The role of enzyme dynamics and tunnelling in catalysing hydride transfer: studies of distal mutants of dihydrofolate reductase

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Residues M42 and G121 of *Escherichia coli* dihydrofolate reductase (*ec*DHFR) are on opposite sides of the catalytic centre (15 and 19 Å away from it, respectively). Theoretical studies have suggested that these distal residues might be part of a dynamics network coupled to the reaction catalysed at the active site. The *ec*DHFR mutant G121V has been extensively studied and appeared to have a significant effect on rate, but only a mild effect on the nature of H-transfer. The present work examines the effect of M42W on the physical nature of the catalysed hydride transfer step. Intrinsic kinetic isotope effects (KIEs), their temperature dependence and activation parameters were studied. The findings presented here are in accordance with the environmentally coupled hydrogen tunnelling. In contrast to the wild-type (WT), fluctuations of the donor–acceptor distance were required, leading to a significant temperature dependence of KIEs and deflated intercepts. A comparison of M42W and G121V to the WT enzyme revealed that the reduced rates, the inflated primary KIEs and their temperature dependences resulted from an imperfect potential surface pre-arrangement relative to the WT enzyme. Apparently, the coupling of the enzyme's dynamics to the reaction coordinate was altered by the mutation, supporting the models in which dynamics of the whole protein is coupled to its catalysed chemistry.

Keywords: hydrogen tunnelling; kinetic isotope effect; enzyme dynamics; dihydrofolate reductase; distal mutation; structure–dynamics–function relationship

1. INTRODUCTION

The role of a protein's active site in enzyme catalysis has been studied since the early days of enzymology and most of its functions are well reasoned and widely accepted (e.g. general acid and base catalysis, orientation of H-bonds and electrostatic dipoles towards tight binding of transition states (TSs), etc.; Jencks 1987; Fersht 1998; Stryer 2002). The role of the whole protein and its dynamics in binding the reactants and releasing the products is also fairly well understood in a per-case manner (e.g. see movie by Sawaya & Kraut (1997) regarding dihydrofolate reductase (DHFR) <http://chem-faculty.ucsd.edu/kraut/dhfr.html>). On the other hand, the role of the whole protein in catalysing the chemical transformation at its active site is far from being resolved and is a matter of extensive ongoing research and substantial debate (Warshel & Parson 2001; Benkovic & Hammes-Schiffer 2003; Garcia-Viloca *et al.* 2003c). The challenge in addressing this issue involves examination of the chemical step (e.g. C–H–C transfer in the case of DHFR) within a complex kinetic cascade (e.g. Fierke *et al.* 1987 for DHFR), and correlating it to the

structural and the dynamic effects of the whole protein. The chemical step in most enzymes is not the rate-limiting step and is hard to study in the context of the enzyme's complex kinetic cascade. The enzyme's structure and dynamics at the TS can be studied by only calculations and computer simulation as no spectroscopic method (e.g. X-ray diffraction, nuclear magnetic resonance (NMR), infrared, electron paramagnetic resonance (EPR), etc.) can capture the small population of systems at that state. Spectroscopic studies of the protein's structure and dynamics with analogues of the reactants, products and putative TSs may provide information that can be indirectly correlated to the catalysed reaction (e.g. Osborne *et al.* 2001; Venkitarishnan *et al.* 2004; McElheny *et al.* 2005 for NMR studies of DHFR). Studies of reactions' TSs require kinetic examination such as rate determination, kinetic isotope effect (KIE) measurements, linear free energy relationship analysis, and/or evaluation of activation parameters (cf. E_a , $\Delta H^\ddagger$, $\Delta S^\ddagger$) and their isotope effects.

DHFR is one of the preferred model systems in studying enzymatic structure–dynamics–function relationships. This is due to the simple chemical transformation it catalyses (C–H–C hydride transfer in single reduction of C=N double bond (scheme 1)) and its simple protein scaffold (approx. 160 amino acids with no SS bonds or bound metals). DHFR is also ubiquitous in almost all organisms and thus an excellent platform for genetic and evolution analysis. Consequently, DHFR has been used as a paradigm for enzymes and

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proteins in general. The structure, dynamics and kinetics of DHFRs from many organisms have been studied by almost every known experimental and theoretical method. In fact, DHFR was commonly used to establish such methods. This wealth of knowledge makes DHFR an ideal system for studying general cutting-edge features in enzymology, such as the role of the enzyme's dynamics in catalysing its chemical step. It is fair to say that the *Escherichia coli* DHFR (*ec*DHFR) is the most extensively studied DHFR and, thus, is the enzyme used in the studies presented here.

In recent years, we developed a method that enables examination of the nature of the hydride transfer step in the DHFR-catalysed reaction (Markham *et al.* 2003, 2004; Agrawal & Kohen 2003; McCracken *et al.* 2003; Sikorski *et al.* 2004). The kinetic cascade associated with the *ec*DHFR reaction is very complex (Fierke *et al.* 1987; Miller & Benkovic 1998a,b; Rajagopalan *et al.* 2002). The methods we developed afford the extraction of information regarding the physical nature of the chemical step within this complex cascade. Features such as H-tunnelling and coupling of the reaction coordinate to its environment can be isolated and examined. In order to examine the coupling of the enzyme's structure and dynamics to its chemical step, we studied two dynamically altered mutants. These mutants are designed at residues far from the active site and are unlikely to affect protein folding, and they are produced by means of site-directed mutagenesis. Such a design minimizes the effects of direct molecular interaction with the ligands or their first solvation residues, and the role of long-range (whole protein) dynamics can be examined. Out of many such remote positions, we chose to test those that are highly conserved in evolution. Common sense suggests that these are more likely to serve an important role in catalysis.

Here, we use the kinetic method established for the wild-type (WT) DHFR from *E. coli* (Sikorski *et al.* 2004) to study a mutant of this enzyme at a position that is 15 Å away from the active site (M42W; figure 1). This residue has been implicated by Sharon Hammes-Schiffer and co-workers (Agarwal *et al.* 2002a,b; Wong *et al.* 2005) as possibly having a dynamic role in catalysis by high-level quantum mechanical/molecular mechanical calculations. Furthermore, simple structural examination (using SYBYL computer package to visualize the protein) suggested that large hydrophobic substitution of the methionine (M) into tryptophan (W) is not likely to alter the structure of the enzyme or its interaction with either ligand (nicotinamide adenine dinucleotide phosphate (NADPH) or 7,8-dihydrofolate (DHF)). The findings with this mutant are compared to the equivalent findings for the WT and other remote mutants (G121V) of DHFR. The comparison reveals interesting and intriguing features regarding the role of remote residues in catalysis and the interpretation of observed rates as discussed later.

2. MATERIAL AND METHODS

(a) Materials

All materials were obtained from Sigma unless indicated. DHF was prepared by dithionite reduction of folic acid as described by Blakely (1960). [1-²H]-Glucose available from

Sigma consists of *ca* 98% deuterium content, which is insufficient for use in competitive KIE measurements. [1-²H]-Glucose was prepared by reduction of δ -gluconolactone with 5% sodium mercury amalgam in 99.96% deuterium oxide (D₂O; Goodman *et al.* 1989). The C1 deuterium content of the product was over 99.9% as determined by ¹H-NMR.

(i) *Synthesis of labelled cofactors for primary kinetic isotope effects*
R [4-²H]-NADPH was prepared through stereospecific reduction of NADP⁺ with 2-propanol-d₈ (greater than 99.7% D at 2C as determined by ¹H-NMR) using alcohol dehydrogenase from *Thermoanaerobium brockii* (*tb*ADH; Jeong & Greedy 1994; Agrawal & Kohen 2003). *R* [4-³H]-NADPH (680 mCi mmol⁻¹) was synthesized by reduction of NADP⁺ with [1-³H]-glucose using glucose dehydrogenase (*GluDH*) from *Cryptococcus uniguttulatus*, followed by oxidation of the resulting NADPH with acetone using *tb*ADH and then a second reduction with unlabelled glucose using *GluDH* as described in more detail elsewhere (McCracken *et al.* 2003). [Ad-¹⁴C]-NADPH (50 mCi mmol⁻¹) was prepared by 2'-phosphorylation of [Ad-¹⁴C]-nicotinamide adenine dinucleotide (NAD⁺) using an NAD⁺ kinase from chicken liver to produce [Ad-¹⁴C]-NADP⁺, followed by reduction with glucose using *GluDH* as described elsewhere (Markham *et al.* 2004). *R* [4,4-²H,³H]-NADPH (680 mCi mmol⁻¹) was prepared by following a three-step procedure: NADP⁺ was reduced to *S* [4-³H]-NADPH with [1-³H]-glucose using *GluDH*, oxidized by acetone using *tb*ADH, and finally the resulting [4-³H]-NADP⁺ was reduced with [1-²H]-glucose using *GluDH*. Prior to the final reduction, *tb*ADH was removed by ultrafiltration. [Ad-¹⁴C, 4-²H₂]-NADPH (50 mCi mmol⁻¹) was prepared from [Ad-¹⁴C]-NADP⁺, which was synthesized as described previously, and then reduced with [1-²H]-glucose to produce 4*S* [Ad-¹⁴C, 4-²H]-NADPH using *GluDH*. 4*S* [Ad-¹⁴C, 4-²H]-NADPH was then oxidized with acetone to produce [Ad-¹⁴C, 4-²H]-NADP⁺ using *tb*ADH, followed by a second reduction with [1-²H]-glucose.

All synthesized cofactors were purified by semi-preparative reverse-phase high-performance liquid chromatography (HPLC) on a Supelco Discovery C18 column (25 cm × 10 mm, 5 µm) as described earlier (Markham *et al.* 2003) and lyophilized for long-term storage at -80 °C.

(ii) Enzyme preparation

The M42W mutant of *E. coli* DHFR (M42W-*ec*DHFR) was expressed, purified and stored as discussed elsewhere (Cameron & Benkovic 1997; Rajagopalan *et al.* 2002).

(b) Methods

(iii) Preparation of samples for competitive kinetic isotope effect experiments

For primary KIE measurements, [Ad-¹⁴C]-NADPH and *R* [4-³H]-NADPH (protium/tritium (H/T) experiments) or [Ad-¹⁴C, 4-²H₂]-NADPH and *R* [4,4-²H,³H]-NADPH (deuterium/tritium (D/T) experiments) were combined in radioactivity ratio close to 1 : 6 (¹⁴C/³H, compensating for the lower efficiency of tritium scintillation counting). Each of the mixtures was copurified by reverse-phase HPLC on a Supelco Discovery C18 column (25 cm × 4.6 mm, 5 µm), divided into aliquots containing 300 000 d.p.m. of ¹⁴C and frozen in liquid nitrogen for short-term storage (less than three weeks) at -80 °C.

(iv) *Competitive kinetic isotope effects*

All the experiments were performed in 50 mM 2-morpholinoethanesulphonic acid, 25 mM Tris, 25 mM ethanolamine and 100 mM NaCl (MTEN) at pH 8.0 under an atmosphere of oxygen over a range of 5–45 °C. In each experiment, one aliquot of the copurified labelled NADPH was thawed just before use. DHF was added to the reaction mixture to a final concentration of 0.85 mM (approximately 200-fold in excess over the 4 µM NADPH). The final volume was brought to 1040 µl by adding MTEN and the pH was readjusted to 8.0 at the experimental temperature. Oxygen was bubbled through the reaction mixture for 5 min to ensure the trapping of the product 5,6,7,8-tetrahydrofolate (THF). Before initiating the reaction, two 100 µl samples ($t=0$) were withdrawn. Two other 100 µl aliquots for infinite time samples ($t=\infty$) were also removed and WTecDHFR (approx. 0.2 unit) was added to each of the latter two samples to ascertain a complete fractional conversion. The reaction was initiated by adding M42W-DHFR. At various time points, 100 µl aliquots were withdrawn at fractional conversions (f) ranging from 25 to 85% as determined from the ^{14}C distribution between NADPH and NADP^+ . All the samples were quenched with methotrexate (final concentration, 1.8 mM). The overall reaction ran between 15 and 40 min and all the quenched samples were immediately frozen and stored in dry ice.

Prior to HPLC–liquid scintillation counter (LSC) analysis, the samples were thawed and bubbled with oxygen for 3 min. The samples were then injected into reverse-phase HPLC for separation of the reactants and products using the analytical method described elsewhere (Markham *et al.* 2003). Fractions (0.8 ml) were collected every minute, mixed with 10 ml Ultima Gold liquid scintillation cocktail (Perkin-Elmer) and stored in the dark for over 24 h before β -emission analysis was performed with a Packard Tricarb Tr2900 LSC for 5 min per sample. Infinite time samples ($t=\infty$) were processed in the same way as the time points (R_t) to estimate background levels of radiation and the ratios of $^3\text{H}/^{14}\text{C}$ at $t=\infty$ (R_∞). Blank samples ($t=0$) were used to assure the quality and purity of the labelled NADPHs. The observed KIEs were calculated according to Melander & Saunders (1987):

$$\text{KIE} = \frac{\ln(1-f)}{\ln[1-f(R_t/R_\infty)]}, \quad (2.1)$$

where the fractional conversion (f) was determined from the ratio of ^{14}C in product to the total ^{14}C , which was calculated from:

$$f = \frac{[\text{Ad-}^{14}\text{C}]\text{NADP}^+}{[\text{Ad-}^{14}\text{C}]\text{NADP}^+ + [\text{Ad-}^{14}\text{C}]\text{NADPH}}, \quad (2.2)$$

and R_t and R_∞ are ratios of $^3\text{H}/^{14}\text{C}$ in the products at various fractional conversions and at 100% conversion, respectively. Each experiment yielded at least five time points and was performed at least in duplicate.

(v) *Intrinsic kinetic isotope effects*

The intrinsic D/T KIEs were derived by numerically solving the following equation (Northrop 1975, 1977, 1991; Kohen 2003, 2005; Cleland 2005):¹

$$\frac{T(V/K)_{\text{D,obs}}^{-1} - 1}{T(V/K)_{\text{H,obs}}^{-1} - 1} = \frac{(k_{\text{D}}/k_{\text{T}})^{-1} - 1}{(k_{\text{D}}/k_{\text{T}})^{-3.34} - 1}, \quad (2.3)$$

where $T(V/K)_{\text{H,obs}}$ and $T(V/K)_{\text{D,obs}}$ are the observed H/T and D/T KIEs, respectively, and $k_{\text{D}}/k_{\text{T}}$ is the intrinsic D/T KIE. The intrinsic H/T KIE is formulated as $(k_{\text{D}}/k_{\text{T}})^{3.34}$ (Streitwieser *et al.* 1958; Kohen 2003). In the procedure, it is assumed that the Swain–Schaad relationship holds or has little temperature dependence for intrinsic primary KIEs

(Northrop 1991; Francisco *et al.* 2002; Kohen 2003; Cleland 2005). In systems where non-classical isotope effects have been suggested from temperature dependency or theoretical simulation, the experimental relationship between primary H/T and D/T KIEs has been shown to be very close to the semi-classical limit (Northrop 1991; Jonsson *et al.* 1994; Bahnson *et al.* 1997; Kohen & Klinman 1999; Chin & Klinman 2000). Several recent gas-phase calculations for a small model reaction also resulted in a Swain–Schaad exponent that did not deviate significantly from its semi-classical value at ambient temperature (300–350 K; Kiefer & Hynes 2003, 2005; Tautermann *et al.* 2004; Smedarchina & Siebrand 2005; Smedarchina *et al.* 2005). Furthermore, even if the magnitude of the exponent (3.3) was altered for a full tunnelling model, it is not expected to change over the narrow temperature range under study and, hence, should not affect the trend manifested in the temperature dependence of the intrinsic isotope effects (Francisco *et al.* 2002; Sikorski *et al.* 2004). To eliminate other common assumptions associated with this procedure (Northrop 1991), the overall reaction needs to be irreversible. Accordingly, the experiments were designed to ensure the irreversibility of the overall reaction by trapping the product THF with oxygen (Markham *et al.* 2003). Values of intrinsic H/T, D/T and (H/D) hydrogen/deuterium KIEs were calculated numerically using the appropriate modification of equation (2.3) (Northrop 1991; Cleland 2005). Standard errors on the intrinsic KIEs were estimated by calculating the intrinsic KIEs from the raw data (without an averaging procedure) followed by standard error analysis as described by Francisco *et al.* (2002).

The isotope effects on the activation parameters for the intrinsic KIEs were calculated by fitting them into the Arrhenius equation for KIEs:

$$k_{\text{L}}/k_{\text{I}} = A_{\text{L}}/A_{\text{I}}e^{\Delta E_{\text{aI-L}}/RT}, \quad (2.4)$$

where L represents H or D and I, D or T. $A_{\text{L}}/A_{\text{I}}$ and $\Delta E_{\text{aI-L}}$ are the isotope effects on the pre-exponential Arrhenius factors and the difference in activation energy between L and I, respectively.

(vi) *Rate measurements*

Initial velocity measurements were obtained under saturating conditions of both the substrate and cofactor (100 µM) by monitoring the decrease in 340 nm absorbance ($\Delta\epsilon_{340} = 13.2 \text{ mM}^{-1} \text{ cm}^{-1}$; Miller & Benkovic 1998a,b). The experiments were carried out over a temperature range of 5–45 °C using a Hewlett-Packard 8453 series UV/vis spectrophotometer equipped with a water-jacketed cuvette holder. All the assays were performed in MTEN buffer (pH 8.0, adjusted at the experimental temperature) containing 1 mM dithiothreitol. In a typical experiment, 30 µl of 100 nM enzyme were pre-incubated with 100 µM cofactor, and the reaction initiated by adding 100 µM substrate. DHF and NADPH saturations were ensured by doubling the concentrations of DHF and NADPH at the temperature extremes, which did not affect the measured rates (thus indicating a true V_{max} measurement). All the measurements were performed at least in triplicate and standard deviations for each temperature set were computed. Calculated k_{cat} values for each temperature were fit to the Arrhenius equation using a nonlinear least-squares regression, in which errors were weighted using the standard deviations.

Scheme 1. The DHFR-catalysed reaction. R≡adenine dinucleotide 2'-P, R'≡(p-aminobenzoyl) glutamate.

3. RESULTS AND DISCUSSION

(a) *Competitive primary kinetic isotope effects*

Mixed labelling experiments were conducted with M42W-*ec*DHFR using the method previously used to study the WT *ec*DHFR (Sikorski *et al.* 2004). Primary H/T and D/T V/K KIE measurements were conducted as described briefly in Sikorski *et al.* (2004) and in more detail in §2*b*. As for the WT enzyme, the intrinsic H/D, H/T and D/T KIEs were calculated numerically from the observed H/T and D/T KIEs using the methodology developed by Northrop (Northrop 1991; Cleland 2005; Kohen 2005). The average intrinsic KIEs and their respective standard errors were then used in calculating the isotope effects on the activation parameters. Tables summarizing the observed and intrinsic primary H/T and D/T isotope effects over a temperature range from 5 to 45 °C are given in the electronic supplementary material (figure 1). Figure 2 presents the observed (open squares) and intrinsic (filled squares) KIEs with G121V-*ec*DHFR. Figure 3 compares the intrinsic H/T KIEs of M42W to those of the WT (Sikorski *et al.* 2004) and G121V (Wang *et al.* 2006). The relations of H/D and D/T follow the same trend as H/T KIEs (not shown). Apparently, the intrinsic KIEs for M42V are slightly larger than those measured with the WT, but somewhat smaller than those measured with G121V. The KIEs of M42W are more temperature-dependent than of either the WT (presenting no temperature dependence within experimental errors) or the G121V mutant (presenting marginal temperature dependence within experimental errors).

Figure 2 reveals that the observed KIEs (in the second order rate constant k_{cat}/K_M) are smaller than their corresponding intrinsic KIEs. This is a common feature that can be rationalized as isotopically insensitive kinetic steps that 'mask' the intrinsic KIEs. Since the KIEs in figure 2 were measured under irreversible reaction conditions, the kinetic complexity can be formulated as follows (Northrop 1991; Cleland 1991):

$$^T(k_{\text{cat}}/K_M)_{\text{L-obs}} = \frac{k_{\text{L}}/k_{\text{T}} + C_{\text{f}}}{1 + C_{\text{f}}}, \quad (3.1)$$

where $^T(k_{\text{cat}}/K_M)_{\text{L-obs}}$ is the L/T KIE in k_{cat}/K_M (L=H or D), $k_{\text{L}}/k_{\text{T}}$ is the intrinsic L/T KIE on the H-transfer step and C_{f} represents the commitment to catalysis. C_{f} is the sum of the ratios between the rate of the forward isotopically sensitive hydride transfer step and each of the rates of the preceding backward isotopically insensitive steps. C_{f} is much smaller for D than for H

(D is more 'rate limiting' to begin with) and the observed D/T KIEs are much closer to their intrinsic values (see figure 2).

Figure 4 compares the C_{f} values of the WT-, G121V- and M42W-DHFRs at a temperature range of 5–45 °C. The data are presented as an Arrhenius plot to appear consistent with figures 2 and 3. None of the intrinsic KIEs of these enzymes are curved (see figure 3 and Sikorski *et al.* 2004; Wang *et al.* submitted). Yet, the temperature dependencies of their observed KIEs and C_{f} are complex. These comparisons demonstrate that the effects on the intrinsic KIEs and the observed ones are not related to each other by any simple or continuous function. This observation further emphasizes the great care that needs to be practiced when analysing measured KIEs and their temperature dependence, and the importance of exposing intrinsic effects when possible.

(b) *Parameters of activation and their isotope effects*

Figure 2 presents Arrhenius plots (KIE on a logarithmic scale versus the reciprocal of the absolute temperature) for the observed and intrinsic KIEs with M42W-DHFR. While the intrinsic KIEs for the WT enzyme appeared temperature-independent, those of M42W are a little larger and more temperature-dependent (figure 3). The temperature dependence was further examined by fitting the intrinsic KIEs to equation (2.4) (the lines in figures 2 and 3), and the fitted isotope effects on the Arrhenius pre-exponential factors ($A_{\text{I}}/A_{\text{H}}$) are summarized in table 1. Table 1 also compares these parameters to those of the WT (Sikorski *et al.* 2004) and the G121V (Wang *et al.* 2006). The reason why $A_{\text{I}}/A_{\text{H}}$ is summarized, but the isotope effects on the slopes are not, is that semi-classical limits for $A_{\text{I}}/A_{\text{H}}$ were calculated using a variety of methods and simulations (Schneider & Stern 1972; Stern & Weston 1974; Bell 1980; Melander & Saunders 1987; Kohen *in press*) making $A_{\text{I}}/A_{\text{H}}$ a useful indicator for H-tunnelling (Kohen 2003). Apparently, all DHFR isozymes discussed here have $A_{\text{I}}/A_{\text{H}}$ larger than unity, but those for M42W are closer to the upper semi-classical limit (within experimental errors) as presented in table 1. Traditionally, an inflated $A_{\text{I}}/A_{\text{H}}$ value indicates tunnelling of both heavy and light isotopes (Kohen & Klinman 1999). Yet the relatively small size of the KIEs and the measured energies of activation cannot be explained by such tunnelling correction, and

Figure 1. Structure of *E. coli* DHFR with bound NADP⁺ (orange) and folic acid (red; PDB 1r×2). Amino acid side chains of M42 and G121 are shown as green and blue spheres, respectively.

Figure 2. Arrhenius plot of observed (open squares) and intrinsic (filled squares) KIEs for the M42W-ecDHFR (from tables 1 and 2 in the electronic supplementary material). H/T KIEs in dark grey, H/D KIEs in light grey and D/T KIEs in black. The lines represent the nonlinear regression to equation (2.4).

‘Marcus-like’ models (discussed in the following section) appear to explain the findings better (Kohen 2005). These models can also be used to rationalize the lack of isotope effects on the activation energy for the WT enzyme and the small, but non-zero, ΔE_a values for both mutants.

(c) Marcus-like models (environmentally coupled tunnelling)

Models assuming a one-dimensional rigid potential surface successfully reproduced temperature-independent large KIEs with no activation energy of the isotopically sensitive step (Kohen *et al.* 1999).

Figure 3. Comparison of the Arrhenius plots of intrinsic H/T KIEs of the wild-type (dark grey; Sikorski *et al.* 2004), G121V (light grey; Wang *et al.* 2006) and M42W (black; table 2 in the electronic supplementary material) DHFRs.

Figure 4. Comparison of the Arrhenius plots of the commitment to catalysis (C_t) on k_{cat}/K_M for the wild-type (dark grey; Sikorski *et al.* 2004), G121V (light grey; Wang *et al.* 2006) and M42W (black; tables 1 and 2 in the electronic supplementary material and equation (3.1)) DHFR.

However, such models cannot explain temperature-independent small KIEs with significant activation energy. Several phenomenological models were developed in recent years that fall under the title Marcus-like models (e.g. Borgis *et al.* 1989; Borgis & Hynes 1993, 1996; Antoniou *et al.* 2002; Knapp & Klinman 2002; Knapp *et al.* 2002; Sutcliffe & Scrutton 2002; Kohen 2003, 2005, in press). All these models were constructed assuming that rates and KIEs can be measured for a single kinetic step (the chemical step), and thus are relevant to the experimental measurements described here. Although these different models were constructed from very different basic principles, they all share several mathematical and

Table 1. Comparative isotope effects on Arrhenius pre-exponential factors.

A_i/A_h	WT	G121V	M42W	S.C. upper limits ^a
H/T	7.0 ± 1.5	6.7 ± 0.8	2.9 ± 1.6	1.6
H/D	3.5 ± 0.5	3.8 ± 0.4	2.1 ± 0.6	1.4
D/T	1.70 ± 0.14	1.76 ± 0.07	1.35 ± 0.16	1.2
rates (s^{-1}) ^b	228	1.4	5.6	

^a Semi-classical upper limits (Stern & Weston 1974; Bell 1980; Melander & Saunders 1987).

^b Pre-steady-state rates of H-transfer at 25 °C and pH 7 (Rajagopalan *et al.* 2002).

physical aspects. They separate the temperature dependence of the reaction rate from that of the KIEs mathematically. This feature enables the rationalization of systems with or without temperature-dependent KIEs whether the barrier for the reaction is significant or not. From the physical point of view, all these models suggest that (i) the hydrogen should be treated quantum mechanically throughout the reaction coordinate (including tunnelling); (ii) fluctuations of the reaction's potential surface occur at a time-scale similar to or slower than the H-transfer rate, and thus determine the overall rate of H-transfer (the solvent coordinate is the reaction coordinate as phrased by Kiefer & Hynes (2003, 2005); and (iii) these fluctuations can be treated as two orthogonal vibrations, one that represents fluctuations in the donor–acceptor distance and the second that represents changes in the system's symmetry (figure 5). Various terms have been coined in these models to characterize H-transfer in an enzymatic system, including 'vibrationally enhanced tunnelling' (Sutcliffe & Scrutton 2002), 'rate-promoting vibrations' (Antoniou *et al.* 2002) and 'environmentally coupled tunnelling' (ECT; Knapp & Klinman 2002; Knapp *et al.* 2002). In this paper, we use the term ECT that was coined by Knapp & Klinman (2002), although the terms coined by others are just as valid. In this model, the changes in the system's symmetry are referred to as system 'rearrangement' (the 'Marcus' term) and fluctuations of the donor–acceptor distance as gating (the 'Frank–Condon' term).

According to this Marcus-like interpretation, the lack of temperature dependence of the KIEs and the large A_i/A_h for the WT *ec*DHFR (figure 3) are rationalized as a perfect rearrangement (the p coordinate in figure 5) of the potential surface, so that the average donor–acceptor distance is optimized for tunnelling and no thermal fluctuations along the q coordinate are needed. For the G121V mutant, the slightly inflated KIEs and their small, but non-zero, temperature dependence would indicate that the rearrangement is not perfect, and the average donor–acceptor distance is longer than the WT enzyme. Accordingly, some thermally activated fluctuations along the q coordinate are required, leading to the slight temperature dependence of the KIEs. For the M42W mutant, the steeper temperature dependence of the KIEs suggests an even less perfect rearrangement and a longer average donor–acceptor distance at the tunnelling conformation. This is interesting because

Figure 5. Illustration of 'Marcus-like' models: energy surface of environmentally coupled hydrogen tunnelling. Two orthogonal coordinates are presented: p , the environmental energy parabolas for the reactant state (R in grey) and the product state (P in black); and q , the H-transfer potential surface at each p configuration. The grey shapes represent the populated states (e.g. the location of the particle). The original Marcus expression would have a fixed q distance between donor and acceptor. Thermal fluctuations of that distance (denoted 'gating' by Knapp & Klinman 2002) lead to the temperature dependency of the KIE.

the M42W mutation reduces the observed H-transfer rate less than the G121V mutation, relative to the WT (Cameron & Benkovic 1997; Rajagopalan *et al.* 2002, table 1). This observation suggests that the work term along the p coordinate (also depicted as reorganizational energy) is larger for G121V, smaller for M42W and even smaller for the WT. In contrast to this trend, the average distance between donor and acceptor (the left panel in figure 5) is perfect for the WT, altered for the G121V mutant, and even less perfect for the M42W mutant. Consequently, H-tunnelling with G121V seems to occur at a conformation closer to that of the WT relative to the M42W.

4. CONCLUSIONS

The present work examined the effect of the M42W *ec*DHFR mutant on the C–H–C transfer step, which is the step where covalent bonds are cleaved and made (cf. the chemical step)². These effects are compared to those of the WT-DHFR and another remote mutant on a residue at the opposite side of the enzyme's active site (G121V-DHFR). The effects of the remote mutation (M42W, 15 Å from the enzyme's active site) on the nature of the hydride transfer along the reaction coordinate were studied by means of intrinsic KIEs and their temperature dependence. The findings were compared to similar examinations of the WT enzyme and another distal mutation at the opposite side of the active site (G121V, 19 Å from the enzyme's active site). This comparison may lead to several conclusions of general interest in enzymology and protein chemistry in general. As demonstrated in figure 3 and table 1 and discussed earlier, the nature of the H-transfer seems to

be altered for the M42W mutant more than for the G121V mutant. The larger temperature dependence of the KIEs and smaller A_h/A_h values for the M42W mutation seem to indicate a longer donor–acceptor distance along the q coordinate (figure 5) than the G121V mutation. This suggests that the work term along the p coordinate is larger for G121V, but H-tunnelling occurs at a conformation closer to that of the WT relative to the M42W. Yet the M42W mutation does not reduce the observed H-transfer rate as much as G121V does, relative to the WT enzyme (Cameron & Benkovic 1997; Rajagopalan *et al.* 2002). This is of general interest suggesting that when a distal mutation alters the catalytic potential surface, no correlation should be expected between effects on the p coordinate ('reorganization') and the q coordinate (gating). It is possible that the H-transfer rate will change dramatically with little change in the nature of the H-transfer step and the temperature dependence of the KIEs, as it is possible that the rate will not be changed, but the nature of the H-tunnelling will be significantly altered.

It is commonly anticipated that efficient quantum mechanical tunnelling and coupling of the tunnelling to the environment are indications of a highly evolved enzymatic system (Knapp & Klinman 2002; Sutcliffe & Scrutton 2002; Klinman 2003; Liang *et al.* 2004). This indeed seems to be the case of the WT *ec*DHFR (Sikorski *et al.* 2004; Wong *et al.* 2005) and several other enzymes (Kohen & Klinman 1998, 1999; Kohen *et al.* 1999; Scrutton 1999; Scrutton *et al.* 1999; Harris *et al.* 2000; Basran *et al.* 2001; Knapp & Klinman 2002; Sutcliffe & Scrutton 2002; Klinman 2003; Francisco *et al.* 2003; Liang *et al.* 2004; Kohen 2005, *in press*). The present findings suggest that this is not due to direct correlation between the reaction rate and the contribution of the tunnelling, since effects on observed rates (e.g. effects on k_{cat} , k_{cat}/K_M , or even pre-steady-state rates) and on the nature of the chemical step do not necessarily correlate. The role of the tunnelling and coupling of the reaction coordinate to its environment may not be directly aimed at speeding up the reaction. Similarly, studies with mutants of lipoxygenase (Rickert & Klinman 1999; Klinman 2003) did not indicate correlation between k_{cat} KIEs and degrees of tunnelling or temperature dependence.

Finally, it is likely that only high-level theoretical studies such as those conducted for the WT and G121V DHFRs (Radkiewicz & Brooks 2000; Agarwal *et al.* 2002a,b; Garcia-Viloca *et al.* 2003a–c; Rod *et al.* 2003; Thorpe & Brooks 2004; Pu *et al.* 2005) may shed light on the role of tunnelling and the whole protein motion/dynamics in catalysis. We hope that the present report will attract the interest of theoreticians and will lead to relevant high-level calculations that will further shed light on the role of such remote residues and the dynamics of the protein in enzyme catalysis. More experiments are under way in an attempt to expose the relations between residues, G121 and M42, and their predicted mutual effect on the H-transfer step with DHFR. Such effects were predicted by Hammes-Schiffer and co-workers (Wong *et al.* 2005) and also addressed by Brooks and co-workers (Radkiewicz & Brooks 2000; Rod *et al.* 2003; Thorpe & Brooks 2004).

Future experimental efforts will examine the nature of H-transfer with double and even triple mutants, and will further probe for correlation between the chemical step and effects on the structure and dynamics of the whole enzyme.

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ENDNOTES

¹Equation (2.3) cannot be solved analytically. Northrop (1991) offers a table (its Appendix 1) that has numerically calculated values for a range of observed KIEs. More recently, we posted on our web site a free of charge JAVA script-based program that can numerically solve equation (2.1) for any experiment of interest (URL: cricket.chem.uiowa.edu/kohen/tools.html).

²For the purpose of this work hydrogen-bonds are not considered covalent bonds.

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)
- Agrawal, N. & Kohen, A. 2003 Microscale synthesis of 2-tritiated isopropanol and 4R-tritiated reduced nicotinamide adenine dinucleotide phosphate. *Anal. Biochem.* **322**, 179–184. (doi:10.1016/j.ab.2003.08.012)
- Antoniou, D., Caratzoulas, S., Kalyanaraman, C., Mincer, J. S. & Schwartz, S. D. 2002 Barrier passage and protein dynamics in enzymatically catalyzed reactions. *Eur. J. Biochem.* **269**, 3103–3112. (and many cited therein) (doi:10.1046/j.1432-1033.2002.03021.x)
- Bahnsen, B. J., Colby, T. D., Chin, J. K., Goldstein, B. M. & Klinman, J. P. 1997 A link between protein structure and enzyme catalyzed hydrogen tunneling. *Proc. Natl Acad. Sci. USA* **94**, 12 797–12 802. (doi:10.1073/pnas.94.24.12797)
- Basran, J., Sutcliffe, M. J. & Scrutton, N. S. 2001 Deuterium isotope effects during carbon-hydrogen cleavage by trimethylamine dehydrogenase. *J. Biol. Chem.* **276**, 24 581–24 587. (doi:10.1074/jbc.M101178200)
- Bell, R. P. 1980 *The tunnel effect in chemistry*. London, UK and New York, NY: Chapman & Hall.
- Benkovic, S. J. & Hammes-Schiffer, S. 2003 A perspective on enzyme catalysis. *Science* **301**, 1196–1202. (doi:10.1126/science.1085515)
- Blakley, R. L. 1960 Crystalline dihydropteroylglutamic acid. *Nature* **188**, 231–232.
- Borgis, D. & Hynes, J. T. 1993 Dynamical theory of proton tunneling transfer rates in solution: general formulation. *Chem. Phys.* **170**, 315–346. (doi:10.1016/0301-0104(93)85117-Q)
- Borgis, D. C. & Hynes, J. T. 1996 Curve crossing formulation for proton transfer reactions in solution. *J. Phys. Chem.* **100**, 1118–1128. (doi:10.1021/jp9522324)
- Borgis, D. C., Lee, S. Y. & Hynes, J. T. 1989 A dynamical theory of nonadiabatic proton and hydrogen atom transfer reaction rates in solution. *Chem. Phys. Lett.* **162**, 19–26. (doi:10.1016/0009-2614(89)85059-6)
- 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)
- Chin, J. K. & Klinman, J. P. 2000 Probes of a role for remote binding interactions on hydrogen tunneling in the horse liver alcohol dehydrogenase reaction. *Biochemistry* **39**, 1278–1284. (doi:10.1021/bi9920331)
- Cleland, W. W. 1991 Multiple isotope effects in enzyme-catalyzed reactions. In *Enzyme mechanism from isotope effects* (ed. P. F. Cook), pp. 247–268. Boca Raton, FL: CRC Press.
- Cleland, W. W. 2005 Enzyme mechanisms from isotope effects. In *Isotope effects in chemistry and biology* (ed. A. Kohen & H. H. Limbach), pp. 915–930. New York, NY: Taylor & Francis/CRC Press.
- Fersht, A. 1998 *Structure and mechanism in protein sciences: a guide to enzyme catalysis and protein folding*. New York, NY: Freeman.
- 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)
- Francisco, W. A., Knapp, M. J., Blackburn, N. J. & Klinman, J. P. 2002 Hydrogen tunneling in peptidylglycine-hydroxylating monooxygenase. *J. Am. Chem. Soc.* **124**, 8194–8195. (doi:10.1021/ja025758s)
- Francisco, W. A., Blackburn, N. J. & Klinman, J. P. 2003 Oxygen and hydrogen isotope effects in an active site tyrosine to phenylalanine mutant of peptidylglycine alpha-hydroxylating monooxygenase: mechanistic implications. *Biochemistry* **42**, 1813–1819. (doi:10.1021/bi020592t)
- Garcia-Viloca, M., Truhlar, D. G. & Gao, J. 2003a Reaction-path energetics and kinetics of the hydride transfer reaction catalyzed by dihydrofolate reductase. *Biochemistry* **42**, 13 558–13 575. (doi:10.1021/bi034824f)
- Garcia-Viloca, M., Truhlar, D. G. & Gao, J. L. 2003b Importance of substrate and cofactor polarization in the active site of dihydrofolate reductase. *J. Mol. Biol.* **327**, 549–560. (doi:10.1016/S0022-2836(03)00123-2)
- Garcia-Viloca, M., Gao, J., Karplus, M. & Truhlar, D. G. 2003c How enzymes work: analysis by modern rate theory and computer simulations. *Science* **303**, 186–195. (doi:10.1126/science.1088172)
- Goodman, M. N., Masuoka, L. K., DeRopp, J. S. & Jones, A. D. 1989 Use of deuterium-labeled glucose in evaluating the pathway of hepatic glycogen synthesis. *Biochem. Biophys. Res. Commun.* **159**, 522–527. (doi:10.1016/0006-291X(89)90024-7)
- Harris, R. J., Meskys, R., Sutcliffe, M. J. & Scrutton, N. S. 2000 Kinetic studies of the mechanism of carbon-hydrogen bond breakage by the heterotetrameric sarcosine oxidase of *Arthrobacter* sp. 1-IN. *Biochemistry* **39**, 1189–1198. (doi:10.1021/bi991941v)
- Jencks, W. P. 1987 *Catalysis in chemistry and enzymology*. New York, NY: McGraw-Hill.
- Jeong, S. S. & Gready, J. E. 1994 A method of preparation and purification of (4R)-deuterated-reduced nicotinamide adenine dinucleotide phosphate. *Anal. Biochem.* **221**, 273–277. (doi:10.1006/abio.1994.1411)
- Jonsson, T., Edmondson, D. E. & Klinman, J. P. 1994 Hydrogen tunneling in the flavoenzyme monoamine oxidase B. *Biochemistry* **33**, 14 871–14 878. (doi:10.1021/bi00253a026)
- Kiefer, P. M. & Hynes, J. T. 2003 Kinetic isotope effects for adiabatic proton transfer reactions in a polar environment. *J. Phys. Chem. A* **107**, 9022–9039. (doi:10.1021/jp030893s)
- Kiefer, P. M. & Hynes, J. T. 2005 Interpretation of primary kinetic isotope effects for adiabatic and nonadiabatic proton transfer reactions in a polar environment. In *Isotope effects in chemistry and biology* (ed. A. Kohen & H. H. Limbach), pp. 549–578. Boca Raton, FL: Taylor & Francis/CRC Press.
- Klinman, J. P. 2003 Dynamic barriers and tunneling. New views of hydrogen transfer in enzyme reactions. *Pure Appl. Chem.* **75**, 601–608.
- Knapp, M. J. & Klinman, J. P. 2002 Environmentally coupled hydrogen tunneling. Linking catalysis to dynamics. *Eur. J. Biochem.* **269**, 3113–3121. (doi:10.1046/j.1432-1033.2002.03022.x)
- Knapp, M. J., Rickert, K. & Klinman, J. P. 2002 Temperature-dependent isotope effects in soybean lipoxygenase-1: correlating hydrogen tunneling with protein dynamics. *J. Am. Chem. Soc.* **124**, 3865–3874. (doi:10.1021/ja012205t)
- Kohen, A. 2003 Kinetic isotope effects as probes for hydrogen tunneling, coupled motion and dynamics contributions to enzyme catalysis. *Prog. React. Kin. Mech.* **28**, 119–156.
- Kohen, A. 2005 Kinetic isotope effects as probes for hydrogen tunneling in enzyme catalysis. In *Isotope effects in chemistry and biology* (ed. A. Kohen & H. H. Limbach), pp. 743–764. New York, NY: Taylor & Francis/CRC Press.
- Kohen, A. In press. Probes for hydrogen tunneling and coupled motion in enzymatic systems. In *Biological aspects of hydrogen transfer* (ed. Schowen R. L., Klinman J. P. & Hynes J. T.), Weinheim, DE: Wiley.
- Kohen, A. & Klinman, J. P. 1998 Enzyme catalysis: beyond classical paradigms. *Acc. Chem. Res.* **31**, 397–404. (doi:10.1021/ar9701225)
- Kohen, A. & Klinman, J. P. 1999 Hydrogen tunneling in biology. *Chem. Biol.* **6**, R191–R198. (doi:10.1016/S1074-5521(99)80058-1)
- Kohen, A., Cannio, R., Bartolucci, S. & Klinman, J. P. 1999 Enzyme dynamics and hydrogen tunneling in a thermophilic alcohol dehydrogenase. *Nature* **399**, 496–499. (doi:10.1038/20981)
- Liang, Z. X., Lee, T., Resing, K. A., Ahn, N. G. & Klinman, J. P. 2004 Thermal activated protein mobility and its correlation with catalysis in thermophilic alcohol dehydrogenase. *Proc. Natl Acad. Sci. USA* **101**, 9556–9561. (doi:10.1073/pnas.040337101)
- Markham, K. A., Sikorski, R. S. & Kohen, A. 2003 Purification, analysis, and preservation of reduced nicotinamide adenine dinucleotide 2'-phosphate. *Anal. Biochem.* **322**, 26–32. (doi:10.1016/j.ab.2003.07.010)
- Markham, K. A., Sikorski, R. S. & Kohen, A. 2004 Synthesis and utility of ¹⁴C labeled nicotinamide cofactors. *Anal. Biochem.* **325**, 62–67. (doi:10.1016/j.ab.2003.10.027)
- McCracken, J. A., Wang, L. & Kohen, A. 2003 Synthesis of R and S tritiated reduced b-nicotinamide adenine dinucleotide 2' phosphate. *Anal. Biochem.* **324**, 131–136. (doi:10.1016/j.ab.2003.09.025)
- McElheny, D., Schnell, J. R., Lansing, J. C., Dyson, H. J. & Wright, P. E. 2005 Defining the role of active-site loop fluctuations in dihydrofolate reductase catalysis. *Proc. Natl Acad. Sci. USA* **102**, 5032–5037. (doi:10.1073/pnas.0500699102)
- Melander, L. & Saunders, W. H. 1987 *Reaction rates of isotopic molecules*, 4th edn. Malabar, FL: Krieger.
- Miller, G. P. & Benkovic, S. J. 1998a Deletion of a highly motional residue affects formation of the Michaelis complex for *Escherichia coli* dihydrofolate reductase. *Biochemistry* **37**, 6327–6335. (doi:10.1021/bi972922t)
- Miller, G. P. & Benkovic, S. J. 1998b Stretching exercises—flexibility in dihydrofolate reductase catalysis. *Chem. Biol.* **5**, R105–R113. (doi:10.1016/S1074-5521(98)90616-0)

- Northrop, D. B. 1975 Steady-state analysis of kinetic isotope effects in enzymatic reactions. *Biochemistry* **14**, 2644–2651. (doi:10.1021/bi00683a013)
- Northrop, D. B. 1977 Determining the absolute magnitude of hydrogen isotope effects. In *Isotope effects on enzyme-catalyzed reactions* (ed. W. W. Cleland, M. H. O'Leary & D. B. Northrop), pp. 122–152. Baltimore, MD: University Park Press.
- Northrop, D. B. 1991 Intrinsic isotope effects in enzyme catalyzed reactions. In *Enzyme mechanism from isotope effects* (ed. P. F. Cook), pp. 181–202. Boca Raton, FL: CRC Press.
- 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)
- Pu, J., Ma, S., Garcia-Viloca, M., Gao, J., Truhlar, D. J. & Kohen, A. 2005 Nonperfect synchronization of reaction center rehybridization in the transition state of the hydride transfer catalyzed by dihydrofolate reductase. *J. Am. Chem. Soc.* **127**, 14 879–14 886. (doi:10.1021/ja054170t)
- Radkiewicz, J. L. & Brooks III, 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)
- Rickert, K. W. & Klinman, J. P. 1999 Nature of hydrogen transfer in soybean lipoxygenase 1: separation of primary and secondary isotope effects. *Biochemistry* **38**, 12 218–12 228. (doi:10.1021/bi990834y)
- Rod, T. H., Radkiewicz, J. L. & Brooks, C. L. 2003 Correlated motion and the effect of distal mutations in dihydrofolate reductase. *Proc. Natl Acad. Sci. 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)
- Schneider, M. E. & Stern, M. J. 1972 Arrhenius preexponential factors for primary hydrogen kinetic isotope effects. *J. Am. Chem. Soc.* **94**, 1517–1522. (doi:10.1021/ja00760a013)
- Scrutton, N. S. 1999 Enzymes in the quantum world. *Biochem. Soc. Trans.* **27**, 767–779.
- Scrutton, N. S., Basran, J. & Sutcliffe, M. J. 1999 New insights into enzyme catalysis. Ground state tunnelling driven by protein dynamics. *Eur. J. Biochem.* **264**, 666–671. (doi:10.1046/j.1432-1327.1999.00645.x)
- Sikorski, R. S., Wang, L., Markham, K. A., Rajagopalan, P. T. R., Benkovic, S. J. & Kohen, A. 2004 Tunneling and coupled motion in the *E. coli* dihydrofolate reductase catalyzed reaction. *J. Am. Chem. Soc.* **126**, 4778–4779. (doi:10.1021/ja031683w)
- Smedarchina, Z. & Siebrand, W. 2005 Generalized Swain–Schaad relations including tunneling and temperature dependence. *Chem. Phys. Lett.* **410**, 370–376. (doi:10.1016/j.cplett.2005.05.102)
- Smedarchina, Z., Fernández-Ramos, A. & Siebrand, W. 2005 Tunneling dynamics of double proton transfer in formic acid and benzoic acid dimers. *J. Chem. Phys.* **122**, 134 309–134 321. (doi:10.1063/1.1868552)
- Stern, M. J. & Weston, R. E. J. 1974 Phenomenological manifestations of quantum-mechanical tunneling. II. Effect on Arrhenius pre-exponential factors for primary hydrogen kinetic isotope effects. *J. Chem. Phys.* **60**, 2808–2814. (doi:10.1063/1.1681448)
- Streitwieser, A., Jagow, R. H., Fahey, R. C. & Suzuki, F. 1958 Kinetic isotope effects in the acetolyses of deuterated cyclopentyl tosylates. *J. Am. Chem. Soc.* **80**, 2326–2332. (doi:10.1021/ja01542a075)
- Stryer, L. 2002 *Biochemistry*, 5th edn. New York, NY: Freeman.
- Sutcliffe, M. J. & Scrutton, N. S. 2002 A new conceptual framework for enzyme catalysis. Hydrogen tunneling coupled to enzyme dynamics in flavoprotein and quino-protein enzymes. *Eur. J. Biochem.* **269**, 3096–3102. (doi:10.1046/j.1432-1033.2002.03020.x) (and many cited therein)
- Tautermann, C. S., Loferer, M. J., Voegelé, A. F. & Liedl, K. R. 2004 Double hydrogen tunneling revisited: the breakdown of experimental tunneling criteria. *J. Chem. Phys.* **120**, 11 650–11 657. (doi:10.1063/1.1753262)
- Thorpe, I. F. & Brooks III, C. L. 2004 The coupling of structural fluctuations to hydride transfer in dihydrofolate reductase. *Proteins* **57**, 444–457. (doi:10.1002/prot.20219)
- Venkitakrishnan, R. P., Zaborowski, E., McElheny, D., Benkovic, S. J., Dyson, H. J. & Wright, P. E. 2004 Conformational changes in the active site loops of dihydrofolate reductase during the catalytic cycle. *Biochemistry* **43**, 16 046–16 055. (doi:10.1021/bi048119y)
- Wang, L., Tharp, S., Selzer, T., Benkovic, S. J. & Kohen, A. Submitted. Effects of a distal mutation on active site chemistry. *Biochemistry* **45**, 1383–1392.
- Warshel, A. & Parson, W. W. 2001 Dynamics of biochemical and biophysical reactions: insight from computer simulations. *Q. Rev. Biophys.* **34**, 563–679.
- Wong, K. F., Selzer, T., Benkovic, S. J. & Hammes-Schiffer, S. 2005 Chemical theory and computation special feature: 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)