Hydride transfer catalysed by *Escherichia coli* and *Bacillus subtilis* dihydrofolate reductase: coupled motions and distal mutations

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This paper reviews the results from hybrid quantum/classical molecular dynamics simulations of the hydride transfer reaction catalysed by wild-type (WT) and mutant *Escherichia coli* and WT *Bacillus subtilis* dihydrofolate reductase (DHFR). Nuclear quantum effects such as zero point energy and hydrogen tunnelling are significant in these reactions and substantially decrease the free energy barrier. The donor–acceptor distance decreases to *ca* 2.7 Å at transition-state configurations to enable the hydride transfer. A network of coupled motions representing conformational changes along the collective reaction coordinate facilitates the hydride transfer reaction by decreasing the donor–acceptor distance and providing a favourable geometric and electrostatic environment. Recent single-molecule experiments confirm that at least some of these thermally averaged equilibrium conformational changes occur on the millisecond time-scale of the hydride transfer. Distal mutations can lead to non-local structural changes and significantly impact the probability of sampling configurations conducive to the hydride transfer, thereby altering the free-energy barrier and the rate of hydride transfer. *E. coli* and *B. subtilis* DHFR enzymes, which have similar tertiary structures and hydride transfer rates with 44% sequence identity, exhibit both similarities and differences in the equilibrium motions and conformational changes correlated to hydride transfer, suggesting a balance of conservation and flexibility across species.

Keywords: hydride transfer; hydrogen tunnelling; enzyme catalysis; molecular dynamics

1. INTRODUCTION

The role of motion in enzyme reactions has been the topic of numerous experimental and theoretical studies (Benkovic & Hammes-Schiffer 2003; Klinman 2003; Tousignant & Pelletier 2004). Dihydrofolate reductase (DHFR) is an ideal system for studies of enzyme motion owing to its relatively small size and well-defined reaction mechanism. DHFR catalyses the reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) using nicotinamide adenine dinucleotide phosphate (NADPH) as a coenzyme. The reaction proceeds via protonation of the N5 nitrogen of the H_2F pterin ring and hydride transfer from the nicotinamide ring of NADPH to the C6 carbon of the pterin ring. Tetrahydrofolate is a necessary precursor for the biosynthesis of purines, pyrimidines and amino acids. As a result, DHFR has been fostered as a pharmacological target for anti-cancer drugs and antibacterial agents (Berg *et al.* 2002).

A wide range of experimental studies has focused on the role of protein motion in DHFR catalysis. X-ray crystal structures of *Escherichia coli* DHFR representing various stages in the reaction pathway show that regions of the protein undergo distinct conformational changes throughout the catalytic process (Sawaya & Kraut 1997). Nuclear magnetic resonance (NMR)

relaxation experiments imply that the binding of the cofactor and the substrate induces conformational changes both near and far from the active site (Falzone *et al.* 1994; Osborne *et al.* 2001; Schnell *et al.* 2004). Furthermore, kinetic measurements of single and multiple mutants have identified non-additive effects that indicate coupling between distal regions in DHFR (Huang *et al.* 1994; Wagner *et al.* 1995; Rajagopalan *et al.* 2002; Wong *et al.* 2005). Measurements of the primary and the secondary kinetic isotope effects (KIEs) and their temperature dependences have also been used as a probe for coupled motion (Maglia & Allemann 2003; Sikorski *et al.* 2004).

Theory and simulation have also provided insights into the role of protein motion in DHFR catalysis. Classical molecular dynamics simulations of the reactant ternary complex for *E. coli* DHFR identified correlated and anticorrelated motions involving many of the same spatial regions as implicated by the dynamic NMR measurements (Osborne *et al.* 2001; Schnell *et al.* 2004). These correlations are absent in the product complex with 5,6,7,8-tetrahydrofolate (Radkiewicz & Brooks 2000). A genomic analysis for sequence conservation across 36 species from *E. coli* to human illustrates that residues both in the active site and distal to the active site are conserved across this wide range of species (Agarwal *et al.* 2002b). The results of this genomic analysis are depicted in figure 1. Hybrid quantum/classical molecular dynamics simulations were used to identify and characterize a network of coupled motions in *E. coli* DHFR

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Figure 1. Three-dimensional structure of *E. coli* DHFR. The residues conserved across numerous species from *E. coli* to human are indicated by a gradient colour scheme (grey to red, where red is the most conserved). NADPH and DHF are in green and magenta, respectively. Reproduced with permission from Agarwal *et al.* (2002b).

(Agarwal *et al.* 2002a,b). These equilibrium motions represent thermally averaged conformational changes of the protein and ligands along the collective reaction coordinate for hydride transfer. Simulations of hydride transfer in *E. coli* DHFR with other computational methods also exhibited some of these conformational changes (Garcia-Viloca *et al.* 2003; Thorpe & Brooks 2003). A detailed analysis of hydrogen bonding, electrostatic potentials and correlated motions along the collective reaction coordinate for hydride transfer indicated significant changes in these properties throughout the protein (Wong *et al.* 2004).

This paper reviews the results from our recent hybrid quantum/classical molecular dynamics simulations of wild-type (WT) and mutant *E. coli* DHFR enzymes and WT *Bacillus subtilis* DHFR enzyme. Our studies of single and multiple mutant *E. coli* DHFR enzymes illustrate that mutations far from the active site can impact the rate of hydride transfer by introducing non-local structural perturbations and altering the conformational sampling of configurations conducive to the chemical reaction (Watney *et al.* 2003; Wong *et al.* 2005). Other simulations of mutant *E. coli* DHFR enzymes have also identified non-local structural effects (Swanwick *et al.* 2004) and alterations in the conformational sampling (Rod *et al.* 2003). *Escherichia coli* and *B. subtilis* DHFR enzymes have a sequence identity of 44%, and the experimentally determined X-ray crystallographic structures and hydride transfer rates are similar (Wilson *et al.* in preparation). Our simulations elucidate the similarities and differences in the network of coupled motions for *E. coli* and *B. subtilis* DHFR and provide insight into the

transferability of these types of motions between different species (Watney & Hammes-Schiffer 2006).

The organization of this paper is as follows. Section 2 describes the methodology for the hybrid quantum/classical molecular dynamics simulations and the analyses. Section 3 summarizes the results and §4 provides the summary and conclusions.

2. MATERIAL AND METHODS

We have developed a hybrid quantum/classical molecular dynamics approach for proton and hydride transfer reactions in enzymes (Billeter *et al.* 2001a,b). This approach includes electronic and nuclear quantum effects, as well as the motions of the entire solvated enzyme. The electronic quantum effects are included with a two-state empirical valence bond (EVB) potential (Warshel 1991), where the two states correspond to the hydrogen being bonded to the donor or acceptor. In VB state 1 the hydride is bonded to its donor carbon, whereas in VB state 2 it is bonded to its acceptor carbon. The diagonal EVB matrix elements are based on the GROMOS force field (van Gunsteren *et al.* 1996) with the modifications described elsewhere (Agarwal *et al.* 2002a). The two EVB parameters, corresponding to the relative energy of the two valence bond states and the coupling between these states, were fit to the experimental free energies of reaction and activation (Fierke *et al.* 1987; Agarwal *et al.* 2002a; Wong *et al.* 2004). The nuclear quantum effects are included by representing the transferring hydrogen nucleus as a three-dimensional vibrational wavefunction (Webb & Hammes-Schiffer 2000). This wavefunction is calculated directly on a grid with a Fourier grid Hamiltonian method for each configuration of the classical nuclei sampled during the molecular dynamics simulation.

The free-energy profile is generated as a function of a collective reaction coordinate comprised of motions from the entire solvated enzyme. This collective reaction coordinate is defined as the difference between the energies of the two VB states averaged over the lowest energy hydrogen vibrational wavefunction:

$$\Lambda(\mathbf{R}) = \langle \Phi_0(\mathbf{r}; \mathbf{R}) | V_{11}(\mathbf{r}, \mathbf{R}) - V_{22}(\mathbf{r}, \mathbf{R}) | \Phi_0(\mathbf{r}; \mathbf{R}) \rangle, \quad (2.1)$$

where $\mathbf{r}$ represents the coordinate of the transferring hydrogen nucleus and $\mathbf{R}$ represents the coordinates of the remaining nuclei. $V_{11}(\mathbf{r}, \mathbf{R})$ and $V_{22}(\mathbf{r}, \mathbf{R})$ are the energies of VB states 1 and 2, respectively, and $\Phi_0(\mathbf{r}; \mathbf{R})$ represents the ground-state hydrogen vibrational wavefunction. This reaction coordinate has been shown to be physically meaningful for two-state charge transfer processes (Marcus 1964; Zusman 1980; Warshel 1982). Moreover, for enzymatic reactions described within the framework of transition state theory, this reaction coordinate leads to estimates of the transmission coefficient (i.e. recrossing factor) that are close to unity (Billeter *et al.* 2001a; Agarwal *et al.* 2002a). Note that this collective reaction coordinate includes motions of the enzyme, the substrate and the cofactor.

A series of mapping potentials is used to sample the relevant range of the collective reaction coordinate. These mapping potentials are defined to be linear combinations of the energies of the two VB states (Warshel 1991):

$$V_{\text{map}}(\mathbf{r}, \mathbf{R}; \lambda) = (1 - \lambda)V_{11}(\mathbf{r}, \mathbf{R}) + \lambda V_{22}(\mathbf{r}, \mathbf{R}). \quad (2.2)$$

As the mapping parameter λ is varied from zero to unity, the reaction progresses from the reactant state to the product state. In this umbrella sampling approach, standard binning techniques are used to generate the segment of the free-energy

Figure 2. The hydride transfer reaction from the NADPH cofactor to the H_3F^+ substrate.

profile for each value of λ . The individual segments are connected using thermodynamic integration or the weighted histogram analysis method (Kumar *et al.* 1992) to form the free-energy profile corresponding to the unbiased Hamiltonian. A free-energy perturbation approach is used to incorporate the nuclear quantum effects into the free-energy profile (Billeter *et al.* 2001b). This procedure allows us to generate the entire adiabatic quantum free-energy profile along the reaction coordinate.

The proton or hydride transfer rate constant is given by the simple expression

$$k = \kappa k_{\text{TST}}, \quad (2.3)$$

where $k_{\text{TST}} = (k_{\text{B}}T/h)\exp(-\Delta G^\ddagger/k_{\text{B}}T)$ is the equilibrium transition-state theory rate constant calculated from the activation free energy $\Delta G^\ddagger$ and κ is the non-equilibrium transmission coefficient accounting for dynamical recrossings of the barrier. Note that k_{TST} can be expressed in an alternative form that is valid for a general choice of reaction coordinate (Watney *et al.* 2005). The free-energy barrier obtained from the adiabatic quantum free-energy profiles is used to calculate the transition-state theory rate constant. The transmission coefficient is calculated by combining the reactive flux scheme for infrequent events (Keck 1960; Anderson 1973; Neria & Karplus 1996; Bennett 1997; Chandler 1998) with a surface-hopping method (Tully 1990; Hammes-Schiffer & Tully 1994, 1995) that includes non-adiabatic transitions among the hydrogen vibrational states.

The system used in our DHFR calculations contains the protein, an NADPH cofactor, a protonated H_2F substrate (H_3F^+) and more than 4000 explicit water molecules in a truncated octahedral periodic box. The initial coordinates for the *E. coli* DHFR simulations were obtained from a crystal structure of *E. coli* DHFR complexed with NADPH^+ and folate (PDB code 1rx2; Sawaya & Kraut 1997). In this crystal structure, the Met20 loop is in the closed conformation, which is thought to be the active form for the hydride transfer step. The initial coordinates for the *B. subtilis* DHFR simulations were obtained from an analogous crystal structure of *B. subtilis* DHFR (Wilson *et al.* in preparation). The initial coordinates for the mutant *E. coli* DHFR enzymes were obtained by using the mutation utility in SwissPDB viewer (Guex & Peitsch 1997) on the WT *E. coli* DHFR crystal structure to determine the most stable rotamer. The reaction studied is the transfer of the pro-*R* hydrogen on the donor carbon of NADPH to the acceptor carbon of H_3F^+ , as depicted in figure 2. The simulation details for the studies of WT *E. coli* (Agarwal *et al.* 2002a; Wong *et al.* 2004), mutant *E. coli* (Watney *et al.* 2003; Wong *et al.* 2005), and WT *B. subtilis* (Watney & Hammes-Schiffer submitted) DHFR are provided elsewhere.

As mentioned earlier, the EVB potential includes two parameters that were fit to two experimentally measured quantities. Specifically, these two parameters were chosen so that the overall rate calculated from the free-energy profile that includes the nuclear quantum effects is in agreement with

the experimentally measured forward and reverse rates for hydride transfer. Kinetic measurements provide forward and backward pH-independent rates for hydride transfer of 950 and 0.55 s^{-1} , respectively, for *E. coli* DHFR (Fierke *et al.* 1987) and 720 and 8 s^{-1} , respectively, for *B. subtilis* DHFR (Wilson *et al.* in preparation). For the simulations of the G121V mutant of *E. coli* DHFR, the two EVB parameters were chosen to be identical to those used for WT *E. coli* DHFR. Maintaining the same EVB parameters for the G121V mutant assumes that the potential-energy surface in the reactive region is not perturbed greatly by this distal mutation. For the triple mutant M42F-G121S-S148A and the corresponding single and double mutants, we found that the EVB potential was not sufficiently flexible to afford transferability of the WT parameters. As a result, the two EVB parameters were reparameterized for these mutants to reproduce the experimentally determined rates for the mutants relative to the WT DHFR (Wong *et al.* 2005). For all of these calculations, no additional parameters were altered to fit any other experimental data. As discussed later, the calculations predicted the KIE for WT *E. coli* DHFR and the increase in the rate for the G121V mutant relative to the WT *E. coli* DHFR to be in agreement with the experimental results.

3. RESULTS

We have applied this hybrid quantum/classical molecular dynamics approach to the hydride transfer reaction catalysed by WT *E. coli* DHFR (Agarwal *et al.* 2002a,b; Wong *et al.* 2004). Inclusion of the nuclear quantum effects of the transferring hydrogen was found to decrease the free-energy barrier by $2.4 \text{ kcal mol}^{-1}$ (Agarwal *et al.* 2002a). Analysis of the nuclear wavefunctions and the splittings between the ground and the excited vibrational states for the transition-state configurations indicated that hydrogen tunnelling plays a significant role in this reaction. The calculated KIE was consistent with the experimental value of 3 (Fierke *et al.* 1987; Agarwal *et al.* 2002a). The transmission coefficient was calculated to be $\kappa = 0.88$, indicating that dynamical barrier recrossings occur but do not significantly impact the overall rate (Agarwal *et al.* 2002a).

To elucidate the role of protein motion, we performed a detailed analysis of these hybrid quantum/classical molecular dynamics simulations (Agarwal *et al.* 2002a,b). Specifically, we analysed the equilibrium, thermally averaged changes in distances and angles along the collective reaction coordinate. Several nanoseconds of molecular dynamics simulations for each biasing potential along the collective reaction coordinate were performed, and the data from the different biasing potentials were combined to generate the entire free-energy profile for the chemical reaction

Figure 3. Selected thermally averaged geometrical properties along the collective reaction coordinate for WT *E. coli* (left), G121V mutant *E. coli* (middle) and WT *B. subtilis* (right) DHFR. From top to bottom, the properties plotted are: the donor–acceptor distance; the angle between the acceptor and methylene amino linkage in H_2F ; the distance between C_γ of Phe31 and C11 of H_2F ; the hydrogen-bonding distance between N of Asp122 and O of Gly15 (black) and between O of Ile14 and carboxamide N of NADPH (grey). The relevant residues are labelled in figure 1. The WT *E. coli* data were obtained from Agarwal *et al.* (2002*b*), the G121V mutant *E. coli* data were obtained from Watney *et al.* (2003), and the *B. subtilis* data were obtained from Watney & Hammes-Schiffer (2005). An improved numerical method for calculating the thermally averaged properties was used for the analysis of the *B. subtilis* data. The *B. subtilis* results obtained with the numerical method used to generate the *E. coli* results are provided in Watney (2005), and the qualitative trends are the same.

and to generate the thermally averaged changes in structural properties along the collective reaction coordinate. Figure 3 depicts selected thermally averaged properties along the collective reaction coordinate. Some of these conformational changes could potentially occur on the experimentally determined millisecond time-scale of hydride transfer. Recently, protein motions on this time-scale were observed in single-molecule fluorescence experiments (Antikainen *et al.* 2005). We also calculated the correlation of distances and angles at the transition state to the degree of dynamical barrier recrossing. These motions occur on the femtosecond to picosecond time-scale, however, and they are not expected to impact the overall rate strongly.

This analysis resulted in the identification and characterization of a network of coupled motions in DHFR (Agarwal *et al.* 2002*a,b*). These equilibrium, thermally averaged motions are located throughout the enzyme and represent conformational changes along the collective reaction coordinate corresponding to reorganization of the environment to facilitate hydride transfer by bringing the donor and acceptor closer, orienting the substrate and cofactor properly and providing a favourable electrostatic environment. These motions are not *dynamically* coupled to the chemical reaction, but rather represent equilibrium

conformational changes that enable hydride transfer. Figure 4 depicts representative configurations from the continuous series of thermally averaged equilibrium configurations sampled along the collective reaction coordinate. The rate-limiting process can be viewed in the context of these conformational changes along the collective reaction coordinate (i.e. the overall rate of transformation from the equilibrium reactant to the equilibrium product configuration).

To further elucidate this network of coupled motions, we analysed the changes in structure, hydrogen bonding and electrostatics along the collective reaction coordinate (Wong *et al.* 2004). Analysis of the thermally averaged reactant, transition state and product structures indicated that the key features of the enzyme structure are retained throughout the reaction, but significant structural changes occur in the loop regions. Furthermore, a number of hydrogen bonds are broken and formed throughout the enzyme during the reaction. Analysis of the electrostatic potential for the thermally averaged reactant, transition state and product structures indicated significant changes throughout the enzyme during the reaction. For example, figure 5 illustrates that the βF – βG and βG – βH loop regions exhibit dramatic changes in the electrostatic potential along the collective reaction

coordinate. As shown in figure 6, both these loop regions are far from the active site. In a more quantitative analysis, the electrostatic contributions from a number of residues distal to the active site were found to change substantially during the reaction. These results imply that significant alterations in hydrogen bonding and electrostatics occur throughout the entire enzyme during the hydride transfer reaction.

We also performed hybrid quantum/classical molecular dynamics simulations for the G121V mutant *E. coli* DHFR enzyme (Watney *et al.* 2003). As depicted in figure 6, this residue is on the exterior of the enzyme and is located more than 12 Å from the active site. Kinetic experiments showed that mutation of residue 121 from glycine to valine decreased the rate of hydride transfer by a factor of 163 (Cameron & Benkovic 1997; Rajagopalan *et al.* 2002). We mutated this residue to valine and followed the same procedure with EVB parameters identical with those used for the WT DHFR simulations. Our calculated free-energy barrier was 3.4 kcal mol⁻¹ greater for the mutant than for the WT enzyme. This increase in the barrier is consistent with the experimental observation of a decrease in the rate by a factor of 163, which corresponds to *ca* 3.0 kcal mol⁻¹ increase in the free-energy barrier when the effects of the transmission coefficient are neglected. We found that the transmission coefficient is not significantly altered by the mutation. Figure 3 depicts selected thermally averaged structural properties along the collective reaction coordinate for WT and G121V mutant *E. coli* DHFR. The donor–acceptor distance decreases to *ca* 2.7 Å at the transition-state configurations for both enzymes to enable hydride transfer. On the other hand, figure 3 illustrates that some of the other motions correlated to hydride transfer in the WT enzyme are attenuated or absent in the mutant. These results suggest that this distal mutation interrupts the network of coupled motions, thereby decreasing the probability of sampling configurations conducive to the hydride transfer and increasing the free-energy barrier.

Subsequent to this initial study, we performed simulations for the triple mutant M42F-G121S-S148A and the corresponding single and double mutants (Wong *et al.* 2005). The locations of these distal residues are depicted in figure 6. Kinetic experiments showed that these distal mutations significantly decrease the rate of hydride transfer and that some of these mutations are non-additive (i.e. the change in the free-energy barrier for the multiple mutation is not equal to the sum of the changes for the single mutations). We analysed these mutants with a rank correlation analysis method, which compares thermally averaged changes of interatomic distances along the collective reaction coordinate to a specified target model (Wong *et al.* 2005). A systematic analysis of the entire system, including the enzyme, cofactor and substrate, indicated that residues throughout the enzyme participate in the network of coupled motions correlated to hydride transfer and that each mutant samples a unique distribution of motions. The observation of non-additive changes to the network of coupled motions provided an explanation for the experimentally observed non-additivity of the rates. This study provided additional evidence that distal

mutations can lead to non-local structural changes (Swanwick *et al.* 2004) and significantly impact the probability of sampling conformations conducive to hydride transfer (Thorpe & Brooks 2003, 2004).

Recently, we applied the hybrid quantum/classical molecular dynamics approach to hydride transfer in *B. subtilis* DHFR (Watney & Hammes-Schiffer submitted). The sequence and structural alignment scheme in PyMOL (DeLano 2002) were used to superimpose the crystal structures of *E. coli* and *B. subtilis* DHFRs. The tertiary structures of the two enzymes are highly similar (Wilson *et al.* in preparation), and the root mean square deviation between C $_{\alpha}$ atoms was determined to be *ca* 1.5 Å. The thermally averaged structures of *E. coli* and *B. subtilis* DHFRs obtained from our simulations for reactant, transition and product states are depicted in figure 7. The root mean square deviation between C $_{\alpha}$ atoms for each of these pairs of thermally averaged *E. coli* and *B. subtilis* DHFR structures is *ca* 2.0 Å. Thus, these simulations indicate that the tertiary structures of both enzymes evolve in a similar manner during the hydride transfer reaction. The simulations also show that inclusion of the nuclear quantum effects of the transferring hydrogen decreases the free-energy barrier for hydride transfer in *B. subtilis* DHFR by 3.3 kcal mol⁻¹, confirming the importance of nuclear quantum effects such as zero point energy and hydrogen tunnelling for this enzyme reaction.

In addition, we performed both a covariance analysis and a rank-correlation analysis of the motions in *E. coli* and *B. subtilis* DHFR. The covariance analysis, which identifies correlations between fluctuations of pairs of atoms about an average structure, was performed for the thermally averaged reactant, transition and product states. The rank-correlation analysis, described earlier, was used to elucidate thermally averaged interatomic distances that increase or decrease monotonically between the reactant and the transition states. These analyses illustrate that *E. coli* and *B. subtilis* DHFR exhibit both similarities and differences in the equilibrium fluctuations and conformational changes that are correlated to hydride transfer. Figure 3 depicts selected thermally averaged structural properties along the collective reaction coordinate for *E. coli* and *B. subtilis* DHFRs. As observed for the G121V *E. coli* mutant, some of the motions correlated to hydride transfer in *E. coli* DHFR are attenuated or absent in *B. subtilis* DHFR. In all three enzymes, however, the donor–acceptor distance decreases to *ca* 2.7 Å at the transition-state configurations to enable hydride transfer. These results suggest a balance between conservation and flexibility in the thermal motions and conformational changes during hydride transfer.

4. CONCLUSIONS

This paper reviews the results from hybrid quantum/classical molecular dynamics simulations of the hydride transfer reaction catalysed by WT and mutant *E. coli* and WT *B. subtilis* DHFRs. Nuclear quantum effects such as zero point energy and hydrogen tunnelling were shown to be significant in these reactions and to lower the free-energy barrier by 2.4–3.3 kcal mol⁻¹. For all

Figure 4. Free-energy profile of the DHFR-catalysed hydride transfer reaction as a function of a collective reaction coordinate that includes motions of the enzyme, substrate and cofactor. The magnitude of the free-energy barrier is determined by the relative probabilities of sampling the transition state and the reactant configurations. The thermally averaged equilibrium structures, as well as the average donor–acceptor distances in angstroms, are provided for selected values of the reaction coordinate. Note that the donor–acceptor distance decreases as the reaction evolves from the reactant to the transition state. The conformational changes along the collective reaction coordinate are attained by equilibrium thermal motions occurring within the confines of the protein fold and reflect a network of coupled motions extending throughout the protein. This network facilitates the hydride transfer reaction by bringing the donor and acceptor closer together, orienting the substrate and cofactor properly and providing a favourable electrostatic environment.

of these systems, the donor–acceptor distance was found to decrease to *ca* 2.7 Å at the transition-state configurations to enable hydride transfer. Our simulations elucidated a network of coupled motions representing conformational changes along the collective reaction coordinate to facilitate hydride transfer by decreasing the donor–acceptor distance, orienting the

substrate and cofactor properly and providing a favourable electrostatic environment for hydride transfer. Distal mutations in *E. coli* DHFR were found to cause non-local structural changes and to significantly impact the probability of sampling configurations conducive to hydride transfer, thereby altering the free-energy barrier and the rate of hydride

Figure 5. Electrostatic potential mapped to the molecular surface of the (a) and (b) β F- β G loop and (c) β G- β H loop. The potentials are given for the reactant state (left), transition state (middle) and product state (right). Blue indicates positive potential and red indicates negative potential. The substrate and cofactor are yellow and green, respectively. This figure was created using GRASP (Nicholls *et al.* 1991). Reproduced with permission from Wong *et al.* (2004).

Figure 6. Three-dimensional structure of WT *E. coli* DHFR with the NADPH cofactor shown in green and the substrate shown in magenta. The important loop regions are identified and the residues involved in the mutant DHFR enzymes studied are labelled with red spheres. Reproduced with permission from Hammes-Schiffer (2006).

Figure 7. Thermally averaged structures for *E. coli* (red) and *B. subtilis* (blue) DHFR at the reactant state (RS), transition state (TS) and product state (PS). Reproduced with permission from Watney & Hammes-Schiffer (2005).

transfer. A comparison of *E. coli* and *B. subtilis* DHFR enzymes, which have similar tertiary structures and hydride transfer rates with 44% sequence identity, elucidated both similarities and differences in the equilibrium motions and conformational changes correlated to hydride transfer.

In conjunction with a vast collection of experimental data, these simulations lead to a general picture in which thermal fluctuations of the enzyme and the ligands enable conformational sampling of configurations that facilitate hydride transfer. These thermal motions are Brownian in nature, but occur within the confines of the enzyme structure. The fast thermal motions are in equilibrium as the reaction proceeds along the collective reaction coordinate, and the overall average equilibrium conformational changes occur on the slower time-scale measured experimentally. Recent single-molecule experiments confirm that at

least some of these thermally averaged equilibrium conformational changes occur on the millisecond time-scale of hydride transfer. In this framework, the experimentally measured hydride transfer rate corresponds to the overall rate of transformation from the equilibrium reactant configuration to the equilibrium product configuration, and the actual transfer of the hydrogen is virtually instantaneous relative to these conformational changes. The implications of this general picture of enzyme catalysis are relevant to protein engineering and drug design.

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REFERENCES

- Agarwal, P. K., Billeter, S. R. & Hammes-Schiffer, S. 2002a Nuclear quantum effects and enzyme dynamics in dihydrofolate reductase catalysis. *J. Phys. Chem. B* **106**, 3283–3293. (doi:10.1021/jp020190v)
- Agarwal, P. K., Billeter, S. R., Rajagopalan, P. T. R., Benkovic, S. J. & Hammes-Schiffer, S. 2002b Network of coupled promoting motions in enzyme catalysis. *Proc. Natl Acad. Sci. USA* **99**, 2794–2799. (doi:10.1073/pnas.052005999)
- Anderson, J. B. 1973 Statistical theories of chemical reactions. Distributions in the transition region. *J. Chem. Phys.* **58**, 4684–4692. (doi:10.1063/1.1679032)
- Antikainen, N. M., Smiley, R. D., Benkovic, S. J. & Hammes, G. G. 2005 Conformation coupled enzyme catalysis: single molecule and transient kinetics investigation of dihydrofolate reductase. *Biochemistry*, **44**, 16 835–16 843.
- Benkovic, S. J. & Hammes-Schiffer, S. 2003 A perspective on enzyme catalysis. *Science* **301**, 1196–1202. (doi:10.1126/science.1085515)
- Bennett, C. H. 1997 *Algorithms for chemical computation*. Washington, DC: American Chemical Society.
- Berg, J. M., Stryer, L. & Tymoczko, J. 2002 *Biochemistry*. New York: Freeman.
- Billeter, S. R., Webb, S. P., Agarwal, P. K., Iordanov, T. & Hammes-Schiffer, S. 2001a Hydride transfer in liver alcohol dehydrogenase: Quantum dynamics, kinetic isotope effects, and the role of enzyme motion. *J. Am. Chem. Soc.* **123**, 11 262–11 272. (doi:10.1021/ja011384b)
- Billeter, S. R., Webb, S. P., Iordanov, T., Agarwal, P. K. & Hammes-Schiffer, S. 2001b Hybrid approach for including electronic and nuclear quantum effects in molecular dynamics simulations of hydrogen transfer reactions in enzymes. *J. Chem. Phys.* **114**, 6925–6936. (doi:10.1063/1.1356441)
- Cameron, C. E. & Benkovic, S. J. 1997 Evidence for a functional role of the dynamics of glycine-121 of *Escherichia coli* dihydrofolate reductase obtained from kinetic analysis of a site-directed mutant. *Biochemistry* **36**, 15 792–15 800. (doi:10.1021/bi9716231)
- Chandler, D. 1998 A story of rare events: From barriers, to electrons, to unknown pathways. In *Classical and quantum dynamics in condensed phase simulations* (ed. B. J. Berne, G. Ciccotti & D. F. Coker), pp. 3–23. Singapore: World Scientific.

- DeLano, W. L. 2002 *The Pymol molecular graphics system*. San Carlos, CA: DeLano Scientific.
- Falzone, C. J., Wright, P. E. & Benkovic, S. J. 1994 Dynamics of a flexible loop in dihydrofolate reductase from *Escherichia coli* and its implication for catalysis. *Biochemistry* **33**, 439–442. (doi:10.1021/bi00168a007)
- Fierke, C. A., Johnson, K. A. & Benkovic, S. J. 1987 Construction and evaluation of the kinetic scheme associated with dihydrofolate reductase from *Escherichia coli*. *Biochemistry* **26**, 4085–4092. (doi:10.1021/bi00387a052)
- Garcia-Viloca, M., Truhlar, D. G. & Gao, J. 2003 Reaction-path energetics and kinetics of the hydride transfer reaction catalyzed by dihydrofolate reductase. *Biochemistry* **42**, 13 558–13 575. (doi:10.1021/bi034824f)
- Guex, N. & Peitsch, M. C. 1997 *Electrophoresis* **18**, 2714. (doi:10.1002/elps.1150181505)
- Hammes-Schiffer, S. 2006 Hydrogen tunneling and protein motion in enzyme reactions. *Acc. Chem. Res.* **39**, 93–100.
- Hammes-Schiffer, S. & Tully, J. C. 1994 Proton transfer in solution: molecular dynamics with quantum transitions. *J. Chem. Phys.* **101**, 4657–4667. (doi:10.1063/1.467455)
- Hammes-Schiffer, S. & Tully, J. C. 1995 Nonadiabatic transition state theory and multiple potential energy surface molecular dynamics of infrequent events. *J. Chem. Phys.* **103**, 8528–8537. (doi:10.1063/1.470162)
- Huang, Z., Wagner, C. R. & Benkovic, S. J. 1994 Nonadditivity of mutational effects at the folate binding site of *Escherichia coli* dihydrofolate reductase. *Biochemistry* **33**, 11 576–11 585. (doi:10.1021/bi00204a020)
- Keck, J. C. 1960 Variational theory of chemical reaction rates applied to three-body recombinations. *J. Chem. Phys.* **32**, 1035–1050. (doi:10.1063/1.1730846)
- Klinman, J. P. 2003 Dynamic barriers and tunneling. New views of hydrogen transfer in enzyme reactions. *Pure Appl. Chem.* **75**, 601–608.
- Kumar, S., Bouzida, D., Swendsen, R. H., Kollman, P. A. & Rosenberg, J. M. 1992 The weighted histogram analysis method for free-energy calculations on biomolecules. I. The method. *J. Comput. Chem.* **13**, 1011–1021. (doi:10.1002/jcc.540130812)
- Maglia, G. & Allemann, R. K. 2003 Evidence for environmentally coupled hydrogen tunneling during dihydrofolate reductase catalysis. *J. Am. Chem. Soc.* **125**, 13 372–13 373. (doi:10.1021/ja035692g)
- Marcus, R. A. 1964 Chemical and electrochemical electron-transfer theory. *Ann. Rev. Phys. Chem.* **15**, 155–196. (doi:10.1146/annurev.pc.15.100164.001103)
- Neria, E. & Karplus, M. 1996 A position dependent friction model for solution reactions in the high friction regime: proton transfer in triosephosphate isomerase (TIM). *J. Chem. Phys.* **105**, 10 812–10 818. (doi:10.1063/1.472889)
- Nicholls, A., Sharp, K. & Honig, B. 1991 *Proteins Struct. Funct. Genet.* **11**, 281. (doi:10.1002/prot.340110407)
- Osborne, M. J., Schnell, J., Benkovic, S. J., Dyson, H. J. & Wright, P. E. 2001 Backbone dynamics in dihydrofolate reductase complexes: role of loop flexibility in the catalytic mechanism. *Biochemistry* **40**, 9846–9859. (doi:10.1021/bi010621k)
- Radkiewicz, J. L. & Brooks, C. L. 2000 Protein dynamics in enzymatic catalysis: exploration of dihydrofolate reductase. *J. Am. Chem. Soc.* **122**, 225–231. (doi:10.1021/ja9913838)
- Rajagopalan, P. T. R., Lutz, S. & Benkovic, S. J. 2002 Coupling interactions of distal residues enhance dihydrofolate reductase catalysis: Mutational effects on hydride transfer rates. *Biochemistry* **41**, 12 618–12 628. (doi:10.1021/bi026369d)
- Rod, T. H., Radkiewicz, J. L. & Brooks III, C. L. 2003 Correlated motion and the effect of distal mutations in dihydrofolate reductase. *Proc. Natl Acad. USA* **100**, 6980–6985. (doi:10.1073/pnas.1230801100)
- Sawaya, M. R. & Kraut, J. 1997 Loop and subdomain movements in the mechanism of *Escherichia coli* dihydrofolate reductase: Crystallographic evidence. *Biochemistry* **36**, 586–603. (doi:10.1021/bi962337c)
- Schnell, J. R., Dyson, H. J. & Wright, P. E. 2004 Effect of cofactor binding and loop conformation on side chain methyl dynamics in dihydrofolate reductase. *Biochemistry* **43**, 374–383. (doi:10.1021/bi035464z)
- Sikorski, R. S., Wang, L., Markham, K. A., Rajagopalan, P. T. R., Benkovic, S. J. & Kohen, A. 2004 Tunneling and coupled motion in the *Escherichia coli* dihydrofolate reductase catalysis. *J. Am. Chem. Soc.* **126**, 4778–4779. (doi:10.1021/ja031683w)
- Swanwick, R. S., Shrimpton, P. J. & Allemann, R. K. 2004 Pivotal role of Gly 121 in dihydrofolate reductase from *Escherichia coli*: the altered structure of a mutant enzyme may form the basis of its diminished catalytic performance. *Biochemistry* **43**, 4119–4127. (doi:10.1021/bi036164k)
- Thorpe, I. F. & Brooks III, C. L. 2003 Barriers to hydride transfer in wild type and mutant dihydrofolate reductase from *E. coli*. *J. Phys. Chem. B* **107**, 14 042–14 051. (doi:10.1021/jp035734n)
- Thorpe, I. F. & Brooks III, C. L. 2004 The coupling of structural fluctuations to hydride transfer in dihydrofolate reductase. *Proteins Struct. Funct. Bioinf.* **57**, 444–457. (doi:10.1002/prot.20219)
- Tousignant, A. & Pelletier, J. N. 2004 Protein motions promote catalysis. *Chem. Biol.* **11**, 1037–1042. (doi:10.1016/j.chembiol.2004.06.007)
- Tully, J. C. 1990 Molecular dynamics with electronic transitions. *J. Chem. Phys.* **93**, 1061–1071. (doi:10.1063/1.459170)
- van Gunsteren, W. F., Billeter, S. R., Eising, A. A., Hunenberger, P. H., Kruger, P., Mark, A. E., Scott, W. R. P. & Tironi, I. G. 1996 *Biomolecular simulation: The GROMOS'96 manual and user guide*. Zurich: VdF Hochschulverlag, ETH Zurich.
- Wagner, C. R., Huang, Z., Singleton, S. F. & Benkovic, S. J. 1995 Molecular basis for nonadditive mutational effects in *Escherichia coli* dihydrofolate reductase. *Biochemistry* **34**, 15 671–15 680. (doi:10.1021/bi00048a011)
- Warshel, A. 1982 Dynamics of reactions in polar solvents: Semiclassical trajectory studies of electron-transfer and proton-transfer reactions. *J. Phys. Chem.* **86**, 2218. (doi:10.1021/j100209a016)
- Warshel, A. 1991 *Computer modeling of chemical reactions in enzymes and solutions*. New York: Wiley.
- Watney, J. B. 2005 In chemistry department, PhD University Park: The Pennsylvania State University.
- Watney, J. B. & Hammes-Schiffer, S. 2006 Comparison of coupled motions in *Escherichia coli* and *Bacillus subtilis* dihydrofolate reductase. *J. Am. Chem. Soc.* **110**, 10 130–10 138.
- Watney, J. B., Agarwal, P. K. & Hammes-Schiffer, S. 2003 Effect of mutation on enzyme motion in dihydrofolate reductase. *J. Am. Chem. Soc.* **125**, 3745–3750. (doi:10.1021/ja028487u)
- Watney, J. B., Soudackov, A., Wong, K. F. & Hammes-Schiffer, S. 2006 Calculation of the transition state theory rate constant for a general reaction coordinate: application to hydride transfer in an enzyme. *Chem. Phys. Lett.* **418**, 268–271.
- Webb, S. P. & Hammes-Schiffer, S. 2000 Fourier grid Hamiltonian multiconfigurational self consistent field: a method to calculate multidimensional hydrogen vibrational

- wavefunctions. *J. Chem. Phys.* **113**, 5214–5227. (doi:10.1063/1.1289528)
- Wilson, M. A., Horswill, A. R., Fan, G., Benkovic, S. J., Ring, D. & Petsko, G. A. In preparation.
- Wong, K. F., Watney, J. B. & Hammes-Schiffer, S. 2004 Analysis of electrostatics and correlated motions for hydride transfer in dihydrofolate reductase. *J. Phys. Chem. B* **108**, 12 231–12 241. (doi:10.1021/jp048565v)
- Wong, K. F., Selzer, T., Benkovic, S. J. & Hammes-Schiffer, S. 2005 Impact of distal mutations on the network of coupled motions correlated to hydride transfer in dihydrofolate reductase. *Proc. Natl Acad. Sci. USA* **102**, 6807–6812. (doi:10.1073/pnas.0408343102)
- Zusman, L. D. 1980 Outer-sphere electron transfer in polar solvents. *Chem. Phys.* **49**, 295–304. (doi:10.1016/0301-0104(80)85267-0)