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Different Reaction Mechanisms for Mesophilic and Thermophilic Dihydrofolate Reductases

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Dihydrofolate reductase (DHFR) is a ubiquitous enzyme that catalyzes the reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) using NADPH as a cofactor. During the DHFR-catalyzed reaction the pro-R hydrogen on C4 of NADPH is transferred to C6 of H_2F with concomitant protonation of N5 of H_2F.[1] Hydride transfer catalyzed by mesophilic DHFR from *Escherichia coli* (EcDHFR) and hyperthermophilic DHFR from *Thermotoga maritima* (TmDHFR) shows a sigmoidal pH profile with pK_a 6.5[2] and 6.0,[3] respectively. The pK_a of the EcDHFR-catalyzed reaction is due to protonation of the substrate itself[4,5] rather than the active-site Asp27 residue.[6]

Residues 9−24 of EcDHFR form a mobile loop (the M20 loop) that controls access to the active site.[7,8] There is evidence that the M20 loop together with the neighboring βFG loop form part of a network of hydrogen bonds and van der Waals interactions that spans the whole enzyme and is central to the catalytic cycle.[8−11] In the Michaelis complex with both H_2F and NADPH bound, the M20 loop adopts the closed conformation, stabilized through hydrogen bonds between residues in the M20 and βFG loops.[8] The pK_a of H_2F protonated on N5 (H_3F^+) increases from 2.6 in solution to 4.0 in the binary complex with EcDHFR and to 6.5 in the ternary complex with the inactive cofactor analogue dihydro-NADPH, proposed to model the Michaelis complex.[4] Computational studies suggested that the proton is donated directly from bulk solvent to N5 of H_2F.[12−14] More recent computational studies showed that closure of the M20 loop of EcDHFR is important to increase the pK_a of H_3F^+ to a physiologically accessible value.[14,15]

Despite only 27% sequence identity, EcDHFR and TmDHFR have similar tertiary structures.[8,16] In contrast to all other known chromosomally encoded DHFRs, TmDHFR forms a stable homodimer.[17] The TmDHFR loop formed by residues 15−24 (M20 loop in EcDHFR) is constrained by the dimer interface and hence is characterized by reduced movement relative to EcDHFR,[16,18] although QM/MM calculations revealed that several of the correlated motions identified previously in EcDHFR[10] are also present within individual subunits of TmDHFR.[18] The "M20 loop" of TmDHFR adopts an open conformation making the active site of TmDHFR more accessible to solvent.[16] Like other thermophilic enzymes, TmDHFR has evolved to be more stable than its mesophilic counterpart at all temperatures,[17] but its catalytic activity is lower.[3]

Here we report results from an investigation into the protonation step during EcDHFR and TmDHFR catalysis. The solvent kinetic isotope effects (SKIEs) were measured for hydride and deuteride transfer at pH 7 in the transient state, and also in the steady state at pH 9.5 for EcDHFR, where hydride transfer is fully rate limiting.[2] Both DHFRs show an inverse SKIE on the chemical step. Proton inventories were linear (Supporting Information) showing that the SKIE arises from a single protonation event,[19] consistent with the DHFR-catalyzed reaction. Interestingly, different multiple isotope effect behavior was observed (Figure 1). For TmDHFR at pH 7 and EcDHFR at pH 9.5, the primary (1°) KIEs were independent of the isotopic composition of the solvent, while the SKIEs were independent of the cofactor isotopic composition. However, the isotope effects in EcDHFR at pH 7 were interdependent; the 1° KIE was suppressed in D_2O, while the SKIE was reduced by deuteration of the cofactor.

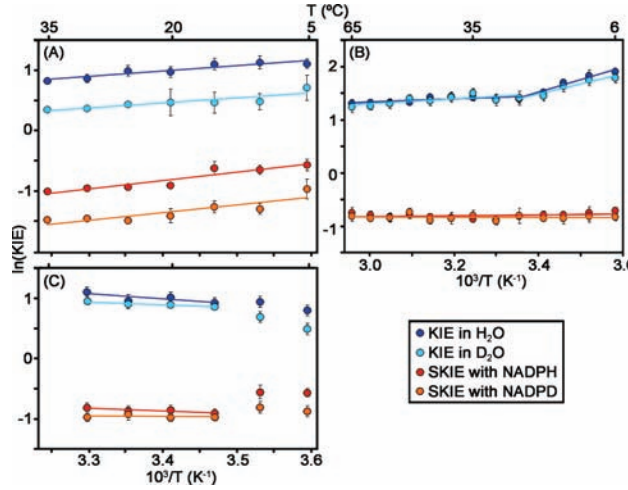

Figure 1. KIEs at pH 7.0 for EcDHFR (A) and TmDHFR (B), as well as for EcDHFR at pH 9.5 (C) plotted on a logarithmic abscissa against the inverse temperature.

Multiple isotope effects can provide information about the timing of the proton and hydride transfer steps of the reaction.[20−22] If the two effects arise on the same step (i.e., if proton and hydride transfer are concerted), then they should be independent of each other if the isotope-dependent step is fully rate-limiting, or the 1° KIE should be increased when the solvent is deuterated if other steps are also relatively slow. If, however, they arise on different steps, then solvent deuteration will suppress the 1° KIE while substrate deuteration will reduce the SKIE. The data for TmDHFR at pH 7 and EcDHFR at pH 9.5 therefore indicate that the isotope effects arise from the same step, and hence hydride and proton transfer are concerted. The KIE for EcDHFR at pH 9.5 is masked by the kinetic complexity below 15 °C,[23] and so analysis was only carried out above this temperature. The isotopic composition of the solvent has no effect on the activation energy (Supporting Information) resulting in temperature independent SKIEs. In EcDHFR at pH 7 on the other hand, the interdependence of the isotope effects suggests a stepwise mechanism in which protonation precedes hydride transfer. The activation energy is increased by 11 kJ mol^{-1} in D_2O for both NADPH and NADPD resulting in temperature dependent SKIEs. In all cases, the inverse SKIE is due to increased

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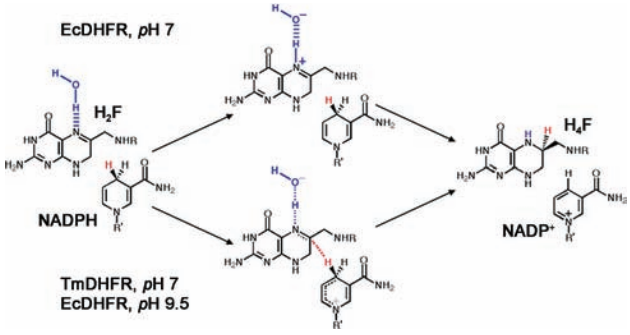
Arrhenius prefactors in D_2O (Supporting Information) suggesting that entropic factors are responsible.

SKIEs may arise from a number of sources including multiple 1° and 2° isotope effects as well as medium effects.[19,24] However, the proton inventories here were linear in all cases and the TmDHFR-catalyzed reaction is known to be insensitive to solution viscosity, while the reaction rate is reduced by a reduction of the dielectric constant (the dielectric constant of D_2O is slightly lower than that of H_2O).[25] Hence it is reasonable to assume that the SKIEs were not due to differences in the viscosity or dielectric constant of H_2O and D_2O. Increased rigidity of the protein due to greater hydrogen bonding strength in D_2O[26] can also be discounted, since this would also reduce the reaction rates rather than increase them. Furthermore, binding effects from the substrate, as seen for yeast alcohol dehydrogenase,[27] are unlikely to play a role as reaction rates and SKIEs were not affected by the substrate concentration (data not shown) and the rate of substrate binding is considerably higher than the measurable limit of the instrument.[2] Therefore, while SKIEs must be interpreted cautiously due to the many consequences of replacing solvent protons with deuterons, the SKIE observed in DHFR catalysis appears to arise predominantly from the reaction itself.

In the steady state at pH 7, hydride transfer is partially rate-limiting for TmDHFR[3] but not EcDHFR.[2] For EcDHFR, both 1° KIEs and SKIEs were close to unity (Supporting Information). For TmDHFR, the 1° KIE was ~3 (in both H_2O and D_2O), similar to that reported previously,[3] while the SKIE was 0.64 (for both NADPH and NADPD). These values are approximately two-thirds of the corresponding values for hydride transfer, showing that in the steady state the SKIE is masked to the same degree as the 1° KIE. Since proton inventories for steady-state TmDHFR turnover were linear, these results suggest that solvent deuteration had no significant effect on the slower physical steps during catalysis by Ec or TmDHFR.

Despite their closely related tertiary structures,[8,16] Ec and TmDHFR follow different reaction mechanisms at physiological pH and this may be a consequence of their different quaternary structures.[16] Modulation of the substrate pK_a by the M20 loop of EcDHFR[15] allows preprotonation (Scheme 1) and leads to increased reactivity with respect to hydride transfer. In TmDHFR, the "M20 loop" is fixed in the open conformation,[16] which exposes the active site to solvent and prevents efficient modulation of the pK_a of its substrate. At high pH, the concentration of H_3F^+ is negligible even when the M20 loop is in the closed conformation, and the reaction follows a concerted pathway. TmDHFR at pH 7 behaves like EcDHFR at pH 9.5, and therefore hydride transfer and protonation occur in a concerted reaction and binding energy may be required to induce electron flow from the cofactor to C6 and N5 of H_2F to facilitate protonation. In this scenario, the higher rates observed for the chemical step in EcDHFR are achieved by active site loop movements that increase the stability of the protonated substrate. Interestingly, the hydride transfer rates of the monomeric DHFR from the moderate thermophile *Bacillus stearothermophilus* are comparable to those of EcDHFR,[28] and our results lead to the testable prediction that the pK_a of H_3F^+ and hence the catalytic activity are efficiently modulated by the M20 loop of this enzyme. In agreement with the results from a theoretical study,[29] it appears that thermostability and decreased general dynamic motions do not necessitate low catalytic activity. Rather the increased thermostability of TmDHFR, which results from dimerization, comes at the cost of a reduction in advantageous loop movements and hence lower reaction rates.

Scheme 1. Mechanisms of H_2F Reduction in EcDHFR and TmDHFR



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Supporting Information Available: Tabulated rate constants and KIEs, proton inventories. This material is available free of charge via the Internet at http://pubs.acs.org.

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