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Methylenetetrahydrofolate Reductase: A Common Human Polymorphism and Its Biochemical Implications

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ABSTRACT: Methlenetetrahydrofolate (CH_2-H_4folate) is required for the conversion of homocysteine to methionine and of dUMP to dTMP in support of DNA synthesis, and also serves as a major source of one carbon unit for purine biosynthesis. This review presents biochemical studies of a human polymorphism in methylenetetrahydrofolate reductase, which catalyzes the reaction shown below. The mutation decreases the flux of CH_2-H_4folate into CH_3-H_4folate, and is associated with both beneficial and deleterious effects that can be traced to the molecular effect of the substitution of alanine 222 by valine. © 2002 The Japan Chemical Journal Forum and John Wiley & Sons, Inc. Chem Rec 2: 4–12, 2002

Key words: flavoprotein; homocysteine; methionine

Introduction

One of the more remarkable chemical syntheses carried out by biological organisms is the *de novo* biosynthesis of methyl groups. Du Vigneaud and Bennett are credited with the initial observations that rats could synthesize methionine from homocysteine in the absence of a source of preformed methyl groups, and this synthesis was later shown to require the presence of folate and cobalamin in the diet. Extensive studies in the laboratories of Stokstad, Huennekens, Buchanan, and Weissbach identified the enzymes responsible for *de novo* synthesis of methyl groups in mammals as methylenetetrahydrofolate reductase and methionine synthase and elucidated the roles of folic acid, riboflavin, and cobalamin as cofactors in these reactions. An excellent history of these early studies is provided by Blakley.[1]

A major source of the methylene group of methylenetetrahydrofolate is the β-carbon of serine, which is transferred to tetrahydrofolate by the action of serine hydroxymethyltransferase, as shown in Equation 1. Alternate sources of the methylene group include formate, which is converted to 10-formyltetrahydrofolate, and thence to methenyl- and finally methylenetetrahydrofolate by the action of formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclohydrolase, and methylenetetrahydrofolate dehydrogenase.[2]

Methylenetetrahydrofolate is reduced to methyltetrahydrofolate by methylenetetrahydrofolate reductase, as shown in Equation 2. The enzyme contains noncovalently bound flavin adenine dinucleotide as a cofactor; the flavin accepts reducing equivalents from NAD(P)H and transfers them to methylenetetrahydrofolate.

Methionine synthase catalyzes the transfer of the nascent methyl group from methyltetrahydrofolate to homocysteine,

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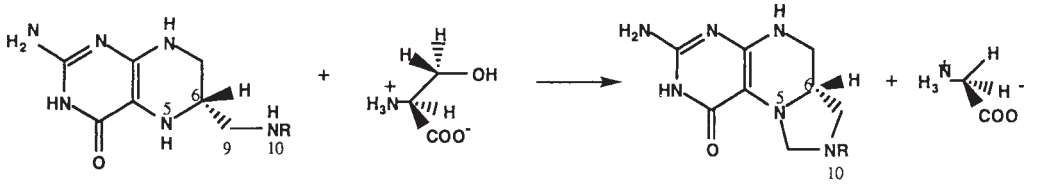
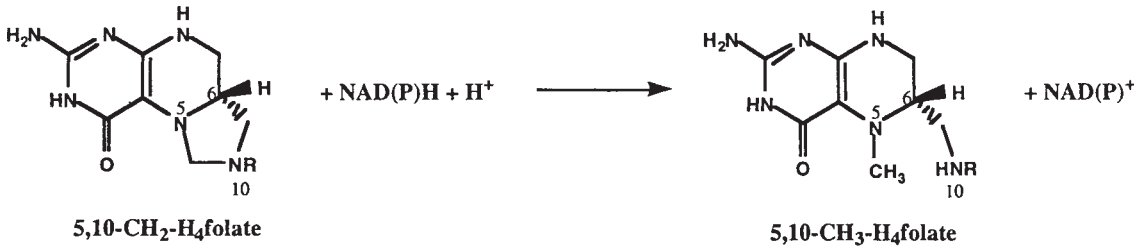


(1)

forming methionine and tetrahydrofolate, as shown in Equation 3. Some prokaryotes have two alternative methionine synthase enzymes, a cobalamin-independent enzyme and a cobalamin-dependent enzyme. In *Escherichia coli*, the former is the product of the *metE* gene, and is often referred to as MetE, while the latter is the product of the *metH* gene. Mammals, including humans, have only the cobalamin-dependent methionine synthase (homologous to MetH), while plants, Archaea, and yeast have only MetE homologues.

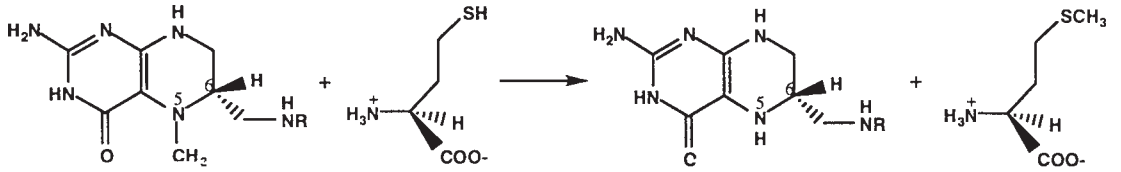
CH_2-H_4folate not only provides one-carbon units for methionine biosynthesis and remethylation of homocysteine, but also for the conversion of dUMP to dTMP in the reaction catalyzed by thymidylate synthase, and for purine biosynthesis following conversion of CH_2-H_4folate to 10-formyl-H_4folate. Regulation of flux through these competing reactions is accomplished in part by allosteric inhibition of MTHFR by adenosylmethionine (AdoMet), a major cellular methyl donor that is derived from methionine and reflects the availability of methionine in the cell. In this instance, AdoMet serves as a regulatory molecule but does not transfer a methyl group to MTHFR.

The pathway from methylenetetrahydrofolate to methionine in humans has become a topic of considerable medical interest with the discovery that elevated levels of homocysteine in human plasma, hyperhomocysteinemia, are associated with significant risks for the development of cardiovascular disease and Alzheimer's disease in adults and for neural tube defects in the developing fetus.[3–5] Mutations in methylenetetrahydrofolate reductase that lead to loss of enzyme function are associated with severe hyperhomocysteinemia and aggressive development of cardiovascular disease.[6] In addition, a common C677T polymorphism in



(2)

Rowena G. Matthews was born in Cambridge, England. She studied with George Wald at Harvard, earning a B.A. in 1963, and with Vincent Massey at the University of Michigan where she earned a Ph.D. degree in Biophysics in 1969. Her postdoctoral training, also at the University of Michigan, was with Charles Williams, Jr. In 1975 she became a member of the faculty at the University of Michigan where she is now G. Robert Greenberg Distinguished University Professor of Biological Chemistry and Senior Scientist in the Biophysics Research Division. She is a fellow of the American Association for the Advancement of Science, received the William Rose Award from the American Society for Biochemistry and Molecular Biology in 2000, and was the 2001 recipient of the Repligen Award for the Chemistry of Biological Processes given by the Biological Chemistry Division of the American Chemical Society. ■



(3)

CH_3-H_4folate **L-homocysteine** **H_4folate** **L-methionine**

the human MTHFR gene that leads to substitution of Ala 222 by Val,[7,8] is associated with mild hyperhomocysteinemia in humans, and may therefore be a risk factor. Mutations leading to loss of function in methionine synthase also lead to severe hyperhomocysteinemia.

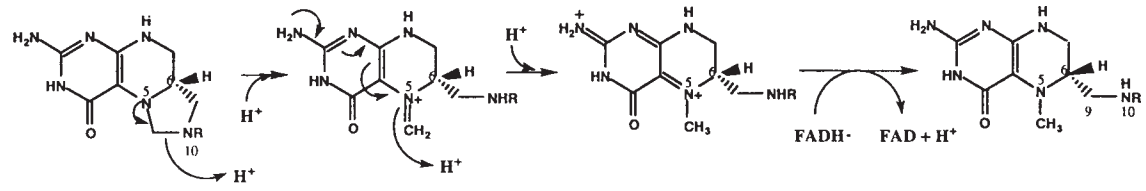
This review is focused on our studies of methylenetetrahydrofolate reductase. The purification and characterization of the porcine enzyme, and particularly the elucidation of peptide sequence, made possible the cloning and sequencing of the gene specifying the human enzyme and the discovery of the common C677T polymorphism. The potential medical importance of this polymorphism, and the emerging recognition of the role of dietary folate supplementation in lowering mild hyperhomocysteinemia in humans, in turn led to studies of methylenetetrahydrofolate reductase from *E. coli*, and construction of a homologue of the C677T mutation in the *E. coli* enzyme. These studies led to the determination of the X-ray structure of *E. coli* methylenetetrahydrofolate reductase. And more recently, we have returned to the mammalian enzymes, developing a system for successful overexpression of the human enzyme and characterizing the biochemical phenotype of the C677T polymorphism in the human enzyme. Our research has been driven by the conviction that understanding cellular biochemistry at the molecular level is a necessary prerequisite for treatment of human disease. Our biochemical studies have been carried out in parallel with complementary studies by structural biologists, geneticists, and physicians, and this interaction has shaped the chemical questions that we seek to answer.

Properties of Mammalian Methylenetetrahydrofolate Reductase

Methylenetetrahydrofolate reductase was initially purified several 100-fold from pig liver and shown to be a flavoprotein.[9] The FAD cofactor was shown to be alternately reduced by NADPH and reoxidized by methylenetetrahydrofolate. The pig liver enzyme was purified to homogeneity in my laboratory with a 32,000-fold enrichment in specific activity as compared to a pig liver homogenate.[10]

My interest in this enzyme was initially engendered by the observation that tetrahydrofolate, like dihydroflavin, is a redox cofactor. I wondered if the reduction of methylene- to methyltetrahydrofolate might exploit the redox properties of the substrate, as shown in Equation 4. The facile catalysis of NADPH-linked reduction of menadione, and of quininoid 6,7-dimethyldihydropterins (**1**) by methylenetetrahydrofolate reductase supported this hypothesis. However, when the flavin cofactor is substituted by 8-demthyl-8-hydroxy-5-deaza-5-carbaflavin adenine dinucleotide (5-deazaFAD), the enzyme catalyzes NADPH-linked reduction of methylenetetrahydrofolate at >50% the rate of native FAD-containing enzyme.[11]

5-DeazaFAD readily accepts and donates hydride ions but is unable to accept or donate single electrons because the semiquinone oxidation state is destabilized.[12,13] Because reduction of 5-deazaFAD leads to incorporation of hydride into a nonexchangeable position on the deazaflavin, the regiospecificity of hydride transfer from deazaflavin hydroquinone to the substrates can be determined.[14] Tritium from (5*S*)-[5-^{3}H]-5-



(4)

CH_2-H_4folate **5-iminium cation** **q- 5-methyl-H_2folate** **CH_3-H_4folate**

deazaFAD is incorporated quantitatively into both $NADP^+$ and methylenetetrahydrofolate, consistent with hydride transfer mechanisms. Furthermore, these studies indicated that each substrate in turn reacts at the *si* face of the flavin cofactor, consistent with the Bi-Bi ping-pong kinetics, in which release of the first product precedes binding of the second substrate, which has been previously observed with this enzyme.

Mammalian MTHFRs are polypeptides of 70–77 kDa, and the porcine enzyme has been shown to be a homodimer.[15] Each subunit contains two distinct regions, an N-terminal region that shares homology with the smaller prokaryotic MTHFRs and is thus presumed to be responsible for catalysis of the overall reaction, and a C-terminal region that is unique to the eukaryotic MTHFRs. The C-terminal region has been shown to bind AdoMet, and is thought to be involved in allosteric regulation of MTHFR activity in response to AdoMet levels in the cell.[16]

Despite the fact that 4 kg of pig liver only yielded about 1 mg of homogenous protein, James Sumner laboriously obtained peptide sequences for about 40% of methylenetetrahydrofolate reductase from pig liver. These peptide sequences allowed the cloning of the human MTHFR cDNA.[7,8]

Methylenetetrahydrofolate Reductase from *E. coli*

Characterization of mammalian MTHFRs has been a challenge because of the very low level of enzyme activity in mammalian tissues. Early attempts at expressing recombinant human MTHFR in bacterial cells produced very low levels of activity, which were not suitable for isolation of enzyme in amounts appropriate for biochemical characterization.[8] We therefore developed procedures for overexpression and purification of histidine-tagged MTHFR from *E. coli*.[17] The bacterial enzyme could be expressed at high levels, yielding about 55 mg of purified enzyme per liter of cell culture and could be easily purified using a nickel affinity column. The purified enzyme is a homotetramer of 33 kDa subunits. Sequence comparisons indicate that the *E. coli* enzyme differs from its mammalian counterpart in that it lacks a C-terminal regulatory domain. The sequence of the N-terminal catalytic domain of the human enzyme is 30% identical to the *E. coli* sequence.[18]

While the mammalian enzyme is a homodimer, the prokaryotic enzyme is a homotetramer.[17] However, the gross structure of the two enzymes may be similar. Scanning transmission electron microscopy revealed that the porcine enzyme consists of four globular domains arranged in a planar rosette; these four domains are presumably the two catalytic domains and the two regulatory domains of the homodimer. The X-ray structure of the enzyme from *E. coli*,[18] to be described in detail below, revealed that the four identical subunits are also arranged in a planar rosette.

The other respect in which the *E. coli* enzyme differs significantly from the human enzyme is in its specificity for NADH rather than NADPH.[17] Mammalian MTHFR enzymes catalyze essentially irreversible reductions of CH_2-H_4folate due to the large standard free energy of the reaction (–6.39 kcal, calculated from reference 19) and the high ratio of NADPH to $NADP^+$ in mammalian cell cytoplasm. Because the NADH/NAD^+ ratio in cell cytoplasm is very low,[20] the NADH-linked reaction is freely reversible.[21] Methylenetetrahydrofolate reductase from higher plants is also NADH-dependent, and although the plant enzymes contain a regulatory domain, they are not inhibited by AdoMet.[21]

An extensive kinetic characterization of the *E. coli* enzyme has been performed using stopped-flow spectrophotometry.[22] Comparison of the rates of flavin reduction by NADH and of flavin reoxidation by CH_2-H_4folate measured in the stopped-flow spectrophotometer with k_{cat} for steady-state turnover in the NADH-CH_2-H_4folate oxidoreductase reaction indicates that the enzyme is kinetically competent to catalyze this reaction by a ping-pong Bi-Bi mechanism (Fig. 1). Reoxidation of the reduced flavin by CH_2-H_4folate is largely rate-limiting in the overall reaction.

The X-ray structure of MTHFR from *E. coli* was determined in collaboration with Brian Guenther and Martha Ludwig.[18] This structure revealed that MTHFR is a $\beta_8\alpha_8$ bar-

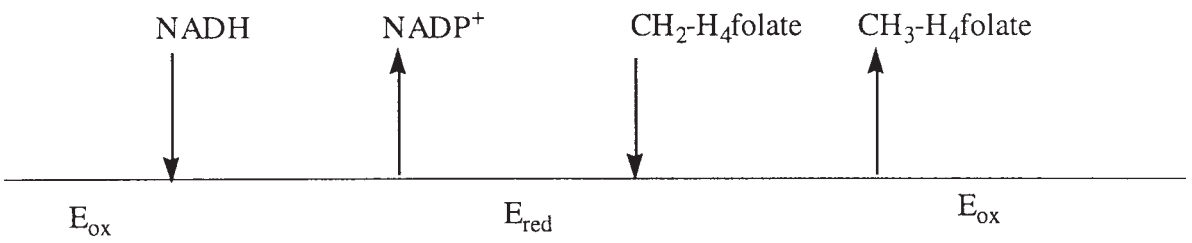


Fig. 1. Kinetic mechanisms for methylenetetrahydrofolate reductase.

rel that binds FAD in the center of the barrel. The MTHFR structure provided a satisfying rationale for the ping-pong kinetics observed with MTHFR. The *re*-face of the FAD is completely shielded by the barrel, and only the *si* face, the face known to react with substrates, is exposed. Thus, the two substrates, NADPH and CH_2-H_4folate, must occupy partially overlapping sites on the *si* face of the flavin, so that binding of one substrate precludes binding of the other. Such kinetic mechanisms are characterized by excess substrate inhibition, which results when binding of NADPH to the reduced enzyme, or of methylenetetrahydrofolate to the oxidized enzyme, results in formation of dead-end complexes.[23]

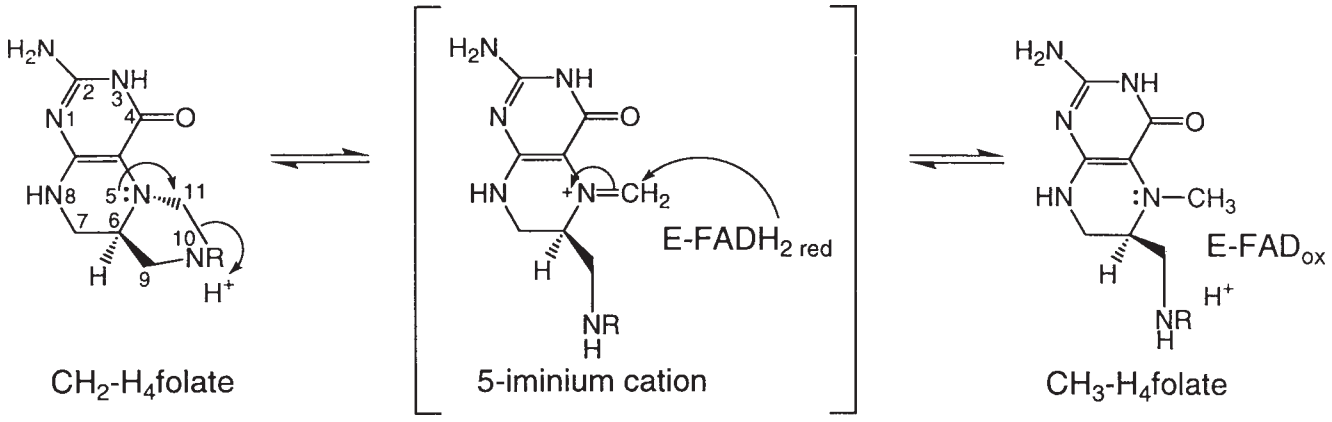
The X-ray structure of methylenetetrahydrofolate reductase provides insights into the role of the protein in facilitating reduction of CH_2-H_4folate. A structure with the substrate bound has not yet been published, but 10-formyl-H_4folate can be positioned on the *si* face of the flavin with the carbon of its formyl group positioned for hydride transfer to N5 of the flavin, and with two invariant active site residues, Gln 183 and Asp120, positioned to form hydrogen bonding interactions with the pterin ring.[24]

The proposed mechanism for reduction of CH_2-H_4folate is shown in Scheme 1, and it is based on model studies of the condensation of formaldehyde with H_4folate, which suggested the formation of a 5-iminium cation as an intermediate in the reaction.[25] Reduction is initiated by a general acid-catalyzed protonation of N10 of CH_2-H_4folate, and opening of the imidazolidine ring to form the 5-iminium cation. This extremely reactive species has never been directly observed in any enzymatic or nonenzymatic reaction, although the N5-carbinolamine formed by attack of water on this species has been detected during the reaction of formaldehyde with H_4folate,[25] and at the active site of thymidylate synthase, an enzyme that catalyzes transfer of the methylene group of CH_2-H_4folate to dUMP.[26] Once formed, the 5-iminium cation can by reduced by hydride transfer from N5 of the reduced flavin to form CH_3-H_4folate.

The X-ray structure of *E. coli* methylenetetrahydrofolate reductase suggests that Glu 28 is positioned appropriately to serve as the general acid catalyst for protonation of N10, either by direct proton transfer or via water. The Glu28Gln mutant of *E. coli* methylenetetrahydrofolate reductase is unable to catalyze the reduction of CH_2-H_4folate.[24] Titration of the mutant oxidized enzyme with CH_3-H_4folate does not result in reduction of the flavin, although a charge transfer complex between CH_3-H_4folate and oxidized flavin indicates that the mutation does not impair binding of the folate.

Methylenetetrahydrofolate Reductase and Human Physiology

The recent construction of a knockout mutation in the murine MTHFR gene has allowed assessment of the physiological role of MTHFR in mice.[6] Plasma total homocysteine levels in heterozygous and homozygous knockout mice are 1.6- and 10-fold higher than those in wild-type littermates, respectively. Both heterozygotes and homozygotes have elevated levels of S-adenosylhomocysteine and decreased levels of AdoMet, and their DNA is hypomethylated. While the heterozygous mice appear normal, the homozygous knockout mice are smaller than their littermates and show developmental retardation. As they mature, they show abnormal lipid deposition in the aorta, suggesting an atherogenic effect of elevated plasma homocysteine in these mice. The phenotypes of homozygous knockout mice resemble those seen in patients with severe methylenetetrahydrofolate reductase deficiency; these patients suffer from developmental delay, and some have thromboses of the arteries and cerebral veins.[27]



Scheme 1. Proposed mechanism for oxidative half-reaction.

Sequencing of the cDNAs from human patients revealed a number of mutations that were associated with loss of function,[28] as well as several polymorphisms that were common in humans with apparently normal function.[8,29] One of these polymorphisms, C677T in the cDNA, leads to substitution of Ala222 by a valine residue;[8] this mutation affects a residue in the catalytic domain of MTHFR that is conserved in both prokaryotic and eukaryotic enzymes.

The allele frequency of the C677T polymorphism is about 33–37% in European whites. About 10% of this population is homozygous for the mutation (i.e., has the TT genotype).[8] The allele frequency of the C677T polymorphism in East Asians is similar to that in European whites, while that frequency in sub-Saharan Africans is 6%.[30] Homozygosity for the C677T mutation is associated with elevated values for serum homocyst(e)ine, particularly in humans with low levels of blood folic acid,[31] and treatment of humans with daily supplements of folic acid leads to decreases in total homocysteine.[32] An elevated level of serum homocyst(e)ine is an established independent risk factor for cardiovascular disease,[3] and is also associated with neural tube defects in the fetus.[33]

Our laboratory has been particularly interested in ascertaining the biological basis for the phenotype of the C677T mutation, and the effect of folate supplementation on lowering serum homocysteine in patients with this polymorphism, and indeed in humans with the normal CC677 genotype. Until very recently, efficient expression systems for human MTHFR did not exist. Therefore, we decided to use the *E. coli* enzyme as a model for the catalytic domain of the human enzyme, and to investigate the structural and catalytic properties of the wild-type enzyme and the enzyme containing a mutation homologous to the human Ala222Val mutation, that is, Ala177Val.

We showed that the Ala177Val mutation does not affect the kinetic parameters for MTHFR. However, it was apparent that the reaction velocity of the mutant enzyme decreased more rapidly than that of the wild-type enzyme. MTHFR is a flavoprotein, and the noncovalently bound FAD is essential for catalysis. Therefore, we diluted a concentrated stock of enzyme into buffer and monitored the rate of flavin release by fluorescence spectroscopy. We found that the rate of activity loss after dilution corresponded to the rate at which flavin was released from the enzyme (C. A. Sheppard, unpublished data). Interestingly, both the wild-type and mutant enzymes lost flavin on dilution, although the mutant lost flavin at a rate 10 times faster than the wild-type enyzme.[18] In each case, the initial rate of flavin loss varied as the square root of the enzyme concentration [Fig. 2(a)]. Such kinetics would be expected if flavin release is accompanied by dissociation of the tetramer into dimers, and if tetramer/dimer equilibrium is established prior to rate-limiting release of FAD, as shown in Figure 3.

These studies suggested that the hyperhomocysteinemia observed in humans homozygous for the C677T polymorphism might result from formation of inactive MTHFR due to flavin dissociation from the mutant enzyme. They also suggested that even the wild-type enzyme was susceptible to flavin loss. It was then of great interest to determine how loss of activity on dilution could be slowed or prevented. We found that CH_3-H_4folate protected both the wild-type and mutant enzymes [Fig. 2(b)] from flavin loss, suggesting that folate supplementation might exert its effect on serum homocysteine levels by maintaining both mutant and wild-type MTHFR in an active holoenzyme form.[18] The X-ray structure of the bacterial enzyme determined in Dr. Martha Ludwig's laboratory has provided a rationale for protection of the enzyme by folates. The positioning of the FAD cofactor with respect to the $\beta_8\alpha_8$ barrel suggested that CH_2-H_4folate would bind with the pteridine ring stacked above the flavin, and this interaction should stabilize the holoenzyme by slowing dissociation of FAD from the enzyme on dilution. Although binding of CH_3-H_4folate would be inhibitory, if we assume that the concentration of the substrate CH_2-H_4folate rises in proportion with the total folate concentration on dietary supplementation with folic acid, the net effect will be to stabilize the enzyme without increasing inhibition by non-substrate folate derivatives.

The four subunits of the tetramer are arranged in an unusual planar rosette (Fig. 4) that is characterized by a twofold rather than a fourfold axis of symmetry. The interactions between subunits B and C and those between A and A′ are much more extensive than the interactions between B and A or C and A′. Thus, the dissociation of the tetramer into dimers rather than monomers on dilution is rationalized by the structure.

The site of the Ala177Val mutation that is homologous to the C677T (Ala222Val) polymorphism in humans is at the bottom of the $\alpha 8\beta 8$ barrel, far from the flavin binding site. The side chain of Ala177 lies inside a tight loop that connects helix 5 with strand 6 of the barrel. Although a structure of the Val177 mutant has not yet been published, this sidechain is more bulky than the methyl group of alanine and cannot be accommodated inside the loop without distortions that propagate helix 5. Residues on helix 5 make extensive interactions with the ADP moiety of the FAD cofactor, suggesting that these interactions are weakened in the Val177 mutant and flavin dissociation is enhanced. Thus, the X-ray structure provides a molecular rationale for the phenotype associated with the Ala177Val mutation.

Nonetheless, one may question the relevance of studies on the bacterial enzyme to the physiology associated with the human polymorphism. Of particular concern is the fact that the bacterial enzyme is a homotetramer of 33 kDa subunits, while the mammalian enzymes are dimers of 77 kDa subunits containing both catalytic and regulatory domains.

Dr. Kazuhiro Yamada, a postdoctoral fellow in my laboratory, has recently succeeded in expressing N-terminal histidine-

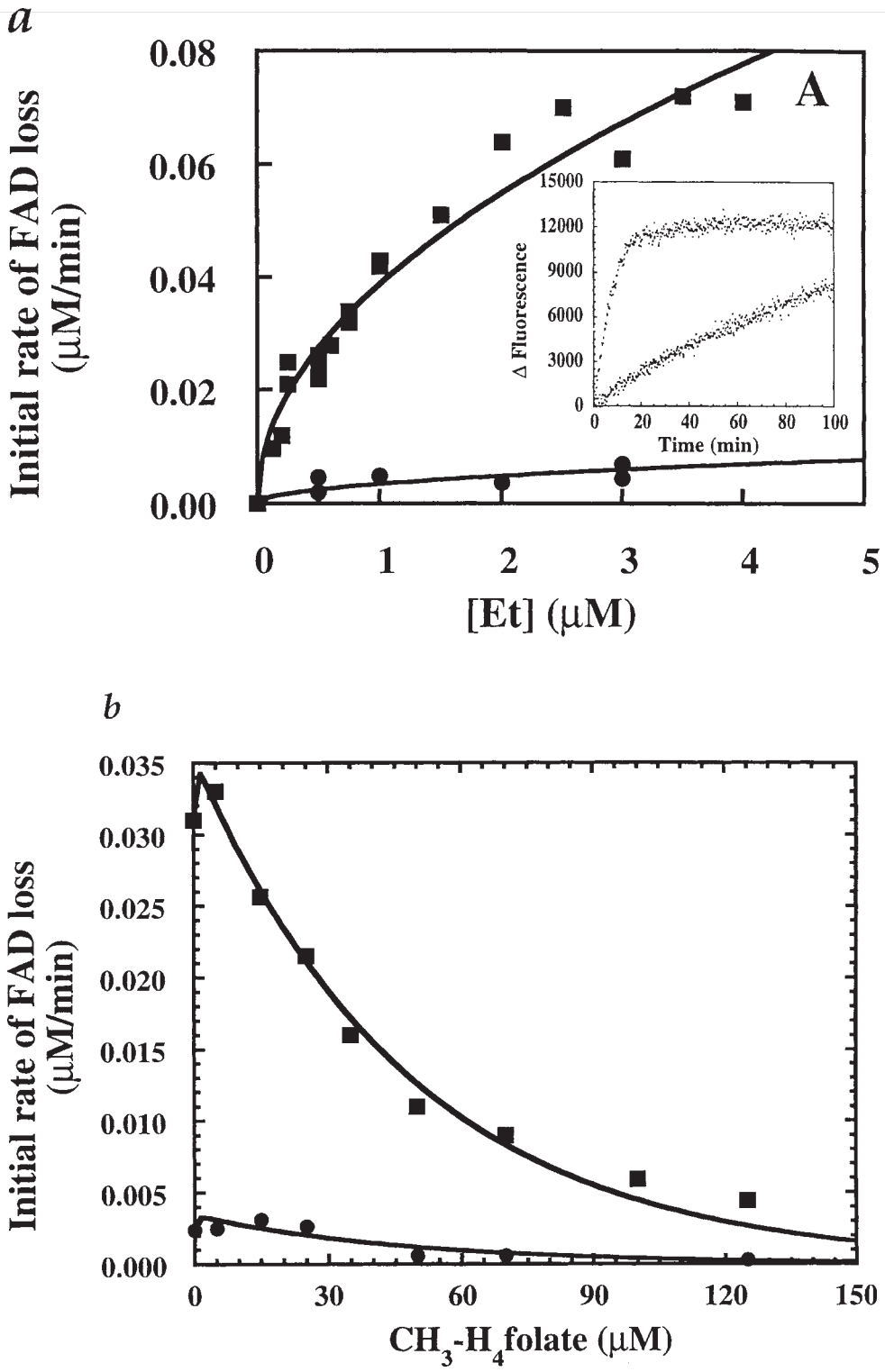


Fig. 2. Reproduced, with permission, from Guenther et al.[18] (a) The Ala177Val mutation in MTHFR from *E. coli* is associated with an enhanced rate of flavin dissociation. After dilution of a concentrated stock (200 μ*M*) of enzyme to the indicated subunit concentrations (Et), the initial rate of FAD dissociation was ascertained for wild-type (◆) and mutant (■) enzymes. The solid lines have been calculated assuming that the rate of flavin release varies as the square root of the enzyme concentration after dilution. Inset: The change in fluorescence versus time is shown for wild-type (lower trace) and mutant (upper trace) enzymes diluted to 500 nm concentration. (b) Protection of wild-type and mutant enzymes against flavin loss after dilution in the presence of CH_3-H_4folate. The initial rate of flavin loss from 500 nm wild-type (●) and mutant (■) enzymes is plotted against the concentration of (6*S*)CH_3-H_4folate monoglutamate present after dilution. The initial rates have been corrected for fluorescence increases associated with the slow oxidation of CH_3-H_4folate catalyzed by the enzyme under aerobic conditions.

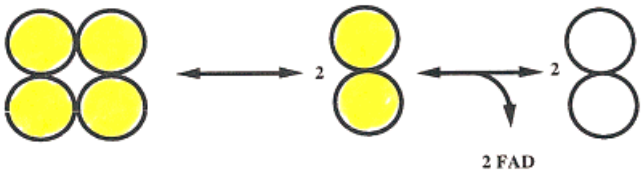


Fig. 3. Kinetic model for the release of FAD associated with the C677T polymorphism. Flavin release occurs on dilution of both wild-type and mutant enzymes, but occurs more rapidly with the mutant.

tagged human methylenetetrahydrofolate reductase at high levels using a baculovirus expression system in insect Sf9 cells. He has constructed the C677T mutant, and has purified both the wild-type and mutant enzymes to homogeneity using nickel affinity chromatography. Comparison of the properties of the wild-type and mutant enzymes has revealed that our studies on the Ala177Val mutant of the bacterial enzyme have provided a very good working model for the effect of the C677T polymorphism on the human enzyme.[34] In particular, the mutation leads to enhanced rates of flavin dissociation, accompanied by dissociation of the dimer into monomers and loss of activity, and CH_3-H_4folate protects the enzyme against loss of activity.

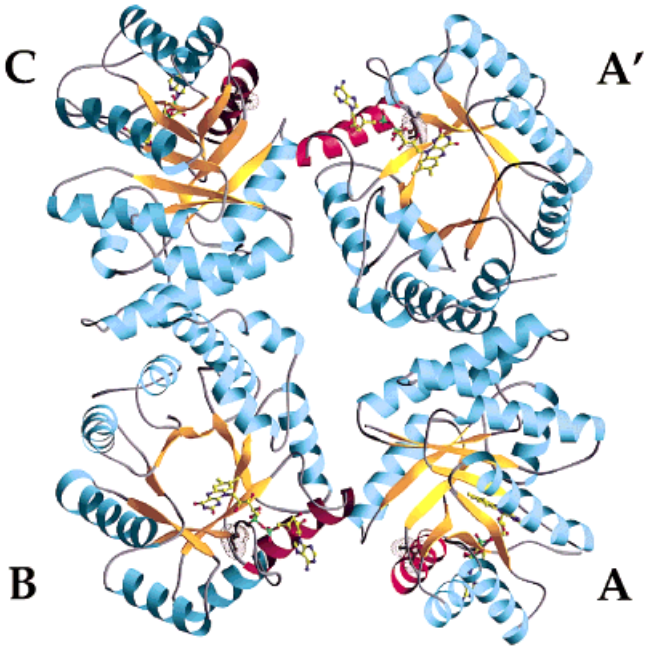


Fig. 4. Reproduced, with permission, from Guenther et al.[18] The tetramer of *E. coli* MTHFR viewed down a local twofold axis. The tetramer consists of four identical subunits that are $\alpha_8\beta_8$ barrels. Ala177 is shown in white and is surrounded by dot surfaces. The Ala177Val mutation is located in a loop between helix 5 (shown in red on each monomer) and strand 6 at the bottom of the barrel; helix alpha 5 may be critical in mediating the effects of mutation at position 177 because this helix is involved in contacts with the ADP-ribose moiety of FAD.

Our studies suggest that the negative effects of the human C677T polymorphism, particularly the mild elevation in serum homocysteine, should be preventable with dietary folate supplementation. They also suggest a rationale for the observation that folate supplementation is effective in lowering serum homocysteine in most humans, regardless of their MTHFR genotype, because the normal enzyme is also susceptible to loss of flavin, and this loss can be retarded by folate supplementation.

Why should a potentially deleterious mutation be so common in humans? Its persistence in the population suggests beneficial properties at least under some conditions. Recent studies have shown that the 677TT genotype is linked to a lower risk of colon cancer and certain childhood leukemias.[35,36] The rationale for the protective effect of the mutation is thought to be that the mutation decreases the flux of CH_2-H_4folate into CH_3-H_4folate, sparing CH_2-H_4folate for the conversion of dUMP into dTMP under folate limiting conditions. While this sparing effect may lead to decreased risks for certain cancers, it probably also is protective to the fetus, and thus might confer benefits that justify the retention of the polymorphism at high levels in many human populations.

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