Probing coupled motions in enzymatic hydrogen tunnelling reactions

Rudolf K. Allemann1, Rhiannon M. Evans and E. Joel Loveridge
School of Chemistry, Cardiff University, Park Place, Cardiff CF10 3AT, U.K.

Abstract
Much work has gone into understanding the physical basis of the enormous catalytic power of enzymes over the last 50 years or so. Nevertheless, the detailed mechanism used by Nature’s catalysts to speed chemical transformations remains elusive. DHFR (dihydrofolate reductase) has served as a paradigm to study the relationship between the structure, function and dynamics of enzymatic transformations. A complex reaction cascade, which involves rearrangements and movements of loops and domains of the enzyme, is used to orientate cofactor and substrate in a reactive configuration from which hydride is transferred by quantum mechanical tunnelling. In the present paper, we review results from experiments that probe the influence of protein dynamics on the chemical step of the reaction catalysed by TmDHFR (DHFR from Thermotoga maritima). This enzyme appears to have evolved an optimal structure that can maintain a catalytically competent conformation under extreme conditions.

Introduction
The enormous catalytic power of enzymes, which can achieve rate enhancements of up to 21 orders of magnitude relative to the uncatalysed reactions, has fascinated scientists for many decades but has long remained mysterious. To a first approximation, Koshland’s induced fit hypothesis [1], which was built on the simple lock and key model proposed more than a century ago by Emil Fischer [2], and its subsequent adaptation to ground-state destabilization by Haldane [3], is still applicable for describing the specificity and selectivity of enzymes. Enzymatic rate accelerations, on the other hand, are generally explained by a lowering of the transition-state energy and/or an increase of the ground-state energy in a static energy barrier. This model, derived from Eyring’s transition-state theory [4,5], has underpinned the design of transition-state analogues for enzyme inhibition and the creation of catalytic antibodies. More recently, however, it has become increasingly clear that repositioning of the relative energies of the ground and transition states along a static energy barrier does not provide a sufficiently full explanation of enzyme catalysis, and the role of quantum mechanical tunnelling by protein dynamics (in addition to contributions from zero-point energies) has become established, at least for enzymes that transfer particles of relatively low mass such as electrons or hydrogen atoms. In several enzyme systems, it has been proposed that protein dynamics are coupled to tunnelling, and a variety of models for active promotion of tunnelling have been invoked, including rate-promoting vibrations [6–10], environmentally coupled tunnelling [11–13], vibrationally enhanced ground-state tunnelling [14,15] and multidimensional tunnelling [16,17].

The ubiquitous enzyme DHFR (dihydrofolate reductase), which catalyses the conversion of 7,8-dihydrofolate into 5,6,7,8-tetrahydrofolate using NADPH as a cofactor, has served as a paradigm for the development of a fundamental understanding of enzyme catalysis through study of the relationship between enzyme structure, dynamics and catalysis of hydrogen-transfer reactions (transfer of H+, H− or H•). Owing to the central role of the enzyme in maintaining intracellular pools of tetrahydrofolate, which acts as a one-carbon carrier during the biosynthesis of purines, thymidylate and several amino acids, DHFR has long been recognized as a target for anticancer and antibacterial drugs. DHFRs from more than 30 organisms from the three domains of life have been characterized. Kinetic measurements have revealed that hydrogen transfer during catalysis by EcDHFR (DHFR from Escherichia coli) occurred with a strong contribution from tunnelling promoted by dynamic motions of the protein [18,19]. Genomic sequence analysis combined with QM/MM (quantum mechanics/molecular mechanics) simulations identified an enzyme-wide network of hydrogen bonds and van der Waals interactions in EcDHFR that was suggested to influence protein dynamics and promote catalysis [20–27].

TmDHFR (DHFR from Thermotoga maritima) is the most thermostable DHFR isolated. Its melting temperature of 83°C is approx. 30°C above that of the E. coli enzyme [28,29]. Despite only 27% sequence identity, the T. maritima and the E. coli DHFRs adopt similar tertiary structures [30,31], although in contrast with all other chromosomally encoded DHFRs that have been characterized, TmDHFR forms a dimer that appears to be largely responsible for its increased thermal stability (Figure 1) [31,32]. Folding of TmDHFR was shown to be a two-state process.
between unfolded monomers and the folded dimer; under no experimental circumstances was a folded monomer observed [32]. Interestingly, in addition to an extensive hydrophobic core in the centre of the dimer interface, intersubunit hydrogen bonds between the ends of $\beta$-strands $\beta F$ and $\beta G$ confer significant stabilization on the dimer. If the mobility of the $\beta F$–$\beta G$ loop is also important in TmDHFR catalysis, as had been suggested for the E. coli enzyme, these interactions might also influence the rate of hydrogen transfer in TmDHFR.

The rates of the hydride transfer reaction catalysed by TmDHFR as a function of temperature were determined by combined QM/MM calculations using EA-VTST/MT (ensemble-averaged variational transition state theory with multidimensional tunnelling) [33–35]. The behaviour of the native TmDHFR dimer was compared with that of the experimentally inaccessible TmDHFR monomer. The results suggest that quantum mechanical tunnelling makes a significant contribution to the reaction rate at all temperatures. In addition, intra- and inter-subunit molecular motions were identified, suggesting that dimerization might be important not only for the thermal stability of TmDHFR but also for its catalytic activity. Here, we report experiments that probe the effects of solvent composition and dimerization on the catalytic activity of TmDHFR.

**Effects of dielectrics and viscosity on TmDHFR catalysis**

Increasing the viscosity and decreasing the dielectric constant of the reaction medium are both known to generally reduce motions within a protein. Computational studies have shown that while increased solvent viscosity affects the protein interior just as much as the surface [36], decreasing the dielectric constant produces a pronounced reduction in motion at the surface but the interior remains highly mobile [37]. Reducing the solvent dielectric constant inhibits motion by strengthening the H-bonding network, which also increases the protein’s stability [38]. It has also been suggested that increased stability is accompanied by a decrease in flexibility [38], although this view has recently been challenged through results obtained in neutron scattering experiments [39].

Given this information, it has recently been proposed that enzymes such as DHFR, in which tunnelling appears to be coupled to long-range protein motions, could be affected by changes to the solvent composition [40]. We recently embarked on a study to test this proposal with TmDHFR, using a range of organic co-solvents [41]. Our study revealed no directly proportional effect from the solvent viscosity on either the steady-state rate ($k_{\text{cat}}$) or the rate of hydride transfer ($k_{\text{H}}$). Both rate constants, however, were decreased in a manner proportional to the dielectric constant, with the exception of $k_{\text{H}}$ in the presence of glycerol (Figure 2). Bulk solvent properties had no significant effect on the KIE (kinetic isotope effect) on either rate constant. CD spectroscopy indicated that neither the secondary structure of TmDHFR nor its thermostability was significantly altered over the range in which kinetics were measured. In combination with computational results, which show that the dimer interface resists thermodenaturation longer than other regions of the enzyme [42], these CD experiments provide strong evidence that dimerization of TmDHFR is not disrupted by the co-solvents used.

The effect of solvent viscosity on the rate of enzymatic reactions may be described by a theoretical model initially developed by Kramers for unimolecular reactions [43]. A later extension of this model predicts that enzymatic reaction rates decrease with increasing solvent viscosity in a manner dependent on internal protein friction [44]. Molecular dynamics studies of TmDHFR revealed intramolecular correlated motions similar to those seen in EcDHFR as well as motions correlated across the subunits of the TmDHFR dimer [45]. These results and Kramers theory predict a dependence of the hydride transfer rates for the TmDHFR catalysed reaction on solvent viscosity. However, our observed rate constants do not fit the predicted dependence on solvent viscosity at any value of the enzyme internal friction, showing that contrary to expectations, solution viscosity does not affect the kinetics of
Enzyme Mechanisms: Fast Reaction and Computational Approaches 351

Figure 2  Plots of $k_H$ and $k_{\text{cat}}$ against solution viscosity (left) and dielectric constant (right) at 20°C
The colors of the symbol indicate the different co-solvents (dark green: no co-solvent; light green: tetrahydrofuran; yellow: sucrose; orange: glycerol; red: ethylene glycol; maroon: propan-2-ol; dark blue: ethanol; and light blue: methanol). In the case of the data for the dielectric constant, lines of best fits are shown. A separate line of best fit is shown for the rate constants of hydride transfer, $k_H$, as a function of glycerol concentration. Data taken from [41].

TmDHFR. The dielectric constant of the solvent, however, had a strong effect on both $k_H$ and $k_{\text{cat}}$, although not on their KIEs. It is most likely that the effect on TmDHFR is due to inhibition of motions critical for catalysis, rather than electrostatic effects on the reaction itself. The absence of an effect from the viscoelastic response of the solvent supports the view that TmDHFR motions are of low amplitude and that this contributes to the low rates of reaction [28,45].

Increasing the concentration of glycerol (and sucrose) led to increased rate constants for hydride transfer, suggesting that these changes were compound specific and not a consequence of changes to the bulk solvent properties. We therefore measured the temperature dependence of the KIEs for the chemical step in the presence of methanol, glycerol and sucrose in the range 6–50°C (Figure 3). Above 25°C, the kinetic breakpoint observed in the absence of co-solvents, the addition of methanol had no effect on the KIE. Below 25°C the KIEs became obscured by kinetic complexity, and no further analysis was performed. In contrast, glycerol led to a reduction of both the KIE in the temperature-independent range, and the temperature of the kinetic breakpoint. The range in which KIEs were temperature-independent, i.e. in which pre-organizational (‘passive’) dynamics dominate, was therefore increased, while below the breakpoint the temperature dependence (determined by the difference in activation energies for H$^-$ and D$^-$ transfer) was increased. Therefore glycerol increases the reliance on ‘gating’ motions at low temperatures, but reduces the range over which these motions dominate.

The equivalent methanol and glycerol solutions used in our study have very similar dielectric constants, whereas the sucrose solutions used were isoviscous to the glycerol solutions. The change in behaviour of the KIEs in the presence of glycerol and sucrose is therefore clearly not dominated by either the viscosity or the dielectric constant of the reaction medium, and must instead be due to specific effects from the two compounds. We speculate that these, and presumably other, polyols may bind to the surface of TmDHFR and disrupt certain interactions, for example by exerting a loosening effect on the edges of the interface, allowing increased motion of the loop regions and an increase in the rate of hydride transfer. Alternatively, polyols may have an effect on the layer of hydration water on the protein surface, and so alter motions ‘slaved’ [46] to this layer.

Is the quaternary structure of TmDHFR important for its catalytic efficiency?
TmDHFR is the most stable DHFR isolated so far. Its stability is dependent both on an optimized composition of the subunits and its dimeric structure [31,32]. Such is
the stability of the dimer that no structured monomers could be detected experimentally in equilibrium or during unfolding [32]. Molecular dynamics simulations of the thermal unfolding of TmDHFR revealed that folding of the TmDHFR subunits and dimerization are intimately linked and not stepwise processes in which folded monomers associate to form the native dimer [42]. A central feature of the dimer interface is a large hydrophobic core that is flanked by intersubunit salt bridges and hydrogen bonds. The mobility of certain residues involved in the dimer interface of TmDHFR has been suggested to be important for catalysis in monomeric DHFRs [47–49].

To address whether the rigidity resulting from dimerization may be responsible for the lower reaction rates observed in TmDHFR when compared with mesophilic enzymes, specific amino acid replacements were introduced into the dimer interface to increase the concentration of folded monomer at equilibrium (Figure 1). Single or double mutations of Ghu156 and Ghu158 to lysine residues to disrupt the main intersubunit salt bridge did not affect dimerization. Similarly, replacement of Val126, located towards the edge of the central hydrophobic patch next to the more hydrophilic periphery, with a glutamate residue also had little effect. Preliminary data indicate, however, that the replacement of Val11, a residue that is positioned more towards the centre of the hydrophobic core of the interface, with aspartate destabilized the dimer interface sufficiently to favour the monomeric form of the enzyme (E.J. Loveridge, R. Rodriguez, R.S. Swanwick and R.K. Allemann, unpublished work). The thermostability of the monomer was reduced relative to the dimer, supporting the hypothesis that oligomerization is required to achieve the necessary high thermal stability of TmDHFR. In contrast, hydride transfer was not significantly impaired by monomerization and hence the reduced rates of the chemical step relative to EcDHFR are not a consequence of the dimeric structure of TmDHFR. Dimerization is, however, clearly crucial to allow the enzyme to maintain a stably folded structure at the high temperatures at which its host survives in the biosphere. In addition, the steady-state rates are somewhat lower in the mutant, suggesting that intersubunit mobility of certain residues involved in the dimer interface of TmDHFR when compared with the monomeric *E. coli* enzyme. The chemical reaction in TmDHFR appears to have been optimized during evolution to withstand environmental changes, perhaps through a protein scaffold that can tolerate motions of higher amplitude than mesophilic DHFRs.

In this sense, TmDHFR may be the more flexible enzyme better suited to accommodate larger fluctuations at increased temperatures [39] while at the same time maintaining a catalytically optimized active site.

**Funding**

This work was supported by the Biotechnology and Biological Sciences Research Council [grant number BB/E008380/1].

**References**


