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Impact of Mutating the Key Residues of a Bifunctional 5,10-Methylenetetrahydrofolate Dehydrogenase-Cyclohydrolase from *Escherichia coli* on Its Activities

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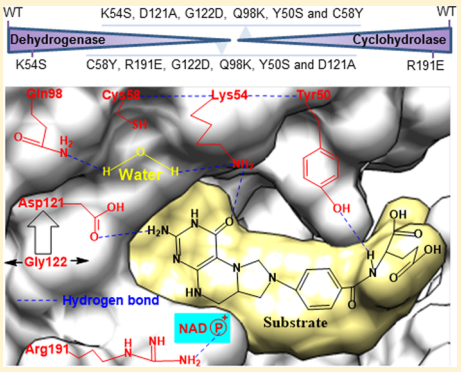
Supporting Information

ABSTRACT: Methylenetetrahydrofolate dehydrogenase-cyclohydrolase (FolD) catalyzes interconversion of 5,10-methylene-tetrahydrofolate and 10-formyl-tetrahydrofolate in the one-carbon metabolic pathway. In some organisms, the essential requirement of 10-formyl-tetrahydrofolate may also be fulfilled by formyltetrahydrofolate synthetase (Fhs). Recently, we developed an *Escherichia coli* strain in which the *folD* gene was deleted in the presence of *Clostridium perfringens fhs* (*E. coli* Δ*folD*/p-*fhs*) and used it to purify FolD mutants (free from the host-encoded FolD) and determine their biological activities. Mutations in the key residues of *E. coli* FolD, as identified from three-dimensional structures (D121A, Q98K, K54S, Y50S, and R191E), and a genetic screen (G122D and C58Y) were generated, and the mutant proteins were purified to determine their kinetic constants. Except for the R191E and K54S mutants, others were highly compromised in terms of both dehydrogenase and cyclohydrolase activities. While the R191E mutant showed high cyclohydrolase activity, it retained only a residual dehydrogenase activity. On the other hand, the K54S mutant lacked the cyclohydrolase activity but possessed high dehydrogenase activity. The D121A and G122D (in a loop between two helices) mutants were highly compromised in terms of both dehydrogenase and cyclohydrolase activities. *In vivo* and *in vitro* characterization of wild-type and mutant (R191E, G122D, D121A, Q98K, C58Y, K54S, and Y50S) FolD together with three-dimensional modeling has allowed us to develop a better understanding of the mechanism for substrate binding and catalysis by *E. coli* FolD.



The one-carbon metabolic pathway is central to the synthesis of thymidylate, glycine, serine, methionine, and purine nucleotides. Methylenetetrahydrofolate dehydrogenase-cyclohydrolase (FolD), a bifunctional homodimeric protein, is a key enzyme in this pathway. The dehydrogenase activity of FolD catalyzes $NADP^+$-dependent oxidation of 5,10-methylene-tetrahydrofolate (5,10-CH_2-THF) to 5,10-methenyl-tetrahydrofolate (5,10-CH^+-THF). The latter is then converted to 10-formyl-tetrahydrofolate (10-CHO-THF) by the cyclohydrolase activity of the enzyme.[1−4] In eukaryotes, these metabolites are also formed by a trifunctional enzyme consisting of dehydrogenase, cyclohydrolase, and synthetase domains. The synthetase activity produces 10-CHO-THF by utilizing THF, formate, and ATP.[5] In many bacteria, the synthetase activity is performed by a monofunctional protein called formyltetrahydrofolate synthetase, Fhs (also known as formate-tetrahydrofolate ligase).[6] Conversely, monofunctional FolD proteins (with dehydrogenase activity) are also found in some bacteria (*Peptostreptococcus* and a few *Clostridium* species) and eukaryotes (yeast) that convert 5,10-CH_2-THF to 5,10-CH^+-THF.[7−10] The metabolite 5,10-CH_2-THF is utilized for the biosynthesis of thymidine, serine, and methionine, and 10-CHO-THF is utilized for formylation of initiator $tRNA^{Met}$ and in purine synthesis.[11] More recently, it was reported that 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP)/5-aminoimidazole-4-carboxamide riboside 5′-triphosphate (ZTP)-bound riboswitch regulates production of 10-CHO-THF and purines in the cell.[12,13]

A number of crystal structures of FolD have been reported. These include the monofunctional NAD^+-dependent enzyme from yeast,[14] bifunctional enzymes with or without an inhibitor and/or a $NADP^+$ complex from *Escherichia coli*,[15] *Pseudomonas aeruginosa*,[16] *Leishmania major*,[17] *Thermoplasma acidophilum*,[18] and *Acinetobacter baumannii*,[19] and the dehydrogenase/cyclohydrolase domains of the human trifunctional enzyme (DC301).[4] FolD proteins from all species are organized in N- and C-terminal domains of α/β folds and connected by two long helices. The N-terminal domain (NTD) contains the catalytic site where the substrate binds and the C-terminal domain (CTD) displays the Rossmann fold for $NADP^+$ cofactor binding.[4,16−21] Residues like Y52, K56, Q100, and D125 in the NTD of DC301 are conserved across the species (Figure 1) and are important for catalysis and substrate

Received: April 15, 2015
Revised: May 15, 2015
Published: May 19, 2015

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DOI: 10.1021/acs.biochem.5b00400
Biochemistry 2015, 54, 3504−3513

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                                                                                  ↓  ↓  ↓
M.t.-2c2y       -MGAIMLDGKATRDEIFGDLKQRVAALDA--AGRTPGLGTILVGDDPGSQAYVRGKHADC 57
A.b.-4b4u       --MALVLDGRALAKQIEENLLVRVEALKAK-TGRTPILATILVGDDGASATYVRMKGNAC 57
E.c.-1b0a       -MAAKIIDGKTIAQQVRSEVAQKVQARIAA-GLRAPGLAVVLVGSNPASQIYVASKRKAC 58
P.a.-4a5o       -MTAQLIDGKAIAANLRQQIAQRVTERRQQ-GLRVPGLAVILVGTDPASQVYVAHKRKDC 58
H.s.-1zn4       ---AVVISGRKLAQQIKQEVRQEVEEWVAS-GNKRPHLSVILVGENPASHSYVLNKTRAA 56
L.m.-4a26       MPSAQIIDGKAIAAAIRSELKDKVAALRELYGGRVPGLASIIVGQRMDSKKYVQLKHKAA 60
H.s.-1dia       MAPAEILNGKEISAQIRARLKNQVTQLKEQVPGFTPRLAILQVGNRDDSNLYINVKLKAA 60
                   * ::.*:    :   :  .*          * *. : **    *  *:  *   .
                                                  ↓
M.t.-2c2y       AKVGITSIRRDLPADISTATLNETIDELNANPDCTGYIVQLPLP--KHLDENAALERVDP 115
A.b.-4b4u       RRVGMDSLKIELPQETTTEQLLAEIEKLNANPDVHGILLQHPVP--AQIDERACFDAISL 115
E.c.-1b0a       EEVGFVSRSYDLPETTSEAELLELIDTLNADNTIDGILVQLPLP--AGIDNVKVLERIHP 116
P.a.-4a5o       EEVGFLSQAYDLPAETSQDDLLALIDRLNDDPAIDGILVQLPLP--AHLDASLLLERIHP 116
H.s.-1zn4       AVVGINSETIMKPASISEEELLNLINKLNNDDNVDGLLVQLPLP--EHIDERRICNAVSP 114
L.m.-4a26       AEVGMASFNVELPEDISQEVLEVNVEKLNNDPNCHGIIVQLPLP--KHLNENRAIEKIHP 118
H.s.-1dia       EEIGIKATHIKLPRTTTESEVMKYITSLNEDSTVHGFLVQLPLDSENSINTEEVINAIAP 120
                  :*: :     *   :   :   :  ** :    * ::* *:     ::     : :
                   ↓↓                                              ↓
M.t.-2c2y       AKDADGLHPTNLGRLVLG--TPAPLPCTPRGIVHLLRRYDISIAGAHVVVIGRGVTVGRP 173
A.b.-4b4u       AKDVDGVTCLGFGRMAMG--EAAYGSATPAGIMTILKENNIEIAGKHAVVVGRSAILGKP 173
E.c.-1b0a       DKDVDGFHPYNVGRLCQR--APRLRPCTPRGIVTLLERYNIDTFGLNAVVIGASNIVGRP 174
P.a.-4a5o       DKDVDGFHPYNIGRLAQR--MPLLRPCTPKGIMTLLASTGADLYGMDAVVVGASNIVGRP 174
H.s.-1zn4       DKDVDGFHVINVGRMCLD--QYSMLPATPWGVWEIIKRTGIPTLGKNVVVAGRSKNVGMP 172
L.m.-4a26       HKDADALLPVNVGLLHYKGREPPFTPCTAKGVIVLLKRCGIEMAGKRAVVLGRSNIVGAP 178
H.s.-1dia       EKDVDGLTSINAGRLARGDLNDCFIPCTPKGCLELIKETGVPIAGRHAVVVGRSKIVGAP 180
                 **.*..   . * :          ..*. *   ::   .    *  .** * .  :* *
                                          ↓
M.t.-2c2y       LGLLLTRRS------ENATVTLCHTGT--RDLPALTRQADIVVAAVGVAHLLTADMVRPG 225
A.b.-4b4u       MAMMLLQ--------ANATVTICHSRT--QNLPELVKQADIIVGAVGKAELIQKDWIKQG 223
E.c.-1b0a       MSMELLLAG--------CTTTVTHRFT--KNLRHHVENADLLIVAVGKPGFIPGDWIKEG 224
P.a.-4a5o       MALELLLGG--------CTVTVTHRFT--RDLADHVSRADLVVVAAGKPGLVKGEWIKEG 224
H.s.-1zn4       IAMLLHTDGAHERPGGDATVTISHRYTPKEQLKKHTILADIVISAAGIPNLITADMIKEG 232
L.m.-4a26       VAALLMKEN--------ATVTIVHSGTSTEDMIDYLRTADIVIAAMGQPGYVKGEWIKEG 230
H.s.-1dia       MHDLLLWNN--------ATVTTCHS--KTAHLDEEVNKGDILVVATGQPEMVKGEWIKPG 230
                :   *            .*.*  *      .:      .*::: * * .  :  : :: *
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Figure 1. Alignment of FolD sequences highlighting the conserved residues. The amino acid sequence (residues 1−224) of *Eco*FolD (E.c., PDB entry 1b0a) is aligned with the FolD sequences of *P. aeruginosa* (P.a., PDB entry 4a5o), *L. major* (L.m., PDB entry 4a26), *Homo sapiens* DC301 (H.s., PDB entry 1dia), *H. sapiens* NMDMC (H.s., PDB entry 1zn4), *Mycobacterium tuberculosis* (M.t., PDB entry 2c2y) and *A. baumannii* (A.b., PDB entry 4b4u) by CLASTALW. The completely conserved residues are denoted with asterisks below the sequence. Residues marked with arrows are the focus of this study.

binding.[4,22] K56 and Q100 form a hydrogen bond between them. However, this interaction is not observed in *E. coli* FolD (*Eco*FolD) (Figure 2A). Many amino acids in the CTD of DC301 are also conserved, but the R173 and S197 residues important in $NADP^+$ binding[23] are not well-conserved across the species, particularly in *Eco*FolD and *P. aeruginosa* FolD (*Pae*FolD) (Figure 1). Superposition of the three-dimensional (3D) structures of DC301 and *Eco*FolD (Figure 2B) reveals that the counterparts of DC301 R173 and S197, crucial for $NADP^+$ binding, are represented by A167 and R191, respectively, in *Eco*FolD.[15] Even though the 3D structures of FolD from different species are similar, they possess different specific activities and affinities for the inhibitors (substrate analogues).[16,19] Furthermore, significant differences in the kinetic properties of *Eco*FolD were reported in the two independent studies.[1,24] Although *E. coli* serves as an important bacterial model, structure−function analysis of *Eco*FolD has not been studied in any detail.

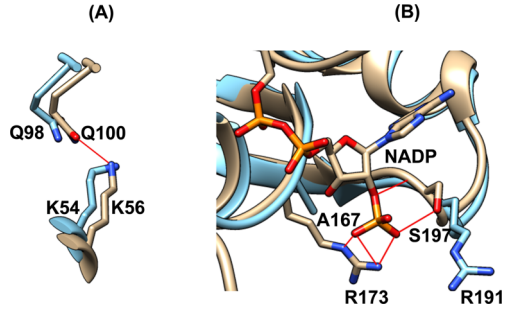

Figure 2. Comparison of the active site residues of *Eco*FolD and DC301. (A) Active site residues Q98 and K54 of *Eco*FolD (PDB entry 1b0a, sky blue) were superimposed on the counterpart residues of DC301 (PDB entry 1dia, tan) with the help of Chimera. Catalytic residue K56 forms a hydrogen bond with the side chain of Q100. The hydrogen bond between the counterpart residues of *Eco*FolD is not seen. (B) The structure of DC301 was superimposed onto that of *Eco*FolD. The $NADP^+$ binding residues of DC301 are R173 and S197, and the counterpart residues in *Eco*FolD are A167 and R191, respectively.

Previously, in a genetic screen,[25] we isolated *E. coli* strains (A48 and B22), which allowed initiation with a mutant initiator tRNA. The strains encoded FolD with G122D[25] and C58Y (unpublished observations) mutations, respectively. To understand the role of these (G122 and C58) and other residues identified as being important from 3D structures of FolD[4,15,19] in *Eco*FolD activity, we have conducted mutational analysis of *Eco*FolD. Our studies have revealed a role for various amino acids that line the substrate binding pocket. In addition, our studies suggest a crucial role for the loop connecting helices 4 and 5 in substrate binding and/or catalysis.

DOI: 10.1021/acs.biochem.5b00400
Biochemistry 2015, 54, 3504−3513

EXPERIMENTAL PROCEDURES

Chemicals, Plasmids, DNA Oligomers, *E. coli* Strains, and Their Growth. (6-*R*,*S*)-5,10-CH_2-THF (calcium salt), and (6-*R*,*S*)-5,10-CH^+-THF chloride were from Schircks Laboratories (Jona, Switzerland). Stock solutions of (6-*R*,*S*)-5,10-CH_2-THF and (6-*R*,*S*)-5,10-CH^+-THF were prepared in N_2-sparged basic buffer [50 mM Tris-HCl (pH 8) and 0.1 M β-mercaptoethanol] and an acidic solution (0.1 M HCl and 0.1 M β-mercaptoethanol), respectively, with minimal light exposure. The quantification of (6-*R*,*S*)-5,10-CH_2-THF by UV spectrophotometry was based upon the reported molar extinction coefficient of 32 mM^{-1} cm^{-1} at 294 nm.[26] The reported kinetic constants of FolD for 5,10-CH_2-THF and 5,10-CH^+-THF are those of the *R*-isomer assuming it to be 50% of the total. *E. coli* strains, plasmids, and DNA oligomers are listed in Tables 1 and 2. Bacteria were grown in Luria-Bertani broth (LB), LB-agar (1.8% agar, Difco), or M9 minimal medium (which includes 0.4% glucose as a carbon source) containing 1 μg mL^{-1} thiamine[27] at 37 °C while being shaken at 200 rpm. Ampicillin (Amp, 100 μg mL^{-1}), kanamycin (Kan, 25 μg mL^{-1}), and tetracycline (Tet, 7.5 μg mL^{-1}) were used as needed.

Table 1. Descriptions of *E. coli* Strains and Plasmids Used in This Study

strain or plasmid	genotype/details	ref
TG1	K-12 *supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5*, ($r_K^-m_K^-$), F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	27
KL16	*E. coli* K12, *thi1*, *relA1*, *spoT1*	49
A48	derivative of KL16 with FolD (G122D)	25
B22	derivative of KL16 with the *folD* gene possessing the C58Y mutation	25, unpublished observations
TG1Δ*folD*::*kan*/p-*fhs*	TG1 deleted for *folD* harboring p-*fhs* (Kan^R, Tet^R)	28
pQE60	expression vector harboring the T5 promoter (Amp^R)	Qiagen
TG1Δ*folD*::*kan*/p-*fhs*/pQE60	TG1Δ*folD*::*kan*/p-*fhs* strain harboring pQE60 (Kan^R, Tet^R, Amp^R)	this study
TG1Δ*folD*::*kan*/p-*fhs*/p-*folD* (wild type or mutants)	TG1Δ*folD*::*kan*/p-*fhs* harboring p-*folD* (wild type or mutants) (Kan^R, Tet^R, Amp^R)	this study

Cloning, Site-Directed Mutagenesis, and Purification of FolD Proteins. All plasmid constructs were made using standard genetic engineering techniques. The wild-type (WT) *folD* from *E. coli* KL16, *folD* (G122D) from *E. coli* A48,[25] and *folD* (C58Y) from *E. coli* B22[25] were amplified by polymerase chain reaction (PCR) using FolD-FP and FolD-RP primers. The amplicons were digested with NcoI and BglII and ligated to similarly digested pQE60 (C-terminal His tag) to give rise to p-*folD* (WT), p-*folD* (G122D), and p-*folD* (C58Y). The other mutations (R191E, D121A, Q98K, K54S, and Y50S) were introduced by site-directed mutagenesis of *folD* in pQE60-*folD* by an inverse PCR method with the primers listed in Table 2. All constructs (WT and the mutants) were verified by DNA sequencing. As FolD is a homodimeric protein, to prevent any contaminations of the mutant proteins with the FolD encoded by the genomic copy of *folD* (upon dimerization with the mutant), purification of FolD proteins was performed with the TG1Δ*folD*::*kan*/pACDH-*fhs* strain (hereafter called Δ*folD*/p-*fhs*). The Δ*folD*/p-*fhs* strain was generated by deleting the *folD* gene with the support of *Clostridium perfringens fhs* on a plasmid.[28] For purification, single colonies of Δ*folD*/p-*fhs* strains harboring plasmid-borne genes (in pQE60) of the WT, R191E, G122D, D121A, Q98K, C58Y, K54S, or Y50S enzyme were inoculated into LB containing Amp, Kan, and Tet and grown overnight. Fresh LB (900 mL) was inoculated with 1% of the overnight culture and grown at 22 °C under medium shaking conditions (~100 rpm) until it reached exponential phase. The cultures were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and allowed to grow for an additional 8 h. The culture was harvested, and the pellet was resuspended in ice-cold buffer [50 mM Tris-HCl (pH 7.6), 40 mM imidazole, and 0.5 M NaCl]. The resuspended cells were lysed by sonication, and the cell homogenate was centrifuged at 100000*g* for 1 h at 4 °C. The supernatant was loaded onto a pre-equilibrated HisTrap HP, 1 mL column (GE Healthcare), followed by washing and elution of proteins using a gradient from 0.04 to 0.5 M imidazole in 30 mL. All proteins except G122D and D121A were purified to near homogeneity as assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The peak fractions of FolD (G122D or D121A) were pooled and dialyzed against 50 mM Tris-HCl (pH 7.6) and 10 mM β-mercaptoethanol. The dialyzed FolD (G122D or D121A) was loaded onto a HiTrap Heparin HP column (1 mL), washed, and eluted with the buffer containing a gradient from 0 to 0.3 M KCl. Pooled fractions of proteins were dialyzed against 50 mM Tris-HCl (pH 7.6), 10 mM β-mercaptoethanol, and 50% (v/v) glycerol and stored at −20 °C.

Enzyme Assays and Kinetics. We purified wild-type *Eco*FolD with or without a His tag, and the activities of the observed His-tagged *Eco*FolD were not compromised (data not

Table 2. List of Primers

primer	5′−3′ sequence
FolD-FP	GTAA*CCATGG*CAGCAAAGATTATTGACGGT
FolD-RP	TCGA*AGATCT*CTCATCCTGTGGATCATGAT
R191E-FP	CACCACTACAGTGACTCACGAATTCACTAAAAATCTGCGTC
R191E-RP	GACGCAGATTTTTAGTGAATTCGTGAGTCACTGTAGTGGTG
D121A-FP	GGACAAAGACGTGGCGGGTTTCCATCCTTAC
D121A-RP	GTAAGGATGGAAACCCGCCACGTCTTTGTCC
Q98K-FP	GATGGCATTCTGGTTAAACTGCCGTTACCG
Q98K-RP	CGGTAACGGCAGTTTAACCAGAATGCCATC
K54S-FP	CAAATTTATGTCGCAAGCAGCCGCAAGGCTTGTGAAGAAG
K54S-RP	CTTCTTCACAAGCCTTGCGGCTGCTTGCGACATAAATTTG
Y50S-FP	CCCTGCATCGCAAATTAGCGTCGCAAGCAAACGC
Y50S-RP	GCGTTTGCTTGCGACGCTAATTTGCGATGCAGGG

DOI: 10.1021/acs.biochem.5b00400
Biochemistry 2015, 54, 3504−3513

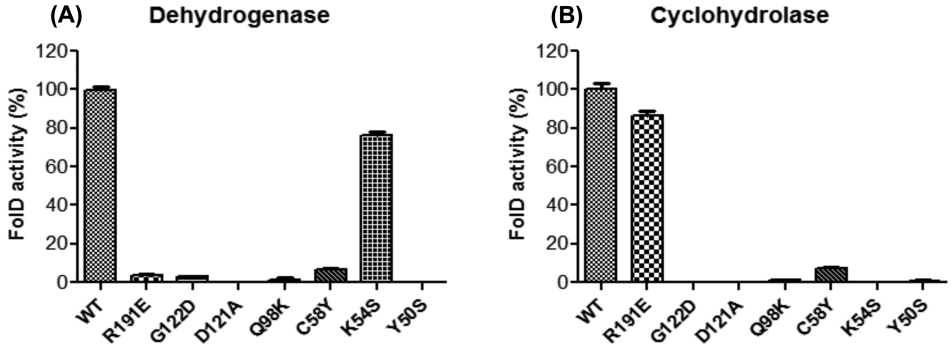

Figure 3. Enzymatic properties of wild-type (WT) and mutant *Eco*FolD proteins. The dehydrogenase (A) and cyclohydrolase (B) activities of the WT and the mutant (R191E, G122D, D121A, Q98K, C58Y, K54S, and Y50S) proteins were assayed as described in Experimental Procedures. The percentage activities of the mutants were determined with respect to the WT protein taken to be 100%. Error bars represent the SEM of at least two replicates.

shown). Therefore, WT FolD and other mutants were purified using a His tag, and used in the assays.[1] Briefly, the dehydrogenase and cyclohydrolase activities were assayed in a buffer consisting of 0.1 M potassium maleate (pH 7.6) and 10 mM β-mercaptoethanol. The kinetic constants of FolD (WT and mutants) with respect to their dehydrogenase activity were determined for 5,10-CH_2-THF and $NADP^+$. In one set of reactions, concentrations of (6-*R*,*S*)-5,10-CH_2-THF were varied from 10 to 2500 μM, keeping the $NADP^+$ concentration at 1 mM in 50 μL reaction mixtures. In another set of reactions, the concentrations of $NADP^+$ were varied from 10 to 2500 μM, keeping the (6-*R*,*S*)-5,10-CH_2-THF concentration at 1.5 mM in 50 μL reaction mixtures. The reaction mixtures were incubated for 2 min (1 h in the case of D121A and G122D mutants) at 30 °C after addition of the enzyme. The reaction was stopped, and NADPH formed in the reaction was destroyed by the addition of 450 μL of 6% perchloric acid, followed by centrifugation at 15000*g* and 4 °C to remove the protein and NADPH precipitates. The supernatant was used to measure the absorbance of the product, 5,10-CH^+-THF (λ_{max} = 350 nm; acidified pH). The kinetic constants for the cyclohydrolase activity were measured with (6-*R*,*S*)-5,10-CH^+-THF by varying its concentration (from 5 to 140 μM) in 0.5 mL reaction mixtures. The enzyme activity was monitored for 30 s after addition of the substrate by measuring the decrease in the absorbance of 5,10-CH^+-THF (λ_{max} = 355 nm; neutral pH) after correcting for the no enzyme background. The amount of 5,10-CH^+-THF was determined using a molar extinction coefficient (24900 M^{-1} cm^{-1}).

***In Vivo* Assessment of FolD Activities.** *E. coli* Δ*folD*/p-*fhs* strains (three or four biological replicates) harboring *folD* or its mutants (R191E, G122D, D121A, Q98K, C58Y, K54S, and Y50S) in the pQE60 plasmid were grown until they reached saturation in 2 mL of LB at 37 °C with required antibiotics. The cultures were diluted (10^{-3}) in M9 minimal medium, and 200 μL volumes were taken in each well of a honeycomb plate. The plate was placed in Bioscreen C growth reader (Oy Growth, Helsinki, Finland), and the culture growth was measured at OD_{600} at 3 h intervals. Mean values with the standard error of the mean (SEM) were plotted versus time.

Circular Dichroism (CD). The CD spectra of the FolD proteins were recorded from 250 to 200 nm at 25 °C using a JASCO J-815 CD spectrometer and a cuvette with a path length of 0.1 cm. The spectrum of proteins in 25 mM potassium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol was collected from the three independent spectral readings for each sample. The baseline for each sample was corrected before spectra were recorded. The calculation of $\Delta\varepsilon$, termed molar circular dichroism, was determined using the method of Dichrocalc,[29] and the percent contents of α-helix and β-strands were calculated using K2D3.[30]

Docking and 3D Modeling. Protein structure homology modeling of mutant FolD proteins was conducted with the latest version of the SWISS-MODEL expert system.[31−33] In brief, the software picks a template from the SWISS-MODEL template library on the basis of the highest-quality alignment and builds a model based on the target−template alignment using Promod-II. The quality of the 3D model of FolD mutants was assessed on the basis of the highest QMEAN score with the apo crystal structure of the wild-type FolD template (PDB entry 1b0a). The structures of *Eco*FolD (WT and modeled structure) were docked with 5,10-CH_2-THF or 5,10-CH^+-THF using SwissDock.[34] Of 256 clusters of ligands bound to *Eco*FolD (WT and modeled structure), the best fit cluster was chosen on the basis of the interactions observed in the structures of DC301 and *A. baumannii* FolD (*Ac*FolD).[4,19,22]

RESULTS

***Eco*FolD Mutants and Their Activities.** All of the reported crystal structures of FolD have hypothesized that the counterparts of D121, and a pair of conserved peptide signatures, $Y^{50}XXXK^{54}$ and S^{47}----Q^{98}--P^{100} of *Eco*FolD (Figure 1), are involved in pterin binding and the hydration reaction. Superimposed structures of DC301 with *Eco*FolD predicted that R191 of *Eco*FolD might be important in $NADP^+$ binding (Figure 2A). Furthermore, in a genetic screen,[25] we identified FolD mutants G122D and C58Y, the dehydrogenase and cyclohydrolase activities of which were compromised. With this information in hand, we cloned, overproduced, and purified WT and mutant (R191E, G122D, D121A, Q98K, C58Y, K54S, and Y50S) *Eco*FolD and assayed them for their dehydrogenase (Figure 3A) and cyclohydrolase (Figure 3B) activities. Except for those of the R191E and K54S proteins, the dehydrogenase and cyclohydrolase activities of all mutants were strongly compromised. Interestingly, while the R191E mutant showed very poor dehydrogenase activity, its cyclohydrolase activity was nearly as good as that of wild-type *Eco*FolD. On the other hand, while K54S retained high activity as a dehydrogenase, its cyclohydrolase activity was negligible.

Kinetic Parameters of *Eco*FolD Proteins. The kinetic constants of WT, C58Y, G122D, and D121A for both the dehydrogenase and cyclohydrolase activities, K54S for its

DOI: 10.1021/acs.biochem.5b00400
Biochemistry 2015, 54, 3504−3513

Table 3. Kinetic Properties of the Dehydrogenase and Cyclohydrolase of FolD[a]

FolD	substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
			Dehydrogenase		
WT	5,10-CH_2-THF	279 ± 39	19 ± 1	1195 ± 65	4.2
	NADP	187 ± 33	16 ± 1	1025 ± 50	5.5
C58Y	5,10-CH_2-THF	302 ± 36	2.5 ± 0.1	155 ± 5	0.5
	NADP	233 ± 21	2.7 ± 0.1	167 ± 4	0.7
K54S	5,10-CH_2-THF	205 ± 12	14 ± 0.2	860 ± 15	4.2
	NADP	143 ± 5	12 ± 0.1	736 ± 5	5
G122D	5,10-CH_2-THF	68 ± 5	0.056	3.5	0.05
D121A	5,10-CH_2-THF	104 ± 17	0.012	0.7	0.007
R191E	5,10-CH_2-THF/NADP	ND	ND	–	–
Q98K	5,10-CH_2-THF/NADP	ND	ND	–	–
Y50S	5,10-CH_2-THF/NADP	ND	ND	–	–
			Cyclohydrolase		
WT	5,10-CH^+-THF	26 ± 7	39 ± 7	2453 ± 329	95
R191E	5,10-CH^+-THF	28 ± 9	55 ± 8	3446 ± 487	124
C58Y	5,10-CH^+-THF	40 ± 9	4.6 ± 0.5	285 ± 31	7
G122D	5,10-CH^+-THF	4.5 ± 1.5	0.025	1.5	0.345
D121A	5,10-CH^+-THF	15 ± 8	0.011	0.6	0.045
Q98K	5,10-CH^+-THF	ND	ND	–	–
K54S	5,10-CH^+-THF	ND	ND	–	–
Y50S	5,10-CH^+-THF	ND	ND	–	–

[a]ND means that the kinetic parameters of the mutants could not be determined because of their very low activities. The details for measuring FolD activities are given in Experimental Procedures.

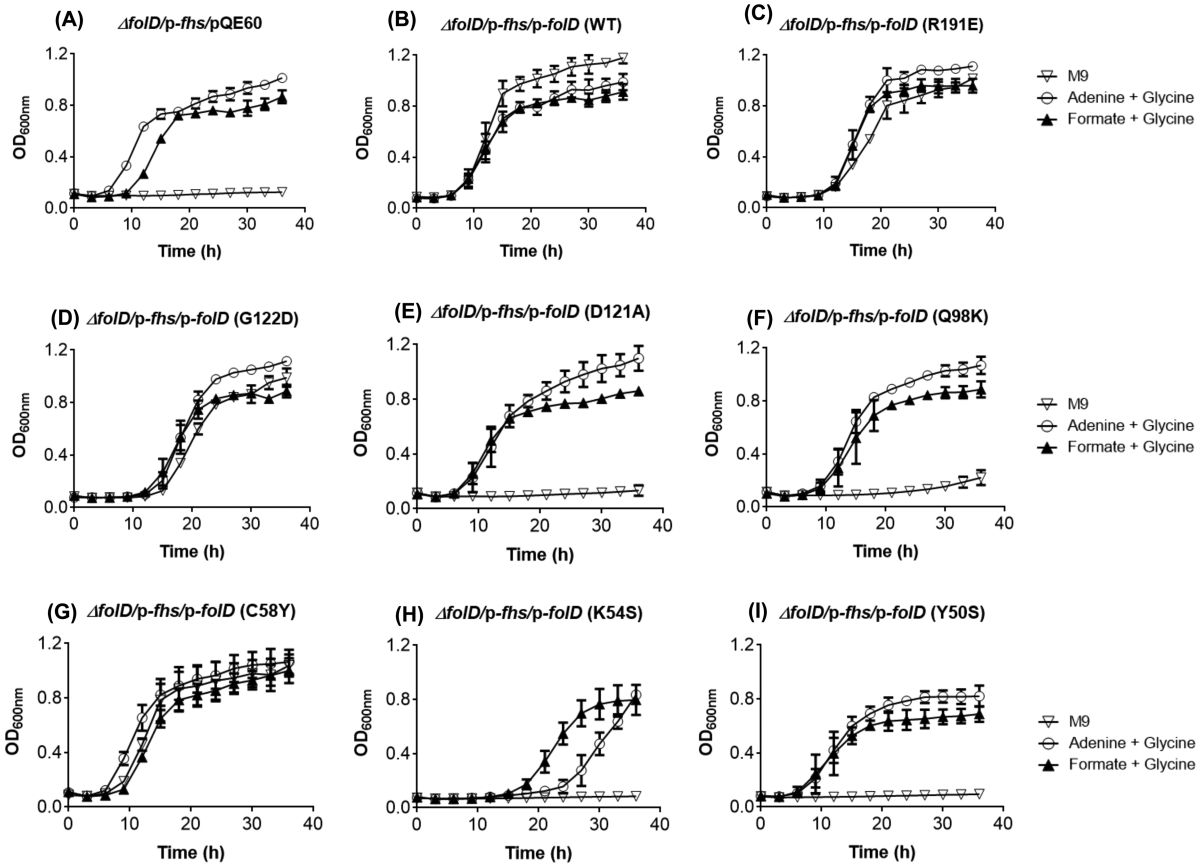

Figure 4. Assessment of the *in vivo* activity of various FolD mutants in the Δ*folD* strain. The Δ*folD* strain harboring the *fhs* gene on the medium copy plasmid was transformed with pQE60 (vector alone) or p-*folD* (wild type or mutants). Growth of the transformants was followed in either M9 minimal medium or medium supplemented with adenine (0.1 mg mL^{-1})/formate (10 mM) and glycine (0.3 mg mL^{-1}). WT, R191E, G122D, and C58Y but not D121A, Q98K, K54S, and Y50S rescued the growth of the Δ*folD*/p-*fhs* strain in M9 medium.

DOI: 10.1021/acs.biochem.5b00400
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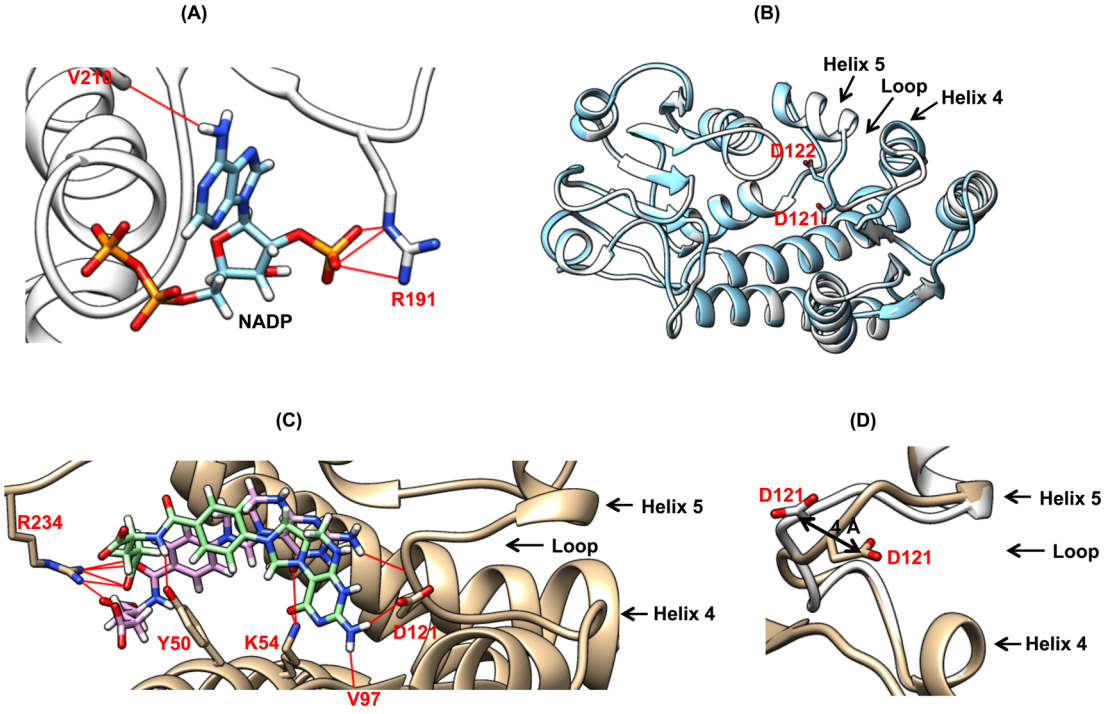

Figure 5. 3D structural modeling of FolD proteins. The structure of *Eco*FolD (PDB entry 1b0a) was docked with $NADP^+$. The NE and NH_2 groups at the side chain of R191 form a hydrogen bond with the 2′-phosphate of $NADP^+$ (A). A subunit of *Eco*FolD (Wt) (PDB entry 1b0a, white) was superimposed with the *Eco*FolD (G122D, light blue) mutant. The orientation of D121 in *Eco*FolD (WT and G122D) and D122 in *Eco*FolD (G122D) is shown (B). 5,10-CH_2-THF (light green) and 5,10-CH^+-THF (light pink) showed hydrogen bond interactions with K54, D121, and R234 of the modeled structure. Besides this, 5,10-CH_2-THF (light green) also formed hydrogen bonds with Y50 and V97 (C). D121 in the loop (connecting helices 4 and 5) of the superimposed structure of *Eco*FolD (PDB entry 1b0a) and the modeled structure may be separated at maximum by 4 Å (D).

dehydrogenase activity, and R191E for its cyclohydrolase activity were determined (Table 3). The kinetic constants of Q98K and Y50S mutants could not be determined because of their negligible activities. The V_{max} of K54S is ∼75% of that of WT FolD, and the k_{cat}/K_m is approximately the same for the mutant and WT proteins. The k_{cat}/K_m of the C58Y mutant is ∼12% for the dehydrogenase and 7% for the cyclohydrolase activities of WT FolD. The kinetic constants of G122D and D121A mutants (Table 3) could be determined by using more than 10 times the amounts of the proteins used in the other cases (0.4 μg for dehydrogenase and 0.2 μg for cyclohydrolase activities).

***In Vivo* Activity of the FolD Mutants.** FolD is an essential protein in *E. coli*. However, recently, we showed that in the presence of Fhs, the *folD* gene could be deleted. We noted that while the Δ*folD*/p-*fhs* strain grew well in rich medium, its growth in M9 minimal medium was dependent on the presence of both the glycine and adenine/formate.[28] Thus, to ensure that the poor or undetectable activities of the FolD mutants were not a consequence of issues related to biochemical purification, we transformed the Δ*folD*/p-*fhs* strain with either an empty plasmid or a *folD* gene (WT or mutants) cloned into the pQE60 plasmid (compatible with *p-fhs*) and checked the growth of the transformants on M9 minimal medium [without any supplements (test) and with supplements of glycine and adenine/formate (control)] to assess *in vivo* activities of the mutants. As shown in Figure 4A–I, we observed that the WT, R191E, G122D, and C58Y but not the D121A, Q98K, K54S, and Y50S mutants rescued the growth of the Δ*folD*/p-*fhs* strain in the M9 medium for its auxotrophy for glycine and adenine/formate. Failure of the K54S mutant to rescue growth in M9 medium in spite of its high dehydrogenase activity suggests that the cyclohydrolase activity of FolD is also essential for the synthesis of 10-CHO-THF in *E. coli*. The Δ*folD*/p-*fhs*/p-*folD* (K54S) mutant showed growth that was much delayed compared to that of Δ*folD*/p-*fhs*/pQE60 (empty vector) (compare panels A and H of Figure 4). This suggests that having high dehydrogenase activity and a lack of cyclohydrolase activity may lead to high accumulation of 5,10-CH^+-THF and 5-formyltetrahydrofolate (5-CHO-THF). 5-CHO-THF is an inhibitor of phosphoribosyl-aminoimidazolecarboxamide formyltransferase (in the purine biosynthetic pathway), serine hydroxymethyltransferase, sarcosine dehydrogenase, dimethylglycine dehydrogenase, FolD, methionyl-tRNA formyltransferase, and dihydrofolate reductase (in the one-carbon metabolism pathway).[35] This may explain the slow growth phenotype of the K54S mutant. More importantly, taken together with the biochemical analyses, these observations suggest that the K54S mutant is a derivative of *Eco*FolD with the monofunctional activity of 5,10-CH_2-THF dehydrogenase.

3D Modeling. For the reliability of 3D modeling, we first investigated if there were any gross changes in the secondary structural elements or the structural integrity of the FolD mutants by CD spectroscopic analysis. The spectral profiles indicated no significant structural changes in any of the mutants (Figure S1 of the Supporting Information). We then built 3D models with the help of SWISS-MODEL. In this analysis also, no overall changes were reflected in the structure of the mutants upon superposing the 3D structures of the mutants with the structure of WT FolD as deposited in the PDB (data not shown). Thus, to probe the role of the mutated positions, the local interactions of the residues were investigated. While

DOI: 10.1021/acs.biochem.5b00400
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*Eco*FolD (PDB entry 1b0a) was docked with 5,10-CH_2-THF and refined by molecular dynamics simulations in an earlier study,[15] the details obtained were not in good agreement with the cocrystal structures of DC301 and *Ac*FolD bound to substrate analogues.[4,19] In our attempts also when *Eco*FolD (PDB entry 1b0a) was docked with the substrate 5,10-CH_2-THF or 5,10-CH^+-THF, none of the 256 clusters that were generated revealed contacts that were in agreement with the structures of DC301 and *Ac*FolD bound to substrate analogues. We then decided to dock *Eco*FolD (PDB entry 1b0a) with $NADP^+$. We observed that the side chain of R191 in the CTD formed hydrogen bonds with the 2′-phosphate (Figure 5A). Why 5,10-CH_2-THF and 5,10-CH^+-THF failed to dock at the NTD of *Eco*FolD (as anticipated from the studies with DC301 and *Ac*FolD) was therefore intriguing. Are some conformational changes and/or domain movements necessary for binding of substrates to the NTD? The possibility of conformational changes and/or domain movements was also suggested by the phenotype of an *E. coli* isolate A48 possessing the G122D mutation. Residue G122 (together with D121) is displayed in the loop connecting helices 4 and 5 on the superimposed structure of *Eco*FolD (G122D) and *Eco*FolD (WT) (Figure 5B). Even though G122 is involved in neither any direct contacts with the substrate nor catalysis, it resulted in a highly compromised FolD activity. As Gly residues are preferred within the loop regions to allow structural flexibility, we decided to model the loop (residues 115−124) using ModLoop[36] to explore if structural flexibility was crucial in substrate binding. The modeled structure was docked with 5,10-CH_2-THF and 5,10-CH^+-THF. Consistent with the DC301 and *Ac*FolD cocrystal structures, the modeled structure now revealed hydrogen bonds between the ϵ-amino group of K54 and the 4-oxo of 5,10-CH_2-THF, the side chain carbonyl of D121 and the 2-amino of 5,10-CH_2-THF, the main chain carbonyl of V97 and the 2-amino of 5,10-CH_2-THF, the hydroxyl group of Y50 and the glutamate tail of 5,10-CH_2-THF, and the side chain group of R234 and the glutamate tail of 5,10-CH_2-THF (Figure 5C). These interactions are similar to those observed in the structures of DC301 and *Ac*FolD bound to substrate analogues.[4,19] Similar interactions involving the ϵ-amino group of K54, the main chain carbonyl of D121, and the side chain group of R234 were also predicted with 5,10-CH^+-THF (Figure 5C). These observations are suggestive of the need for conformational changes in the loop connecting helices 4 and 5 for binding of 5,10-CH_2-THF or 5,10-CH^+-THF to *Eco*FolD, and that G122 in the loop might be playing an important role during these structural transitions. The D121 residue in the loop of the superimposed structure of *Eco*FolD (PDB entry 1b0a) and modeled structure may be separated at maximum by 4 Å (Figure 5D). The modeling of other mutants also predicted that while K54 of *Eco*FolD does not form a hydrogen bond with Y50 (Figure 6A), the K54S mutant formed a hydrogen bond with the main chain carbonyl of Y50 (Figure 6B). Likewise, while the C58 residue does not form a hydrogen bond with P34, L36, and V64 (Figure 6C), the C58Y mutant forms hydrogen bonds with them (Figure 6D).

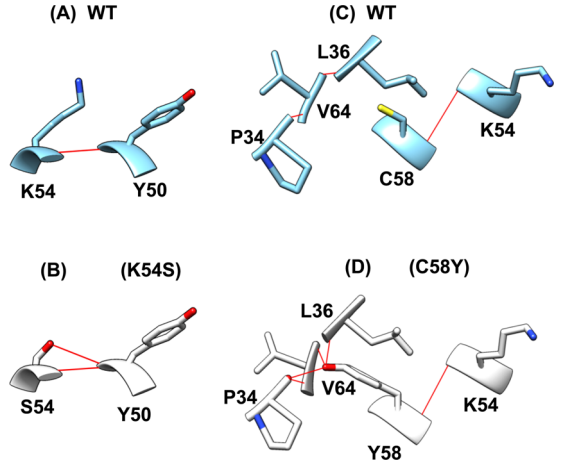

Figure 6. Roles of mutated amino acids in K54S and C58Y mutants determined by 3D modeling. The 3D structures of K54S and C58Y mutants were generated by SWISS-MODEL and Chimera using the wild-type template (PDB entry 1b0a). K54 is shown in the 3D model (A). An additional hydrogen bond is formed between the hydroxyl group of S54 (mutated from K54) and the main chain carbonyl of Y50 (B). C58 in *Eco*FolD forms a hydrogen bond with the main chain carbonyl of K54 (C). Y58 (mutated from C58) forms an additional hydrogen bond with the main chain carbonyl of V64 and P34 and the main chain amide of L36 (D).

DISCUSSION

FolD is a well-studied enzyme in both prokaryotic and eukaryotic organisms. In the first report, the specific activity for dehydrogenase of *Eco*FolD (purified using multiple steps followed by polyacrylamide disc gel electrophoresis) was 31.2 μmol min^{-1} mg^{-1}.[24] Subsequently, with a 2000-fold purified enzyme, specific activities of 200 μmol min^{-1} mg^{-1} for dehydrogenase and 33 μmol min^{-1} mg^{-1} for cyclohydrolase (~16.5% of dehydrogenase activity) were reported.[1] However, the study used different buffers for the dehydrogenase and cyclohydrolase assays. In our study, we used the *E. coli* Δ*folD*/p-*fhs* strain to purify FolD and its mutants to ensure no contaminating presence of the host FolD to determine K_m, V_{max}, k_{cat}, and k_{cat}/K_m (Table 3). When the same buffer conditions were kept for the dehydrogenase and cyclohydrolase assays, WT protein V_{max} values were found to be 19 and 39 μmol min^{-1} mg^{-1} for the dehydrogenase and cyclohydrolase activities, respectively. Thus, in our study, the dehydrogenase activity is more similar to that in the first report[24] and the cyclohydrolase activity to that in the second report.[1] With the exception of the FolD from a purine-requiring organism, *Leishmania*,[3] FolD proteins from other organisms, e.g., human (cytoplasmic or mitochondrial), *A. baumannii*, *Photobacterium phosphoreum*, yeast, porcine, and rabbit liver,[19,22,37−40] possess approximately equal or higher specific activities for the cyclohydrolase activity than for the dehydrogenase activity.

FolD structures have revealed the presence of N- and C-terminal domains of the α/β fold connected by two long helices[4,15−17,19,20] and identified the conserved motifs, YX_3K and S-Q-P, important for substrate binding and catalysis. Further, the inhibitors (substrate analogues) bound to DC301 and *Ac*FolD identified the counterparts of K54 and Q98 (*Eco*FolD) to bind a well-ordered water molecule.[4,15,19] The K54S mutation led to a complete loss of the cyclohydrolase activity but retention of ~75% of its dehydrogenase activity (Figure 3). Although mutations of the corresponding K56 in DC301 also resulted in a complete loss of cyclohydrolase activity, the K56S mutant retained only ~2% dehydrogenase activity.[4] Thus, our observations suggest that the role of K54 (in *Eco*FolD) can be partially fulfilled by Ser for the dehydrogenase activity and that its efficiency is context-dependent. Furthermore, as proposed for DC301,[22] for the

DOI: 10.1021/acs.biochem.5b00400
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dehydrogenase activity of *Eco*FolD, the lone pair of electrons present on the ε-amino group of K54 may increase the electron density in the tetrahydropteridine ring to assist in the abstraction of a hydride from the -CH_2- group of 5,10-CH_2-THF to $NADP^+$. The reasons for maintaining high dehydrogenase activity in the K54S mutant (*Eco*FolD) compared to that in the K56S mutant (DC301) remain unclear. However, it may be that the closeness of S54 (in the K54S mutant) with S53 and R55 (whose counterparts in DC301 are V55 and L57, respectively) might increase the electron density in the tetrahydropteridine ring for hydride transfer. The loss of cyclohydrolase activities in the K54S and K56S mutants suggests that the Lys side chain is important for maintaining a well-ordered water molecule that is required during the cyclohydrolase reaction.[22] The importance of a Lys residue for the cyclohydrolase reaction at the corresponding position was also reflected in yeast FolD, where there is a Thr in place of this Lys, which lacks cyclohydrolase activity.[41]

The structure of FolD bound with inhibitors (substrate analogues) of DC301 identified hydrophobic stacking of Y52 with the inhibitors.[4] To test the importance of stacking of Y50 with the substrate, we generated the Y50S mutant; this resulted in the loss of both the dehydrogenase and cyclohydrolase activities. However, such a mutation in DC301 preserved ∼30% of the cyclohydrolase activity, suggesting that in DC301 the primary role of this Tyr (Y52) is in stacking with the *p*-aminobenzoyl moiety, and perhaps not in catalysis.[4] However, a complete loss of the cyclohydrolase activity in the Y50S mutant (*Eco*FolD) suggests that it may also have a role in catalysis. A role of Y50 in activating water molecule was also supported by the crystal structure of *Ac*FolD.[19]

The conserved Q100 (DC301) forms a hydrogen bond with K56 and the water molecule.[4] The Q100K mutant showed no detectable cyclohydrolase activity and only a weak dehydrogenase activity of ∼10%.[22] Q98 and K54 (*Eco*FolD) are also supposed to form a complex with a well-ordered water molecule. The observation that the Q98K mutant lost both the dehydrogenase and cyclohydrolase activities *in vitro* and failed to function *in vivo* suggests that at least in the context of *Eco*FolD, these contacts are crucial.

The structures of DC301 and *Ac*FolD bound to substrate analogues showed that D125 (DC301) and D120 (*Ac*FolD) form hydrogen bonds with the C_2 amino group of the substrate analogues.[4,19] Mutation of D121 (*Eco*FolD) to A, similar to the D125A mutation (DC301),[22] showed loss of both dehydrogenase and cyclohydrolase activities, suggesting an important role of this residue. Both modeling (Figure 5C,D) and kinetic studies suggest that D121 might be involved in binding and/or positioning the substrate. Aspartate residues have also been identified as being important in such a role in other 5,10-CH_2-THF-utilizing enzymes, methylenetetrahydrofolate reductase[42] and thymidylate synthase.[43] The G122D mutation resulted in highly compromised dehydrogenase and cyclohydrolase activities (Table 3), yet the mutant was able to rescue the growth defect of the Δ*folD* strain, suggesting residual activity of the mutant is sufficient if it is overexpressed along with Fhs. The poor biochemical activities could be a consequence of this mutant being unstable *in vitro*. In fact, longer storage of this protein resulted in complete loss of activities as dehydrogenase and cyclohydrolase, suggesting a structural role for G122. Glycine is unique for its conformational adaptability to fit in the tight turns. The essentiality of glycine in the Gly-X-Gly-X-X-Gly motif, for example, in protein kinases is well-documented.[44] The fact that the Gly residue is the smallest amino acid might allow flexibility of the loop to accommodate the substrates in the wild-type protein. The flexibility of the loop might be restricted in the G122D mutant. Conformational changes from an "open" unliganded state to the "closed" complexed state for the correct orientation of substrates upon 5,10-CH_2-THF or substrate analogue binding to thymidylate synthase have been documented.[45−47]

The structures of FolD from different species identified $NADP^+$ binding Rossmann fold at the CTD. R173 and S197 of DC301 and their counterparts in *Ac*FolD and *L. major* FolD (*Lm*FolD) are involved in binding $NADP^+$ via the 2′-phosphate.[4,18,19] In mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase (NMDMC), residues corresponding to R173 and S197 of DC301 are represented by R166 and R198, respectively (Figure 1). Of these, R166 is involved in P_i binding at the NAD^+ binding site, and R198 may be facilitating this binding.[48] However, these residues in *Eco*FolD are represented by A167 and R191, respectively. The side chain of R191 was predicted to form a hydrogen bond with the 2′-phosphate of $NADP^+$ (Figure 5A). A167 is not involved in binding with $NADP^+$. The observation that mutation of R191 to E resulted in the loss of dehydrogenase activity but not cyclohydrolase activity lends further support to the idea that R191 is important in $NADP^+$ binding. However, as the R191E mutant rescued the growth defect of the Δ*folD* strain (Figure 4C), the weak dehydrogenase activity it possessed was adequate *in vivo*, at least when it is expressed from a high copy vector (in the presence of Fhs).

The C58Y mutant retained ∼10% of the dehydrogenase and cyclohydrolase activities. C58 forms a hydrogen bond with the main chain carbonyl of K54. The additional hydrogen bond formed by Y58 in the mutant with the main chain carbonyl of V64 and P34 and amide of L36 might impact residues like K54 and Q98, resulting in decreased activities.

In conclusion, the mutational and 3D modeling of *Eco*FolD for the role of Y50, K54, C58, Q98, D121, G122, and R191 has highlighted commonalities and distinctiveness of their roles in the mechanism of catalysis by FolD.

■ ASSOCIATED CONTENT

Supporting Information

Supporting materials along with additional experimental results. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00400.

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Funding

This work was funded by grants from the Department of Science and Technology (DST) and the Department of Biotechnology (DBT), New Delhi, India. U.V. is a J. C. Bose fellow of the DST (New Delhi, India). S.S. is a Dr. D. S. Kothari postdoctoral fellow of the University Grants Commission (New Delhi, India).

Notes

The authors declare no competing financial interest.

DOI: 10.1021/acs.biochem.5b00400
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■ ACKNOWLEDGMENTS

We thank our laboratory colleagues for their suggestions on the manuscript.

■ ABBREVIATIONS

FolD, methylenetetrahydrofolate dehydrogenase-cyclohydrolase; Fhs, formyltetrahydrofolate synthetase; 5,10-CH_2-THF, 5,10-methylene-tetrahydrofolate; 5,10-CH^+-THF, 5,10-methenyl-tetrahydrofolate; 10-CHO-THF, 10-formyl-tetrahydrofolate; ZMP, 5-aminoimidazole-4-carboxamide ribonucleotide; ZTP, 5-aminoimidazole-4-carboxamide riboside 5′-triphosphate; NTD, N-terminal domain; CTD, C-terminal domain; DC301, dehydrogenase/cyclohydrolase domains of the human trifunctional enzyme; *Eco*FolD, *E. coli* FolD; *Pae*FolD, *P. aeruginosa* FolD; IPTG, isopropyl β-D-1-thiogalactopyranoside; 5-CHO-THF, 5-formyltetrahydrofolate; *Ac*FolD, *A. baumannii* FolD; *Lm*FolD, *L. major* FolD; NMDMC, mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase; PDB, Protein Data Bank.

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DOI: 10.1021/acs.biochem.5b00400
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