Hydrogen tunnelling in enzyme-catalysed H-transfer reactions: flavoprotein and quinoprotein systems

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It is now widely accepted that enzyme-catalysed C–H bond breakage occurs by quantum mechanical tunnelling. This paradigm shift in the conceptual framework for these reactions away from semi-classical transition state theory (TST, i.e. including zero-point energy, but with no tunnelling correction) has been driven over the recent years by experimental studies of the temperature dependence of kinetic isotope effects (KIEs) for these reactions in a range of enzymes, including the tryptophan tryptophylquinone-dependent enzymes such as methylamine dehydrogenase and aromatic amine dehydrogenase, and the flavoenzymes such as morphinone reductase and pentaerythritol tetranitrate reductase, which produced observations that are also inconsistent with the simple Bell-correction model of tunnelling. However, these data—especially, the strong temperature dependence of reaction rates and the variable temperature dependence of KIEs—are consistent with other tunnelling models (termed full tunnelling models), in which protein and/or substrate fluctuations generate a configuration compatible with tunnelling. These models accommodate substrate/protein (environment) fluctuations required to attain a configuration with degenerate nuclear quantum states and, when necessary, motion required to increase the probability of tunnelling in these states. Furthermore, tunnelling mechanisms in enzymes are supported by atomistic computational studies performed within the framework of modern TST, which incorporates quantum nuclear effects.

Keywords: H-tunnelling; transition state theory; stopped-flow kinetics; kinetic isotope effect; computational simulation

1. INTRODUCTION

Enzymes are extremely efficient catalysts that can achieve rate enhancements up to 10^{21} over the uncatalysed reaction rate (Lad *et al.* 2003). Our quest to understand the physical basis of this catalytic power—pivotal to our understanding of biological reactions and exploitation of enzymes in chemical, biomedical and biotechnological processes—is challenging, and has involved sustained and intensive research efforts for over 100 years (for reviews, see Cannon & Benkovic 1998; Cleland *et al.* 1998; Neet 1998; Warshel 1998; Benkovic & Hammes-Schiffer 2003). However, our understanding of how enzymes achieve phenomenal rate enhancements is far from complete. Recent years have seen new and important activity in this area, and these studies include roles of protein

‘motion’ (Cameron & Benkovic 1997; Rajagopalan *et al.* 2002; Benkovic & Hammes-Schiffer 2003), active site pre-organization (for reviews, see Cannon *et al.* 1996; Warshel 1998) and, in particular, the role of quantum mechanical tunnelling in enzymic hydrogen transfer (for reviews, see Antoniou *et al.* 2002; Knapp & Klinman 2002; Sutcliffe & Scrutton 2002; Liang & Klinman 2004). Understanding the factors that drive this H-tunnelling reaction, i.e. transfer occurs *through* the energy barrier separating reactant from product, is key to understand a large number of reactions in biology; C–H bond cleavage occurs in approximately 50% of all biological reactions, and all these reactions are likely to involve tunnelling to some degree. Such non-classical behaviour is expected to transfer a light particle, such as the H-nucleus, over short distances since the de Broglie wavelength is 0.63 Å for protium and 0.45 Å for deuterium (assuming an energy of 20 kJ mol^{−1}).

Quantum tunnelling studies of H-transfer focused initially on deviations from values predicted by

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semi-classical models (in which zero-point energies (ZPEs), but not tunnelling, have been taken into account), namely kinetic isotope effects (KIEs), Swain–Schaad relationships (Kohen & Jensen 2002) ($\exp((\ln(k_{\text{H}}/k_{\text{T}}))/(\ln(k_{\text{D}}/k_{\text{T}}))) > 3.26$, where k_{H} , k_{D} and k_{T} are the rates of transfer of protium, deuterium and tritium, respectively) or Arrhenius pre-factor ratios (much greater than unity for a reaction proceeding purely by tunnelling and less than unity for moderate tunnelling). Early examples in which H-tunnelling was inferred from measurements of KIEs include the quinoprotein bovine serum amine oxidase (Grant & Klinman 1989), the Zn^{2+} -dependent yeast alcohol dehydrogenase (Cha *et al.* 1989) and horse liver alcohol dehydrogenase (Bahnsen *et al.* 1993), and the flavin-dependent monoamine oxidase (Jonsson *et al.* 1994). These studies were shown to be consistent with the so-called Bell tunnel correction model of semi-classical transfer, which invokes tunnelling just below the classical transition state (TS; Bell 1980). This correction model accommodates minor corrections to the rate of a reaction and predicts inflated KIEs and Arrhenius pre-factor ratios to be less than unity (the so-called Kreevoy criteria for tunnelling; Kim & Kreevoy 1992) when KIEs are calculated as a function of temperature. When new enzyme-catalysed reactions were studied, a range of primary and secondary KIE values, Arrhenius pre-factor ratios and temperature dependences of rates and KIEs were observed. Studies from our own group (Basran *et al.* 1999, 2001a,b, 2003; Kohen *et al.* 1999; Harris *et al.* 2000), Klinman (Knapp *et al.* 2002) and more recently from Kohen (Sikorski *et al.* 2004) and Alleman (Maglia & Allemann 2003) have indicated that the simple Bell-correction model cannot adequately account for observed KIEs in a number of enzyme systems. This has led to the so-called full tunnelling models (see for example, Kuznetsov & Ulstrup 1999; Knapp & Klinman 2002; Knapp *et al.* 2002), akin to the established models for electron transfer, which are consistent with the strong temperature dependence of reaction rates, the variable temperature dependence of KIEs and the observed range of the Arrhenius pre-factor ratio. Given that protein motion is thought to be an important factor in driving quantum tunnelling in enzymic H-transfer reactions, computational methodology for identifying the residues important in creating reaction-promoting vibrations in enzymes has also been developed (Agarwal *et al.* 2002b; Mincer & Schwartz 2003).

Computational simulations of the reaction (i.e. the breaking and making of bonds) are performed within the framework of modern transition state theory (TST), i.e. including quantum nuclear effects such as H-tunnelling and, in the more complete applications, including sampling over protein configurations. The potential energy is described either by a hybrid quantum mechanics/molecular mechanics (QM/MM) potential (for a review, see Friesner & Guallar 2005; Mulholland 2005) or the empirical valence bond approach (Warshel 1991; Hammes-Schiffer 2004). In the former method, which we used in the study of methylamine dehydrogenase (MADH), atoms involved in the reaction are treated quantum mechanically and the rest of the system is treated classically using

molecular mechanics. Contributions of hydrogen tunnelling to the rate constant can then be calculated by different approaches (Garcia-Viloca *et al.* 2004; Hammes-Schiffer 2004; Olsson *et al.* 2004). Given the larger number of degrees of freedom involved, it is still very challenging for these atomistic methods to address the temperature dependence of the kinetics, especially of the KIEs. Thus, most of the studies have been limited to a single temperature. The results of the first temperature dependence studies, which have been presented recently (Hatcher *et al.* 2004; Olsson *et al.* 2004; Pu *et al.* 2005), are encouraging.

Our studies, in which we have investigated H-tunnelling in a number of quinoprotein and flavoprotein enzymes, have provided evidence—when interpreted within the framework of phenomenological Marcus-like tunnelling models—consistent with H-transfer by quantum tunnelling from the vibrational ground state of the reactive C–H bond of the substrate. This involves *either* H-tunnelling in which the KIE is temperature-independent—we interpret this to correspond to the absence of gated motion (i.e. no ‘compression’ of the transfer distance by substrate and/or protein fluctuations) *or* H-transfer in which the KIE is temperature-dependent—we interpret this to correspond to the involvement of gated motion. Our work (Basran *et al.* 2001a) has also highlighted the importance of energy barrier shape in determining the rates of H-transfer, and the concomitant values of KIEs, obtained in experimental studies.

2. STOPPED-FLOW METHODS TO ACCESS THE H-TUNNELLING STEPS IN QUINOPROTEINS AND FLAVOENZYMES

The quinoprotein and flavoprotein enzymes are ideally suited to studies of H-transfer during substrate oxidation using stopped-flow methods. Analysis using the steady-state approach is often compromised by the inability to focus on a single chemical step, owing to the existence of multiple barriers for binding, product release and a number of chemical steps, each of which may contribute to the overall catalytic rate. Using the stopped-flow method, the chemical step can often be isolated and the kinetics of C–H bond breakage determined without complications arising from other events in the catalytic sequence. With flavoprotein and quinoprotein enzymes, the reactions catalysed are conveniently divided into reductive and oxidative half-reactions. Enzyme reduction occurs through the breakage of substrate or coenzyme C–H bonds. The kinetics of bond breakage is conveniently followed by absorbance spectrophotometry, since the reaction is concomitant with the reduction of the redox centre. Thus, the alternative redox states of flavins and quinoprotein centres provide a readily available spectroscopic probe for following the kinetics of C–H bond breakage. The oxidative half-reaction usually involves long-range electron transfer to acceptor proteins (e.g. cytochromes, copper proteins or other flavoproteins), but in the case of flavoproteins it can also involve H-transfer to a second substrate. The absorbance changes associated with the oxidation of the flavin again provide a readily available signal for monitoring

H-transfer to the oxidizing substrate. The ability to interrogate each half-reaction by stopped-flow methods simplifies the kinetic analysis substantially and this makes these enzymes attractive targets in the studies of H-transfer employing KIEs as probes of enzymic H-tunnelling. Here, we present our work on the tryptophan tryptophylquinone (TTQ)-dependent quinoprotein amine oxidases such as MADH and aromatic amine dehydrogenase (AADH), and the flavoenzymes such as morphine reductase (MR) and pentaerythritol tetranitrate (PETN) reductase. We have additionally studied H-tunnelling in the flavoenzymes such as trimethylamine dehydrogenase (Basran *et al.* 2001b) and heterotetrameric sarcosine oxidase (Harris *et al.* 2000).

3. TEMPERATURE DEPENDENCE OF ISOTOPE EFFECTS AS PROBES OF H-TUNNELLING

The temperature-dependent behaviour of KIEs (i.e. temperature-dependent versus temperature-independent) is a key experimental result when considering the nature of tunnelling. To explain the experimental range of results obtained for enzymic systems, it has been suggested that protein/substrate motions modulate H-tunnelling. The realization that thermally induced vibrations (Antoniou & Schwartz 1998) in the protein scaffold (i.e. a thermally fluctuating energy surface) might drive H-tunnelling led to the formulation of the theoretical model of Kuznetsov & Ulstrup (1999), analogous to the electron transfer theory (Marcus & Sutin 1985), and is illustrated in figure 1. This model has been adopted by Knapp & Klinman (Knapp & Klinman 2002; Knapp *et al.* 2002), and is of the form:

$$k_{\text{tunnel}} = (\text{const.}) \times \left[\exp \left\{ \frac{-(\Delta G^\circ + \lambda)^2}{4\lambda RT} \right\} \right] \times (\text{F.C. Term}) \times (\text{Active Dynamics Term}). \quad (3.1)$$

Here, k_{tunnel} is the tunnelling rate constant; const., an isotope-independent term describing electronic coupling; the term in square parentheses is an environmental energy term relating the driving force of the reaction, ΔG° , and the reorganizational energy, λ (R is the gas constant and T , the temperature in K); F.C. Term, the Frank–Condon nuclear overlap along the hydrogen coordinate (HC), arises from the overlap between the initial and the final states of the hydrogen's nuclear wave function. In the simplest limit, when only the lowest vibrational level is occupied, the F.C. Term is temperature-independent. Temperature-dependent 'gating' (or 'active') dynamics, which can be likened to 'squeezing' of the HC (figure 1) potential energy barrier, can modulate the F.C. Term.

This model predicts that if the gating term dominates (i.e. energy of gating is lesser than thermal energy), then the observed KIE can be temperature-dependent since this leads to different transfer distances for the heavy and light isotopes (Knapp & Klinman 2002). In this regime, the ratio of Arrhenius pre-factors ($A_{\text{H}}/A_{\text{D}}$) is predicted to be less than unity. Alternatively, if the Frank–Condon term dominates (i.e. energy of gating is greater than thermal energy),

Figure 1. Representation of the model for the hydrogen transfer reaction used to interpret the experimental data (see text and references Benkovic & Hammes-Schiffer (2003), Kohen & Klinman (1998) and Kohen & Klinman (1999) for more details). The three axes are: E , energy; q , environmental coordinate (from which the transferred hydrogen atom is excluded) and HC, hydrogen coordinate. The four vertical panels show the potential energy curve as a function of HC for three values of the environmental coordinate: q^R is for the reactant, q^* is for the transition state and q^P is for the product. The grey spheres represent the ground state vibrational wave function of the hydrogen nucleus. The panel labelled M shows a Marcus-like view of the free-energy curves as functions of this environmental coordinate. The motions of the environment modulate the symmetry of the double well, thus allowing the system to reach a configuration with (nearly) degenerate quantum states ($q=q^*$), from which the hydrogen is able to tunnel (F.C. Term in equation (3.1)). The difference between panels *a* and *b* is a gating motion that reduces the distance between the two wells along the HC-axis (r_{H}) away from its equilibrium value (r_0). This motion increases the probability of tunnelling at the (nearly) degenerate configuration q^* (active dynamics term in equation (3.1)).

then the KIE will be temperature-independent. In the latter scenario, occupation of excited vibrational levels could result in some temperature dependence (Knapp *et al.* 2002). However, the Boltzmann distribution at 298 K suggests that tunnelling should be predominantly from the vibrational ground state of the nuclear wave function of hydrogen. In the regime in which the energy of gating is roughly equal to the thermal energy, gating would play some role in modulating the tunnelling probability; the temperature-dependent KIEs would be observed and the $A_{\text{H}}/A_{\text{D}}$ values would decrease (compared with the regime where the Frank–Condon term dominates), and could approach unity (Knapp & Klinman 2002).

Summarizing, within the framework provided by this model (Kuznetsov & Ulstrup 1999; Knapp & Klinman 2002; Knapp *et al.* 2002), the temperature dependence of the KIE can be interpreted as follows: (i) H-tunnelling in which the KIE is temperature-independent corresponds to either the absence of, or at least no detectable contributions from, gated motion (i.e. no significant motion along HC in figure 1) and (ii) H-tunnelling in which the KIE is temperature-dependent corresponds to the involvement of gated motion (i.e. motion along HC in figure 1). According to this, two types of motions need to be considered in enzymic H-tunnelling: (i) those which

facilitate attaining a nuclear configuration compatible with tunnelling (i.e. a configuration with degenerate quantum states), termed 'passive dynamics' and (ii) those which enhance the probability of tunnelling once (i) has been attained, termed 'active dynamics'.

Although these phenomenological models set a simple conceptual framework to interpret the experimental data, it is not possible to completely decouple motions, nor their effects on H-tunnelling (Mincer & Schwartz 2004), into active and passive dynamics. Moreover, in the cases where the KIE is temperature-dependent (of which, from our own work, there are only two examples to date; Basran *et al.* 2001a, 2003), the reaction can be taking place partly via the over-the-barrier route and partly by tunnelling; both these over/through the barrier explanation and/or the dominance of active gating would be consistent with a temperature-dependent KIE. Thus, it is neither possible to map directly from the kinetic data to a detailed picture of the concomitant changes (motions) at the atomic level, nor to the nature of the (free) energy barrier separating the reactants from the products. It should also be noted that the experimentally accessible temperature range is quite narrow. Thus, it is not always possible to show unambiguously that a given KIE is truly temperature-independent. Recent computational studies using the ensemble-averaged variational transition state theory with multidimensional tunnelling corrections (EA-VTST/MT) framework (Pu *et al.* 2005) illustrate how, even with different tunnelling behaviour for H and D, nearly temperature-independent KIEs can be observed in the temperature range of 5–45 °C.

A key role for dynamics has, however, been advocated from the theoretical studies and experimental observations, for example, Bruno & Bialek (Bruno & Bialek 1992), Klinman (Liang *et al.* 2004), Benkovic (Falzone *et al.* 1994; Epstein *et al.* 1995; Cameron & Benkovic 1997; Osborne *et al.* 2001), Hammes-Schiffer (Billeter *et al.* 2001; Agarwal *et al.* 2002a,b; Hammes-Schiffer 2002) and Schwartz (Antoniou & Schwartz 1998; Antoniou & Schwartz 2001; Caratzoulas & Schwartz 2001; Antoniou *et al.* 2002; Caratzoulas *et al.* 2002; Mincer & Schwartz 2003; Schwartz 2003). Care must be taken when interpreting these studies, as terms such as 'dynamics' and 'protein motions' are used by different authors to refer to very different events. For example, Schwartz uses the term 'promoting vibrations' to refer to vibrations on the sub-picosecond time-scale (Antoniou & Schwartz 2001) that are coupled to the reaction coordinate and result in changes in the (quantum) free-energy barrier. Hammes-Schiffer uses the term 'promoting motions' to refer to motions averaged over Schwartz's faster 'promoting vibrations'. These promoting motions occur on the much longer time-scale of the chemical reaction being catalysed; these thermally averaged motions affect the free energy. The term 'dynamical motions' refers to those that influence barrier recrossing (Benkovic & Hammes-Schiffer 2003). A similar definition has been used by Warshel (Villa & Warshel 2001; Warshel & Villa-Freixa 2003). A more general context of atomic and molecular motion has also been used by others (for example, by Karplus in Garcia-Viloca *et al.* (2004)).

Although protein/substrate motions effect H-tunnelling, the notion that enzymes have evolved to optimize H-tunnelling by acquiring strategies to increase the probability of transfer during evolution remains controversial (see, for example, a recent News Feature in Nature; Ball 2004). Warshel claims (Warshel & Villa-Freixa 2003; Olsson *et al.* 2004) that the dynamical effects of a reference reaction in solution will be of the same magnitude as those in the enzyme and, thus, they will not contribute to explain the catalytic power of the enzyme (Villa & Warshel 2001). In a study employing an adenosylcobalamin-dependent diol dehydratase model reaction, it is also argued by Finke & Doll (Doll & Finke 2003; Doll *et al.* 2003) that this B₁₂-dependent enzyme (which breaks a cobalt–carbon bond) exploits the same level of quantum mechanical tunnelling that is available in the reaction occurring in the absence of the enzyme (i.e. there is no 'compressive' motion that preferentially enhances H-tunnelling in the enzyme over the reaction in solvent). Moreover, Siebrand & Smedarchina (2004) have questioned the statistical significance of data reported over a relatively narrow temperature range for reactions of wild-type and mutant lipoxxygenase; these data were originally presented as evidence for gated motion in this enzyme (Knapp *et al.* 2002). The same authors, on theoretical grounds, also argue that flexible proteins are 'ill-equipped to cause strong local compression' (Siebrand & Smedarchina 2004, p. 4186). One needs to be aware of these issues, but our view is that it is not logical to generalize them on the basis of a small number of studies and hence a case-by-case analysis is appropriate.

4. H-TUNNELLING IN FLAVOENZYMES MORPHINONE REDUCTASE AND PENTAERYTHRITOL TETRANITRATE REDUCTASE

We have used the formulation of Knapp & Klinman (described earlier) to interpret the anomalous temperature dependencies of H-transfer in flavin mononucleotide (FMN)-containing MR and the homologous PETN reductase. In particular, we have studied the reactions of (i) PETN reductase with nicotinamide adenine dinucleotide phosphate (NADPH; Basran *et al.* 2003); (ii) MR with nicotinamide adenine dinucleotide (NADH; Basran *et al.* 2003); and (iii) MR in the oxidative half-reaction with 2-cyclohexenone (Basran *et al.* 2003), using stopped-flow and steady-state kinetic methods with protiated and deuterated nicotinamide coenzymes.

(a) *Reductive half-reaction in morphinone reductase and PETN reductase*

The temperature-dependent behaviour of the primary KIE for flavin reduction in MR and PETN reductase by nicotinamide coenzyme indicates that quantum mechanical tunnelling plays a major role in hydride transfer. In PETN reductase, the KIE is essentially temperature-independent in the experimentally accessible range, and this contrasts with the strongly temperature-dependent reaction rates (table 1). The data are, therefore, consistent with a tunnelling

Table 1. Tunnelling regimes and associated parameters in various flavoprotein and quinoprotein enzymes.

enzyme	substrate	A^H/A^D	ΔH^H (kJ mol ⁻¹)	ΔH^D (kJ mol ⁻¹)	KIE ^a	passive ^b	gated ^b	reference
MADH ^c	methylamine	13.3	44.6 ± 0.5	45.0 ± 0.5	16.8 TI ^a	✓		Basran <i>et al.</i> (1999)
MADH ^c	ethanolamine	0.57	43.5 ± 0.6	51.9 ± 1.1	14.7 TD ^a		✓	Basran <i>et al.</i> (2001a)
AADH ^c	tryptamine			53.5 ± 1.2	54.7 TI ^a	✓		Basran <i>et al.</i> (2001a)
AADH ^c	dopamine	9.4	50.9 ± 0.7	51.6 ± 0.7	12.9 TI ^a	✓		Basran <i>et al.</i> (2001a)
AADH ^c	benzylamine	3.7	68.1 ± 1.4	67.1 ± 0.9	4.8 TI ^a		✓	Basran <i>et al.</i> (2001a)
MR ^c	2-cyclohexenone	3.7	17.6 ± 0.9	17.1 ± 0.9	3.5 TI ^a	✓		Basran <i>et al.</i> (2003)
MR ^c	NADH	0.126	35.3 ± 0.5	43.5 ± 0.8	3.9 TD ^a		✓	Basran <i>et al.</i> (2003)
PETNR ^c	NADPH	4.1	36.4 ± 0.9	36.6 ± 0.9	4.1 TI ^a	✓		Basran <i>et al.</i> (2003)

^a TI, temperature-independent KIE; TD, temperature-dependent KIE; KIE values for enzyme–substrate combinations displaying a temperature-dependent (TD) KIE (i.e. reactions involving gated motion; Knapp & Klinman 2002; Knapp *et al.* 2002) are given at 298 K.

^b The terms ‘passive’ (i.e. the KIE is (almost) temperature-independent) and ‘gated’ (i.e. the KIE is temperature-dependent) dynamics are taken from the work of Knapp & Klinman (Knapp & Klinman 2002; Knapp *et al.* 2002). See text for a discussion on the current limitations of this, and other, interpretations of factors affecting the temperature (in)dependence of KIEs.

^c Enzyme abbreviations: MADH, methylamine dehydrogenase; AADH, aromatic amine dehydrogenase; MR, morphinone reductase; PETNR, pentaerythritol tetranitrate reductase.

mechanism governed by passive dynamics and from the vibrational ground state of the reactive C–H/D bond. In MR, however, both the reaction rates, with NADH and the KIE, are temperature-dependent. Analysis using the Eyring equation (i.e. a plot of $\ln(k/T)$ versus $1/T$) suggests that hydride transfer has a major tunnelling component, which unlike in PETN reductase would be classed as active dynamics (table 1). We have suggested that these differences arise from PETN reductase being relatively more rigid than MR, which would make gating more dominant in MR, predicting in turn that the KIE would be more temperature-dependent in MR than in PETN reductase. In addition, the active site of PETN reductase might be more optimally configured for hydride transfer than that of MR, thus requiring little (or no) vibrational assistance through gated motion. In other words, the active site of PETN reductase appears ideally configured to transfer a hydride ion from NADPH to FMN, and the nuclear reorganization associated with H-tunnelling (i.e. passive dynamics) is the major dynamic component. We have compared the high-resolution crystal structures of MR (Barna *et al.* 2002) and PETN reductase (Barna *et al.* 2001) in an attempt to provide insight into why gating is potentially more important in MR. Analysis of the structures of each enzyme suggests that the key factor could be a double-stranded anti-parallel β -sheet D, against which the NAD(P)H coenzyme is thought to bind (Barna *et al.* 2002). This region harbours arginine residues, which are important in the recognition of the 2'-phosphate of NADPH (PETN reductase), and a glutamate residue required to form a H-bond with the 2'-OH group of NADH (MR). The position of this sheet diverges at Leu-133 (PETN reductase)/Val-138 (MR) and converges again at Ile-141 (PETN reductase)/Gly-146 (MR). In addition, there is an insertion of a glycine residue (Gly-133) in MR immediately before the start of β -sheet D. These differences are consistent with MR being more mobile at physiological temperatures in this region than PETN reductase, which in turn might assist in a ‘squeezing’ or ‘compression’ of the HC energy barrier in MR (figure 1). This suggestion is consistent with the

temperature factors of MR (all C_α temperature factors greater than 40; PDB accession code 1GWJ; Berman *et al.* 2000) and PETN reductase (all C_α temperature factors less than 20; PDB accession code 1GVQ) in this region. The next stage of our work to test this hypothesis is to obtain structural information for the coenzyme complexes at high resolution and to perform a more detailed theoretical analysis involving QM/MM, variational transition state theory with multi-dimensional tunnelling (VTST/MT) and molecular dynamics studies.

(b) Oxidative half-reaction in morphinone reductase

The oxidative half-reaction of MR with the substrate 2-cyclohexenone and NADH at saturating concentrations is fully rate limiting in steady-state turnover. This has enabled us to investigate the potential tunnelling regimes in this part of the reaction cycle. Reduction of 2-cyclohexenone involves hydride transfer from reduced flavin (FMNH₂) and protonation. Thus, two H-transfer reactions are involved in this oxidative half-reaction (scheme 1). We have demonstrated that across the experimentally accessible temperature range, the KIE for hydride transfer from reduced flavin to the α/β unsaturated bond of 2-cyclohexenone is temperature-independent, in contrast to strongly temperature-dependent reaction rates. In contrast, a large solvent isotope effect (SIE) accompanies the oxidative half-reaction, which is also temperature-independent in the experimentally accessible range. Moreover, double isotope effects indicate that hydride transfer from the flavin N5 atom to 2-cyclohexenone and the protonation of 2-cyclohexenone are coupled. Both the temperature-independent KIE and SIE suggest that (i) gated motion is not required to compress the energy barrier and (ii) this reaction proceeds by ground state quantum tunnelling. Our work with MR was, therefore, the first to show that both passive and active dynamics are a feature of H-tunnelling within the same native enzyme; in the reductive half-reaction we suggest that barrier compression is required to facilitate hydride transfer from NADH to FMN, whereas in the oxidative half-reaction

Scheme 1. Proposed scheme for the oxidative half-reaction of morphinone reductase. The identity of the proton donor in the oxidative half-reaction is not known.

the active site is configured to catalyse hydride and proton transfer in a concerted fashion without vibrational assistance through gated motion.

5. H-TUNNELLING IN QUINOPROTEINS METHYLAMINE DEHYDROGENASE AND AROMATIC AMINE DEHYDROGENASE

(a) *Experimental studies of substrate oxidation in tryptophan tryptophylquinone-dependent amine dehydrogenases*

Our work on H-tunnelling in quinoprotein enzymes has focused on TTQ-dependent MADH (Basran *et al.* 1999, 2001a) and AADH (Basran *et al.* 2001a). Earlier studies on the quinoprotein bovine serum amine oxidase (a topa quinone (TPQ)-dependent amine oxidase) also demonstrated deviation from semi-classical behaviour, thus suggesting a role for H-tunnelling (Grant & Klinman 1989). In this case, the data were interpreted in terms of the tunnelling correction models of Bell. In MADH, TTQ reduction is concerted with the breakage of C–H bond from an iminoquinone intermediate that forms rapidly in the reductive half-reaction (scheme 1; Chen *et al.* 1992; Davidson *et al.* 1992; Hyun & Davidson 1995b). The rate of breakage of this bond is accessible using stopped-flow spectrophotometry. The reduction of TTQ cofactor is associated with a large KIE (McWhirter & Klapper 1989; Brooks *et al.* 1993; Basran *et al.* 1999; e.g. $\text{KIE} = 16.8 \pm 0.5$ at 298 K; Basran *et al.* 1999) when methylamine and deuterated methylamine are used as substrates. The value of KIE is, therefore, larger than the higher value expected for reactions described by semi-classical TST (i.e. including ZPE but with no tunnelling correction), and is clearly suggestive of tunnelling. Our studies on TTQ reduction in MADH indicated that the value of KIE was temperature-independent in the experimentally accessible region, but significantly the reaction rate was strongly temperature-dependent. These findings are consistent with the dissipative tunnelling models (Kuznetsov & Ulstrup 1999; Knapp & Klinman

2002; Knapp *et al.* 2002). Interpreted in the framework of these models, the oxidation of methylamine by MADH is dominated by environmental reorganization associated with tunnelling, and no further motions (gated motions) are required to explain the kinetic data. It is interesting to note that an earlier study (Krishtalik 1986) had observed temperature-independent KIE values (approx. 2–3) in steady-state reactions catalysed by serine proteases performed in deuterated solvent, and these were suggested to indicate tunnelling (note, however, that the effect of D₂O on the reaction dynamics is potentially complicated owing to the exchange of protons throughout the protein scaffold). The data were modelled on earlier theoretical treatments of H-tunnelling propounded by Dogonadze and co-workers in which thermal vibrations bring the solvent into a configuration favourable to tunnelling. Moreover, at the time of our own work with MADH, similar observations were made with the thermophilic alcohol dehydrogenase of *Bacillus stearothermophilus* (Kohen *et al.* 1999), which also indicates that the Bell-correction model for H-tunnelling was inappropriate for these enzyme-catalysed reactions. We extended our work with MADH to study the tunnelling behaviour with non-physiological substrates. In our work on the oxidation of ethanolamine by MADH, we found evidence for quantum tunnelling (table 1). In this case, however, the KIE was temperature-dependent, inconsistent with a reaction dominated by passive dynamics, but consistent with significant active dynamics (Basran *et al.* 2001a).

Our more recent studies have focused on the related TTQ-dependent enzyme AADH, for which Davidson and co-workers (Hyun & Davidson 1995a) observed a large KIE with the aromatic substrate dopamine. Using similar stopped-flow approaches to those reported for studies with MADH, we have studied the oxidation of three different substrates: dopamine, benzylamine and tryptamine. We have shown that the rate of TTQ reduction by dopamine in AADH has a large, temperature-independent KIE ($\text{KIE} = 12.9 \pm 0.2$), consistent with tunnelling involving passive, but not gated, dynamics. We have also demonstrated that H-transfer is compromised with benzylamine as substrate and that the KIE is deflated (4.8 ± 0.2), although the KIE remains temperature-independent. As with dopamine, however, the reaction rates are strongly temperature-dependent. Tryptamine is a ‘fast’ substrate for AADH, thus the rate of TTQ reduction could only initially be determined at low temperatures using the stopped-flow method with protiated substrate. It is said that an exceptionally large KIE (54.7 ± 1.0 ; single measurement at 4 °C) is observed for the breakage of substrate C–H/D bond, and the reaction rate (for C–D bond breakage) is strongly temperature-dependent. The exceptionally large KIE for this substrate suggests a major role for H-tunnelling in substrate oxidation, and by comparison with studies using dopamine and benzylamine we inferred that only passive dynamics is involved in H-tunnelling. Our very recent studies (Masgrau *et al.* 2006) have utilized a stopped-flow instrument with a smaller dead time (0.5 ms), and confirmed that the KIE with tryptamine

does not show a significant temperature dependence in the experimentally accessible range.

We have used KIEs as a key indicator of H-tunnelling, and used the phenomenological Marcus-like models to interpret the experimental results. However, as mentioned earlier, it is not possible to map directly from the KIE to the nature of either (at the atomic level) the protein/substrate motions or (the shape of) of the barrier through which tunnelling occurs. It is clear, however, that for a chemical reaction such as proton transfer in AADH (Basran *et al.* 2001a) and MADH (Faulder *et al.* 2001), the shape of the barrier is complex in nature and cannot be adequately represented by the rectangular barrier used to model electron transfer. To gain insight into the details of the reaction at the atomic level, computational methods can be used.

(b) Computational studies of substrate oxidation in tryptophan tryptophylquinone-dependent amine dehydrogenases

From the earlier discussion, it is clear that the factors which give rise to the observed kinetic data for these systems are far from trivial. To date, several computational studies elucidating how these enzymes might work and facilitate tunnelling have been published on MADH (Faulder *et al.* 2001; Alhambra *et al.* 2001, 2002; Tresadern *et al.* 2002, 2003a,b). Both methylamine and ethanolamine substrates have been studied. The reaction step modelled corresponds to the C–H bond breakage involving abstraction by the active site base (Asp 428) of a proton from iminoquinone (figure 2). As mentioned earlier, this proton transfer has been attributed to an elevated experimental KIE, which was taken as an indicator of H-tunnelling. In all these cases, the calculation of the rate constants and the H-tunnelling contributions was performed within the framework of VTST/MT corrections (Truhlar *et al.* 1985; Truhlar *et al.* 2001; Gao & Truhlar 2002) and a hybrid QM/MM scheme (figure 3) was used to describe the potential energy surface. Within the QM/MM approach (Warshel & Levitt 1976; Field *et al.* 1990 and for a recent review, see Friesner & Guallar 2005; Mulholland 2005), a small region in the active site containing the atoms involved in the breaking and forming of bonds is treated by quantum mechanical methods, whereas the rest of the solvated enzyme is described by a molecular mechanics force field. Therefore, this approach allows the atomic-level modelling of the reaction process in the presence of the enzyme (see Gao & Truhlar 2002 for a review). The VTST/MT rate constant for a unimolecular reaction can be expressed as:

$$k(T) = \kappa(T) \frac{k_B T}{h} \exp\left(\frac{-\Delta G^{\text{TS},0}(T)}{RT}\right), \quad (5.1)$$

where $(\Delta G^{\text{TS},0}(T))$ is the standard-state molar free energy of activation at temperature T , with the quantized vibrational energy included; $\kappa(T)$, the transmission coefficient, which accounts for quantum nuclear effects on the reaction coordinate and dynamical recrossing; T , k_B , h and R are the temperature, the Boltzmann, Planck and the gas constants, respectively.

Figure 2. (a) The structure of the tryptophan tryptophylquinone (TTQ) cofactor. (b) Reaction mechanism of the reductive half-reaction of MADH. Steps enclosed in the box represent binding steps. A similar scheme has been proposed for the reaction of AADH with aromatic primary amines. The tunnelling step is denoted k_3 .

Our (preliminary) work on MADH-methylamine (Faulder *et al.* 2001) used Gaussian 94 (Frisch *et al.* 1995) and AMBER (Pearlman *et al.* 1995) for the QM/MM calculations, with the semi-empirical Parameterized Model 3 (PM3) method (Stewart 1989) and the link-atom approach (Singh & Kollman 1986; Stewart 1989; Field *et al.* 1990; Maseras & Morokuma 1995; for interfacing this QM region with the rest of the protein modelled by MM). A reactant complex was found using a single protein configuration, optimizing the QM region embedded in the frozen MM environment, and the corresponding saddle point and product complex were identified. From this saddle point, the minimum energy path (MEP) was calculated downhill until the energy approached that of the reactant and the product complex using POLYRATE v. 7.4 (Steckler *et al.* 1997); for the QM region, the first and second derivatives of the energy were also calculated along this MEP. The rate constant was then calculated including a multidimensional treatment of H-tunnelling. The results (figure 4) suggested that approximately 96% of the reaction proceeds by tunnelling, with the remaining approximately 4% proceeding via the classical over-the-barrier route. It was also shown that tunnelling needs to be included to obtain a KIE value comparable

Figure 3. Schematic of the active site in methylamine dehydrogenase, illustrating the QM/MM partition used in the calculations and the mechanism corresponding to the tunnelling reaction. The QM region is shown on a white background and part of the surrounding MM region on a grey background, with link atoms circled; hydrogen bonds are depicted by dashed lines.

to the experimental one, and that a multidimensional (i.e. more comprehensive) treatment of tunnelling also improved the result: a value of 6.1 was obtained when tunnelling was omitted, 9.6 when tunnelling was included by a simple one-dimensional model (i.e. the Wigner correction; Wigner 1932; Truhlar *et al.* 1985) and 11.1 when the small-curvature tunnelling (Liu *et al.* 1993) was used. Thus, our calculated value was in qualitative agreement with the corresponding experimental value of 16.8 ± 0.5 (all at 298 K; Basran *et al.* 1999).

In the same year, an independent study on the MADH-methylamine system was published (Alhambra *et al.* 2001; Alhambra *et al.* 2002). The authors determined an even larger degree of tunnelling for the H-transfer step by increasing the rate of reaction 100-fold, i.e. 99% of the H-transfer reactions proceeding via tunnelling and 1% of the reactions proceeding via the classical over-the-barrier route (again at 298 K). Their predicted KIE of 18.3 at 298 K is in good agreement with the experimental result (Basran *et al.* 1999) and also larger than our calculated value. Despite the qualitative agreement between their work and ours, the schemes followed differ. The main differences are that they used specific reaction parameters (PM3-SRP; González-Lafont *et al.* 1991) to improve the PM3 potential energy surface, and that they employed the EA-VTST/MT approach (Truhlar *et al.* 2001; Gao & Truhlar 2002). In short, this approach derives the activation free energy and the tunnelling transmission coefficient by averaging over protein configurations instead of using a single MEP.

Despite the differences in the methods used, the picture emerging from our work and that of others for this proton transfer is qualitatively the same. First, the two heavy atoms (C on the iminoquinone and O on the Asp) approach each other until the distance is reduced sufficiently for (a significant amount of) tunnelling to

Figure 4. Energetics along the reaction path for proton tunnelling. Potential energy relative to reactant (solid black line), and minimum energy path (including zero-point energy; solid grey line) calculated to obtain the KIEs for proton transfer. The reaction coordinate, s , is the mass-weighted difference in position from the transition state (1 bohr = 0.529 Å), with $s=0$ at the top of the barrier and negative at the reactant side.

take place through the potential energy surface (note that the tunnelling probability is related inversely to the tunnelling distance). Tunnelling is further enhanced by a corner-cutting mechanism as the C–H (and later the O–H) stretching modes become coupled to the reaction coordinate. This, together with a reaction coordinate dominated by the motion of the light particle (H-nucleus), results in a shorter effective tunnelling path, which is responsible for an enhancement of the tunnelling probability. The importance of this corner-cutting effect in modulating tunnelling has been recognized for a long time and was used in the work of Tresadern *et al.* (2002) to explain the different tunnelling behaviour among MADH, liver alcohol dehydrogenase and soybean lipoxygenase. Although similar potential energy barriers were obtained for three hydrogen transfer reactions, these three enzymes exhibit different degrees of tunnelling. The authors rationalized this in terms of different donor–acceptor distance behaviour during the reaction, and linked this to corner-cutting and different curvature of the reaction paths.

Tresadern *et al.* (2003a,b) have also studied the reaction of MADH with ethanolamine, which turned out to be a much more complex system as a number of substrate conformers were identified, differing by the position of the hydroxyl group of ethanolamine. When the most stable conformer (denoted EA(A) by the authors; the hydroxyl group hydrogen bonded to a water molecule, which was in turn hydrogen bonded to another residue) was studied, the results did not agree completely with the experimental observations. Indeed, contrary to the experiment, more tunnelling was obtained for ethanolamine than for methylamine. When the other conformer was considered (denoted EA(B); the hydroxyl group of ethanolamine hydrogen bonded to the non-reactive oxygen atom of the Asp428; figure 3), the energy profile and the kinetic results were significantly different. A higher barrier, a more positive energy of reaction and (concomitantly) much smaller

tunnelling effects were obtained. The authors speculated that as the two conformers were close in energy, they both could be contributing to the observed kinetic data, in particular, the temperature dependence of the KIE. Thus, they suggest that the less stable structure, EA(B), with a smaller KIE, would become more important at higher temperatures. The possible contribution to the observed kinetic data of different reactant conformers is certainly an interesting observation and an issue to be considered when studying the reactivity of any system (Zhang *et al.* 2003).

Summarizing, these computational studies confirm that over-the-barrier processes are not the only mechanism employed by MADH, particularly with methylamine, and H-tunnelling enhances the rate of transfer significantly. A good agreement between the calculated and the experimental KIE is obtained when tunnelling is included. Moreover, a multidimensional treatment of tunnelling is necessary to reproduce the high experimental KIE values, as simpler tunnelling models are insufficient. Thus, in accord with the experimental observations, these computational studies suggest that these H-transfer reactions invoke tunnelling. Indeed, the degree of tunnelling calculated for MADH-methylamine is larger than that for other protein systems[†], the next largest one being 85% in xylose isomerase (Alhambra *et al.* 2000; Garcia-Viloca *et al.* 2003).

6. CONCLUSIONS

Quantum tunnelling of hydrogen has emerged over the recent years as a means by which enzymes catalyse reactions involving hydrogen transfer. The increasing body of experimental and computational evidences suggest that H-tunnelling is likely to be adopted extensively by enzymes. The temperature-dependent behaviour of KIEs has revealed that enzymes can catalyse reactions by 'pure' quantum tunnelling. KIEs give insight at the macroscopic level, but not at the atomic level. Computational studies are used to give insight into the atomic details of the mechanisms used by the enzymes that invoke quantum tunnelling. Protein dynamics is thought to modulate these tunnelling reactions, and a picture of how enzymes achieve this is beginning to emerge through, for example, phenomenological Marcus-like models and modern TST (which incorporates quantum nuclear effects). However, many details at the atomic level remain to be discovered, and these will be probed in greater detail as more refined kinetic (e.g. at cryogenic temperatures) and computational techniques are developed.

[†]NOTE ADDED IN PROOF

One very recent computational study of H-tunnelling in AADH (Masgrau *et al.* 2006) reveals that with tryptamine the degree of tunnelling is calculated to exceed 99.9%.

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