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A role for glutamine 183 in the folate oxidative half-reaction of methylenetetrahydrofolate reductase from *Escherichia coli*☆

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ABSTRACT

The flavoprotein methylenetetrahydrofolate reductase (MTHFR) from *Escherichia coli* catalyzes a ping-pong reaction with NADH and 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to produce NAD^+ and 5-methyltetrahydrofolate (CH_3-H_4folate). This work focuses on the function of the invariant, active-site aminoacyl residue Gln183. X-ray structures of the enzyme complexes E_{red}(wild-type)•NADH and E_{ox}(Glu28Gln)•CH_3-H_4folate indicate that Gln183 makes key hydrogen-bonding interactions with both NADH and folate in their respective half-reactions, suggesting roles in binding each substrate. We propose that the polarity of Gln183 may also aid in stabilizing the proposed 5-iminium cation intermediate during catalysis in the oxidative half-reaction with folate. We have prepared mutants Gln183Ala and Gln183Glu, which we hypothesize to have altered charge/polarity and hydrogen bonding properties. We have examined the enzymes by steady-state and stopped-flow kinetics and by measurement of the flavin redox potentials. In the reductive half-reaction, NADH binding affinity and the rate of flavin reduction have not been hindered by either mutation. By contrast, our results support a minor role for Gln183 in the oxidative half-reaction. The Gln183Ala variant exhibited a 6–10 fold lower rate of folate reduction and bound CH_2-H_4folate with 7-fold lower affinity, whereas the Gln183Glu mutant displayed catalytic constants within 3-fold of the wild-type enzyme.

1. Introduction

Methylenetetrahydrofolate reductase (MTHFR)[1] catalyzes the flavin adenine dinucleotide (FAD)-dependent reduction of 5,10-methylenetetrahydrofolate (CH_2-H_4folate) by NAD(P)H, as shown in Equation (1).

$$CH_2-H_4 \text{ folate} + NAD(P)\ H + H^+ \leftrightarrow CH_3-H_4 \text{ folate} + NAD(P)^+ \quad (1)$$

MTHFR is a key enzyme in folate metabolism; the enzyme is the sole provider of 5-methyltetrahydrofolate (CH_3-H_4folate), the cosubstrate required by methionine synthase for the conversion of homocysteine to methionine. Defects in MTHFR lead to elevation of blood homocysteine, hyperhomocyst(e)inemia, which has been associated with an increased risk for cardiovascular disease and Alzheimer's disease in adults, and of neural tube defects in the fetus (reviewed in Ref. [1]).

We have investigated MTHFR from *Escherichia coli* as a model for the catalytic domain of the human enzyme. The *E. coli* enzyme differs from the characterized pig, human, and yeast MTHFRs in its preference for NADH over NADPH as substrate and by the absence of a regulatory domain. Within the catalytic domain shared by *E. coli* MTHFR and these eukaryotic enzymes, there is ~30% amino acid sequence identity, suggesting both a common reaction mechanism and structure. As reviewed recently [1], the chemical mechanisms of these enzymes are indeed similar.

The reaction catalyzed by *E. coli* MTHFR can be divided into a reductive half-reaction and an oxidative half-reaction, shown as Equations (2) and (3), respectively, in which the FAD cofactor acts as an intermediate electron acceptor and donor.

$$E{-}FAD_{ox} + NADH \rightarrow E{-}FAD_{red} + NAD^+ \quad (2)$$

Abbreviations: CH_2-H_4folate, 5,10-methylenetetrahydrofolate; CH_3-H_4folate, 5-methyltetrahydrofolate; DHFR, dihydrofolate reductase; *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; E_m, midpoint potential; FAD, flavin adenine dinucleotide; H_2folate, dihydrofolate; H_4folate, tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; *p*ABA, *p*-aminobenzoate; PCA, protocatechuate; PCD, protocatechuate dioxygenase

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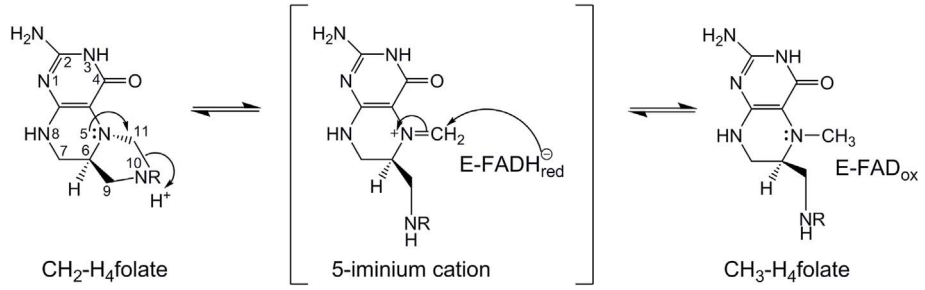

Scheme 1. Proposed mechanism for the oxidative half-reaction.

$$E{-}FAD_{red} + CH_2{-}H_4\ folate + H^+ \leftrightarrow E{-}FAD_{ox} + CH_3{-}H_4\ folate \quad (3)$$

The enzyme employs a ping-pong bi-bi kinetic mechanism, in which the release of NAD^+, the product of the first half-reaction, precedes binding of the substrate CH_2-H_4folate in the second half-reaction. Rapid-reaction kinetic studies have established that the enzyme catalyzes these half-reactions at rates consistent with the observed rate of overall turnover [2].

Previous studies with the porcine enzyme have revealed stereochemical and mechanistic details of the MTHFR reaction. In the reductive half-reaction, the nicotinamide moiety of NAD(P)H binds at the *si* face of the enzyme-bound FAD. The *pro*-4S hydrogen is stereospecifically removed from NAD(P)H [3] and transferred as a hydride to N5 of the FAD [4]. The product $NAD(P)^+$ then dissociates from the active site. In the oxidative half-reaction (Scheme 1), CH_2-H_4folate also binds at the *si* face of the FAD, replacing $NAD(P)^+$ [4]. An early proposal advocated that CH_2-H_4folate is first activated by undergoing protonation and imidazolidine ring opening to form a reactive 5-iminium cation intermediate [5], and we postulate that in the active site, an equilibrium may exist between this intermediate and the CH_2-H_4folate substrate. Although the 5-iminium has not been observed directly in an enzymatic or nonenzymatic reaction, the 5-iminium cation has been hypothesized to form during the nonenzymatic synthesis of CH_2-H_4folate from formaldehyde and H_4folate [6]. Furthermore, a structure has been obtained of 5-$HOCH_2$-H_4folate, the product of the reaction of the 5-iminium cation with water, in complex with thymidylate synthase [7], an enzyme that also employs CH_2-H_4folate as a substrate. Transfer of a hydride from N5 of the reduced FAD [4] to the C11 methylene at the more sterically accessible face of the 5-iminium cation (opposite the hydrogen at C6) [5] generates the CH_3-H_4folate product of MTHFR.

Our studies to further investigate the chemical mechanism of MTHFR have employed the enzyme from *E. coli* because of its ready availability and known X-ray structures of free and ligand-bound enzyme [8–10]. MTHFR is a homotetramer, in which each 33 kDa subunit is a $\beta_8\alpha_8$ barrel. The FAD cofactor is bound in the center of the barrel with only the *si* face of the isoalloxazine ring exposed to solvent [8], consistent with the known stereochemistry of the reaction [4]. Structures of E_{red}(wild-type)•NADH (Fig. 1A) and E_{ox}(Glu28Gln)•CH_3-H_4folate (Fig. 1B) complexes reveal that the ligands occupy partially overlapping sites at the *si* face of the FAD [9]. Thus, the binding of one substrate to the enzyme prevents the binding of the other substrate, consistent with the ping-pong bi-bi kinetic mechanism [2]. However, the structures reveal that the active site conformations of NADH and CH_3-H_4folate are remarkably different.

In the E_{red}(wild-type)•NADH complex (Fig. 1A), the NADH is bound in an unusual, highly folded conformation, stabilized by π-π aromatic stacking interactions. A four-layer sandwich exists where both the adenine and nicotinamide rings of the NADH are wedged between the isoalloxazine ring of the FAD and the aromatic side chain of Phe 223. The N5 of the FAD is 3.5 Å from the C4 of the nicotinamide in an orientation favorable for hydride transfer [9]. By contrast, in the E_{ox}(Glu28Gln)•CH_3-H_4folate complex (Fig. 1B), the CH_3-H_4folate is bound in an extended, L-shaped conformation with the pterin ring perpendicular to the plane of the *p*-aminobenzoate (*p*ABA) ring and the monoglutamate tail extending out from the binding pocket. Like the nicotinamide of NADH, the pterin is stacked against the flavin

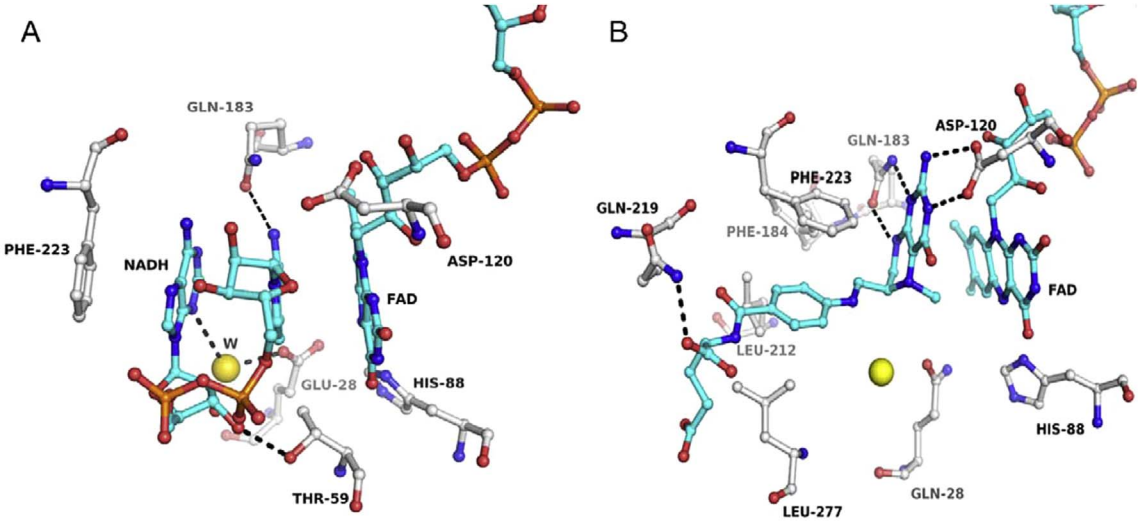

Fig. 1. Structures of *E. coli* MTHFR [9]. **(A)** Structure of bound NADH and its interactions with wild-type reduced MTHFR (PDB entry 1ZPT). NADH adopts a highly folded conformation and is sandwiched between the aromatic side chain of Phe223 and the FAD isoalloxazine ring. Hydrogen bonds between NADH and Thr59, Gln183, and the solvent are indicated by dashed lines. **(B)** Structure of bound CH_3-H_4folate and its interactions with Glu28Gln MTHFR (PDB entry 1ZP4). The CH_3-H_4folate is bound in an extended conformation, with its pterin ring stacked against the flavin isoalloxazine ring. Hydrogen bonds between CH_3-H_4folate and Asp120, Gln183, and Gln219 are indicated by dashed lines.