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Protein Motions and the Activation of the C-H Bond Catalyzed by Dihydrofolate Reductase

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Abstract

The role of protein motions in enzymatic C-H→C transfer is an area of great contemporary debate. An effective tool in probing such a role is the temperature dependence of the intrinsic kinetic isotope effects for the enzyme-catalyzed reaction. The outcome of those experiments is interpreted within the context of phenomenological Marcus-like models of hydrogen tunneling. The current review focuses on recent studies of dihydrofolate reductase (DHFR) and how the role of protein motions in the catalyzed reaction has been demonstrated. The motions in DHFR are controlled by local effects of active site residues, global effects involving remote residues across the enzyme and appear to be preserved during the evolution of the enzyme from bacteria to human.

Introduction

Dihydrofolate reductase (DHFR) has emerged as a model system in many investigations of the role of protein motions in enzymatic C-H→C transfer, and enzyme catalysis in general. DHFR catalyzes the reduction of 7,8-dihydrofolate (DHF) to *S*-5,6,7,8-tetrahydrofolate (THF) through a hydride transfer of pro-*R* hydrogen from the C4 atom of NADPH to the C6 position of DHF. DHFR is critical in maintaining the intercellular pool of THF, which is required for the biosynthesis of many essential molecules including thymine. Due to its pharmacological importance, small size (18 kDa), and relative ease of study the enzyme has been the focus of many experimental and theoretical studies [3–6]. The current review focuses on the enzyme from *Escherichia coli* and outlines the recent advances made in elucidating the nature of the catalyzed hydride transfer. A particular focus is placed on the role of protein motions, how both active site and remote amino acid residues modulate these motions and the evolutionary preservation of protein dynamics to optimize the DHFR catalyzed reaction.

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Temperature Dependence of Intrinsic Kinetic Isotope Effects (KIEs) and the Marcus-Like Model

A wealth of information concerning the nature of the hydride transfer reaction catalyzed by DHFR has been gained through measurements of the temperature dependence of the KIEs for wild-type (WT) and site-directed mutant forms of the enzyme [1,7–11]. Such results are best interpreted through full tunneling Marcus-like models of C-H transfer (also referred to as vibrationally enhanced tunneling, environmentally coupled tunneling and other terms found in literature) [4,6,12–19]. The Marcus-like model, adapted from Ruldoph Marcus' landmark theory of electron tunneling [20], is based on three principles. First, the hydrogen should be treated quantum mechanically throughout the reaction coordinate and therefore it tunnels under the barrier once the needed conditions are there. Second, the model states that the rate of hydrogen transfer is determined by fluctuations of the electronic potential surface of the reaction. Finally, these fluctuations comprise two orthogonal coordinates: a rearrangement coordinate that adjusts the energy levels of the reactant and product while reaching the tunneling ready state (TRS), and a 'gating' coordinate that represents the fluctuations of the hydrogen donor and acceptor distance (DAD) at the TRS. These principles are depicted in Figure 1.

The electronic potential surface of the reaction is not altered upon isotopic substitution of the reactants, so KIEs afford the opportunity to probe a minimally perturbed reaction coordinate. More importantly, it has been demonstrated by several theoretical studies that the temperature dependence of the KIE is highly sensitive to changes in DAD fluctuations at the TRS of the reaction [6,12,16,18,21–23]. At optimal DADs, there is a sufficient wave function overlap between donor and acceptor at the TRS so that all isotopes of H can tunnel (middle panels of A and B in Figure 1). If this is also a narrow and accurate distribution of DADs, the KIEs will be temperature independent, as most often observed for WT enzymes with their physiological substrates [6,12,16,22–26]. Under non-optimal reaction conditions, which can often be induced through site directed mutagenesis of the enzyme, the average DAD at the TRS is too long for efficient tunneling of heavier isotopes and the DADs' distribution is broader (poorly organized TRS), which result in inflated and temperature dependent KIEs. This larger temperature dependency is due to thermally activated motions of the protein scaffold that at high temperature lead to a shorter DAD, from which all isotopes can tunnel (small KIE), but at low temperature are confined to a DAD that is too long for heavy isotope tunneling (large KIE). The temperature dependence of the KIE therefore provides an indirect, but very powerful probe of the involvement of enzyme motions in the catalyzed H transfer.

KIEs as a Probe of Local Effects on DHFR Catalyzed Chemistry

The KIEs for WT DHFR have been shown to be temperature independent, which suggests that the enzyme has well defined active site dynamics that maintain a short and narrowly distributed DAD for hydride tunneling [8]. To gain insights into how the dynamics of the active site of DHFR are maintained, I14 was studied trough a series of mutations that were chosen to decrease the side chain while not significantly perturbing the electrostatics of the active site [7,9]. As shown in Figure 2, I14 is positioned behind the nicotinamide ring of NADPH and assists in maintaining an optimal DAD between the cofactor (H donor) and the

DHF substrate (H acceptor). Such a role of a hydrophobic residue has also been seen in morphonone reductase [27], horse liver alcohol dehydrodrogenase [28,29] and TauD [30]. I14A was mutated to V, A and G and the temperature dependence of the intrinsic H/T KIEs were measured for each mutant, along with other kinetic parameters. The study afforded both an explanation as to how DHFR maintains an optimal DAD for H tunneling and an important test to Marcus-like models. As seen in Figure 2, the isotope effects on the activation energy for catalyzed reaction (i.e., the temperature dependence of the intrinsic KIEs given by the slopes in the Arrhenius plots) progressively increased as the size of the amino acid at position 14 decreased. Molecular dynamics (MD) simulations of the WT and mutants reveled that the increased flexibility induced upon mutation resulted in populations of the enzyme active complexes whose average DAD is longer and has a broader distribution. Importantly, MD simulations confirmed the predictions of Marcus-like models, which state that longer and more broadly distributed DADs result in steeper temperature dependencies of the KIE.

KIEs as a Probe of Global Effects on DHFR Catalyzed Chemistry

The studies of I14 serve as a framework for understanding the roles of remote residues whose functions are not as easily explained from 3D structures of the enzyme alone. Several remote residues were proposed to participate in a ‘global dynamic network’ based on quantum mechanical/molecular mechanical (QM/MM) calculations, MD simulations and bioinformatic analysis looking at co-evolution of the enzyme (Figure 3) [4,31–35]. Initial kinetic studies on G121 and M42 mutants implicated a synergy between the two residues in the catalyzed chemistry [36]. This was shown by measuring single turnover rates of G121V and M42W DHFRs and comparing the results to a G121V/M42W double mutant. The effects of the single mutants on single-turnover rates were smaller than the combined effects of both mutations on G121V/M42W DHFR [36]. The temperature dependence of the intrinsic H/T KIEs were measured for each of the mutants and the double mutant, in order to establish a possible link between these remote residues and hydride tunneling occurring at the active site [10,11]. The G121V and M42W single mutants exhibited only a modest increase in the temperature dependence of the KIE, whereas the G121V/M42W double mutant was steeply temperature dependent (Figure 3). Recently, another residue predicted to be part of that network [4,31–35], F125, has been also tested, and F125M and its double mutants with G121V and M42W indicated that this residue is also part of the same network [2]. By contrast, W133, which was predicted to comprise the global network of coupled motions based solely on bioinformatic analysis [34], was found to have no effect on the hydride transfer reaction. This confirms that results obtained with G121, M42 and F125 are associated with a functional role of a dynamic network and not a general role of all residues in the enzyme. These results suggest that motions across the entire protein provide the environmental reorganization needed to maintain the short and narrowly distributed DAD needed for efficient tunneling. The outcome confirmed the predictions of high level QM/MM simulations [4,31,33,35], suggesting those residues are part of the same dynamic network coupled to the chemical step. Importantly, the examination of the temperature dependence of intrinsic KIEs with the double mutants accords with the synergy between the remote residues that was suggested by single-turnover measurements [2,36,37], although those rates may involve more microscopic rate constants than just the C-H activation step.

KIEs as a Probe of Conservation of Functional Motions in Enzyme Evolution

A recent analysis of over 200 DHFR sequences from organisms ranging from bacteria to humans have identified phylogenetic coherent events (PCE) in the course of evolution from bacteria to human [38]. In particular the genomic analysis identified N23 and G51 (the numbering corresponds to the *E. coli* enzyme) as two such PCEs. Residue N23 was previously studied through a diproline insertion to create a N23PP *E. coli* DHFR (with or without S148A, which was found to have no added effect). NMR relaxation experiments indicated a restriction of msec timescale motions in this mutant as compared to the WT and both steady-state and single turnover rates were significantly decreased [39]. Based on the PCEs identified in ref [38], where N23PP was only present after another insertion at G51, a more 'humanized' form of *E. coli* DHFR (N23PP/G51PEKN) was created. The kinetic parameters of N23PP/G51PEKN were restored to that of the WT and QM/MM simulations showed that the mutant had as short and narrow distribution of DADs as WT *E. coli* DHFR. This is in contrast to the results of N23PP DHFR, which exhibits a long and broad distribution of DADs as shown by two different QM/MM calculations [38,40]. The Marcus-like model therefore predicts that N23PP should have steeply dependent KIEs, while the N23PP/G51PEKN mutant should exhibit the temperature independent KIEs of the WT. As shown in Figure 4, this is exactly what was observed [1]. The results demonstrate that the dynamics of DHFR are evolutionary preserved, which is interesting given that the chemical step is far from being rate limiting in the DHFR reaction [41].

It should be noted that the studies of N23PP/S148A was not met without controversy. Empirical valence bond (EVB) calculations suggested that the impaired rates of the mutant are due to a increased reorganization energy for the reaction [42]. Furthermore, studies of the temperature dependence of the KIEs on single turnover rates [43] and a study on an isotopically labeled 'heavy N23PP/S148A DHFR' [40] failed to detect any effect of the altered dynamics on the single turnover rates. However, single-turnover rates reflect not only hydride transfer, but a combination of microscopic rate constants, which results in what is known as kinetic complexity [1]. This kinetic complexity masks effects on the chemical step (C-H→C transfer in this case), a problem that can be alleviated by studying intrinsic KIEs as in Figure 4 above [44,45]). Thus, while the mutation may have an effect on the reorganization energy as suggested by the EVB calculations [42], it also influences the dynamics at the TRS and the chemical step of the DHFR catalyzed reaction.

Conclusions

The temperature dependence of intrinsic KIEs, analyzed using Marcus-like models, has been applied to elucidate various aspects of the hydride transfer reaction catalyzed by DHFR. In the framework of these models, the observed temperature independent KIEs indicate that the WT enzyme activates and reorganize the reactants to an optimized TRS with well defined and narrow distribution of DADs, most suitable for H-tunneling. This orientation is achieved through motions of active site residues, solvent molecules, and residues across the protein. Those remote and active site residues constitute a dynamic network of functional correlated motions, directly affecting the formation of the TRS. Phylogenetic analysis of DHFR sequences and functional studies of mutants generated based on those studies suggest that

the functional motions under study are evolutionarily conserved. The studies outlined here demonstrate the structural and dynamic role of residues across the protein in enzyme catalyzed C-H→C transfer. The findings may apply not only to other enzymes catalyzing hydride transfer, but may indicate a general role of the whole protein in enzyme catalysis.

Acknowledgments

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Highlights

- The role of protein motions in catalysis by dihydrofolate reductase is reviewed.
- Marcus-like models explain the temperature dependence of kinetic isotope effects.
- Both local and global motions contribute to dihydrofolate reductase catalysis.
- The functional motions of dihydrofolate reductase are evolutionarily conserved.

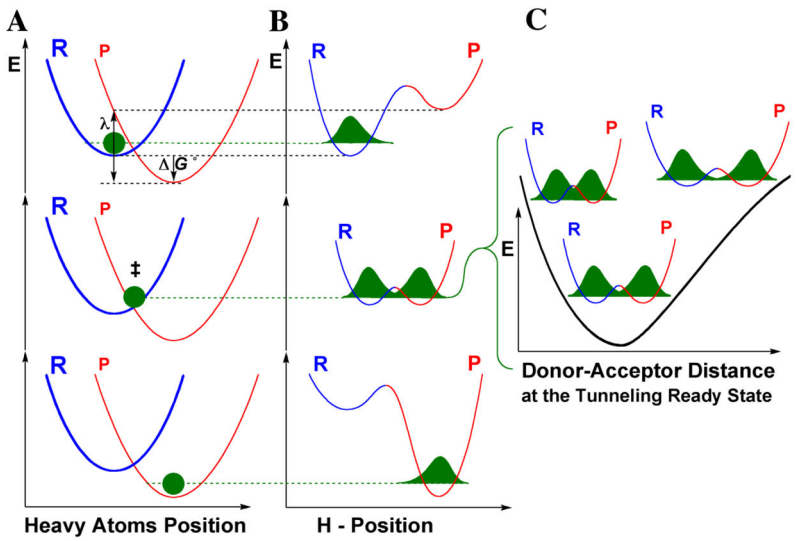

Figure 1. Marcus-Like models of hydrogen tunneling

Three slices of the potential energy surface (PES) along components of the collective reaction coordinate showing the effect of heavy-atom motions on the zero point energy (ZPE) in the reactant (blue) and product (red) potential well. Panel A shows the heavy atom coordinate, and Panel B shows the H-atom position, which is orthogonal to the heavy atom coordinate. In the top panels the hydrogen is localized in the reactant well, and the ZPE of the product state is higher than that of the reactant state. Heavy atom reorganization brings the system to the tunneling ready state (TRS) (middle panels A and B), where the ZPEs in the reactant and product wells are degenerate and the hydrogen can tunnel between the wells. Further heavy atom reorganization breaks the transient degeneracy and traps the hydrogen in the product state (bottom panels). The rate of reaching the TRS depends on the reorganization energy (λ) and driving force ($\Delta G°$). Panel C shows the effect of donor and acceptor distance (DAD) sampling on the wavefunction overlap at the TRS. Reprinted with permission from Annual Reviews [6].

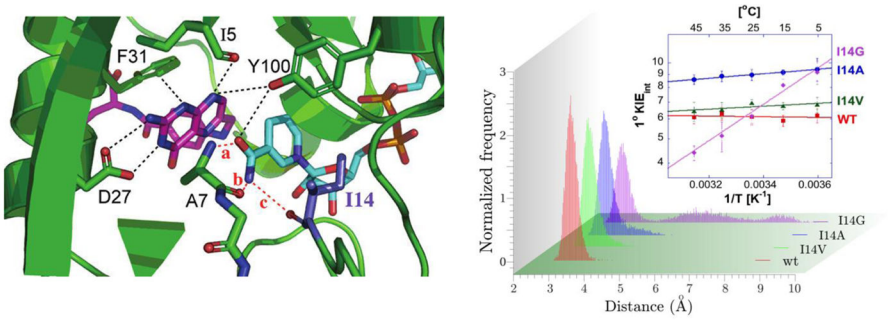

Figure 2. Role of I14 on Local Motions in DHFR

Left Panel: Active Site of *E.coli* DHFR (PDB ID 1RX2) where the nicotinamide is in blue, the folate in magenta and I14 highlighted as metallic blue. *Right Panel*. Histogram showing the normalized frequency versus DAD for WT (red), I14V (green), I14A (blue) and I14G (purple) [7]. The inset shows Arrhenius plots for the intrinsic H/T KIEs for the WT and mutants. Reprinted with permission from the American Chemical Society.

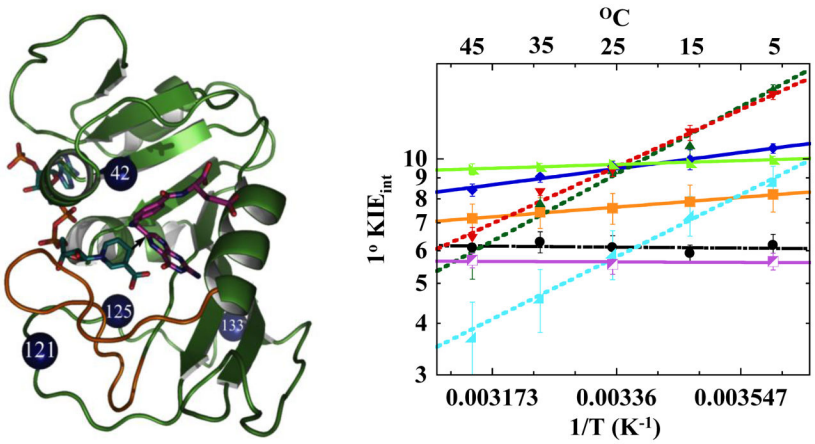

Figure 3. Roles of Distal Residues on the DHFR catalyzed reaction

Left Panel. Structure of WT-DHFR (PDB Code 1RX2), with folate in magenta and NADP in light blue. A black arrow marks the hydride's path from C4 of the nicotinamide to C6 of the folate, and the residues reviewed here are marked as blue spheres. *Right Panel*. Arrhenius plots of intrinsic H/T KIEs of WT (black) and distal DHFR mutants: W133F (magenta), M42W (orange), G121V (light green), F125M (dark blue), M42W-F125M (dark green), G121V-F125M (red), M42W-G121V (light blue) [2]. Reprinted with permission from the American Chemical Society.

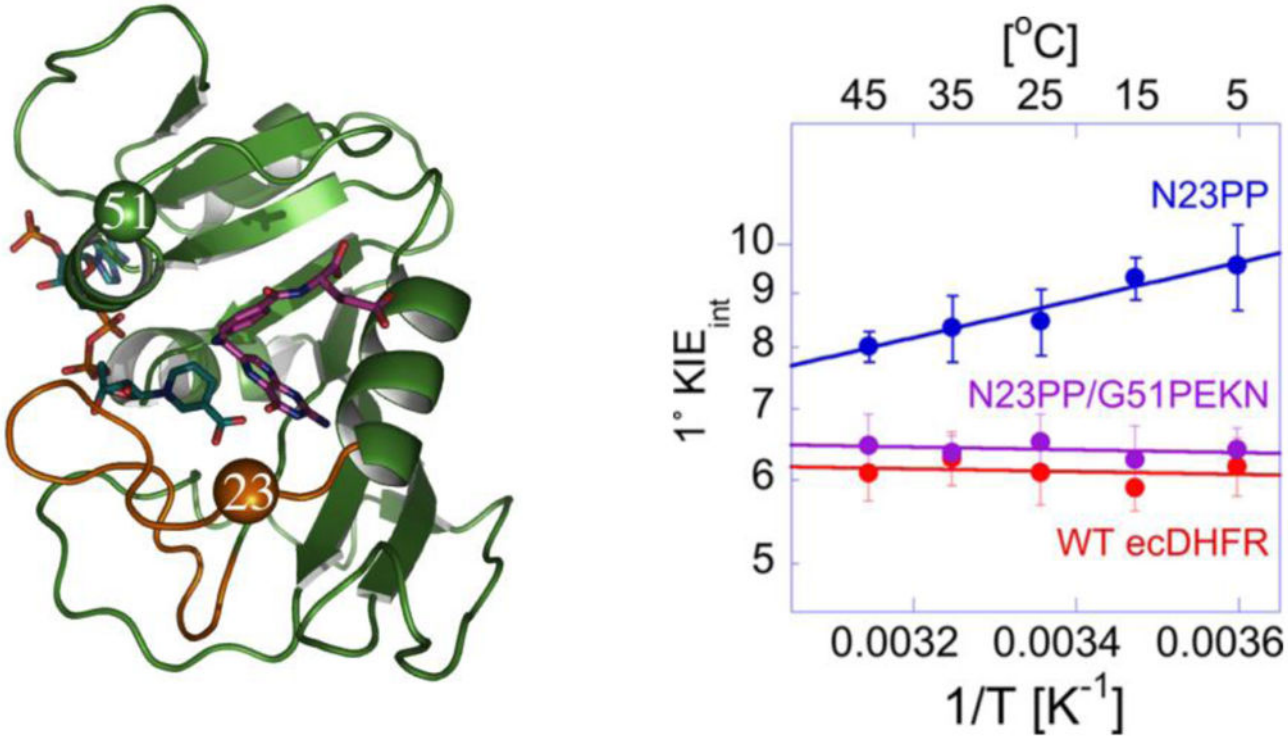

Figure 4. Evolutionary Preservation of DHFR Dynamics

Left Panel: Structure of WT DHFR showing the positions of N23 and G51. The Met-20 loop is shown in brown, folate is shown in purple, and the nicotinamide ring of NADPH is in blue. Residues N23 and G51 are shown as brown and green spheres, respectively. *Right Panel:* Arrhenius plots for the intrinsic H/T KIEs for the WT, N23PP, N23PP/G51PEKN [1]. Reprinted with permission from the American Society of Biochemistry and Molecular Biology.