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Tunneling and Coupled Motion in the *Escherichia coli* Dihydrofolate Reductase Catalysis

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Dihydrofolate reductase from *Escherichia coli* (*ec*DHFR) has been a paradigm of enzymatic systems in many experimental and theoretical studies.[1,2] Kinetic studies of *ec*DHFR have shown that its kinetic cascade is complex, that product release is rate-determining at neutral pH (12.5 s^{-1}), and that the hydride transfer rate (the chemical step) is 950 s^{-1}.[3] In this communication, we examine the nature of this hydride transfer reaction using intrinsic primary (1°) and secondary (2°) kinetic isotope effects (KIEs) and their temperature dependences.

Extensive computational studies on the mechanism of *ec*DHFR have been carried out by quantum mechanical/molecular mechanical (QM/MM) and free-energy perturbation methods giving insight into the nature of this hydride transfer.[4−8] QM/MM studies by Hammes-Schiffer and co-workers have suggested a network of coupled promoting motions that participate in the reaction mechanism.[2,9] From the experimental point of view, it is of interest to explore the effect of such dynamics on the nature of the hydride transfer. The study presented here offers an in-depth examination of that nature within the DHFR complete kinetic cascade.

Pre-steady-state studies with a thermophilic DHFR from *Thermotoga maritime* resulted in a steep temperature dependency between 5 and 20 °C that indicates tunneling of the protium isotope (observed KIE close to 6 with intercept much smaller than unity) and classical temperature dependency between 20 and 65 °C (observed KIE close to 4 with intercept close to unity).[10,11]

In the current study, mixed labeling experiments[12] were conducted with the *ec*DHFR to examine coupling between 1° and 2° hydrogens, and intrinsic KIEs were calculated from observed H/T and D/T *V*/*K* KIEs. Competitive *V*/*K* kinetic isotope effect studies were carried out with wild-type *ec*DHFR using six different labeling patterns of the NADPH cofactor. Mixtures of 4*R* [4-^{3}H]-NADPH with [Ad-^{14}C]-NADPH and 4*R* [4,4-^{2}H,^{3}H]-NADPH with [Ad-^{14}C, 4-$^2H_2$]-NADPH were used for 1° H/T and 1° D/T KIE studies, respectively. Mixtures of 4*S* [4-^{3}H]-NADPH with [Ad-^{14}C]-NADPH and 4*S* [4,4-^{3}H,^{2}H]-NADPH with [Ad-^{14}C, 4-$^2H_2$]-NADPH were used for 2° H/T and D/T KIE studies, respectively. ^{14}C labeling of NADPH that was not tritiated was used to facilitate LSC analytical analysis.[13,14] The synthesis, purification, and storage of these labeled cofactors has been presented before.[15−18]

All experiments were carried out at pH = 9.0 because at this pH the chemistry is more rate-determining at 25 °C.[3] The observed 1° *V*/*K* KIEs were H/T = 4.81 ± 0.06 and D/T = 1.65 ± 0.01 over a temperature range from 45 to 15 °C. Below 15 °C both observed KIEs decreased with temperature (Figure 1A). The observed values were used to calculate intrinsic KIEs following the equation $(^{T}(V/K)_{H,obs}{}^{-1} - 1)/(^{T}(V/K)_{D,obs}{}^{-1} - 1) = ((k_H/k_T)^{-1} - 1)/((k_H/k_T)^{-1/3.34} - 1)$,[20,21] where $^{T}(V/K)_{H,obs}$ and $^{T}(V/K)_{D,obs}$ are the observed H/T and D/T KIEs and k_H/k_T is the intrinsic H/T KIE. The same procedure also afforded calculations of intrinsic D/T and H/D KIEs (Figure 1B).[19] Intrinsic KIEs were calculated throughout the temperature range as described recently by Francisco et al. for peptidylglycine-hydroxylating monooxygenase.[22]

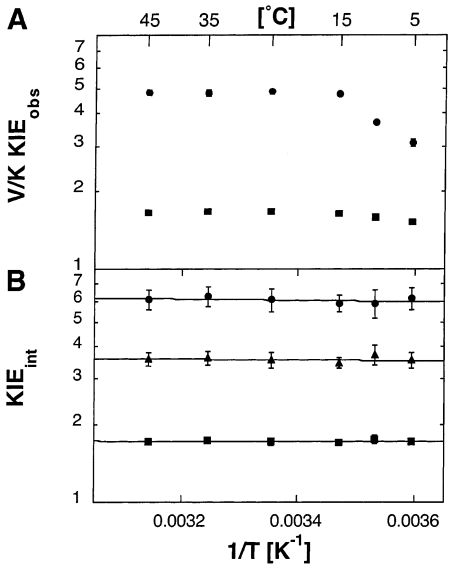

Figure 1. Arrhenius plot of observed (A) and intrinsic (B) 1° KIEs for hydride transfer from NADPH(D) to H_2F. H/T KIEs ●, D/T KIEs ■, and H/D KIEs ▲ for DHFR.[19] Curve fitting was carried out as a least root-mean-square, standard deviations weighted, exponential regression.

Fitting these intrinsic KIEs to the Arrhenius equation resulted in no temperature dependency (ΔE_a H/T = −0.1 ± 0.3, D/T = −0.03 ± 0.09, and H/D = −0.07 ± 0.2 kcal/mol) and in isotope effects on Arrhenius preexponential factors which were A_H/A_T = 7.4 ± 4, A_H/A_D = 4.0 ± 1.5, and A_D/A_T = 1.8 ± 0.3. These values are all larger than the semiclassical limits (1.73, 1.41, and 1.22, respectively).[21,23,24] Preliminary initial velocity studies resulted in an energy of activation (E_a) of 3.7 ± 0.3 kcal/mol,[25] which is in accordance with a pre-steady-state study from which E_a between 3 and 6 kcal/mol can be calculated.[10] Taken together, these data can only be rationalized by models with an extensive tunneling contribution and environmentally coupled tunneling.[1,21,26−29] This is because theoretical models that use tunneling correction explain the lack of temperature dependence of KIEs by assuming no temperature dependence on the reaction rates for both light and heavy isotopes (full, or extensive, tunneling models[21,30]). The fact that E_a is larger than zero can be explained by models that invoke an isotopically insensitive, thermally activated step (e.g., environmental rearrangement) and an isotopically sensitive but temperature-independent H-transfer step (tunneling). Several terms have been coined to describe such models, including "vibrationally enhanced

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ground-state quantum tunneling",[31] "rate-promoting vibrations",[32] and "environmentally coupled tunneling".[28,29]

The reason that the observed KIE (KIE_{obs}) is smaller than the intrinsic KIE (KIE_{int}) is that kinetic steps other than the H-transfer step mask the KIE_{int}. In the current work, the KIEs were measured under irreversible reaction conditions, and the kinetic complexity can be formulated as: $KIE_{obs} = [KIE_{int} + C_f]/[1 + C_f]$, where the forward commitment to catalysis (C_f) is the ratio of the forward isotopically insensitive step over preceding reverse steps.[20] Commitments to catalysis were calculated for the observed *V*/*K* KIE. Above 15 °C all these commitments were close to 0.35, and at temperatures below 15 °C the commitment increased (with $C_f = 0.83$ at 10 °C and $C_f = 1.52$ at 5 °C). Additionally, the intrinsic H/D KIE at 25 °C afforded the commitment on the pre-steady-state hydride transfer rate. Benkovic and co-workers measured pre-steady-state kinetics of wild-type and mutant *ec*DHFR using UV absorbance and fluorescence resonance energy transfer (FRET) at 25 °C. The KIE measured for the H-transfer was 3.0 ± 0.1 at 25 °C.[3,33] The intrinsic KIE we report here is 3.5 ± 0.2 at 25 °C. These observed and intrinsic KIEs result in commitment on the pre-steady-state H-transfer rate of $C_f = 0.25$. This commitment can be rationalized by including two kinetic steps in the pre-steady-state hydride transfer rate. The isotopically insensitive flip of the nicotinamide ring in and out of the active site and the hydride transfer itself. This finding demonstrates that the current method exposes the chemical step better than previous methods. Hammes-Schiffer and co-workers calculated an intrinsic KIE of 3.4,[9] which is larger than any KIE measured in the past and is, within experimental error, equal to the one reported here.

Experimental 2° KIEs for DHFR are reported here for the first time (to the best of our knowledge). These measurements were carried out at 25 °C, pH = 9.0, and *V*/*K* values of 1.149 ± 0.005 for H/T KIE and 1.058 ± 0.003 for D/T KIE were measured. These values were then corrected for the commitment (calculated above for 1° KIE) and 1.25% protium contamination in the deuterated NADPH (from ^{1}H NMR), using the method of Cha et al.[34] The corrected values were 1.194 ± 0.007 for 2° k_H/k_T and 1.052 ± 0.007 for 2° k_D/k_T. The Swain−Schaad exponent calculated from these 2° KIEs is 3.5 ± 0.5. This exponent provides no indication of coupled motion between the 1° and 2° hydrogens for hydride abstraction from NADPH.[12] Such coupled motion was suggested for hydride extraction from NADH by Huskey and Schowen[35] to rationalize a large 2° KIE for horse-liver alcohol dehydrogenase.[36] Using the Swain−Schaad relationship, it is possible to calculate an intrinsic 2° H/D KIE of 1.13. Interestingly, the same value was recently predicted from QM/MM calculations by Garcia-Viloca, Gao, and Truhlar for the same enzyme.[6] The theoretical predictions were published prior to the experimental value.

The two QM/MM studies mentioned here are complementary. Reference 6 used ensemble-averaged variational transition-state theory with reaction coordinates based on minimum energy paths, with multidimensional tunneling contribution, and with 31 atoms quantum mechanical. Reference 9 used mixed quantum/classical molecular dynamics valence bond energies as the reaction coordinate, a transmission coefficient accounting for barrier recrossing, and the hydride nucleus represented as a 3-D quantum classical wave function.

In summary, the experimental studies described above are consistent with environmentally coupled hydrogen tunneling in the H-transfer step of the *ec*DHFR catalysis. Such phenomenon was suggested from various theoretical studies but has never been observed before with *ec*DHFR. A mixed labeling experiment examined possible 1°−2° coupled motion on the C4 position of nicotinamide for the first time. In contrast to studies of alcohol dehydrogenase,[26,30] no such coupling was evident from the 2° Swain−Schaad exponents, and the ability to measure such phenomenon for these ubiquitous cofactors was demonstrated. Interestingly, two KIEs that were recently predicted from theoretical calculations turned out to be in good agreement with our measurements (1° KIE[9] and 2° KIE[6]). Finally, the methods described here appear to expose the H-transfer step in the DHFR reaction better than previous methods, opening the door to in-depth investigation of mutants with altered dynamics.[2]

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Supporting Information Available: Tables presenting raw data and more experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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