CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200801804

Solvent Effects on Environmentally Coupled Hydrogen Tunnelling During Catalysis by Dihydrofolate Reductase from *Thermotoga maritima*

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Abstract: Protein motions may be perturbed by altering the properties of the reaction medium. Here we show that dielectric constant, but not viscosity, affects the rate of the hydride-transfer reaction catalysed by dihydrofolate reductase from *Thermotoga maritima* (TmDHFR), in which quantum-mechanical tunnelling has previously been shown to be driven by protein motions. Neither dielectric constant nor viscosity directly alters the kinetic isotope effect of the reaction or the mechanism of coupling of protein motions to tunnelling. Glycerol and sucrose cause a significant increase in the rate of hydride transfer, but lead to a reduction in the magnitude of the kinetic isotope effect as well as an extension of the temperature range over which "passive" protein dynamics (rather than "active" gating motions) dominate the reaction. Our results are in agreement with the proposal that non-equilibrium dynamical processes (promoting motions) drive the hydride-transfer reaction in TmDHFR.

Keywords: dielectric constants · dihydrofolate reductase · enzyme catalysis · enzymes · viscosity

Introduction

While structural studies of enzymes have significantly influenced our view of catalysis and provided insight into the molecular basis of the often exquisite specificity and selectivity of enzyme-catalysed reactions, the role of protein dynamics in enzymatic reactions remains a key question in enzymology. It is now generally accepted that hydrogen tunnelling (hydrogen atom, proton or hydride) is a common feature of many enzymatic reactions, but the effect of protein dynamics, the coupling of specific protein motions to the reaction coordinate, remains an issue of debate.[1–6] Experimental evidence for promoting motions in enzymatic hydrogen-transfer reactions is mainly derived from unusual temperature dependences of (mostly) primary kinetic isotope effects (KIEs).[7,8]

Dihydrofolate reductase (DHFR) catalyses the reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) by reduced nicotinamide adenine dinucleotide phosphate (NADPH). During the reaction, the C4 *pro-R* hydrogen of NADPH is transferred most likely as hydride to the *Re* face of C6 of H_2F with concomitant protonation of the N5 of H_2F.[9] H_4F carries one-carbon units in various oxidation states and is essential for the production of purine bases, deoxythymidine triphosphate, methionine and, in prokaryotes, pantothenic acid. Due to this central position in metabolism and their pharmacological importance, DHFRs from more than thirty species and all three kingdoms of life have been characterised with respect to their structure and function. DHFR from the hyperthermophile *Thermotoga maritima* (TmDHFR) is the only chromosomal DHFR known to have a dimeric structure (Figure 1),[10] and the lower rates of the TmDHFR-catalysed reaction relative to those of the *E. coli* enzyme (EcDHFR) are believed to be due to impairment of catalytically important motions as a result of dimerisation.[11] Molecular dynamics simulation of the thermal unfolding of TmDHFR showed that the dimer interface was only disrupted after loss of structure of the subunits.[12]

DHFR has been widely used for the study of the relationship between enzyme structure, dynamics and catalysis. Hydride transfer in DHFR may be explained by environmentally coupled quantum-mechanical tunnelling, in which dynamic structural fluctuations of the enzyme are critical for catalysis.[7,8,13] The primary KIE for the hydride transfer by EcDHFR is temperature independent at high pH[14] and temperature dependent at pH 7,[15] which suggests an alteration in dynamics with pH. At pH 7, TmDHFR shows biphasic kinetics with a breakpoint in the primary KIE at 25 °C.[16]

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200801804.

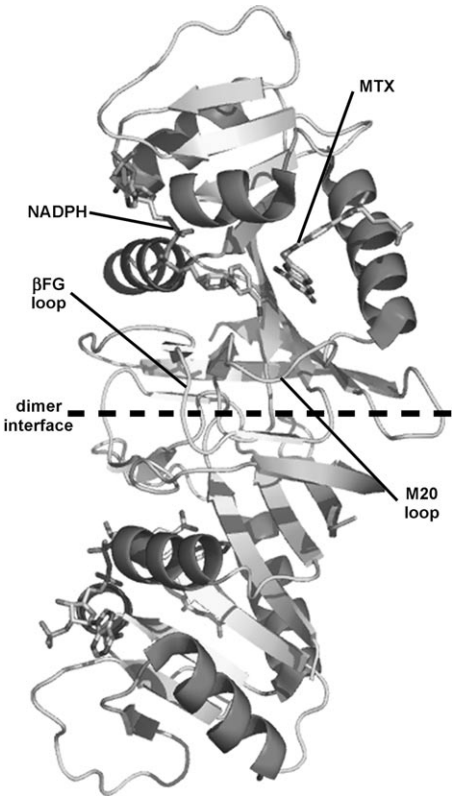

Figure 1. Cartoon of the structure of TmDHFR indicating the dimeric nature of the hyperthermophilic enzyme (PDB 1D1G).[10] The cofactor NADPH and substrate analogue methotrexate (MTX) are shown as sticks. The βFG and M20 loops are indicated for one subunit (see main text for details).

The KIE is independent of temperature above the breakpoint, and becomes temperature dependent below it. A similar breakpoint in the temperature dependence of the primary (and also secondary) KIE has been observed for the thermophilic alcohol dehydrogenase from *Bacillus stearothermophilus*,[17] matched by a change in protein flexibility.[18] This has been explained by preorganisational ("passive") dynamics, which lead to an active-site conformation conducive to the reaction, dominating above the breakpoint. At lower temperatures, the enzyme relies on gating motions that compress the hydrogen coordinate for efficient hydride transfer.[18]

In EcDHFR, a number of studies have shown the βFG loop to be important for promoting hydride transfer. It has been suggested that hydride transfer is promoted through a network of interactions that span the whole protein and include the βFG loop.[2,19–22] In particular, motions of this loop are coupled to those of the nearby M20 loop, which forms contacts with the bound cofactor. Site-directed mutagenesis of Gly121, a highly mobile residue in the βFG loop over 19 Å from the active site, to bulkier residues, such as valine, sharply reduces the hydride-transfer rate.[23] We have shown that this is due to non-local structural effects that may disrupt the network of coupled motions.[24] Computational studies of the dynamics of TmDHFR have revealed correlations between the movement of the βFG and M20 loops of TmDHFR even in this dimeric protein.[25] The correlations are reduced in the (hypothetical but computationally accessible) monomer, which suggests that dimerisation may in fact improve catalysis and stability in TmDHFR. Inter-subunit correlated motions were observed, which are also likely to be important for catalysis.[25] The βFG loop forms part of the dimer interface in TmDHFR, restricting its movement.[10] This is likely to reduce the amplitude of correlated motions with the M20 loop.

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.[26] Both increasing the viscosity and decreasing the dielectric constant of the reaction medium are known to generally reduce protein motion. Computational studies have suggested that, while increasing the viscosity of the solvent reduces motion in the protein interior just as much as at the surface,[27] altering the dielectric constant produces a pronounced effect at the surface but this is not transmitted to the interior, which remains highly mobile.[28] Reducing the dielectric constant of the solvent inhibits protein motions by strengthening the H-bonding network, making the protein more stable and less flexible.[29]

Results and Discussion

Effect of cosolvent on TmDHFR catalysis: To test the proposal that DHFR could be affected by changes to the solvent composition, the steady-state rate (k_{cat}) and the rate of hydride transfer (k_H) for the TmDHFR-catalysed reaction in the presence of organic cosolvents were initially measured at 20 °C (see Figure 2 and the Supporting Information). k_H was also determined at 40 °C (see the Supporting Information), that is, above the kinetic breakpoint seen in the absence of cosolvents.[16] Increasing amounts of cosolvent led to a reduction of both rates, except in the case of glycerol (and sucrose to a lesser extent). While the steady-state rate was also reduced in the presence of glycerol, the rate constant for hydride transfer was increased (see Figure 2 and the Supporting Information). Neither rate constant was reduced in a manner directly proportional to the medium viscosity, but both (except k_H in the presence of glycerol) were decreased in a manner proportional to the dielectric constant. The KIE on both k_H and k_{cat} did not alter proportionally to the solvent properties (see the Supporting Information).

Because of the extremely tight binding of TmDHFR to its ligands, K_m values could not be determined accurately in most cases. K_m values for both NADPH and H_2F were below 1 μM, except for the K_m for NADPH in the presence of 50% ethanol ((1.3 ± 0.2) μM), 50% isopropanol ((1.1 ± 0.2) μM), 50% ethylene glycol ((1.7 ± 0.3) μM) and 50% glycerol ((3.4 ± 0.3) μM). This indicates that all k_{cat} values reported here were measured under saturating conditions. Kirsch has developed methods for determining the degree by which

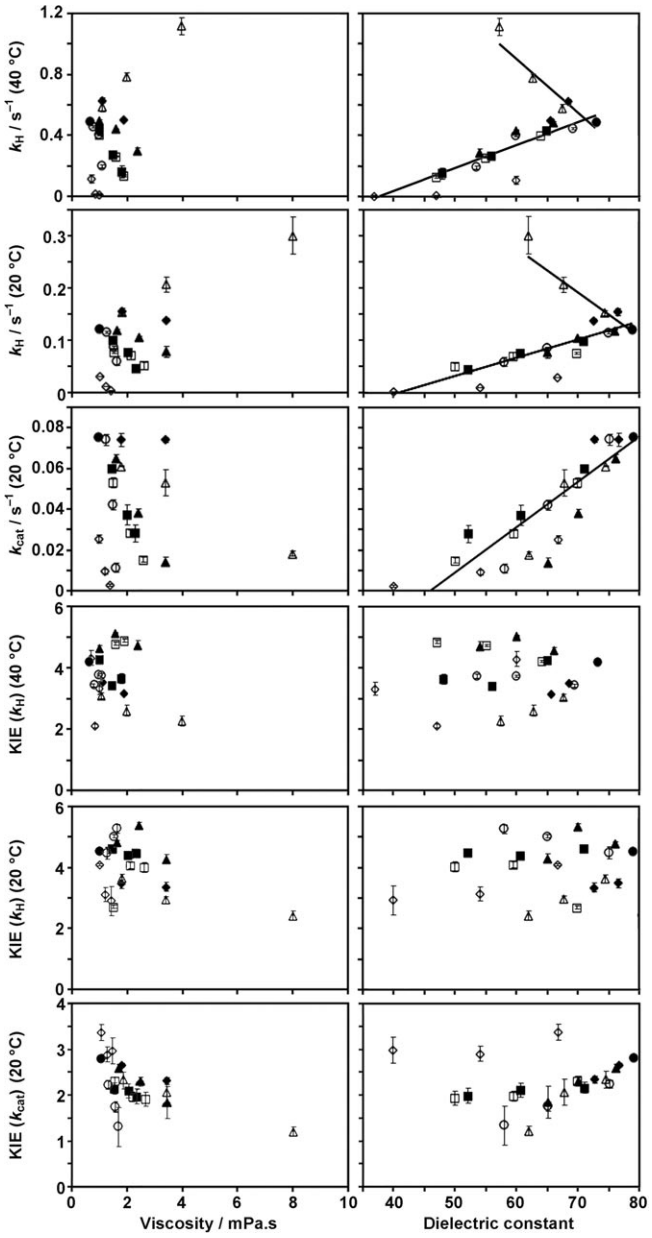

Figure 2. Plots of k_H, k_{cat} and their KIEs against solution viscosity (left) and dielectric constant (right). Symbols represent the different cosolvents, where ● = no cosolvent, ○ = methanol, ■ = ethanol, □ = isopropanol, ▲ = ethylene glycol, △ = glycerol, ◆ = sucrose and ◇ = THF. In the case of the dielectric constant data, lines of best fit are shown. A separate line of best fit for the hydride-transfer rate constants k_H as a function of the glycerol concentration is shown.

an enzymatic reaction is controlled by diffusion of substrates, using the viscosity dependence of k_{cat}/K_m. However, as K_m values could not be accurately determined here, such an approach is not appropriate in this case.[30,31]

Circular dichroism (CD) spectroscopy indicated that effects on catalysis were unlikely to be due to extensive structural changes (see the Supporting Information). In all cases, even in 50% cosolvent, the CD spectra of TmDHFR were virtually identical to that obtained in buffer only. The temperature dependence of the CD spectra showed that secondary structure was maintained up to 60 °C in 50% methanol, higher than the temperature range over which kinetics were measured. Glycerol and sucrose led to significant increases in the thermal stability of TmDHFR (the melting temperature increased from 83 to 92 and >98 °C in 50% sucrose and glycerol), as seen for many proteins.[32] This, combined with computational results showing that the dimer interface resists thermodenaturation longer than other regions of the enzyme,[12] provides strong evidence that the cosolvents used do not disrupt dimerisation of TmDHFR.

Kramers developed a theoretical model relating the influence of solvent viscosity to the rate of unimolecular reactions.[33] This was later extended to enzymatic reactions, which predict that reaction rates decrease with increasing solvent viscosity in a manner dependent on internal protein friction.[34] While this decrease is seen in several cases,[35–38] other enzymes appear to either have such high internal friction that their behaviour is independent of bulk viscosity, or be so affected by other solvent effects that the influence of viscosity becomes negligible.[26,39–41] For the reductive half-reaction of morphinone reductase, absence of a viscosity effect was interpreted as indicating the absence of long-range promoting motions,[26] supported by the detection of a promoting motion localised in the active site of the related aromatic amine dehydrogenase by computational analysis.[42] In contrast, TmDHFR is clearly affected by bulk solvent properties and this may be due to the existence of longer-range gating motions as has been proposed earlier.[21] 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.[25] These results and Kramers theory predict a dependence of the hydride-transfer rates for the TmDHFR-catalysed reaction on solvent viscosity.

Here we have shown that contrary to this prediction, solution viscosity does not affect the kinetics of TmDHFR. Our data do not fit the Kramers–Ansari equation for the dependence of the rate constant on solvent viscosity at any value of the enzyme internal friction (Figure 3). While increasing concentrations of organic cosolvents led to altered rate constants for both steady-state turnover and for hydride transfer, there was no general dependence on solvent viscosity. The dielectric constant of the solvent, however, had a strong effect on both k_H and k_{cat}, but not on their KIEs. The decrease in k_{cat} cannot be due exclusively to the reduction in the partially rate-limiting[11] hydride-transfer rate as glycerol increases k_H, yet decreases k_{cat}. The effect on k_{cat}, therefore, cannot be due to electrostatic effects on the reaction itself, and as the secondary structure of the enzyme is also unchanged, it is likely that inhibition of enzyme motions is responsible, affecting a partially rate-limiting physical component of the reaction cycle. As these are unaffected by the viscoelastic response of the solvent, this supports the view that motions in TmDHFR are of low amplitude and that this contributes to the low rates of reaction.[11,25]

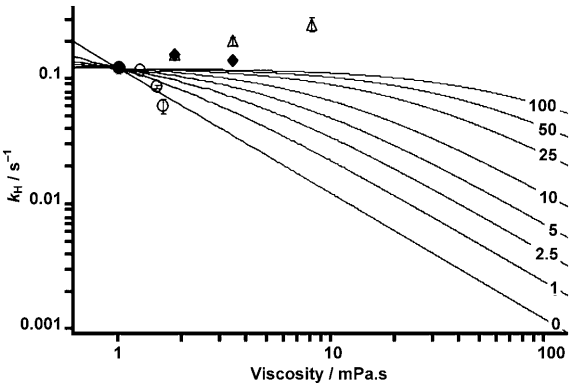

Figure 3. Rate constants for TmDHFR-catalysed hydride transfer in the presence of no cosolvent (●), methanol (○), glycerol (△) and sucrose (◆) plotted against viscosity. Both axes are on logarithmic scales. The rate-constant data are modelled (——) according to the Kramers–Ansari equation[34] for increasing values of protein internal friction (indicated on lines). The experimental data do not fit the model at any protein internal friction.

In other enzymes, such as formate dehydrogenase,[43] porcine pancreatic lipase[44] and trypsin,[45] the rate of hydride transfer increases as the dielectric constant decreases due to strengthening of interactions between the charges moved during the reaction. For TmDHFR, the hydride-transfer rate decreases with decreasing dielectric constant and hence the effect on k_H is also likely to be due to inhibition of motions critical for catalysis rather than electrostatic effects on the reaction itself. Reducing the dielectric constant will increase the strength of electrostatic interactions, such as the important inter-subunit salt bridge between Lys129 and Glu136/Glu138,[10] which may further decrease the flexibility of regions of the enzyme around the dimer interface. This is likely to exert effects on the physical steps of the reaction by altering the flexibility of the M20 loop, and also the chemical step of the reaction, by disrupting the network of promoting motions.

Effect of cosolvent on the temperature dependence of the KIE: Increasing the glycerol (and sucrose) concentration 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. The effects of cosolvents on the kinetics of the TmDHFR-catalysed reaction were, therefore, further analysed by measuring the temperature dependence of the KIEs for the chemical step in the presence of methanol, glycerol and sucrose in the range 6–50 °C (Figures 4 and 5). Above 25 °C, the kinetic breakpoint observed in the absence of cosolvents, the addition of methanol had no effect on the KIE. Below 25 °C, a breakpoint was introduced into the Arrhenius plot for hydride as well as deuteride transfer, leading to a decrease in the KIE with decreasing temperature that depended on the methanol concentration. Since CD spectroscopy indicated that methanol does not induce a major structural change (vide supra), this is likely a consequence of kinetic complexity that masks the true rate of the chemical step in the presence of methanol. Competitive KIE experiments should be able to unmask the intrinsic KIE in the presence of methanol and so determine whether there is any significant effect on the KIE (and therefore dynamics) of the reaction at lower temperatures.

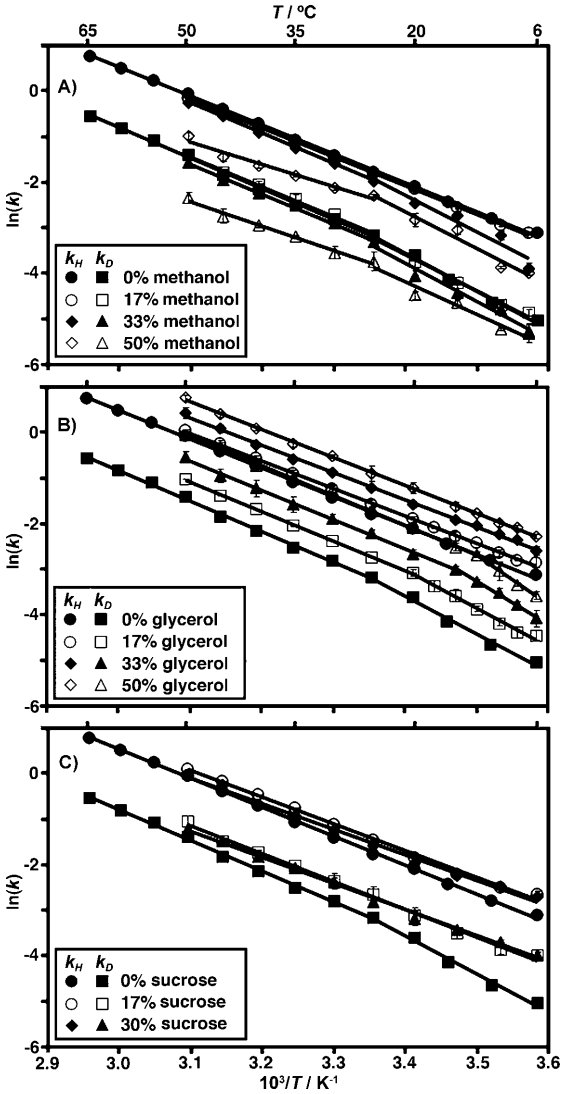

Figure 4. Arrhenius plots for TmDHFR-catalysed hydride and deuteride transfer in the presence of varying concentrations of methanol (A), glycerol (B) or sucrose (C).

The effects of glycerol and sucrose were different from that of methanol (Figures 4 and 5). Addition of glycerol increased the rates of both hydride and deuteride transfer up to around 2.5-fold for 50 % glycerol (see the Supporting Information), ruling out inhibition of the chemical step of the TmDHFR-catalysed reaction. Glycerol led to a reduction of the KIE in the temperature-independent range (Table 1) and remarkably also moved the kinetic breakpoint to lower temperatures, increasing the range in which KIEs were temperature independent. While the range of the temperature-dependent region was reduced by addition of glycerol, the temperature dependence within this region increased. The

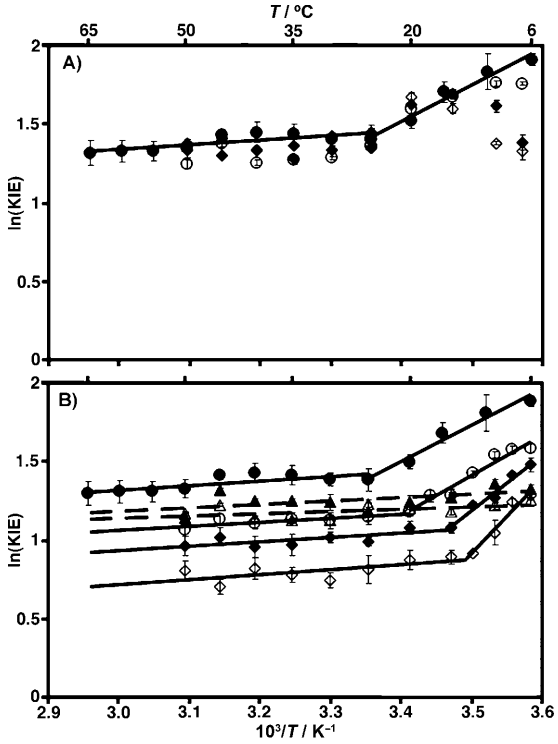

Figure 5. KIEs for TmDHFR-catalysed hydride/deuteride transfer on a logarithmic abscissa versus the inverse temperature in the presence of varying concentrations of methanol (A) or glycerol and sucrose (B). A) ● = Buffer only, ○ = 17% methanol, ◆ = 33% methanol, ◇ = 50% methanol. B) ● = Buffer only, ○ = 17% glycerol, ▲ = 17% sucrose, ◆ = 33% glycerol, △ = 30% sucrose, ◇ = 50% glycerol.

Table 1. Activation energies and Arrhenius prefactors for the region of TmDHFR catalysis showing temperature-independent KIEs, in the presence of varying concentrations of glycerol and sucrose.

Cosolvent	E_a^H [kJ mol^{-1}]	ΔE_a [kJ mol^{-1}]	A_H [s^{-1}]	A_H/A_D	KIE
none	53.5 ± 0.4	2.5 ± 1.0	(4.11±0.68) × 10^8	1.56 ± 0.47	3.9 ± 0.2
17% glycerol	52.5 ± 0.4	1.8 ± 1.0	(3.31±0.55) × 10^8	1.55 ± 0.58	3.1 ± 0.1
33% glycerol	52.1 ± 1.1	2.1 ± 1.7	(3.97±1.68) × 10^8	1.22 ± 0.82	2.7 ± 0.1
50% glycerol	51.7 ± 0.8	2.7 ± 1.5	(4.69±1.39) × 10^8	0.77 ± 0.45	2.2 ± 0.1
17% sucrose	48.9 ± 1.1	1.9 ± 1.7	(8.89±4.00) × 10^7	1.68 ± 1.12	3.6 ± 0.2
30% sucrose	46.5 ± 1.2	1.2 ± 1.6	(2.94±1.46) × 10^7	2.02 ± 1.32	3.3 ± 0.2

Table 2. Activation energies and Arrhenius prefactors for the region of TmDHFR catalysis showing temperature-dependent KIEs, in the presence of varying concentrations of glycerol.

Cosolvent	E_a^H [kJ mol^{-1}]	ΔE_a [kJ mol^{-1}]	A_H [s^{-1}]	A_H/A_D
none	49.9 ± 1.7	18.5 ± 6.7	(7.66±0.50) × 10^7	(2.41±0.30) × 10^{-3}
17% glycerol	48.7 ± 2.1	21.4 ± 4.0	(7.14±0.35) × 10^7	(5.03±0.37) × 10^{-4}
33% glycerol	48.4 ± 4.4	29.6 ± 5.8	(9.05±0.92) × 10^7	(1.28±0.15) × 10^{-5}
50% glycerol	51.2 ± 3.9	40.4 ± 5.0	(4.04±0.34) × 10^8	(1.01±0.09) × 10^{-7}

activation energy for deuteride transfer increased with increasing glycerol concentration, doubling the difference in activation energy within the experimental range and leading to a more inverse ratio of Arrhenius prefactors (Table 2). Therefore, while glycerol reduces the range over which "gating" motions dominate, the reliance on these motions at low temperatures is increased. This does not provide direct information on the effect on the "gating" motion, as this cannot be isolated from other protein motions. It is clear, however, that large-scale motions of TmDHFR are affected by glycerol. Together, these results and those obtained in the presence of methanol imply that "gating" motions are important under all conditions but may be masked by larger-scale motions dominating the effect on the KIE at higher temperatures.

Sucrose also decreased the KIE, but to a lesser extent than isoviscous concentrations of glycerol. More interestingly, sucrose led to a complete loss of the breakpoint, giving temperature-independent KIEs. In the case of horse liver alcohol dehydrogenase, a KIE breakpoint has been observed at sub-zero temperatures.[46] The breakpoint for TmDHFR in the presence of sucrose is, therefore, most likely shifted to outside the range studied rather than actually lost.

The change in behaviour of the KIEs in the presence of glycerol and sucrose is clearly not dominated by the dielectric constant. Equivalent methanol and glycerol concentrations have similar dielectric constants but methanol (which causes little change in viscosity) does not affect the KIE, at least above 20 °C. Furthermore, the change in the behaviour of the KIEs is not purely related to the medium viscosity, as isoviscous glycerol and sucrose solutions do not produce the same effect. These observations underline that the dramatic difference between the trend in k_H against dielectric constant for glycerol compared to other cosolvents is most likely a structure-specific effect rather than one related to a bulk solvent property. From its bulk properties, glycerol would be expected to reduce the rate of reaction to a similar degree to methanol.

Movement of the breakpoint in the temperature dependence of the KIE shows that preorganisational ("passive") dynamics dominate over a wider temperature range. Glycerol and sucrose, therefore, appear to activate motions in TmDHFR that are important for hydride transfer, whilst inhibiting other motions as expected from their bulk solvent properties. Glycerol and sucrose, therefore, allow TmDHFR to adopt a conformation more conducive to tunnelling (of both hydride and deuteride), reducing the reliance on "gating" motions. We speculate that the polyols may bind to the surface of the protein and disrupt certain interactions that normally rigidify TmDHFR, 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, they may specifically affect the layer of hydration water on the protein surface,

and so alter motions "slaved" to this layer.[47] To date, TmDHFR dynamics have not been studied except by computational methods,[25] even in the absence of cosolvents, and this study raises important questions about these motions and how they relate to catalysis. In the future, NMR spectroscopy, X-ray crystallography, elastic incoherent neutron scattering or terahertz spectroscopy might provide direct experimental evidence on the effects of polyols on TmDHFR structure and dynamics.

Conclusion

We have shown that the TmDHFR-catalysed reaction is sensitive to changes in the solvent dielectric constant, but not its viscosity, and that the dielectric constant of the solvent has an effect on large-scale motions of TmDHFR. Neither parameter affects either the magnitude or temperature-dependence of the KIE, which shows that the mechanism of coupling of protein motions to the reaction is unaffected by bulk solvent composition. Specific effects from polyols, such as glycerol and sucrose, were observed that imply that these compounds alter the dynamics of TmDHFR to promote the formation of a configuration conducive to the tunneling reaction. These results support the proposal that non-equilibrium dynamical processes (or "gating" motions) promote the chemical step of the TmDHFR-catalysed reaction.

Experimental Section

General: NADPH was purchased from Melford. TmDHFR, NADPD and dihydrofolate were prepared as described previously.[11] In brief, TmDHFR was purified by heating the crude cellular lysate of TmDHFR-containing *E. coli* cells to 75 °C for 30 min to precipitate native proteins, followed by cation exchange chromatography on SP-sepharose resin. NADPD was synthesised by enzymatic reduction of $NADP^+$ by using the alcohol dehydrogenase from *Thermoanaerobium brockii* with perdeuterated isopropanol as the deuteride source. Dihydrofolate was prepared by dithionite reduction of folate.

Circular dichroism (CD) experiments were performed on an Applied Photophysics Chirascan spectrometer at a protein concentration of 10 μM in 5 mM potassium phosphate (pH 7.0) containing 50 % organic cosolvent. Protein unfolding was followed by monitoring the CD signal at 222 nm between 20 and 98 °C, applying a temperature gradient of 0.2 °C min^{-1}.

All kinetic experiments were performed in 100 mM potassium phosphate buffer, pH 7.0, containing 100 mM NaCl. Cosolvent concentrations of 17, 33 and 50 % (volume cosolvent per final solution volume) were used. As the rate of the DHFR-catalysed reaction is pH sensitive,[11,48] the pH was adjusted after the addition of cosolvent to ensure consistency. Details of dielectric constants and viscosities of solvent mixtures are given in Table S1 (see the Supporting Information).

Pre-steady state kinetic experiments were performed by using an Applied Photophysics stopped flow instrument with 2.5 mL drive syringes. TmDHFR (10 μM) was pre-incubated with NADPH or NADPD (4 μM) for at least five minutes and the reaction started by rapid mixing with an equal volume of dihydrofolate (100 μM). Fluorescence energy transfer was used to monitor the reactions, by excitation at 292 nm and detection by using a 400 nm cut-off filter.

Steady-state rates at 20 °C were measured spectrophotometrically by following the decrease in absorbance at 340 nm during the reaction. In a typical experiment, TmDHFR (1 μM) was pre-incubated with NADPH (100 μM) for one minute to avoid hysteresis. The reaction was started through the addition of dihydrofolate (100 μM final concentration). Concentrations of substrate and cofactor were varied to demonstrate that the used concentrations were indeed saturating. Most K_m values were <1 μM and, therefore, difficult to measure to any greater accuracy.

Acknowledgement

This work was supported by the UK Biotechnology and Biological Sciences Research Council (BBSRC) (grant BB/E008380/1 to E.J.L. and R.K.A.), the Engineering and Physical Sciences Research Council (EPSRC-DTG award to R.M.E.) and Cardiff University.

[1] S. J. Benkovic, S. Hammes-Schiffer, *Science* **2003**, *301*, 1196–1202.
[2] D. D. Boehr, D. McElheny, H. J. Dyson, P. E. Wright, *Science* **2006**, *313*, 1638–1642.
[3] S. J. Benkovic, S. Hammes-Schiffer, *Science* **2006**, *312*, 208–209.
[4] M. H. M. Olsson, W. W. Parson, A. Warshel, *Chem. Rev.* **2006**, *106*, 1737–1756.
[5] K. Henzler-Wildman, D. Kern, *Nature* **2007**, *450*, 964–972.
[6] J. Pineda, S. D. Schwartz, *Phil. Trans. R. Soc. B* **2006**, *361*, 1433–1438.
[7] M. J. Knapp, J. P. Klinman, *Eur. J. Biochem.* **2002**, *269*, 3113–3121.
[8] Z. D. Nagel, J. P. Klinman, *Chem. Rev.* **2006**, *106*, 3095–3118.
[9] P. A. Charlton, D. W. Young, B. Birdsall, J. Feeney, G. C. K. Roberts, *J. Chem Soc. Chem. Commun.* **1979**, 922–924.
[10] T. Dams, G. Auerbach, G. Bader, U. Jacob, T. Ploom, R. Huber, R. Jaenicke, *J. Mol. Biol.* **2000**, *297*, 659–672.
[11] G. Maglia, M. H. Javed, R. K. Allemann, *Biochem. J.* **2003**, *374*, 529–535.
[12] J. Y. Pang, R. K. Allemann, *Phys. Chem. Chem. Phys.* **2007**, *9*, 711–718.
[13] R. K. Allemann, R. M. Evans, L. H. Tey, G. Maglia, J. Y. Pang, R. Rodriguez, P. J. Shrimpton, R. S. Swanwick, *Phil. Trans. R. Soc. B* **2006**, *361*, 1317–1321.
[14] R. S. Sikorski, L. Wang, K. A. Markham, P. T. R. Rajagopalan, S. J. Benkovic, A. Kohen, *J. Am. Chem. Soc.* **2004**, *126*, 4778–4779.
[15] R. S. Swanwick, G. Maglia, L. H. Tey, R. K. Allemann, *Biochem. J.* **2006**, *394*, 259–265.
[16] G. Maglia, R. K. Allemann, *J. Am. Chem. Soc.* **2003**, *125*, 13372–13373.
[17] A. Kohen, R. Cannio, S. Bartolucci, J. P. Klinman, *Nature* **1999**, *399*, 496–499.
[18] Z. X. Liang, T. Lee, K. A. Resing, N. G. Ahn, J. P. Klinman, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9556–9561.
[19] M. R. Sawaya, J. Kraut, *Biochemistry* **1997**, *36*, 586–603.
[20] J. L. Radkiewicz, C. L. Brooks, *J. Am. Chem. Soc.* **2000**, *122*, 225–231.
[21] P. K. Agarwal, S. R. Billeter, P. T. R. Rajagopalan, S. J. Benkovic, S. Hammes-Schiffer, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 2794–2799.
[22] J. B. Watney, P. K. Agarwal, S. Hammes-Schiffer, *J. Am. Chem. Soc.* **2003**, *125*, 3745–3750.
[23] C. E. Cameron, S. J. Benkovic, *Biochemistry* **1997**, *36*, 15792–15800.
[24] R. S. Swanwick, P. J. Shrimpton, R. K. Allemann, *Biochemistry* **2004**, *43*, 4119–4127.
[25] J. Pang, J. Z. Pu, J. L. Gao, D. G. Truhlar, R. K. Allemann, *J. Am. Chem. Soc.* **2006**, *128*, 8015–8023.
[26] S. Hay, C. R. Pudney, M. J. Sutcliffe, N. S. Scrutton, *Angew. Chem.* **2008**, *120*, 547–550; *Angew. Chem. Int. Ed.* **2008**, *47*, 537–540.
[27] R. Walser, W. F. van Gunsteren, *Proteins* **2001**, *42*, 414–421.
[28] R. Affleck, C. A. Haynes, D. S. Clark, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 5167–5170.
[29] D. S. Hartsough, K. M. Merz, *J. Am. Chem. Soc.* **1993**, *115*, 6529–6537.
[30] J. M. Goldberg, J. F. Kirsch, *Biochemistry* **1996**, *35*, 5280–5291.

[31] A. C. Brouwer, J. F. Kirsch, *Biochemistry* **1982**, *21*, 1302–1307.
[32] S. N. Timasheff, *Annu. Rev. Biophys. Biomol. Struct.* **1993**, *22*, 67–97.
[33] H. A. Kramers, *Physica* **1940**, *7*, 284–304.
[34] A. Ansari, C. M. Jones, E. R. Henry, J. Hofrichter, W. A. Eaton, *Science* **1992**, *256*, 1796–1798.
[35] A. P. Demchenko, O. I. Rusyn, E. A. Saburova, *Biochim. Biophys. Acta* **1989**, *998*, 196–203.
[36] B. Gavish, M. M. Werber, *Biochemistry* **1979**, *18*, 1269–1275.
[37] J. S. Zhou, N. M. Kostic, *J. Am. Chem. Soc.* **1993**, *115*, 10796–10804.
[38] C. J. Feng, R. V. Kedia, J. T. Hazzard, J. K. Hurley, G. Tollin, J. H. Enemark, *Biochemistry* **2002**, *41*, 5816–5821.
[39] E. W. Westhead, B. G. Malmstrom, *J. Biol. Chem.* **1957**, *228*, 655–671.
[40] G. D. Dzingeleski, R. Wolfenden, *Biochemistry* **1993**, *32*, 9143–9147.
[41] A. I. Voznesensky, J. B. Schenkman, *Eur. J. Biochem.* **1992**, *210*, 741–746.
[42] L. Masgrau, A. Roujeinikova, L. O. Johannissen, P. Hothi, J. Basran, K. E. Ranaghan, A. J. Mulholland, M. J. Sutcliffe, N. S. Scrutton, D. Leys, *Science* **2006**, *312*, 237–241.
[43] A. P. Demchenko, O. I. Rusyn, A. M. Egorov, V. I. Tishkov, *Biochim. Biophys. Acta* **1990**, *1039*, 290–296.
[44] H. Park, K. S. Lee, S. M. Park, K. W. Lee, A. Y. Kim, Y. M. Chi, *J. Microbiol. Biotechnol.* **2005**, *15*, 587–594.
[45] H. Park, Y. M. Chi, *J. Microbiol. Biotechnol.* **1998**, *8*, 656–662.
[46] S. C. Tsai, J. P. Klinman, *Biochemistry* **2001**, *40*, 2303–2311.
[47] P. W. Fenimore, H. Frauenfelder, B. H. McMahon, F. G. Parak, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16047–16051.
[48] C. A. Fierke, K. A. Johnson, S. J. Benkovic, *Biochemistry* **1987**, *26*, 4085–4092.

Received: September 2, 2008
Published online: October 16, 2008

