [20]. Thus, pig liver MTHFR can catalyze the individual half-reactions at rates sufficiently fast to account for catalytic turnover, and the enzyme is kinetically competent to catalyze these reactions by a ping-pong Bi-Bi mechanism. These results also demonstrate that reoxidation of reduced flavin by CH$_2$-H$_4$folate is substantially rate-limiting in the physiological NADPH-CH$_2$-H$_4$folate oxidoreductase reaction (Eq (3)). CH$_3$-H$_4$folate-linked reduction of the FAD is rate-determining in the CH$_3$-H$_4$folate-menadione oxidoreductase reaction (Eq (1)) as is the NADPH-linked reduction in the NADPH-menadione oxidoreductase reaction (Eq (4)) [20].

**Table 2. Rapid-reaction kinetic constants**

<table>
<thead>
<tr>
<th></th>
<th>Porcine $^c$</th>
<th>$E. coli$ $^d$</th>
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<tbody>
<tr>
<td><strong>Reductive Half-Reaction with NADH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ for NADH (µM)</td>
<td>28</td>
<td>32 $^e$</td>
</tr>
<tr>
<td>$k_2'$ (s$^{-1}$)</td>
<td>160</td>
<td>55 $^e$</td>
</tr>
<tr>
<td><strong>Oxidative Half-Reaction with Menadione</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_5$ (M$^{-1}$ s$^{-1}$)</td>
<td>1.7 x 10$^7$</td>
<td>1.0 x 10$^7$</td>
</tr>
<tr>
<td>At 140 µM (s$^{-1}$) (calculated)</td>
<td>2300</td>
<td>1400</td>
</tr>
<tr>
<td><strong>Oxidative Half-Reaction with CH$_2$-H$_4$folate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ for CH$_2$-H$_4$folate (µM)</td>
<td>88</td>
<td>11 $^f$</td>
</tr>
<tr>
<td>$k_5'$ (s$^{-1}$)</td>
<td>50</td>
<td>10.3 $^f$</td>
</tr>
<tr>
<td><strong>Reductive Half-Reaction with CH$_2$-H$_4$folate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ for CH$_2$-H$_4$folate (µM)</td>
<td>20</td>
<td>n.d. $^g$</td>
</tr>
<tr>
<td>$k_3$ (s$^{-1}$)</td>
<td>5.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

$^a$ Rate constants were determined at 25 °C in 50 mM potassium phosphate buffer (pH 7.2) containing 0.3 mM EDTA and 10% glycerol.

$^b$ Definition of rate constants are given in Figure 3. The net rate constant for reduction, $k_2'$, is defined as $(k_2 k_3)/(k_2 + k_3)$. The net rate constant for oxidation, $k_5'$, is defined as $(k_3 k_6)/(k_5 + k_6)$.

$^c$ Porcine enzyme, from ref 20

$^d$ $E. coli$ enzyme, from ref 63

$^e$ From slow phase of reductive half-reaction

$^f$ From slow phase of oxidative half-reaction

$^g$ Not determined.

### 2.1.c. Chemical Mechanism

Stereochemical and kinetic isotope analyses were performed to investigate the reductive half-reaction. In the NADPH-menadione oxidoreductase assay, use of [4($S$)-$^3$H]NADPH as substrate led to the release of 90% of the total tritium to solvent, whereas use of [4($R$)-$^3$H]NADPH resulted in the release of only 3-4% of the isotope [27]. From these experiments, it was concluded that the pro-$4S$ hydrogen is stereospecifically removed from NADPH. Most likely, the exchange of tritium with solvent occurs after transfer of the 4S tritium from NADPH to N5 of the enzyme-bound FAD. A full primary kinetic isotope effect of 4.8 on $V_{max}$ and $V_{max}/K_m$ was obtained in the NADPH-menadione oxidoreductase assay, by comparing the rates of oxidation of [4($S$)-$^2$H]NADPH and [4($S$)-$^1$H]NADPH [27]. Furthermore, when the assay was run in tritiated water, no incorporation of solvent tritium was observed into the residual NADPH. Taken together, these results suggest that the chemistry step ($k_2'$) is slow compared to the dissociation of Eox·NADPH ($k_1$) and the release of NADP$^+$ product ($k_3$) [27], giving evidence that the oxidation of NADPH is largely rate-limiting within the reductive half-reaction.

Studies of MTHFR reconstituted with deazaflavin demonstrated hydride transfer as the mechanism of flavin reduction in the reductive half-reaction [28]. As described above, the reaction of MTHFR with NADPH is essentially irreversible. Employment of the FAD analog 8-demethyl-8-hydroxy-5-deaza-5-carbaflavin adenine dinucleotide (Em$_m$ = -0.350 V at pH 7.0 [29]), however, permits the reverse reaction to occur appreciably. In the experiment, MTHFR was reconstituted with either ($S$)- or ($R$)-[5-$^3$H]-8-OH-5-deaza-FAD hydroquinone and reacted with NADP$^+$. Tritium from the 5S stereoisomer was quantitatively incorporated into the NADPH product, generating (4S)-[4-$^3$H]-NADPH; no transfer of tritium occurred with (5R)-[5-$^3$H]-8-OH-5-deaza-FAD hydroquinone.
Four mechanisms have been postulated for the reversible interconversion of CH2-H4folate and CH3-H4folate in the oxidative half-reaction (Fig. (4)) [13]. In all cases, it is proposed that CH2-H4folate first undergoes protonation and imidazolidine ring opening to form a 5-iminium cation intermediate [32]. Although this reactive species has not been directly observed in an enzymatic or nonenzymatic reaction, the 5-iminium cation has been postulated to form during the condensation of formaldehyde and H4folate to synthesize CH2-H4folate [33]. Moreover, a structure has been obtained of 5-HOCH2-H4folate, the product of the reaction of a 5-iminium cation with water, at the active site of thymidylate synthase [34], an enzyme that also utilizes CH2-H4folate as a substrate (See Fig. (1)). The first two pathways involve intramolecular oxidoreductions to form either 5-methyl-7,8-H2folate (5-methyl-H2folate) (Pathway (1)) or 5-methyl-quinonoid-H2folate (quinonoid-5-methyl-H2folate) (Pathway (2)), which is reduced to CH3-H4folate by the transfer of two electrons from reduced FAD. Pathway (3) describes direct transfer of a hydride to the exocyclic methylene group of the 5-iminium cation. Alternatively, pathway (4) involves one-electron chemistry, generation of an amine radical cation, followed by electron transfer to form CH3-H4folate (Fig. (4)).

The possible formation of a 5-CH3-H4folate intermediate (pathway (1) in Fig. (4)) was suggested by the observation of H2folate as a potent inhibitor of MTHFR [25]. The 5-iminium cation could be converted to 5-CH3-H4folate by tautomerization involving removal of the C-6 hydrogen; reduction of the pteridine ring would then lead to CH2-H4folate. To test this mechanism, [methylen-14C, 6-3H]-H4folate was reduced by NADPH and the CH3-H4folate product analyzed [25]. No evidence of tritium transfer from 6 to the methyl group of CH3-H4folate was found, thus eliminating the possibility of pathway (1). Additional experiments were carried out to further investigate the source of hydrogen for CH3-H4folate methyl formation. Specifically examined was whether exchange of methylene protons with solvent occurs during reduction of CH2-H4folate to CH3-H4folate. In early studies, when [methylen-13C]-H4folate in buffered H2O was reduced with NADPH, the CH3-H4folate product had the same specific activity as the initial CH2-H4folate, indicating no release of tritium to solvent [25]. Consistent with this result, partitioning studies showed that less than 8.5% of the methylene protons are exchanged with solvent during reduction of CH2-H4folate to CH3-H4folate [35]. When [methylen-13C]-H4folate was reduced in the presence of D2O solvent, one deuterium was incorporated per methyl group [35]. Finally, a near maximum kinetic isotope effect on the incorporation of solvent hydrogen into the methyl group indicates that the hydrogen required for reduction comes from the solvent [35]. These results are inconsistent with pathway (1), but do not distinguish between pathways (2), (3), and (4) of Fig. (4).

Mechanistically, the major question is whether reducing equivalents enter CH2-H4folate at the pterin ring (pathway (2)) or at the exocyclic methylene group via hydride transfer (pathway (3)) or one-electron chemistry (pathway (4) (Fig. (4)). Lending support to the second mechanism was the observation that MTHFR catalyzes the reduction of quinonoid H2pterins without a N5-substituent, at rates similar to that of its physiological CH2-H4folate substrate [36]. A one-electron mechanism for reduction of CH2-H4folate has also been hypothesized (pathway (4)) based on the facile reduction of menadione by MTHFR. The enzyme, however, does not stabilize the FAD semiquinone, and this species has not been observed during reduction of enzyme with NADPH or CH2-H4folate or during reoxidation of photoreduced enzyme with CH2-H4folate [17].
Fig. (4). Four possible mechanisms for the reduction of CH$_2$-H$_4$folate to CH$_3$-H$_4$folate catalyzed by MTHFR [13]. All mechanisms are hypothesized to begin by ring opening of CH$_2$-H$_4$folate to generate a 5-iminium cation. In pathway (1), this intermediate undergoes tautomerization to form 5-methyl-7,8-dihydrofolate (5-CH$_3$-H$_2$folate), and in pathway (2), intramolecular oxidoreduction yields 5-methyl-quinonoid-H$_2$folate (quinonoid-5-CH$_3$-H$_2$folate). These species are subsequently reduced to CH$_3$-H$_4$folate. In pathway (3), the 5-iminium cation is reduced by transfer of a hydride from the reduced FAD to the exocyclic methylene group to produce CH$_3$-H$_4$folate. In pathway (4), the cation intermediate is reduced by transfer of an electron and a proton to yield an amine radical cation, with subsequent donation of an electron to form CH$_3$-H$_4$folate. Reproduced, with permission, from Matthews and Drummond [13].

Experiments with deazaflavin-containing MTHFR were carried out to differentiate between the three remaining pathways (Fig. (4)) [28]. When enzyme reconstituted with (5S)-[5-3H]-8-OH-5-deaza-FAD hydroquinone was reacted with CH$_2$-H$_4$folate under anaerobic conditions, tritium from the C5 of the deazaflavin was transferred to the exocyclic methylene group of CH$_2$-H$_4$folate, bound at the $si$ face of the flavin. No tritium was released to solvent from C5 of the reduced deazaflavin, eliminating pathway (2) as a possible mechanism. To determine the activity of MTHFR in the presence of 8-OH-5-deaza-FAD, the NADPH-menadione, NADPH-quinoid-H$_2$folate, and NADPH-CH$_2$-H$_4$folate oxidoreductase assays were performed. While a 52% increase in activity was observed in the physiological NADPH-CH$_2$-H$_4$folate oxidoreductase reaction compared to that of the FAD-reconstituted enzyme, essentially no increase occurred when either menadione or quinonoid-H$_2$folate served as electron acceptors. Since 5-deazaflavins are generally unable to catalyze one-electron transfers, these results establish that both half-reactions of MTHFR proceed by hydride transfer: reduction of enzyme by NADPH and reoxidation of enzyme by CH$_2$-H$_4$folate [28].
2.1.d. Allosteric Regulation by AdoMet

Early studies by Kutzbach and Stokstad [16] suggested AdoMet as an allosteric inhibitor of the mammalian enzyme. They showed that reactions of MTHFR are inhibited by AdoMet and that AdoHcy could partially reverse this inhibition. Heat treatment could desensitize the enzyme to the inhibitory effect of AdoMet with only partial loss of catalytic activity [16]. Using homogeneous porcine MTHFR, Daubner and Matthews [37] demonstrated that AdoMet inhibits the MTHFR reactions in Eq (1), (3), and (4). The onset of inhibition, however, is slow, as also seen by Stokstad [16]; thus, all assays were performed after a 10 minute preincubation of the enzyme with AdoMet [37].

The addition of AdoMet to the enzyme results in spectral perturbations of the enzyme-bound FAD, with increased absorbance centered at 503 nm [17]. An apparent $K_d$ of 5 µM for AdoMet was estimated from the spectral changes [38]. AdoHcy binds to purified MTHFR with a $K_d$ of 3 µM and alleviates the inhibition caused by AdoMet [37], confirming the results of Stokstad [16].

STEM, limited proteolysis, and photoaffinity labeling experiments investigated AdoMet binding to porcine MTHFR. STEM analysis showed a dimer of identical 77 kDa subunits with each subunit containing two globular domains of approximately equal size – four domains arranged in a planar rosette [18]. Consistent with these results, limited proteolysis with trypsin cleaved the 77 kDa subunits into an N-terminal fragment of 40 kDa and a C-terminal fragment of 37 kDa. During proteolysis, loss of catalytic activity was not observed, but the enzyme was desensitized to inhibition by AdoMet [18]. When this desensitized and partially digested enzyme was subjected to photolabeling with 8-azido-S-adenosylmethionine, the label was observed on the C-terminal 37 kDa fragment, indicating the location of the AdoMet binding site [39]. Taken together, these results suggest that the MTHFR enzyme is composed of two domains – an N-terminal domain, which contains the requirements for catalysis, and a C-terminal domain, responsible for AdoMet binding and regulation. It follows that, upon cleavage of the enzyme into these two domains, AdoMet sensitivity is lost due to its inability to sufficiently stabilize the inactive state of the enzyme. AdoMet inhibition, thus, appears to require specific interaction between the two domains of the enzyme - the N-terminal catalytic domain and the C-terminal regulatory domain [39].

To investigate the effect of AdoMet on individual half-reactions of the enzyme, experiments were carried out in which enzyme was preincubated with AdoMet prior to reaction with substrate in the stopped-flow spectrophotometer. In the presence of AdoMet, the rate of reduction of enzyme by NADPH was 50,000-fold slower than in its absence [40]. Smaller effects were observed on the half-reaction with folate. Incubation of reduced enzyme with AdoMet resulted in a 12,000-fold decrease in rate of reoxidation of the enzyme-bound FAD by CH$_2$-H$_4$folate, and the rate of reduction of enzyme by CH$_3$-H$_4$folate was reduced 3000-fold in the presence of AdoMet [41].

Early observations by Matthews and coworkers had suggested that MTHFR could exist in active and inactive forms, even in the absence of AdoMet [20]. In the stopped-flow apparatus, reduction of enzyme by NADPH was biphasic with fast and slow phases accounting for 75% and 25%, respectively, of the total absorbance change. To explain the data, they hypothesized that the enzyme could exist in two states, an active R form and an inactive T form, and that 25% of the enzyme preparation was in the inactive state and, therefore, catalyzed flavin reduction at a much slower rate.

To study the interactions between enzyme, NADPH, and AdoMet independent of the folate substrate, the NADPH-menadione oxidoreductase assay was employed. Supporting the hypothesis presented above, preincubation of enzyme with NADPH in the absence of AdoMet led to enhanced activity in the assay, suggesting that NADPH was able to recruit active enzyme forms [42]. Activity was also boosted when enzyme was preincubated with AdoMet and increasing concentrations of NADPH, and under these conditions, the dependence on the concentration of AdoMet was sigmoidal, indicative of cooperativity [42]. By contrast, increasing concentrations of AdoMet in the preincubation mixture resulted in recruitment of inactive enzyme forms [42]. The antagonistic roles for AdoMet and NADPH were further demonstrated in kinetic experiments. A clear biphasic pattern was observed, with AdoMet or NADPH able to influence the amplitude and rate of the slow and fast phases [42]. To determine whether AdoMet also has an antagonistic relationship with CH$_3$-H$_4$folate, the CH$_3$-H$_4$folate-menadione oxidoreductase assay was performed in the presence of AdoMet. The data show that the inhibitory effect of AdoMet on this reaction is similar to that observed with the NADPH-menadione oxidoreductase reaction [41].

Jencks and Matthews proposed the following model to explain the interactions of MTHFR with AdoMet and NADPH consistent with their data [42]. The dimeric enzyme exists in two quaternary states, an active R form and an inactive T form, to which the ligands, AdoMet and NADPH, bind with opposing affinities. The binding of AdoMet to the R state greatly decreases the affinity of this state for NADPH and slightly increases the rate of interconversion between R and T states. Alternatively, the binding of NADPH to the R form decreases the affinity of this form for AdoMet. Thus, the biphasic inhibition by AdoMet can be explained by rapid binding of AdoMet to the R state enzyme, causing the fast phase, and a comparatively slower interconversion between the R and T states.
causing the slow phase. Finally, studies examining the effect of AdoHcy on relieving AdoMet inhibition in the NADPH-menadione oxidoreductase assay suggest that AdoHcy and AdoMet compete for the same site on the both the R and T state enzymes. However, AdoHcy itself does not activate the enzyme nor does it affect the R to T transition in the absence of AdoMet [12].

2.2. Human MTHFR

In 1990, Rozen, Kang, and coworkers purified MTHFR 30,000-fold from human cadaver liver [43]. SDS/PAGE revealed a band of 39 kDa, whereas Superose 12 gel filtration chromatography demonstrated 150 kDa as the molecular mass for the native protein. MTHFR in fresh liver extract was inhibited by AdoMet, but the purified enzyme was not. When both 75 kDa and 39 kDa bands were observed by SDS/PAGE analysis of fresh human lymphocyte extracts [43], the authors reasoned that the 39 kDa band had resulted from autolysis of the human liver enzyme and that insensitivity to AdoMet had occurred, similar to what had been observed for the porcine enzyme [18]. They concluded that human MTHFR is a dimer of 77 kDa subunits [43], same as the porcine liver enzyme [18].

The availability of peptide sequences from porcine MTHFR allowed the isolation of a 2.2 Kb cDNA for human MTHFR [6, 44]. Expression of the cDNA in Escherichia coli yielded an active enzyme, with a subunit size of 70 kDa by SDS/PAGE and Western analyses [6, 45]. Human MTHFR was also produced in the yeast Saccharomyces cerevisiae [46]. Expression levels in both E. coli and S. cerevisiae, however, were low and insufficient for full biochemical characterization of the enzyme. In 2001, human MTHFR was successfully expressed as an N-terminal histidine-tagged enzyme using a baculovirus expression system and Sf9 insect cells [47]. A two-step, 62-fold purification generated 3.5 mg homogeneous protein per 1.5 L culture. The purified enzyme contains one FAD per subunit, and it migrates as a 70 kDa band by SDS/PAGE, as in the expression systems mentioned above. This smaller 70 kDa size, however, stands in contrast to the 77 kDa subunit size demonstrated by purification of MTHFR from pig [17] or human liver [43]. An explanation was found when characterization of the complete cDNA and gene structure of MTHFR revealed two different isoforms 70 kDa and 77 kDa, resulting from alternative splicing, both of which produce active enzyme [48].

Expressed in insect cells, the 70 kDa isoform of MTHFR is active and is regulated by AdoMet [47]. In the NADPH-CH$_2$-H$_4$folate oxidoreductase assay at 25 °C and pH 6.7, the enzyme demonstrated a $k_{cat}$ of 16 s$^{-1}$ and a $K_m$ for CH$_2$-H$_4$folate of 10 µM [47], similar to the kinetic parameters for the pig enzyme (see Table (1)). AdoMet was confirmed as an inhibitor of human MTHFR. Additional studies into regulation revealed that the human enzyme is multiply phosphorylated on a serine-rich N-terminal extension region [49]. Among the seven phosphorylation sites, Thr 34 appears to be the first amino acid to be phosphorylated, i.e. the priming site. Removal of phosphate by treatment with alkaline phosphatase led to a 1.2-fold more active enzyme, which was 40% less sensitive to inhibition by AdoMet. Taken together, the results suggest that phosphorylation plays a critical role in allosteric regulation of MTHFR by AdoMet. The effect of phosphorylation may be to alter the equilibrium of R and T states of the enzyme, favoring the inactive T state, the form to which AdoMet preferentially binds [42].

2.3. Yeast, Arabidopsis, and Leishmania MTHFRs

In recent years, eukaryotic MTHFRs from yeast, Arabidopsis, and Leishmania have also been characterized. Different from the eukaryotic pig and human MTHFRs discussed above, fungi and plants each have two enzymes. Two MTHFR-encoding genes from S. cerevisiae [50], Schizosaccharomyces pombe [51], Aspergillus nidulans [52], Fusarium graminearum [53], and Arabidopsis thaliana [54] have been identified. Phylogenetic analyses and sequence alignments [50, 52, 53, 55] have shown the two fungal proteins (referred to here as MET12 and MET13) to be about 30% identical to each other (and about 30% identical to human MTHFR) with the MET12 protein containing an additional conserved sequence motif [53]. In each case, single disruption of the MET13 gene resulted in methionine auxotrophy, whereas single disruption of the MET12 gene gave variable or no methionine auxotrophy [50-53]. Measurement of enzyme activity in crude cell extracts showed both MET12 and MET13 proteins to be functional MTHFRs, with MET13 contributing the majority of activity [50-53]. S. cerevisiae MET13 was overexpressed in the met12 met13 double disruptant strain RRY3 [56]. Enzyme assays of desalted yeast extracts demonstrated that MET13 MTHFR uses NADPH as the reductant and is inhibited by AdoMet [56], similar to the pig and human enzymes. Significant substrate inhibition by CH$_2$-H$_4$folate was observed in the NADPH-CH$_2$-H$_4$folate oxidoreductase assay, as observed for porcine MTHFR [25], and the following kinetic parameters at 30 °C and pH 7.2 were estimated: $K_m$ (NADPH), 21 µM; $K_m$ (CH$_2$-H$_4$folate), 11 µM; and $K_i$ (CH$_2$-H$_4$folate), 455 µM [56]. Homogeneous MET13 protein has not yet been obtained. A molecular mass of 68.5 kDa has been calculated from the amino acid sequence, and alignment with the human enzyme indicates a similar two domain structure [50].
Fig. (5). Alignment of MTHFR amino acid sequences. Shown are the sequences for Met 13 from *S. cerevisiae* (yMTHFR/Met13) [50], MTHFR1 from *A. thaliana* (AtMTHFR1) [54], MTHFR from *H. sapiens* (hMTHFR) [6, 44], MTHFR from *T. thermophilus HB8* (tMTHFR) [88], and MetF from *E. coli* (eMTHFR/MetF) [61].

Conserved amino acids between the species are highlighted in bold.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
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<tr>
<td><em>A. thaliana</em></td>
<td>MKVUVOKTVKSRPQ-CQPT 17</td>
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<td><em>H. sapiens</em></td>
<td>MKIKDLLKARRGP-...L 14</td>
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<tr>
<td><em>E. coli</em></td>
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</table>
contradiction to the fungal enzymes, one of the two MTHFRs from *Arabidopsis thaliana* (AtMTHFR-1) has been purified as a histidine-tagged protein by nickel affinity chromatography [54]. The enzyme migrates as a 64 kDa band on SDS/PAGE and has an apparent molecular mass of 141 kDa by size exclusion chromatography, suggesting a dimer of 66 kDa subunits [54]. Unexpectedly, AtMTHFR-1 uses NADH preferentially over NADPH and is not sensitive to AdoMet inhibition [54], despite having a C-terminal domain homologous to that of the human, pig, and yeast enzymes. Just purified, plant AtMTHFR-1 loses activity readily; its kinetic parameters in the NADH-CH$_2$-H$_4$folate oxidoreductase assay at 30 °C and pH 7.2 were determined to be: $K_m$(NADH), 2.7 µM; $K_m$(CH$_2$-H$_4$folate), 287 µM; and $k_(cat)$(CH$_2$-H$_4$folate), 873 µM [56]. The specificity of the *A. thaliana* enzyme for NADH and a high NADH/NADPH ratio in plant cells suggest that the MTHFR reaction may be reversible under physiological conditions [54], in contrast to the deemed irreversible situation in the human and pig enzymes.

Lastly, eukaryotic MTHFR from the protozoan parasite *Leishmania major* has been overexpressed in *E. coli* and purified [57]. The enzyme has a calculated mass of 34.8 kDa, and sequence alignment shows 32% identity to human MTHFR in the N-terminal domain, but a complete absence of the C-terminal domain. As expected, the *L. major* enzyme is insensitive to AdoMet, and interestingly, it uses NADH or NADPH equally well as reductant. In the NADH-CH$_2$-H$_4$folate oxidoreductase assay at 37 °C and pH 7.2, kinetic parameters $K_m$(CH$_2$-H$_4$folate) and $k_(cat)$ were determined to be 15 µM and 11.1 s$^{-1}$, respectively [57].

3. BIOCHEMICAL CHARACTERIZATION OF BACTERIAL MTHFRS

### 3.1. *E. coli* MTHFR

MTHFR activity was first observed in crude *E. coli* cell extracts in 1961 by Buchanan and coworkers [58]. Subsequently, MTHFR was partially purified and shown to catalyze the reduction of CH$_2$-H$_4$folate to CH$_3$-H$_4$folate in the presence of NADH and FAD, suggesting it to be a flavoprotein [59]. Further purification led to an enzyme preparation that had lost the ability to utilize NADH and needed a flavin reductase to generate the reduced FAD for the reaction [60]. No additional characterization occurred until 1999 when Matthews and coworkers successfully overexpressed the cloned *metF* gene [61] in *E. coli* [62]. When purification proved difficult due to enzyme instability, they overexpressed MTHFR with a C-terminal histidine tag and purified it to homogeneity in high yield (50 mg/L culture) [62]. *E. coli* MTHFR is a homotetramer, in which each ~33 kDa subunit contains a noncovalently bound FAD [62]. An alignment of MTHFR sequences from human, *S. cerevisiae*, *A. thaliana*, and *E. coli* (Fig. (5)) shows that the bacterial enzyme differs from eukaryotic MTHFRs in the absence of a C-terminal regulatory domain. Within the N-terminal catalytic domain, however, *E. coli* MTHFR demonstrates ~30% identity to the eukaryotic enzymes.

The purified bacterial enzyme uses NADH as reductant and catalyzes the three oxidoreductase reactions (Eq (1), (3), and (4)) with kinetic parameters (Table (1)) [62, 63] generally comparable to those of porcine MTHFR. Marked inhibition was observed in the NADH-CH$_2$-H$_4$folate oxidoreductase reaction. Stopped-flow studies demonstrate that the *E. coli* enzyme can catalyze the individual half-reactions at rates consistent with the observed rate of catalytic turnover (Table (2)) [63]. As expected, there is no evidence that the bacterial enzyme is allosterically regulated by AdoMet [62]. Using a redox dye method developed by Massey [64], the midpoint potential of *E. coli* MTHFR was determined to be -237 mV [63]. Significantly, anaerobic titration of the enzyme with CH$_3$-H$_4$folate gave an $E_m$ of -223 mV [63]; the identical value was obtained for the porcine enzyme using the same method [22]. Gel filtration was used to investigate the stability of the histidine-tagged *E. coli* MTHFR. At low enzyme concentrations, the tetrameric enzyme was found to dissociate into dimers [65]. A later study showed that an enzymatically active dimer could be stabilized upon treatment of the enzyme with 1 M urea [66].

X-ray structures of *E. coli* MTHFR, free [65] and ligand-bound [67, 68], are available. MTHFR is a homotetramer, in which each subunit is a $\beta_8\alpha_8$ barrel. The tetramer (Fig (6)) is unusual in that it displays only C2 symmetry with the four polypeptide chains being held together by two types of interfaces. The FAD cofactor is bound in the center of the barrel with only the si face of the isoalloxazine ring exposed to solvent (Fig. (7)). Structures of the E$_m$(wild-type)$\cdot$NADH and E$_m$(Glu28Gln)$\cdot$CH$_2$-H$_4$folate complexes reveal that the ligands occupy partially overlapping sites at the si face of the FAD [67]. Thus, the binding of one substrate precludes the binding of the other, consistent with the ping-pong mechanism.

The availability of *E. coli* MTHFR and the known three-dimensional structures make it ideal for further investigation into the chemical mechanism as well as for examination of the catalytic roles of specific amino acid...
Fig. (6). Structure of the *E. coli* MTHFR tetramer viewed down the local 2-fold axis (PDB entry 1B5T). Each monomer is a β8α8 barrel, and the monomers are arranged in a planar rosette. The bound FAD is shown in ball-and-stick mode. The tetramer has 2-fold symmetry and is held together by two types of interfaces, A-B (A'-C) and B-C (A-A'). Reproduced, with permission, from Guenther et al [65].

Fig. (7). Structure of the *E. coli* MTHFR monomer (PDB entry 1ZP3). The FAD cofactor is bound in the center of the barrel and is shown in ball-and-stick mode. The β-strands are shown as arrows and the α-helices as coils. The view is toward the *si* face of the flavin isoalloxazine ring and along the substrate binding groove, showing the truncation of helix α8 and strand β8. Reproduced, with permission, from Pejchal et al [67].

Fig. (8). Structure of bound NADH and its interactions with wild-type reduced MTHFR (PDB entry (1ZP3)) [67]. NADH adopts a highly folded conformation and is sandwiched between the aromatic side chain of Phe 223 and the FAD isoalloxazine ring. Hydrogen bonds between NADH and Thr 59, Gln 183, and the solvent are indicated by dashed lines. A stopped-flow investigation of the bacterial reductive half-reaction demonstrated the formation of pre-steady state complexes [63], which had not been observed for porcine MTHFR [20]. Under anaerobic conditions at pH 7.2 and 25 °C, the reduction of the *E. coli* enzyme by NADH occurs in two exponential phases. In a fast phase, a transient, long-wavelength species appears rapidly at 550-650 nm. In a slow phase, the intermediate species decays at 550 nm, concomitant with a decrease in absorbance at 450 nm corresponding to flavin reduction. This slow phase is hyperbolically dependent on the concentration of NADH and has a limiting rate constant of 55 s⁻¹ (Table (2)). Taken together, the data at 450 and 550 nm are consistent with rapid formation of an Eox•NADH charge-transfer complex followed by its decay which occurs at the same rate as flavin reduction. The structure of the Ered(wild-type)•NADH complex (Fig (8)) [67] provides insight into the mechanism of the reductive half-reaction. The NADH is bound in an unusual, highly folded conformation, stabilized by π-π aromatic stacking interactions; the adenine ring is stacked against the nicotinamide ring and, in turn, the nicotinamide is stacked against the isoalloxazine of the FAD in an orientation that is favorable for hydride transfer. In addition to *E. coli* MTHFR, at least three examples have been reported of folded NADH in an orientation competent for hydride transfer within flavoproteins [69-71]. In MTHFR, the folded conformation of NADH is supported by the aromatic side chain of Phe 223, which stacks against the adenine ring, forming the outer layer of a 4-way sandwich. In the three cases cited, a 3-layer instead of a 4-layer stacking exists as the enzymes contain a Leu or Ile in the equivalent position of Phe 223 in *E. coli* MTHFR [69-71]. Curiously in MTHFR, Phe 223 is only partially conserved; the porcine enzyme, for example, contains a Leu at the corresponding position. Furthermore, the lack of flavin-pyridine
nucleotide charge-transfer complexes in the pig liver enzyme [20] suggests that the involvement of Phe 223 in the NADH stacking may be important for charge-transfer absorbance in MTHFR. In support of this view, charge-transfer complexes were not observed when Phe 223 was replaced by Leu or Ala in E. coli MTHFR [72].

The first structure of E. coli MTHFR [65] revealed an unusual Asp residue; invariant Asp 120 is positioned within 3.8 Å of the N1-C2=O position of the FAD isoalloxazine ring. A potentially negative charge at this location is in contrast to most flavoproteins, which typically have a positively-charged residue or the positive end of a helix dipole near the N1-C2=O position [73]. The predominant positive charge here is thought (1) to elevate the flavin redox potential to make the reduction more thermodynamically favorable and (2) to stabilize the anionic form of the reduced flavin hydroquinone [73]. A negatively-charged Asp 120 at the N1-C2=O position in MTHFR would, therefore, be expected to have opposite effects on the flavin redox chemistry: (1) to lower the redox potential and (2) to preferentially stabilize the neutral flavin hydroquinone to prevent unfavorable electrostatic repulsion. To test these proposals, Asp 120 in MTHFR was replaced by the following amino acids in order of decreasing negative-charge density: Asn, Ser, Ala, Val, and Lys [74]. Increases in redox potential were 17-19 mV (Asp120Asn, -Ser, -Ala), 23 mV (Asp120Val), and 30 mV (Asp120Lys) at 25 °C and pH 7.2. Moreover, the rates of flavin reduction by NADH were increased slightly, 1.3-1.5-fold [74]. Work is in progress to evaluate the effect of the mutations on the protonation state of the flavin hydroquinone. Although these results are consistent with a role for Asp 120 in modulation of the flavin redox properties, the changes due to the mutations are less than expected. The structure of the Ered(wild-type)•NADH complex [67] in comparison to that of the free enzyme [65] suggests an explanation. When NADH binds to the enzyme, Asp 120, a part of loop L4, moves from a “closed” conformation above the N1-C2=O position of the isoalloxazine to an “open” position with the 120 side chain moved away from the FAD by more than 1.0 Å [67]. In this open conformation, electrostatic repulsion between the negatively-charged Asp and the reduced FAD would be lessened during the reductive half-reaction. Thus, Asp 120 has less influence on the redox properties of MTHFR than predicted, and the Asp 120 mutants show small effects.

Fig. (9). Oxidative half-reaction catalyzed by MTHFR. CH2-H4folate binds at the si face of the reduced FAD. Protonation of N10 is proposed to lead to opening of the 5-membered imidazolidine ring and to formation of the 5-iminium cation. Transfer of a hydride from N5 of the reduced FAD to the exocyclic methylene group (C11) yields the product CH3-H4folate.

Kinetic, structural, and site-directed mutagenesis studies have also revealed mechanistic details of the oxidative half-reaction of E. coli MTHFR. Reduction of CH2-H4folate has different requirements than the reductive half-reaction involving NADH, but yet must be carried out in essentially the same active site as dictated by the ping pong kinetics. The substrate CH2-H4folate, the nitrogen analogue of an acetal, is not well activated for hydride attack. Thus, the reaction is believed to be initiated by acid-catalyzed protonation of N10, and opening of the imidazolidine ring to generate the 5-iminium cation intermediate (Fig. (9)). The oxidative half-reaction of E. coli MTHFR has been examined in the stopped-flow spectrophotometer under anaerobic conditions at pH 7.2 and at 25 °C [75] (Table (2)) and at 4 °C [74]. The reaction of reduced enzyme with CH2-H4folate at 25 °C, monitored by the increase in absorbance at 450 nm, exhibits two phases, each accounting for 50% of the total absorbance change: a fast phase with an observed rate constant of 34 s⁻¹ and a slow phase hyperbolically dependent on substrate concentration with a maximal rate constant of 10.3 s⁻¹ and an apparent K_d of 11 µM [63]. The origin of these biphasic kinetics is not yet understood; see discussion in [63] and [74]. The kcat for the NADH-CH2-H4folate oxidoreductase reaction is 10.4 s⁻¹ at 25 °C [63], indicating that the slow phase of the oxidative half-reaction is rate-limiting in overall turnover. At 4 °C, the kinetics of the oxidative half-reaction become more complex [74] (Table (2)). Reaction traces at 450 and 337 nm are best fit to three exponential phases. At 450 nm, an initial lag occurs
Fig. (10). Structure of bound CH$_3$-H$_4$folate and its interactions with Glu28Gln MTHFR (PDB entry 1ZP4) [67]. The CH$_3$-H$_4$folate is bound in an extended conformation, with its pterin ring stacked against the flavin isoalloxazine ring. Hydrogen bonds between CH$_3$-H$_4$folate and Asp 120, Gln 183, and Gln 219 are indicated by dashed lines.

Fig. (11). Stereoview of the superposition of the wild-type MTHFR-NADH complex (gray, PDB entry 1ZPT) and the Glu28Gln MTHFR-CH$_3$-H$_4$folate complex (white, PDB entry 1ZP4) [67]. The ligands occupy partially overlapping sites at the si face of the FAD. The L4 loop moves as a rigid unit to adopt two conformations with different orientations of Asp 120. The side chain of Phe 223 positions itself to stack either against the adenine ring of NADH or against the pABA ring of CH$_3$-H$_4$folate. Reproduced, with permission, from Pejchal et al [67] and Moon et al [72].
in the human and pig enzymes, has no effect on the CH$_2$-H$_4$folate binding affinity and actually accelerates the oxidative half-reaction by 3-fold [72].

Two functional roles for Asp 120 in the oxidative half-reaction have been suggested by the structure of the oxidized enzyme in complex with CH$_3$-H$_4$folate [67]. First, bidentate hydrogen bonding interactions between the carboxylate of Asp 120 and the N3 and N2-amino groups of the pterin ring may facilitate folate binding. Significantly, hydrogen-bonding interactions between an Asp and the pterin ring have been identified in thymidylate synthase [76], and in several other folate-dependent enzymes [77-85]. Second, the negatively-charged Asp 120 may also promote the formation and/or stabilization of the proposed 5-iminium cation intermediate. Asp 120 has already been described above as a conformationally mobile amino acid, which has different conformations in the free and NADH-bound enzymes. The structure of the E$_{ox}$(Glu28Gln)•CH$_3$-H$_4$folate complex shows Asp 120 in a closed conformation, like in the free enzyme. The carboxylate of Asp 120 side chain has moved back in toward the flavin, and toward the pterin ring of CH$_3$-H$_4$folate (see Fig. (11)) [67], most probably to aid the oxidative half-reaction. Indeed, consistent with these structural data, functional roles for Asp 120 in the binding of CH$_2$-H$_4$folate and in folate catalysis have been demonstrated [74].

Replacement of Asp 120 with Asn, Ser, Ala, Val, and Lys, residues of varying hydrogen bonding potential, increases the $K_d$ for CH$_2$-H$_4$folate up to 15-fold. The mutants also showed a 100-300-fold decreased rate in the oxidative half-reaction consistent with a role for Asp 120 in catalysis, perhaps in electrostatic stabilization of the 5-iminium cation [74].

In the proposed mechanism of the oxidative half-reaction (Fig. (9)), protonation of N10 by a general acid catalyst on the enzyme would enhance its leaving-group ability and, thereby, facilitate opening of the imidazolidine ring and concomitant formation of the 5-iminium cation intermediate. Structural data reveal a conserved Glu (Glu 28) positioned near N10 of CH$_3$-H$_4$folate and in coordination with a water molecule [67] (Fig. (10)). In analogy to Glu 60 of *E. coli* thymidylate synthase [86, 87], Glu 28 was postulated to serve as the general acid catalyst for N10 protonation, likely via water. Consistent with this hypothesis, a Glu28Gln mutant of MTHFR was unable to catalyze the reduction of CH$_2$-H$_4$folate to CH$_3$-H$_4$folate. The mutant does bind CH$_3$-H$_4$folate, however, as indicated by a perturbation of the flavin spectrum [75] and the E$_{ox}$(Glu28Gln)•CH$_3$-H$_4$folate structure [67]. In summary, structural and mechanistic studies of *E. coli* MTHFR suggest that the active site residues Phe 223, Asp 120, and Glu 28 are significant players in the reaction.

### 3.2. *Thermus thermophilus* HB8 MTHFR

Recently, Yamada and coworkers successfully overexpressed MTHFR from *Thermus thermophilus* HB8 in *E. coli* and purified the enzyme to homogeneity in good yield (25 mg/L culture) [88]. The enzyme is a homodimer of 33 kDa subunits; its amino acid sequence can be aligned with the full *E. coli* sequence and the N-terminal catalytic domain of the eukaryotic MTHFRs (Fig 5). Anaerobic titrations demonstrated full reduction of homogeneous enzyme with NADH or with CH$_3$-H$_4$folate. The purified enzyme showed activity in each of the three oxidoreductase assays (Eq (1), (3), and (4)) with the kinetic parameters given in Table (1) [88]. Curiously, significant substrate inhibition was observed in the CH$_2$-H$_4$folate-menadione oxidoreductase reaction (Eq (1)), but not in the NADH-CH$_2$-H$_4$folate oxidoreductase reaction (Eq (3)), as had been seen for the pig [25] and *E. coli* [63] enzymes. The X-ray structure of the purified enzyme revealed a surprising result: a dimer containing a holo-subunit with FAD noncovalently bound and an apo-subunit without FAD present; thus, one FAD is bound per dimer [88]. The structure of the holo-subunit is very similar to the *E. coli* MTHFR determined previously [65]. The apo-subunit, however, crystallized into a previously unobserved, closed structure. In the absence of FAD, the center of the barrel has become completely buried by helix a7a and the loop containing Asp 109 (Asp 120 in *E. coli* enzyme).

Treatment of the purified *T. thermophilus* HB8 enzyme with FAD created a “FAD-replete” MTHFR, which crystallized as a homodimer of two holo-subunits, each containing FAD [88]. Upon binding of FAD to the apo-subunit, the subunit rotates ~50° with respect to the holo-subunit, causing a large conformational change. Both FAD cofactors in the holo-subunits are reduced completely when titrated with NADH, and the enzyme demonstrates full activity in the NADH-menadione oxidoreductase assay. Titration with CH$_2$-H$_4$folate results in full reduction of the enzyme, suggesting that the added FAD is bound in the proper orientation. By contrast, the FAD-replete homodimer shows only ~50% activity in the CH$_3$-H$_4$folate-menadione and the NADH-CH$_2$-H$_4$folate oxidoreductase assays. An examination of the active site structure provides a reasonable explanation for this reduction in folate activity. Asp 109, located above the N1-C2=O position of the FAD, is the equivalent of Asp 120 of *E. coli* MTHFR. As described above, Asp 120 hydrogen bonds to the folate pterin ring and it plays a significant role in both folate binding and catalysis [74, 75]. In the bacterial enzyme, the loop containing Asp 120 occupies closed and open positions, respectively, in the NADH-bound and CH$_3$-H$_4$folate-bound structures (Fig. (11)) [67]. In the *Thermus*
FAD-replete structure, the Asp 109 loop appears to be in a similarly closed position, suggesting that the aspartate is not properly situated for reaction with folate [88].

3.3. *Clostridium formicoaceticum* and *Peptostreptococcus productus* MTHFRs

Lastly, MTHFRs from *Clostridium formicoaceticum* and *Peptostreptococcus productus* have been characterized [89, 90]. However, these enzymes bear little resemblance to the eukaryotic and bacterial MTHFRs described thus far in this review. The enzyme from *C. formicoaceticum* has been purified to homogeneity, and it is an iron-sulfur, zinc-containing flavoprotein with an α₄β₄ oligomeric structure (~237 kDa). Under anaerobic conditions which are necessary for stability, *C. formicoaceticum* MTHFR catalyzes the reduction of CH₂-H₄folate in the presence of reduced ferredoxin as reductant [89]. The enzyme from *P. productus* is stable to oxygen and is isolated as an octamer of 32 kDa subunits containing 4 FAD per octamer. In catalyzing the CH₂-H₄folate to CH₃-H₄folate conversion, the purified enzyme uses NADH as reductant [90], similar to the *E. coli* and *T. thermophilus* HB8 MTHFRs. No further characterization of the enzymes has appeared in the literature.

4. MEDICAL SIGNIFICANCE OF MTHFR

4.1. MTHFR and Human Physiology

Deficiency in MTHFR is a relatively rare human disease with varying severity [5]. A number of mutations associated with severe deficiency in patients have been identified, enabled by the availability of the human cDNA [44, 91-94]. These patients usually have 0-20% of wild-type enzyme activity, very elevated levels of homocysteine in their blood and urine, and low levels of methionine. Hyperhomocyst(e)inemia likely occurs from insufficient CH₃-H₄folate for remethylation of homocysteine to produce methionine. Individuals present with a wide range of clinical symptoms, including developmental delay, vascular complications, and psychiatric abnormalities. The most severely affected patients do not survive [5].

A variant form of MTHFR deficiency characterized by mild hyperhomocyst(e)inemia and present in patients with coronary heart disease was first described by Kang in 1991 [95] and later by Blom in 1995 [96]. Individuals with this mild deficiency were identified by having a thermolabile form of the enzyme. Thermolability is determined by using the CH₃-H₄folate-menadione oxidoreductase assay (Eq (1)); it is the difference between the enzyme activity measured in crude lymphocyte extracts after heating to 46 °C for 5 minutes and the activity measured after heating to 37 °C for the same amount of time. In 1995, using a sample of 40 patients with premature vascular disease, it was demonstrated that a common polymorphism of MTHFR, a C677T mutation which leads to the substitution of Ala 222 with Val in the amino acid sequence, was associated with mildly elevated levels of blood homocysteine, reduced enzyme activity, and increased thermolability in lymphocyte extracts [6]. Taken together, the data suggest that the C677T polymorphism is likely the mild form of MTHFR deficiency first recognized by Kang in the cardiac patients.

The C677T polymorphism is common in most human populations. About 10-15% of North American whites are homozygous for the mutation (TT genotype) [6, 97, 98]. The prevalence in several European populations (e.g., France, Hungary) [6, 99] is similar to that in North American whites, but some areas of lower frequency (~6%) also exist (e.g., Finland, Russia) [99]. Values as high as 25% have been reported for Hispanic Americans [100].

Once identified and shown to be highly prevalent in the world’s populations, the C677T polymorphism became the subject of intense study. Enzyme activity and homocysteine levels were measured in groups of individuals homozygous and heterozygous for the mutation. Generally, 677CT heterozygotes were intermediate in their properties compared to the TT and the CC (wild-type) homozygotes. Lymphocytes from heterozygous and homozygous mutant individuals typically showed specific activities 40% and 70% of the wild-type value, as described by Christensen *et al* [101]. Additional studies demonstrated that 677TT mutant homozygotes have ~25% higher homocysteine levels than the CC homozygotes, particularly when the folate status is low [97, 102-106]. Significantly, in these individuals, folate supplementation has resulted in lower homocysteine levels [103, 105].

A second, common polymorphism in MTHFR, A1298C, resulting in replacement of a glutamate with an alanine at position 429 in the regulatory domain of the protein, was first identified in human ovarian carcinomas [107]. The laboratories of Blom [108] and Rozen [109] subsequently characterized the mutation, which had a frequency of ~10% in the small Dutch and Canadian populations analyzed. Lymphocyte extracts from homozygous 1298CC individuals showed 60% of wild-type enzyme activity, although a similar reduction was observed in extracts from individuals heterozygous for both 1298AC and 677TT mutations. The A1298C polymorphism did not result in higher plasma homocysteine in its homozygous state.
4.1.a. MTHFR and Cardiovascular Diseases

The relationships between the C677T MTHFR polymorphism, plasma homocysteine concentrations, and cardiovascular disease have been examined extensively since 1995. Some studies have found a positive association between the MTHFR 677TT mutant genotype and various cardiovascular diseases [110-114], supporting the hypothesis that moderate hyperhomocysteinemia is a causal factor for cardiovascular disease. However, other reports have found no association [104, 106, 115, 116]. If hyperhomocysteinemia were a cause of cardiovascular disease, we would expect that intervention trials using folate supplementation to lower homocysteine levels would significantly reduce disease risk. However, large folate therapy intervention trials have shown little benefit to high-risk patients [117, 118]. A number of pathogenetic mechanisms have been proposed to explain a possible role for elevated homocysteine in vascular injury. These include the promotion of endothelial oxidative damage, platelet activation and aggregation, inflammation, proliferation of vascular smooth muscle cells, and impairment of DNA methylation (reviewed in [119]).

4.1.b. MTHFR and Neural Tube Defects

The MTHFR C677T polymorphism has been identified as a genetic risk factor for neural tube closure defects (NTDs) (reviewed in [4]). These birth defects are congenital malformations of the central nervous system and include the conditions anencephaly and spina bifida, where, respectively, the anterior and posterior end of the neural tube does not close. Early studies established a link between elevated homocysteine levels of mothers and incidence of NTDs in their children [120, 121]. In 1995, Blom and coworkers first demonstrated in a Dutch population that the homozygous 677TT genotype in infants with spina bifida or their mothers was associated with increased occurrence of this defect [122]. Similar results were found for NTDs in an Irish population [123]. A meta-analysis in 2006 indicated that the TT genotype in the affected child confers an overall 1.9-times increase in NTD risk [124]. However, a more recent meta-analysis showed a positive association with non-Latin European populations only [125]. The A1298C polymorphism has also been linked to NTDs in one study [108], but a recent meta-analysis demonstrated that there is no significant association between the mutation and NTD susceptibility in white populations [126]. Elucidation of the metabolic basis for NTDs first focused on the role of homocysteine, which is elevated in the C677T individuals. It has been well established that folic acid supplementation in mothers reduces the risk of their offspring being born with a NTD by more than 50% [127], presumably due to the lowering of homocysteine levels. Indeed, since the mandatory fortification of enriched grain products with folic acid in 1998 [128], the prevalence of NTDs in the United States has declined significantly [129]. To explain the association between TT MTHFR and folate-responsive NTDs, Blom has put forth the methylation hypothesis [124]. Insufficient CH$_3$H$_4$folate due to a dysfunctional MTHFR causes homocysteine levels to rise and AdoMet levels to fall, which leads to decreased methylation in the cell at a time of rapid growth, resulting in NTDs. Homocysteine is not a cause, itself, of NTDs, but rather a biomarker of disturbed one-carbon metabolism. In support of this proposal, Blom and coworkers found that treatment of cultured chicken embryos with methylation inhibitors causes a dose-dependent delay in neurulation [130]. Interestingly, the prevalence of the C667T polymorphism cannot fully explain the decrease in NTD risk in response to folic acid supplementation, suggesting that the underlying mechanisms of NTDs are not completely known [4, 124].

4.1.c. MTHFR and Psychological Disorders

Since dementia and Alzheimer’s disease could be logically linked to vascular disease, these disorders have also been investigated for a possible association with homocysteineinemia and the MTHFR polymorphisms. Elevated levels of homocysteine are indeed found in Alzheimer’s disease patients compared to controls [3], and a recent meta-analysis showed a significant association between the C677T mutation and Alzheimer’s disease in East Asians, although not in Caucasians [131]. Several possible explanations have been offered for the function of homocysteine in this complex neurodegenerative disorder (reviewed in [131]). For example, by promoting vascular lesions and atherosclerosis, homocysteine may reduce blood supply to the brain and thereby hasten neuron apoptosis. A systematic review found that folic acid and other B vitamin supplementation did not significantly improve cognitive function [132], but further investigation is needed. Meta-analyses examining the link between the C677T polymorphism and psychiatric disorders yielded a significant association with depression [133, 134].

4.1.d. MTHFR and Cancer

The findings that individuals homozygous for 677TT exhibit lower levels of genomic DNA methylation under low folate status [135] and that global DNA hypomethylation is observed in early tumorigenesis [136], suggested that the C667T mutation could be connected with the development of cancer. Indeed, a recent, large-scale meta-analysis found a significant correlation between the polymorphism and an increased risk for both esophageal
and gastric cancers [7]. By contrast, according to meta-analyses, the C667T mutation confers a protective effect on colorectal cancer [7] and on acute lymphoblastic leukemia in children [8] and adults [9]. These results may be explained by considering that the substrate of MTHFR, CH$_2$-H$_4$folate, not only provides one-carbon units for methionine biosynthesis and AdoMet-dependent methylation reactions, but also for dTMP synthesis and purine biosynthesis (Fig. (1)). Thus, the reduction of MTHFR activity caused by the C677T polymorphism may lead to increased availability of CH$_2$-H$_4$folate and, therefore, more efficient DNA synthesis. In addition, as first described by Ames and coworkers [137], elevated CH$_2$-H$_4$folate levels would cause a decrease in the amount of uracil in DNA, leading to a smaller probability of DNA strand breakage and cancer, as a result of uracil misincorporation. The findings suggest that the protective effect of the C677T mutation may be most beneficial in tissues such as colorectal epithelium or bone marrow, with high levels of DNA synthesis [7]. Moreover, these studies suggest that the C677T polymorphism may be so prevalent in the population because reduction of MTHFR activity may be more tolerable than uracil misincorporation.

### 4.2 Mouse models of MTHFR

To investigate the *in vivo* pathogenetic mechanisms of MTHFR deficiency, knockout mice homozygous and heterozygous for *Mthfr* were generated [138, 139]. In 2001, Rima Rozen and coworkers incorporated a targeted disruption in the *Mthfr* gene in BALB/c mice, and characterized the phenotypes of the heterozygous and homozygous *Mthfr*-deficient animals [138]. The *Mthfr*+/- mice showed normal growth and similar survival to that of wild-type mice. By contrast, the *Mthfr*+/- mice developed more slowly, had ~20% lower body weights, and exhibited a significantly reduced survival rate (26% at 5 weeks) [140] than their wild-type and heterozygous litter mates. The homozygous null mice were void of MTHFR enzyme activity in all tissues, whereas the heterozygotes showed 60% residual activity, similar to the 40% value observed in patients homozygous for the C677T polymorphism [101]. In the *Mthfr*+/- and *Mthfr*+ mice, the plasma total homocysteine levels were 1.6- and 10-fold higher, respectively, than the wild-type controls. AdoMet levels were decreased, but AdoHcy levels were elevated considerably, with global DNA hypomethylation observed in both heterozygotes and homozygotes. An altered distribution of folates was seen with the proportion of CH$_2$-H$_4$folate significantly reduced in plasma, liver, and brain of the null mice. Because the very low survival rate of the BALB/c *Mthfr*+/- mice prevented a thorough examination of the homozygous genotype, a second *Mthfr* mouse model on a C57B1/6 genetic background was generated and characterized [139]. Indeed, enhanced survival (81% at 5 weeks) and an overall less severe phenotype were observed, although the levels of homocysteine are increased 25% in these C57B1/6 *Mthfr*+/- mice compared to the homozygous BALB/c mice. Overall, in comparing the generated mouse models to human patients, the heterozygous knockout mice appear to be a good animal model for individuals homozygous for the C677T polymorphism, whereas the homozygous null mice are a good model for severely MTHFR-deficient individuals. Moreover, the use of two different genetic backgrounds allows investigation of the variable phenotypes within MTHFR deficiency.

Recently, the heterozygous and homozygous *Mthfr*-deficient animals have been valuable in examining the role of MTHFR and MTHFR polymorphisms in cardiovascular disease, neural tube defects, and protection against cancer. Studies of vascular function in *Mthfr*+/- BALB/c mice showed that the heterozygous genotype sensitizes mice to hyperhomocysteinemia induced by a low folate diet, but that the effects on endothelial function are influenced mainly by diet rather than *MTHFR* genotype [141, 142]. The heterozygous mice were also examined as a model for NTDs. *Mthfr*+/- or *Mthfr*+/- BALB/c females fed control or folate-deficient diets were mated with *Mthfr*-/- males, and the embryos were examined at 10.5 days post coitum [143]. Delay in embryonic growth, smaller embryos, and a low frequency of the NTD exencephaly were observed in offspring of the heterozygous mothers fed low dietary folate, but these effects were also seen in some *Mthfr*+/- animals on low folate. Thus, although no direct association can be made between maternal MTHFR genotype and the embryonic phenotypes observed, these studies do highlight the importance of genotype in conjunction with adequate folate in the prevention of adverse pregnancy outcomes. Lastly, in a study where *Mthfr*+/- animals were mated with mice predisposed to tumorigenesis (*Apc*min/+), *Mthfr* deficiency was found to reduce adenoma formation, in support of a role for C677T MTHFR in reducing cancer risk [144].

### 4.3 Biochemical Characterization of MTHFR Polymorphisms

#### 4.3.a C677T Polymorphism

Frosst et al first identified the common C677T mutation, which leads to the substitution of Ala 222 with a valine in the human MTHFR enzyme [6]. Ala 222 is located in the catalytic domain of the human enzyme, and this amino acid is highly conserved amongst MTHFR enzymes. However, as shown in the sequence alignment (Fig. (5)), the Ala is not invariant as a glycine occurs at this position in the *S. cerevisiae* MET13 MTHFR [50] and a leucine occurs in the *T. thermophilus* HB8 enzyme [88]. Ala222Val mutant and wild-type human MTHFRs were
increase the levels of all folic acid derivatives in the cell, and this increased intracellular concentration of folate both mutant and wild-type MTHFRs are protected by folates, the more dramatic effect would be expected for the TT would be expected to stabilize MTHFR against flavin dissociation and loss of activity. While the data show that structures of wild-type [65] and Ala177Val mutant [68] individuals due to their increased propensity for flavin loss.

β

observed a significantly decreased growth rate of assay (Eq (3)). However, the Ala177Val variant is thermolabile [65], a result confirmed by a later study which lowered the melting temperature at 60 µM by 7.1 °C for the mutant compared to the wild-type enzyme, and both proteins denature at progressively lower temperatures as they are diluted [65]. These results suggest the formation of an altered oligomeric state of the enzyme, which was tested by subjecting the mutant and wild-type enzymes to gel filtration chromatography on Superose 12. At a concentration of 60 µM, both proteins elute as tetramers, however, when diluted to 0.5 µM, the proteins elute as dimers. Furthermore, dissociation to dimers is accompanied by loss of enzyme activity and release of the FAD coenzyme. Free FAD is fluorescent, whereas FAD bound to MTHFR is not. By monitoring the release of FAD by fluorescence spectroscopy, it was shown that the Ala177Val mutant loses its FAD at an 11-fold faster rate than does wild-type MTHFR. Significantly, for both enzymes, the initial rate of flavin loss varies as the square root of the protein concentration, suggesting the kinetic model for FAD release depicted in Fig. (12). In the mechanism, a rapid, reversible holoenzyme tetramer/dimer equilibrium is established, which is followed by ratereducing irreversible loss of FAD from the holoenzyme dimers. Taken together, the results suggest that the Ala177Val mutation has no influence on the kinetic parameters of bacterial MTHFR, but rather reduces enzyme stability and affinity for FAD, and thus increases the tendency to form inactive enzyme via flavin dissociation, compared to the wild-type enzyme [65].

![Fig. (12)](image)

**Fig. (12).** Proposed kinetic model for FAD dissociation in diluted *E. coli* MTHFR. The homotetrameric enzyme dissociates into holodimers in a rapid and reversible step, which is followed by rate-limiting flavin release. Reproduced, with permission, from Yamada and Matthews [10].

In view of the use of folate supplementation to reduce homocysteine levels, it was of interest to examine the effect of added folate in experiments with the Ala177Val variant enzyme. When CH3-H4folate is added upon dilution of the mutant and wild-type enzymes, it slows the rate of flavin dissociation and loss of activity, and the Ala177Val variant requires a higher concentration of CH3-H4folate to reduce the rate of FAD loss to the same value as the wild-type enzyme. Protection was also observed in the presence of CH2-H4folate and H2folate [65]. These results provide a reasonable explanation for the observed reduction in homocysteine levels in TT individuals with high folate status. Dietary supplements of folic acid are likely to increase the levels of all folic acid derivatives in the cell, and this increased intracellular concentration of folate would be expected to stabilize MTHFR against flavin dissociation and loss of activity. While the data show that both mutant and wild-type MTHFRs are protected by folates, the more dramatic effect would be expected for the TT individuals due to their increased propensity for flavin loss.

Insight into the Ala177Val phenotype and the protection of the enzyme by folates has come from X-ray structures of wild-type [65] and Ala177Val mutant [68] *E. coli* MTHFRs. Ala 177 is located at the bottom of the βαα9 barrel; its side chain lies in a tight loop which connects helix α5 with strand β6. While Ala 177 does not contact the bound FAD directly, helix α5 contains several residues which interact with the cofactor. Upon mutation of Ala 177 to the bulkier valine, helix α5 is displaced, altering interactions between the adenine and adenine phosphate portions of FAD and residues Asn 168, Arg 171, and Lys 172 and introducing two solvent water molecules. Together, these structural perturbations are consistent with the observed weaker binding of FAD by the Ala177Val mutant. Since the loss of FAD from the mutant is linked to dissociation of the tetramer into dimers [65], the wild-type and Ala177Val MTHFR structures were also examined for differences in intersubunit contacts in the tetrameric enzyme [68]. As described above, the *E. coli* MTHFR tetramer has two-fold rather than four-fold symmetry and is held together by two types of interfaces. A comparison of the areas of subunit contact predicts that
This review summarizes a large body of literature on methylenetetrahydrofolate reductase. Several methods including steady-state and stopped-flow kinetics, mutagenesis, and structural determination, have led to significant advances in the understanding of the biochemistry of this medically important enzyme. A picture has begun to emerge of how the amino acids at the active site carry out the direct reduction of CH₂-H₄folate following NAD(P)H-linked reduction of the FAD. Biochemical characterization of the common C677T polymorphism has begun to emerge of how the amino acids at the active site carry out the direct reduction of CH₂-H₄folate following significant advances in the understanding of the biochemistry of this medically important enzyme. A picture has begun to emerge of how the amino acids at the active site carry out the direct reduction of CH₂-H₄folate following significant advances in the understanding of the biochemistry of this medically important enzyme. 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been central to our understanding of the molecular basis of this mutation. In human patients, the polymorphism is linked to a number of diseases, through its association with hyperhomocyst(e)inemia. However, homocysteine levels can be influenced by genetic, nutritional, and physiological factors. Particularly in the case of the C677T mutation, the folate status of the patient is critical for predicting phenotypic outcomes. The mouse model for MTHFR deficiency has proven very useful in this context because of the controlled conditions and detailed metabolic measurements possible. An understanding of the determinants for hyperhomocyst(e)inemia including the role of MTHFR will be necessary for successful prevention or treatment of these complex diseases.

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ABBREVIATIONS:

AdoHcy = adenosylhomocysteine
AdoMet = S-adenosylmethionine
H\textsubscript{2}folate = 7,8-dihydrofolate
CH\textsuperscript{+}=H\textsubscript{4}folate = 5, 10-methenyltetrahydrofolate
CH\textsubscript{2}-H\textsubscript{4}folate = 5,10-methylenetetrahydrofolate
CH\textsubscript{3}-H\textsubscript{4}folate = 5-methyltetrahydrofolate
10-CHO-H\textsubscript{4}folate = 10-formyltetrahydrofolate
E\textsubscript{m} = midpoint potential
FAD = flavin adenine dinucleotide
Hcy = homocysteine
H\textsubscript{2}folate = tetrahydrofolate
MTHFR = methylenetetrahydrofolate reductase
pABA = p-aminobenzoate
STEM = scanning transmission electron microscopy

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