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Studies of the Flavin Enzyme Methylene tetrahydrofolate Reductase (MTHFR) (Updated for Summer 2012)

We are interested in the structures and reaction mechanisms of enzymes containing the redox coenzyme flavin. "Flavin enzymes" have the ability to catalyze a variety of biochemical reactions. Flavins can participate in one or two electron transfers and can exist in three redox states: oxidized, one-electron reduced semiquinone, and two-electron reduced hydroquinone (Figure 1). A fundamental question in flavin research is how do specific interactions between the flavin and its protein environment control chemical reactivity and mechanism (1).

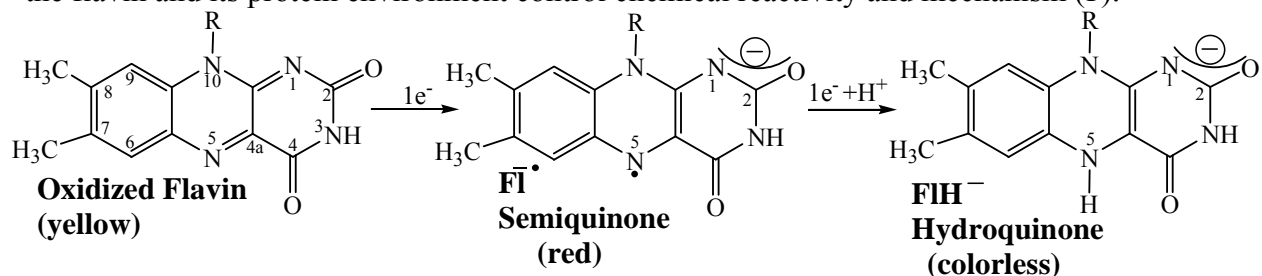


Figure 1: Flavin Redox States

In our research, we investigate how the chemical mechanism is influenced by the amino acids surrounding the flavin in the enzyme methylenetetrahydrofolate reductase (MTHFR). As shown in Figure 2 below, MTHFR catalyzes the NAD(P)H-dependent reduction of 5,10-methylenetetrahydrofolate (CH₂-H₄folate) to 5-methyltetrahydrofolate (CH₃-H₄folate) using the flavin adenine dinucleotide (FAD) form of flavin. MTHFR is a key enzyme in folate metabolism; in humans, deficiency in MTHFR has been correlated with increased risk for

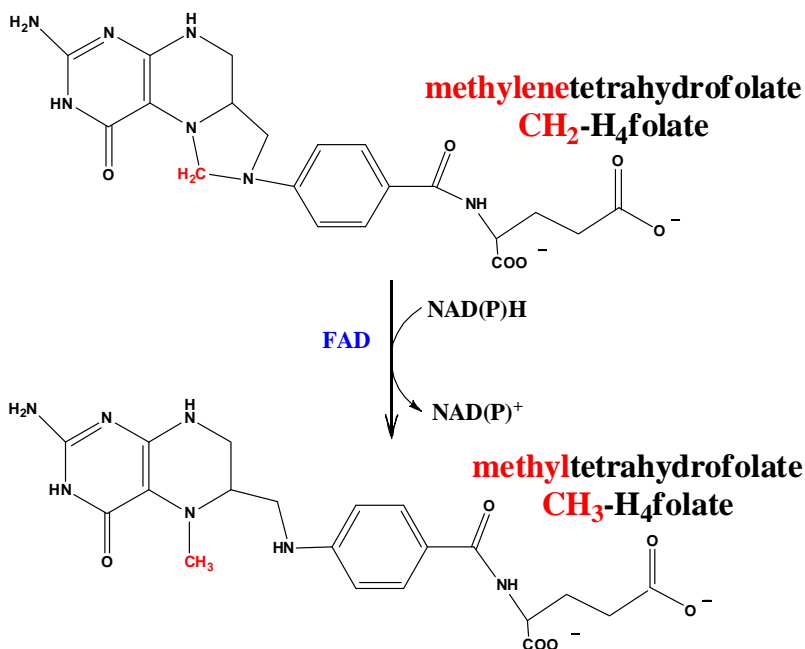


Figure 2: Reaction of Methylene tetrahydrofolate Reductase

cardiovascular disease (2) and for neural tube defects in the fetus (3).

We study MTHFR from *Escherichia coli* as a model system for the catalytic domain of the human enzyme. In contrast to human MTHFR, the bacterial enzyme utilizes NADH rather than NADPH. Otherwise, the enzymes are believed to have a common chemical and kinetic mechanism. The catalyzed reaction can be divided into two half-reactions: a reductive half-reaction in which the enzyme-bound FAD is reduced by NADH (Figure 3) and an oxidative half-reaction in which the reduced

FAD is reoxidized by CH₂-H₄folate (Figure 4).

Studies by Matthews and co-workers have demonstrated that the reductive half-reaction occurs by transfer of a hydride from the C4 position of NADH to the N5 atom of the FAD to form NAD⁺ and reduced FAD (4) (Figure 3).

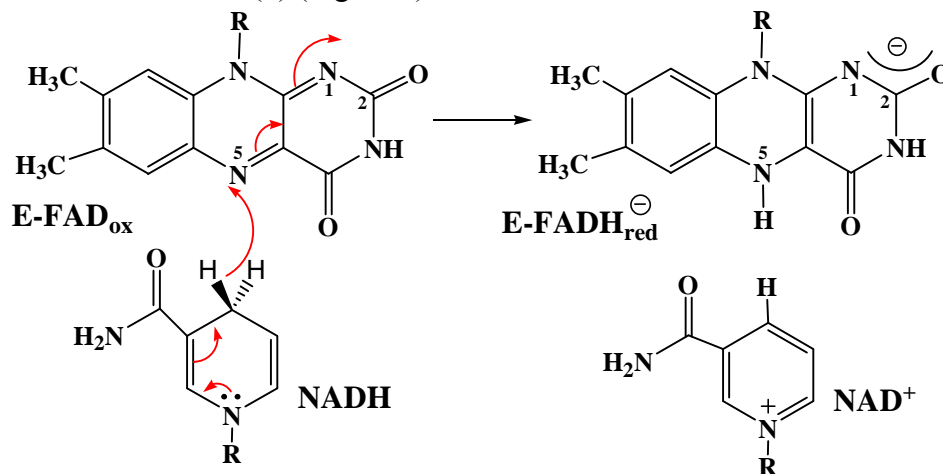


Figure 3: Reductive Half-Reaction of MTHFR

In the oxidative half-reaction, it is proposed that CH₂-H₄folate is first converted to a 5-iminium cation intermediate by protonation at N10 and concomitant opening of the 5-membered imidazolidine ring. Transfer of a hydride from the N5 of the reduced FAD to the exocyclic methylene (C11) produces CH₃-H₄folate and reoxidized FAD (4) (Figure 4).

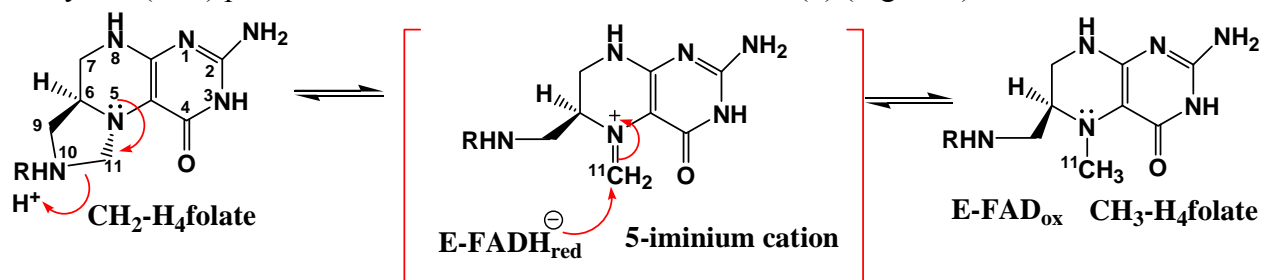


Figure 4: Oxidative Half-Reaction of MTHFR

We are interested in elucidating the chemical steps in these two half-reactions as well as in determining the roles of the active-site amino acids. The 3-dimensional structure of *E. coli* MTHFR has been solved by X-ray crystallography (5), and two enzyme structures with bound substrates (NADH or CH₃-H₄folate) have also been determined (6). These structural data have revealed the amino acids present at the active site which interact with the bound substrates. From 2000-2011 with generous internal support from Grinnell as well as with ACS-PRF-G funds, my research has examined how three different amino acids Aspartate 120, Phenylalanine 223, and Glutamine 183 participate in the reaction of *E. coli* MTHFR. The work on Aspartate 120 was published in 2005 (7). Experiments investigating the function of Phenylalanine 223 within MTHFR were completed during my 2007-08 sabbatical and the manuscript describing this work was published in August 2009 (8). Since my sabbatical and with the support of the college, we have been continuing our work on the Glutamine 183 mutants. In addition, we have begun examining the pH-dependence of the two half-reactions and the overall enzyme reaction.

These studies should shed light on any protonation/deprotonation steps in the mechanism as well as on the ionization states of the amino acids that are required for optimum enzyme activity.

Project 1: Kinetic Characterization of Gln 183 MTHFR Mutants

The structure of reduced enzyme in complex with NAD(H) (Figure 5) (6) shows NAD(H) in an unusual folded conformation; a four way sandwich has been assembled among the rings of Phe 223 – adenosine of NAD(H) – nicotinamide of NAD(H) - isoalloxazine of FAD. The carboxamide of Gln 183 is hydrogen bonded in a bidentate manner to the carboxamide oxygen and nitrogen atoms of the nicotinamide, suggesting that this amino acid makes critical binding interactions with the NADH substrate. The polar amide group of Gln 183 may also aid in stabilizing the charged transition state associated with the hydride transfer, suggesting a role for Gln 183 in NADH catalysis.

A function for Gln 183 in binding of the second substrate, CH₂-H₄folate, is indicated by the structure of MTHFR in complex with the product CH₃-H₄folate (Figure 6) (6). In the structure, the carboxamide nitrogen and oxygen atoms of Gln 183 participate in bidentate hydrogen bonding with the N1 and N8 atoms of the folate. These interactions suggest a role for Gln 183 in folate binding, and, perhaps, in catalysis of the oxidative half-reaction.

For this project, mutant enzymes Gln183Glu and Gln183Ala have been prepared by Andriana Nikolova ('07). We are now in the process of assessing the influence of the Gln 183 side chain modifications by comparing the kinetic properties of the mutant enzymes to those of wild-type MTHFR by both steady-state and pre-steady-state kinetics. Three steady-state assays are performed in order to determine the kinetic parameters k_{cat} and K_m . The NADH-menadi

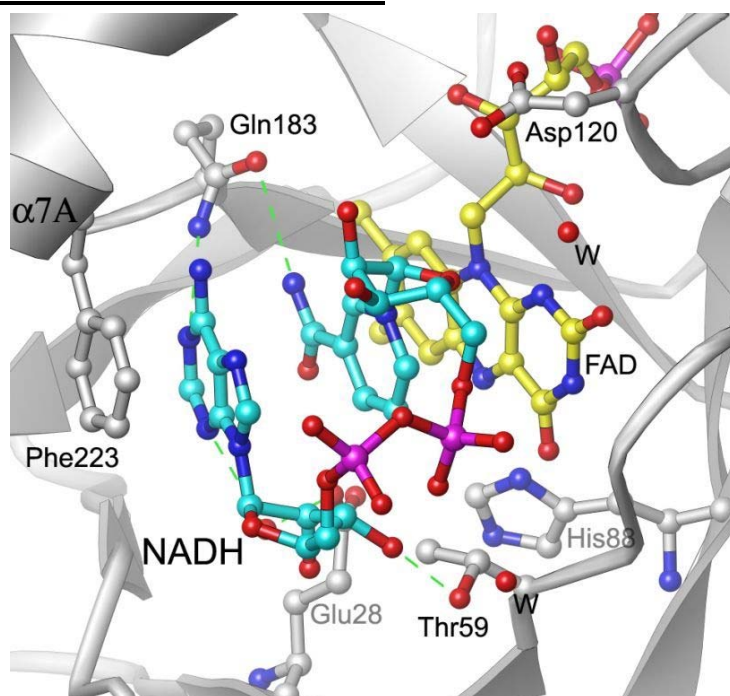


Figure 5: *E. coli* MTHFR with NADH Bound

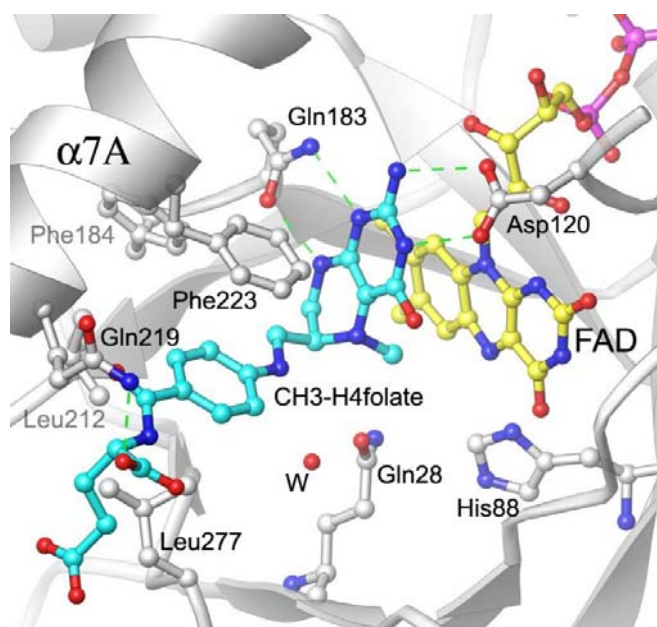


Figure 6: *E. coli* MTHFR with CH₃-H₄folate Bound

assay measures enzyme reduction by NADH and the CH₃-H₄folate-menadione assay measures enzyme reduction by CH₃-H₄folate. These assays use menadione as an alternate electron acceptor in place of the second substrate. A third assay, the physiological NADH-CH₂-H₄folate oxidoreductase reaction, is carried out under oxygen-free conditions because of the susceptibility of CH₂-H₄folate to oxygen. Performing these assays at 25 °C, Amber Jolly ('06) and Sirui Cao ('08) obtained preliminary results on the Glu183Ala and Glu183Glu mutants. However, due to instability of the enzymes, in particular the Glu183Glu mutant, these assays need to be repeated at 4 °C. Our current (25 °C) data suggests that Gln 183 plays a major role in the folate half-reaction (effect of 10-fold on k_{cat}), but not in the NADH half-reaction.

Another part of this project is the examination of the individual half-reactions under anaerobic conditions in a stopped-flow spectrophotometer. Reduction of enzyme-bound flavin or oxidation of reduced flavin can be followed directly at 450 nm. As described in detail for the wild-type enzyme (9), these studies will yield the rate constants of the individual half-reactions (k_{red} and k_{ox}) as well as the substrate dissociation constants (K_d). If the mutations disrupt substrate binding as we have predicted, the K_d values would be expected to increase compared to that of the wild-type enzyme. If catalysis is also impaired in the mutants, the individual rate constants k_{red} and k_{ox} as well as k_{cat} may decrease. David Satzer ('11) and I carried out a preliminary set of stopped-flow experiments for a week in summer 2009 at the University of Nebraska, Lincoln in collaboration with Dr. Donald Becker of the department of biochemistry, and I returned in summer 2010 to perform additional experiments. Unfortunately, we are encountering enzyme instability issues, even at the lower 4 °C temperature, as well as problems arising from the anaerobic setup at Nebraska. My plan is to travel to the laboratory of my collaborator Dr. David Ballou at the University of Michigan to use his instrument and anaerobic system.

Some biochemistry experience would be helpful, but not required, for this project. Techniques to learn: (1) Protein purification, (2) Enzyme steady-state kinetic assays, (3) Anaerobic (oxygen-free) technique, and (4) Stopped-flow enzyme kinetics to determine rate constants and K_d 's.

Project 2: Study of the pH dependence of Wild-type and Mutant MTHFR Reactions

Studies on the pH-dependence of the enzyme reaction should inform us about any protonation/deprotonation steps in the mechanism as well as about the required ionization states of the catalytic amino acids. The proposed mechanism of the NADH reductive half-reaction (Figure 3) shows no proton transfers, but a negatively-charged amino acid could help stabilize the positive charge developing in the transition state as the reaction proceeds to the NAD⁺ product. In the folate oxidative half-reaction, it is proposed that protonation of the N10 of CH₂-H₄folate aids ring opening and iminium cation formation. In particular, Glu 28, located near N10 of the folate (given as Gln 28 in Figure 6), has been proposed as the general acid catalyst responsible for this protonation. Interestingly, Glu 28 appears to be part of a putative protonation triad extending down into the β/α barrel that also involves the highly conserved His 273 and Ser 26. These three residues are within hydrogen bonding distance of each other and a water molecule is positioned near Ser 26. We postulate that protonation of N10 of CH₂-H₄folate may occur by proton transfer from water through this protonation triad. Structural analysis (6) also suggests that within the protein, the location of Asp 120, a critical amino acid in folate binding and catalysis (9), changes as a function of pH.

Our goal in this project is to characterize biochemically the pH effects on the reactions catalyzed by MTHFR. We will study the wild-type enzyme first, followed by select mutant enzymes. Last summer, Jingjing Wang ('13) measured the k_{cat} and K_m of the wild-type enzyme as a function of pH using the CH₃-H₄folate-menadione assay. This assay is performed in the reverse of the physiological direction and allows examination of the rate-limiting folate half-reaction. Her results show that as pH increases, both k_{cat} and k_{cat}/K_m increase with an optimum at pH 8-10 and an inflection point near pH 6, consistent with the need for deprotonation of the folate N10 in the reaction. During the fall semester, Jingjing examined the effect of pH on the rate-limiting NADH reductive half-reaction by performing the NADH-menadione assay. Her results show an optimum pH of 6-9 and an inflection point near pH 5. During the current spring semester, she is measuring the pH dependence of the physiological NADH-CH₂-H₄folate assay. For the coming summer, a new student working on this project will first finish the physiological assay studies. He/she will then examine the pH dependence of a Glu28Gln mutant enzyme (previously constructed by Prof. Trimmer (10)).

Some biochemistry experience would be helpful, but not required, for this project.

Techniques to learn: (1) Protein purification, (2) UV-vis spectrophotometry, (3) Enzyme steady-state kinetic assays, and (4) Anaerobic (oxygen-free) technique.

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