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Loop Interactions during Catalysis by Dihydrofolate Reductase from *Moritella profunda*

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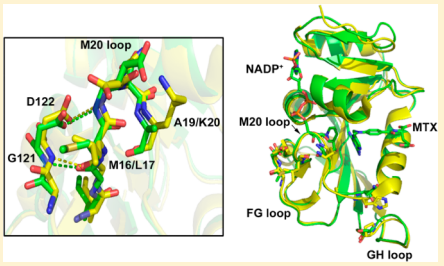
Supporting Information

ABSTRACT: Dihydrofolate reductase (DHFR) is often used as a model system to study the relation between protein dynamics and catalysis. We have studied a number of variants of the cold-adapted DHFR from *Moritella profunda* (MpDHFR), in which the catalytically important M20 and FG loops have been altered, and present a comparison with the corresponding variants of the well-studied DHFR from *Escherichia coli* (EcDHFR). Mutations in the M20 loop do not affect the actual chemical step of transfer of hydride from reduced nicotinamide adenine dinucleotide phosphate to the substrate 7,8-dihydrofolate in the catalytic cycle in either enzyme; they affect the steady state turnover rate in EcDHFR but not in MpDHFR. Mutations in the FG loop also have different effects on catalysis by the two DHFRs. Despite the two enzymes most likely sharing a common catalytic cycle at pH 7, motions of these loops, known to be important for progression through the catalytic cycle in EcDHFR, appear not to play a significant role in MpDHFR.



Many proteins exist simultaneously in multiple conformations, and the importance of protein conformational movements in ligand binding and release is well-established. The role of protein motions in the physical steps and, more controversially, the chemical step of the reaction catalyzed by dihydrofolate reductase (DHFR) has been studied extensively.[1−12] DHFR catalyzes the stereospecific reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) by nicotinamide adenine dinucleotide phosphate (NADPH). The structure of DHFR contains three mobile loops, namely, the M20 loop [residues 9−24 in DHFR from *Escherichia coli* (EcDHFR)], the FG loop (residues 116−132 in EcDHFR), and the GH loop (residues 142−149 in EcDHFR), the movements of which are important for EcDHFR catalysis.[3,4] Depending on the bound ligand(s), two different conformations have been observed for the M20 loop of EcDHFR: closed and occluded (Figure 1).[3] In the substrate complexes, E·NADPH and E·NADPH·DHF, the M20 loop adopts the closed conformation, which is stabilized by hydrogen bonding interactions between Asp122 (HN and Oδ) in the FG loop and Gly15 (O) and Glu17 (HN) in the M20 loop (Figure 1).[3] Asp122 is extensively conserved among bacterial DHFRs, along with a glycine residue in the preceding position (Supporting Information). Mutations at positions 121 and 122 of EcDHFR, which perturb the ability of the enzyme to form the closed conformation and instead favor the occluded conformation, have been studied previously.[12−21]

In the three product complexes, E·NADP$^+$·THF, E·THF, and E·NADPH·THF, the M20 loop of EcDHFR switches to the occluded conformation (ref 3 and DOI: 10.1021/bi500507v). The hydrogen bonds stabilizing the closed conformation are lost, and two new hydrogen bonds are formed between Asn23 (HN and O) in the GH loop and Ser148 (Oγ and HN) in the M20 loop.[3] At pH 7, release of THF from the mixed ternary complex E·NADPH·THF and the accompanying return of the M20 loop to the closed conformation are the rate-limiting steps of the catalytic cycle.[22] Residues involved in stabilizing the occluded conformation in EcDHFR are less well conserved (Supporting Information). It has been shown previously that the EcDHFR-S148A variant cannot form the occluded conformation.[5] Similarly, DHFR from the psychrophilic bacterium *Moritella profunda* (MpDHFR), which has a proline residue in this position, does not form an occluded conformation despite following the same catalytic cycle as EcDHFR (DOI: 10.1021/bi500507v).

MpDHFR shows extensive conservation of catalytically important residues compared to EcDHFR.[23,24] The hydrogen bond formed between Asp122 and Glu17 of EcDHFR[3] is also present between the complementary residues of MpDHFR, and Gly123 in MpDHFR is in a position and an orientation very similar to those of Gly121 in EcDHFR (Figure 1).[23] In addition, alignment of the EcDHFR and MpDHFR amino acid sequences shows only three significant differences in the M20 loop (Figure 1 and Supporting Information). These are Met16 (replaced by Leu17 in MpDHFR), Ala19 (replaced by Lys20 in MpDHFR), and Asn23 (replaced by His24 in MpDHFR). We have shown previously that MpDHFR-H24N has a pH dependence in the steady state similar to that observed for wild-type MpDHFR with similar k_{cat} values, suggesting that this mutation did not affect the steady state rate-limiting step.[25]

Here, we describe the effect of site-directed mutagenesis of the M20 and FG loops of EcDHFR and MpDHFR. Mutations in the M20 loop were created at positions 16 and 19 in

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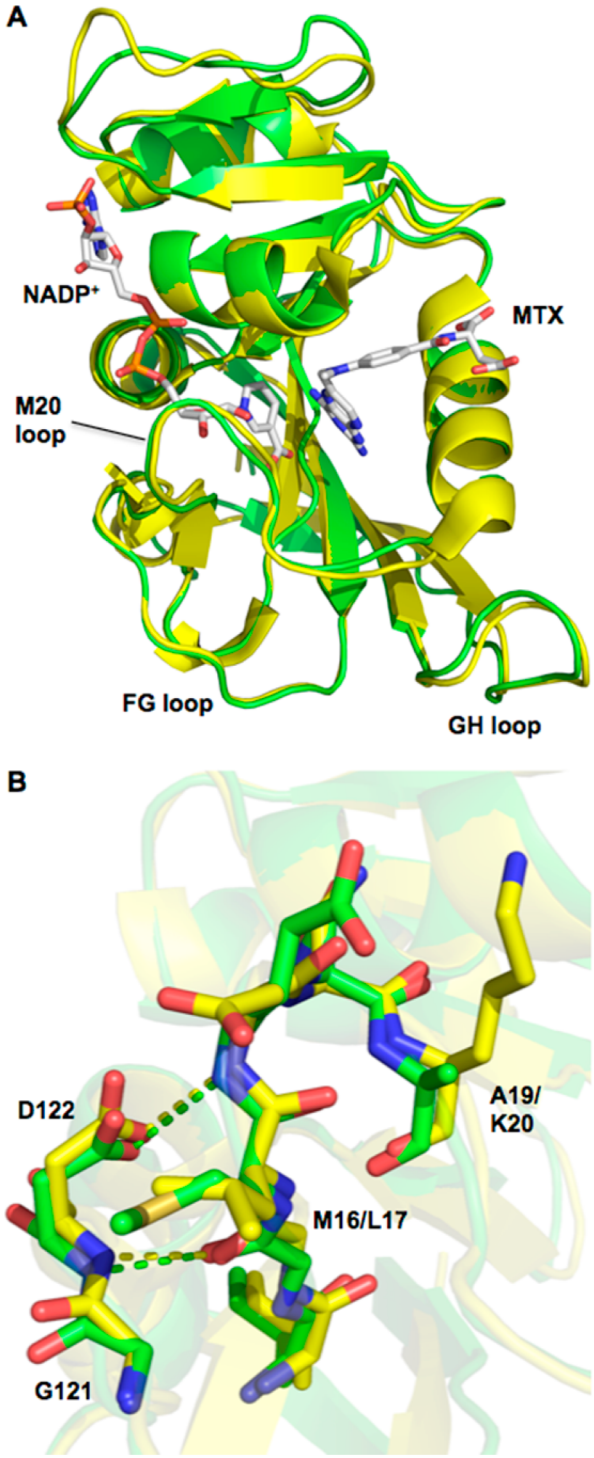

Figure 1. Cartoon representations of EcDHFR (green, Protein Data Bank entry 1DRE)[3] and MpDHFR (yellow, Protein Data Bank entry 3IA4)[23] showing the positions of the residues substituted in this work (A) and hydrogen bonding between D122 in the FG loop and E/D17 and G15 in the M20 loop (EcDHFR numbering) in the closed conformation (B). Ligands and amino acid residues are shown as sticks.

EcDHFR and the equivalent positions 17 and 20 in MpDHFR (Figure 1) to explore the role of these residues in catalysis. The differences in these positions are the most dramatic changes to the M20 loop between the two enzymes. Mutations in the FG loop targeted Gly123 and Asp124 of MpDHFR; the corresponding residues, Gly121 and Asp122, are involved in stabilizing the closed conformation of EcDHFR.[3,13,14] Our results demonstrate important differences between MpDHFR and EcDHFR and show that, in contrast to EcDHFR, loop motions in MpDHFR are not important for progression through the catalytic cycle.

MATERIALS AND METHODS

Chemicals. $NADP^+$ and NADPH were purchased from Melford. Dihydrofolate was prepared by dithionite reduction of folate.[26] 4-(*R*)-NADPD was prepared as described previously.[27] All DHFRs were produced as reported previously[25] and purified by anion exchange chromatography on Q-Sepharose resin followed by size exclusion chromatography on a Superdex 75 column. The concentrations of NADPH and NADPD were determined spectrophotometrically using an extinction coefficient of 6200 M^{-1} cm^{-1} at 339 nm. An extinction coefficient of 28000 M^{-1} cm^{-1} was used to determine the concentration of DHF at 282 nm.

Site-Directed Mutagenesis. The *Pfu* site-directed mutagenesis kit (Stratagene) and the following primers were used to generate the different MpDHFR and EcDHFR variants: EcDHFR-M16L, 5′-GATCGCGTTATCGGCCTG-GAAAACGCCATGCCG-3′; EcDHFR-A19K, 5′-GGCATGG-AAAACAAAATGCCGTGGAACCTG-3′; EcDHFR-M16L/A19K, 5′-GATCGCGTTATCGGCCTGGAAAACAAAATG-CCGTGGAAC-3′; MpDHFR-L17M, 5′-CCGCGTGATTGG-TATGGATAATAAGATGCCG-3′; MpDHFR-K20A, 5′-GAT-TGGTTTAGATAATGCGATGCCGTGGCAT-3′; MpDHFR-L17M/K20A, 5′-AACCGCGTGATTGGTATGGA-TAATGCGATGCCGTGGCAT-3′; MpDHFR-M43W, 5′-GC-AAACCTATCGTTTGGGGTCGTAATACCTTTG-3′; MpDHFR-D124N, 5′-GAATTAACCACCGAAGGGAATAC-TTGGTTC-3′; MpDHFR-G123V, 5′-GAATTAACCACCGA-AGTGGATACTTGGTTC-3′. The replaced bases are underlined.

Primers for the creation of MpDHFR-P150S and EcDHFR-S148P are given in the preceding paper (DOI: 10.1021/bi500507v).

Successful mutagenesis to form MpDHFR-M43W required additional silent mutations to the flanking codons, designed using the Graphical Codon Usage Analyzer.[28]

Steady State Kinetic Measurements. Turnover rates were measured spectrophotometrically on a JASCO V-660 spectrophotometer by following the decrease in absorbance at 340 nm during the reaction (ε_{340} = 11800 M^{-1} cm^{-1}).[29] Initial rates were determined under saturating conditions (100 μM NADPH and DHF) at pH 7 in 100 mM potassium phosphate containing 100 mM NaCl and 10 mM β-mercaptoethanol.

Pre-Steady State Kinetic Measurements. Hydride transfer rate constants were measured under single-turnover conditions using an Applied Photophysics stopped-flow spectrophotometer. The enzyme (final concentration of 20 μM) was preincubated with NADPH (final concentration of 8 μM) for at least 5 min in 100 mM potassium phosphate (pH 7.0) containing 100 mM NaCl and 10 mM β-mercaptoethanol, and the reaction was initiated by rapidly mixing the solution with DHF (final concentration of 200 μM) in the same buffer. Loss of fluorescence resonance energy transfer from the enzyme to NADPH during the reaction was observed by exciting the sample at 292 nm and measuring the emission using an output filter with a 400 nm cutoff. Both EcDHFR and MpDHFR exist in multiple conformations in the apoenzyme

but form a single major conformation on binding NADPH.[9,18,30] Preincubation with NADPH eliminates contributions to the kinetics from these alternative apoenzyme conformations and ensures consistent kinetics.

RESULTS AND DISCUSSION

M20 Loop Variants. EcDHFR-M16L, EcDHFR-A19K, EcDHFR-M16L/A19K, MpDHFR-L17M, MpDHFR-K20A, and MpDHFR-L17M/K20A were prepared to investigate the effect of exchanging residues in the M20 loops of EcDHFR and MpDHFR. These changes were also combined with the EcDHFR-S148P and MpDHFR-P150S mutations (DOI: 10.1021/bi500507v) to generate EcDHFR-M16L/S148P, EcDHFR-A19K/S148P, EcDHFR-M16L/A19K/S148P, MpDHFR-L17M/P150S, MpDHFR-K20A/P150S, and MpDHFR-L17M/K20A/P150S.

The temperature dependence of transfer of hydride and deuteride from NADPH/D to DHF and the resulting kinetic isotope effects (KIE) were measured under single-turnover conditions at pH 7 for all M20 loop variants. The six EcDHFR variants all have hydride transfer rate constants and KIEs similar to those of wild-type EcDHFR[31] (Supporting Information), indicating that the mutations did not affect the chemical step. The rate constants and KIEs for the MpDHFR variants were similar to or slightly higher than those of wild-type MpDHFR at most temperatures (Supporting Information). However, the kinetic isotope effect was decreased at a low temperature in all variants, as seen in wild-type EcDHFR at pH 9.5,[31] demonstrating increased kinetic complexity under these conditions.

However, while the mutations did not significantly affect the hydride transfer step in either enzyme, their effects on the steady state rate constant (k_{cat}) at pH 7 and 20 °C differed. While the values of k_{cat} observed for the MpDHFR variants were similar to those of wild-type MpDHFR, a decrease in k_{cat} was observed for all EcDHFR M20 loop variants (Table 1). It has previously been shown that the L20M variant of DHFR from the thermophile *Geobacillus stearothermophilus* (BsDHFR) also has kinetics similar to that of the wild-type enzyme.[32] K_M values were largely unchanged for the EcDHFR variants but slightly elevated for NADPH in the MpDHFR variants (Supporting Information). The steady state rate constant of wild-type EcDHFR at pH 7 is limited by the release of THF from the E·NADPH·THF mixed ternary complex.[22] The same is true of wild-type MpDHFR (DOI: 10.1021/bi500507v). The decrease in k_{cat} observed for the EcDHFR variants suggests that the M16L and A19K mutations affect the motion of the M20 loop, which has been shown to be important for progression through the catalytic cycle in EcDHFR.[3,4] The effect of the mutations on k_{cat} is comparable to that seen when the interactions between the M20 and GH loops were perturbed in the EcDHFR-S148P variant (DOI: 10.1021/bi500507v). On the other hand, the complementary mutations in MpDHFR did not significantly alter the steady state rate constant. These observations suggest that, in contrast to EcDHFR,[3,4] MpDHFR does not use large-scale motions of these loops to control the physical steps of the catalytic cycle. Experiments using cosolvents have shown that none of these three DHFRs use large-scale loop motions in the chemical step of catalysis.[9,32,33]

Table 1. Steady State Turnover Numbers (k_{cat}) Measured at pH 7 and 20 °C during Catalysis by the EcDHFR and MpDHFR M20 Loop Variants

enzyme	k_{cat} (s^{-1})	enzyme	k_{cat} (s^{-1})
EcDHFR	6.9 ± 0.1	MpDHFR[25]	14.8 ± 0.8
EcDHFR-M16L	0.5 ± 0.01	MpDHFR-L17M	21.6 ± 1. 6
EcDHFR-M16L/A19K	1.7 ± 0.1	MpDHFR-L17M/K20A	9.6 ± 1.0
EcDHFR-A19K	2.2 ± 0.3	MpDHFR-K20A	18.1 ± 0.7
EcDHFR-M16L/S148P	0.4 ± 0.01	MpDHFR-L17M/P150S	10.0 ± 0.8
EcDHFR-A19K/S148P	2.2 ± 0.4	MpDHFR-K20A/P150S	9.8 ± 1.0
EcDHFR-M16L/A19K/S148P	0.7 ± 0.1	MpDHFR-L17M/K20A/P150S	12.7 ± 2.0

FG Loop Variants. MpDHFR-G123V and MpDHFR-D124N were prepared to investigate the effect of disrupting the interactions between the FG and M20 loops of MpDHFR, which are necessary to stabilize the closed conformation in EcDHFR.[3]

As was seen for the M20 loop variants, the temperature dependence of the hydride transfer rate constant and its KIE were not significantly affected for MpDHFR-D124N (Supporting Information). Slight differences observed in MpDHFR-G123V are likely due to the reduced thermostability of this variant, which showed suppression of the hydride transfer rate constant above 15 °C and a large decrease above 25 °C (Supporting Information). This is consistent with studies of EcDHFR-G121V, which showed that this mutant gives a gradual loss of circular dichroism signal with an increase in temperature rather than displaying cooperative unfolding.[19] In contrast, both FG loop variants showed a >30-fold decrease in the steady state rate constant, giving k_{cat} values of 0.47 ± 0.04 s^{-1} for MpDHFR-G123V and 0.41 ± 0.08 s^{-1} for MpDHFR-D124N. MpDHFR-G123V also has K_M values for both NADPH and DHF very similar to those of wild-type MpDHFR (Supporting Information).

The results for MpDHFR-G123V and MpDHFR-D124N are very different from the data reported previously for the corresponding variants of EcDHFR. The hydride transfer rate constants in EcDHFR-D122N[13] and EcDHFR-G121V[14] are 25- and 160-fold lower than in wild-type EcDHFR, respectively. In both cases, the magnitude of k_{cat} is not greatly altered, but a significant kinetic isotope effect is observed on k_{cat}, demonstrating a rate-limiting contribution from the chemical step. In addition, the affinities for $NADP^+$, DHF, and THF were not affected by either mutation, while the affinity for NADPH decreases 3-fold in EcDHFR-D122N[13] and 32-fold in EcDHFR-G121V.[14] Substitution of Asp122 was also found to change the preferred catalytic pathway.[13] While loss of THF from the E·NADPH·THF mixed ternary complex is the rate-limiting step in wild-type EcDHFR,[22] this step and loss of THF from the binary DHFR·THF complex both contributed to the steady state turnover rate constant k_{cat} in the D122N variant.[13] In contrast, the G121V substitution did not affect the overall form of the catalytic cycle.[14] The steady state rate constants for both MpDHFR FG loop variants are comparable to the rate constant for dissociation of THF from the E·THF binary complex in wild-type MpDHFR (DOI: 10.1021/bi500507v), suggesting that both have altered catalytic pathways compared to that of wild-type MpDHFR.

The G121V variant has been extensively studied in EcDHFR. Substitution of Gly121 with valine (or other bulky side chains) has a dramatic effect on the kinetics of EcDHFR, decreasing the hydride transfer rate constant by up to 1000-fold and resulting

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in an additional kinetic step in the reaction mechanism.[14] The G121V mutation in EcDHFR causes structural perturbation by destabilizing the contacts between the M20 and the FG loops,[12,19] such that EcDHFR-G121V adopts the occluded conformation in the E·NADP$^+$·folate complex in contrast to wild-type EcDHFR, which forms the closed conformation in the same complex.[18] On the other hand, the transition state of EcDHFR-G121V, modeled by the E·NADPH·MTX complex for NMR and X-ray studies,[3,21] has been shown to form a closed complex (as is necessary for catalysis to occur) but with significant excursions of the FG and M20 loops away from the accurate alignment required for reaction.[21] This is in agreement with previous computational studies, which showed that the G121V mutation leads to an altered transition state structure in EcDHFR, with a concomitant increase in the activation free energy barrier.[17,20]

These results suggest that EcDHFR-G121V is capable of adopting the closed conformation but that the conformational fluctuations are different from those of the wild-type enzyme. This has also been seen for EcDHFR-M42W,[34] which also has a hydride transfer rate constant much lower than that of wild-type EcDHFR.[35] The MpDHFR-M43W variant, on the other hand, showed a similar K_M (Supporting Information) and only a slightly reduced k_{cat} (3.31 ± 0.10 s^{-1}) compared to the wild-type enzyme. The MpDHFR-M43W/G123V variant gave kinetic parameters similar to those of MpDHFR-G123V with a k_{cat} of 0.30 ± 0.01 s^{-1} and similar K_M values (Supporting Information). In EcDHFR, the M42W/G121V double mutation causes a 1700-fold decrease in the rate constant for hydride transfer, revealing that the two mutations have nonadditive effects.[35] This is clearly not seen in MpDHFR, particularly as its G123V, M43W, and M43W/G123V variants all give KIEs on k_{cat} of unity, demonstrating no rate-limiting contribution of the hydride transfer step to the steady state rate constant.

The ensemble of conformational substates within the closed Michaelis complex determines the electrostatic preorganization within the active site, and hence the rate constant for hydride transfer.[8] While the dynamic properties of EcDHFR clearly affect the conformational motions that are important for the turnover of substrate to product, our current and previous[7−9,33,36] results suggest that no direct correlation exists between these motions and the chemical step. The decrease in the hydride transfer rate constant for the FG variants in EcDHFR[13,14] is therefore most probably not due directly to impaired loop motions, but to alterations to the conformational ensemble caused by the mutations. When the closed conformation is destabilized by mutations in the FG loop, the occluded conformation becomes the ground state of EcDHFR in the Michaelis complex. Reaction can then only occur from a minor population of the overall ensemble,[21] as NADPH and DHF are held in positions unsuitable for reaction in the occluded conformation.[3]

As MpDHFR lacks the key residue that stabilizes the occluded conformation (DOI: 10.1021/bi500507v), loss of hydrogen bonds that stabilize the closed conformation in MpDHFR will simply lead to formation of a destabilized closed conformation rather than favoring an occluded conformation. The closed conformation therefore remains the ground state, and the hydride transfer rate constants are unaffected. Mutations in the FG loop in positions 123 and 124 therefore affect only the physical steps of the catalytic cycle in MpDHFR. This strongly suggests that MpDHFR does not use loop dynamics to modulate the active site configuration even within the closed conformation. Our results also strongly suggest that the network of coupled motions identified in EcDHFR[37] is not present in MpDHFR.

The absence of large-scale loop motions is unlikely to be a specific adaptation of the psychrophilic enzyme. DHFRs from many other organisms, including the mesophilic bacterium *Lactobacillus casei* and the thermophile *Geobacillus stearothermophilus*, lack the key residue that stabilizes the occluded conformation (DOI: 10.1021/bi500507v). The inability of MpDHFR to form the occluded conformation and the resulting differences between the conformational ensembles of EcDHFR and MpDHFR likely explain the difference in the effect of mutations in the FG loop on the hydride transfer rate constant. Similarly, the presence or absence of large-scale loop motions does not appear to have a large effect on steady state turnover. EcDHFR and MpDHFR have the same rate-limiting step in the catalytic cycle and have similar values of k_{cat} despite their different conformational behavior. In EcDHFR, progression between steps of the catalytic cycle that do not require conformational changes is instead driven by changes in the dynamic behavior of the ligand binding regions.[4] It is therefore likely that MpDHFR uses this mechanism throughout its catalytic cycle.

CONCLUSIONS

EcDHFR is known to use loop motions to control progression through the catalytic cycle.[3,4] However, we have shown that conformational fluctuations are not directly involved in the chemical step of the reaction[7,8] and that the occluded conformation does not affect the chemical step of the reaction and only modifies the binding of the enzyme to ligands (DOI: 10.1021/bi500507v). Here, we provide evidence that the dynamic nature of EcDHFR and its effects on the catalytic cycle are not observed for the homologue from the psychrophile *M. profunda*.

Mutations in the M20 loop most probably disturb its motions in EcDHFR, causing a sharp decrease in the turnover numbers for all the EcDHFR variants studied here. This reflects the physical steps of the catalytic cycle, without perturbing the hydride transfer reaction itself. On the other hand, none of the MpDHFR variants showed a large difference in the turnover number, suggesting that the M20 loop dynamics observed in EcDHFR do not control the catalytic cycle in MpDHFR. In addition, in contrast to the reported data from similar mutations for EcDHFR,[13,14] mutation at positions 123 and 124 of MpDHFR did not affect the hydride transfer step of the catalytic cycle. Instead, the two variants show a sharp decrease in the steady state rate constant, a consequence of changes to the physical steps of the catalytic cycle.

The reactions catalyzed by MpDHFR and EcDHFR most likely follow the same catalytic cycle at pH 7 (DOI: 10.1021/bi500507v), and the nature of the chemistry in both enzymes is similar. However, the data presented here, together with those presented in the preceding paper (DOI: 10.1021/bi500507v), provide strong evidence that MpDHFR uses a mechanism different from that of EcDHFR to modulate the formation of different ligand complexes and progression through the catalytic cycle. In particular, the conformational cycling between the closed and occluded forms found in EcDHFR does not appear to drive progression through the catalytic cycle in MpDHFR.

■ ASSOCIATED CONTENT

Supporting Information

Alignment of the amino acid sequences of EcDHFR and MpDHFR; temperature dependencies and Arrhenius plots of the pre-steady state rate constants ($k_{H/D}$) and their KIEs during catalysis by EcDHFR, MpDHFR, and different variants; tabulated data of the temperature dependence of $k_{H/D}$ and KIE at pH 7; and Michaelis constants (K_M) and steady state turnover rates (k_{cat}) of different DHFR variants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

E.M.B. performed the bulk of the experimental work. R.M.E. and J.G. performed additional experimental work. E.M.B., E.J.L., and R.K.A. designed the experiments and wrote the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DHFR, dihydrofolate reductase; EcDHFR, DHFR from *E. coli*; MpDHFR, DHFR from *M. profunda*; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); DHF, dihydrofolate; THF, tetrahydrofolate; MTX, methotrexate; KIE, kinetic isotope effect.

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