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Enzymology takes a quantum leap forward

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Abstract

Enzymes are biological molecules that accelerate chemical reactions. They are central to the existence of life. Since the discovery of enzymes just over a century ago, we have witnessed an explosion in our understanding of enzyme catalysis, leading to a more detailed appreciation of how they work. A key breakthrough came from understanding how enzymes surmount the potential-energy barrier that separates reactants from products. The genetic engineering revolution has provided tools for dissecting enzyme structure and enabling design of novel function. Despite the huge efforts to redesign enzyme molecules for specific applications, progress in this area has been generally disappointing. This stems from our limited understanding of the subtleties by which enzymes enhance reaction rates. Based on current dogma, the vast majority of studies have concentrated on understanding how enzymes facilitate passage of the reaction over a static potential-energy barrier. However, recent studies have revealed that passage through, rather than over, the barrier can occur. These studies reveal that quantum mechanical phenomena, driven by protein dynamics, can play a pivotal role in enzyme action. The new millennium will witness a flurry of activity directed at understanding the role of quantum mechanics and protein motion in enzyme action. We discuss these new developments and how they will guide enzymology into the new millennium.

Keywords

hydrogen tunnelling; enzymes; quantum mechanics; transition-state theory; isotope effect

1. Introduction

Enzymes facilitate life via a plethora of reactions (Fersht 1985). Not only do they sustain life, they are also involved in a myriad of processes that affect our everyday lives. These include applications in medicine, household detergents, fine chemical synthesis, the food industry, bioelectronics and the degradation of chemical waste. Over many years, much effort has been expended in the quest to create enzymes for specific biotechnological roles. Prior to the early 1980s, the only methods available for changing enzyme structure were those of chemical modification of functional groups (Hirs 1967) or ‘forced evolution’ (Rigby *et al*. 1974). These methods have now been surpassed by knowledge-based (i.e. rational) site-directed mutagenesis, and the grafting of biological function into existing enzyme molecules (‘retrofitting’; see Hecht (1996)). More recently, gene-shuffling techniques (Stemmer 1994a) have been used to generate novel enzymes. Rational redesign of enzymes is a logical approach to producing better enzymes. However, with a few notable exceptions (see, for example, Clarke *et al*. 1989a; b; Nixon *et al*. 1998; Scrutton *et al*. 1990), rational approaches have been generally

unsuccessful, reiterating our poor level of understanding of how enzymes work. This has led to a more ‘shotgun’ approach to redesign, involving random mutagenesis, producing modest success, but dependent on being able to ‘pull out’ an improved enzyme from a very large collection of randomly modified enzymes (Graham *et al*. 1994; Stemmer 1994b). However, development of a suitable test to identify an improved enzyme is intrinsically very difficult; the rational approach cannot, therefore, be ignored.

Enzymes are large biological molecules, usually proteins, that speed up chemical reactions. Molecules that speed up chemical reactions, but are unchanged afterwards, are known as catalysts. The substances that enzymes act on are known as substrates. Enzymes exhibit remarkable specificity for their substrate molecules, and can approach ‘catalytic perfection’ (Albery & Knowles 1976). A popular approach to modelling catalysis has been to visualize an energy barrier that must be surmounted to proceed from reactants to products (figure 1). The greater the height of this energy barrier, the slower the rate of reaction. Enzymes (like other catalysts) reduce the amount of energy required to pass over this barrier, thereby increasing reaction rate. The structure of the reactant at the top of the barrier is energetically unstable, and is known as the transition state. The energy required to pass over the barrier is the activation energy; the barrier is surmounted by thermal excitation of the substrate. This classical over-the-barrier treatment, known as transition state theory (TST), has been used to picture enzyme-catalysed reactions over the last 50 years (Kraut 1988). However, recent developments indicate that this ‘textbook’ illustration is fundamentally flawed (at least in some circumstances).

TST considers only the particle-like properties of matter. However, matter (especially those particles with smaller mass) can also be considered as having wave-like properties: this is known as the wave–particle duality of matter. For enzyme-catalysed reactions, an alternative picture to TST has emerged from considering the wave–particle duality of matter. One important feature of the wave-like properties of matter is that it can pass through regions that would be inaccessible if it were treated as a particle, i.e. the wave-like properties mean that matter can pass through regions where there is zero probability of finding it. Thus, the pathway from reactants to products in an enzyme-catalysed reaction may not need to pass over the barrier, as in TST with particle-like behaviour, but could pass through the barrier. This passing through the barrier (quantum tunnelling; figure 2) can be likened to passing from one valley to an adjacent valley via a tunnel, rather than having to climb over the mountain between. As the analogy suggests, this can significantly lower the energy required to proceed from reactants to products. Thus, quantum tunnelling may play an important role in driving enzyme-catalysed reactions, especially for the transfer of small nuclei, such as hydrogen.

Quantum tunnelling is the established mechanism for enzyme-mediated transfer of the much smaller electron (DeVault 1980; Marcus & Sutin 1985). Proteins are electrical insulators; nevertheless, electrons can travel large distances (up to *ca*. 3×10^{-9} m) through them. This apparent paradox, of an electron passing through an electrical insulator, can be understood in terms of the wave-like properties of the electron. Thus, the electron can pass via quantum tunnelling through regions from which it would be excluded by its particle-like nature.

In contrast to electron transfer via quantum tunnelling, the ground-breaking work of Klinman and colleagues provides the only experimental indication of H-tunnelling in enzyme molecules (currently five in total; see Bahnson & Klinman (1995)). This arises conceptually because the mass of the H nucleus is approximately 1840 times greater than that of the electron. The probability of tunnelling decreases with increasing mass, which significantly reduces the probability of hydrogen versus electron tunnelling. Nevertheless, for those enzyme-catalysed reactions with a large activation energy, quantum tunnelling is an attractive means of transferring hydrogen from reactant to product. Until recently, quantum tunnelling was thought

to be significant only at very low (cryogenic) temperatures. However, deviations from classical TST behaviour have been seen recently, implying that H-tunnelling may be significant at physiological temperatures. These results have, in the main, been modelled as hybrid 'over' (TST) and 'through' (quantum-tunnelling) barrier transfer reactions, i.e. quantum correction models of TST (Bell 1980).

Our own studies have revealed, for the first time, that quantum tunnelling can be the sole means by which an enzyme catalyses H-transfer during C–H bond breakage (Basran *et al*. 1999). The reaction pathway does not pass up the energy barrier prior to tunnelling, as with the quantum correction models of TST, but tunnels through the barrier from the starting (or ground) state. Paradoxically, reaction rates (as with TST) are still highly dependent on temperature. This observation is inconsistent with a pure 'ground-state' tunnelling reaction, since the probability of tunnelling (and, thus, rate of reaction) is a function of barrier width, but is independent of temperature. This apparent paradox is resolved by taking into account the temperature-dependent natural breathing of enzyme molecules, which distorts the structure of the protein to produce the geometry required for nuclear tunnelling (achieved by reducing the width of the barrier between reactants and products). In this dynamic view of enzyme catalysis, it is thus the width, and not the height (as with TST), of the energy barrier that controls the reaction rate.

The important criterion thus becomes the ability of the enzyme to distort and thereby reduce barrier width, and not stabilization of the transition state with concomitant reduction in barrier height (activation energy). We now describe theoretical approaches to enzymatic catalysis that have led to the development of dynamic barrier (width) tunnelling theories for H-transfer. Indeed, enzymatic H-tunnelling can be treated conceptually in a similar way to the well-established quantum theories for electron transfer in proteins.

2. Enzyme catalysis in the classical world

In the classical world (and biochemistry textbooks), TST has been used extensively to model enzyme catalysis (Glasstone *et al*. 1941; Johnson *et al*. 1974; Kraut 1988). The basic premise of TST is that the single-step reaction converting substrate to product, S → P, is instead treated as a two-step reaction over a static potential-energy barrier,

$$\mathrm{S} \rightleftharpoons \mathrm{X}^{\ddagger} \overset{k}{\rightarrow} \mathrm{P}, \tag{2.1}$$

where $X^{\ddagger}$ is the transition state, which is treated as being in *quasi*-equilibrium with the ground state of the substrate at the foot of the energy barrier. An equilibrium constant, $K^{\ddagger} = \exp(-\Delta G^{\ddagger}/RT)$ can be defined, where $\Delta G^{\ddagger}$ (the Gibbs free energy[†]) is that required to surmount the barrier, T is the absolute temperature, and R is the gas constant. It can also be shown that the rate constant for the reaction, k, is related to the degree by which the transition state is populated as follows:

$$k = \left(\frac{k_{\mathrm{B}}T}{h}\right) \exp\left(\frac{-\Delta G^{\ddagger}}{RT}\right). \tag{2.2}$$

Where k_{B} is the Boltzmann constant, and h is Planck's constant. These simple mathematical relationships probably account for the popularity of TST in modelling enzyme catalysis.

[†]The Gibbs free energy is equivalent to the activation energy, ΔE_{a}, discussed elsewhere (providing that $\Delta E_{\mathrm{a}} \gg RT$, which is the case in the systems discussed herein; $\Delta H = \Delta E_{\mathrm{a}} - RT$). Note that by convention, enzymologists consider reactions on the macroscopic scale and use the macroscopic Gibbs free energy; this is equivalent to considering the potential energy on the microscopic scale.

TST has been useful in providing a rationale for the so-called 'kinetic isotope effect' (KIE) (More O'Ferrall 1975; Van Hook 1971). The KIE is used by enzymologists to probe various aspects of mechanism (Klinman 1978). Importantly, measured KIEs have also been used to monitor non-classical behaviour in enzyme-catalysed H-transfer reactions (Bahnson & Klinman 1995). The KIE arises because of the differential reactivity of, for example, a C–H (protium), a C–D (deuterium) and a C–T (tritium) bond. The electronic, rotational and translational properties of the H, D and T atoms are identical. However, by virtue of the larger reduced mass of C–T compared with that of C–D and C–H, the zero-point vibrational energy of C–H is greater than that of C–D, which is greater than that of C–T (figure 1). In the transition state, one vibrational degree of freedom is lost, which leads to differences between isotopes in $\Delta G^{\ddagger}$. This leads, in turn, to an isotope-dependent difference in rate: the lower the mass of the isotope, the lower $\Delta G^{\ddagger}$, and, thus, the faster the rate. The KIEs, therefore, have different values depending on the isotopes being compared: $k_H/k_D \approx 7$ and $k_H/k_T \approx 15$ at 25 °C (Schneider & Stern 1972).

For a single barrier, the classical theory places an upper limit on the observed KIE. However, with enzyme-catalysed reactions, the value of the KIE is often less than the upper limit. This can arise because of the complexity of enzyme-catalysed reactions. For example, enzymes often catalyse multi-step reactions, involving transfer over multiple barriers. In the simplest case, the highest barrier will determine the overall reaction rate. However, in the case where two (or more) barriers are of similar height, each will contribute to determining the overall rate; if transfer over the second barrier does not involve breakage of a C–H bond, it will not be an isotope-sensitive step, thus leading to a reduction in the observed KIE. An alternative rationale for reduced KIEs has also been discussed in relation to the structure of the transition state. For isoenergetic reactions (i.e. the substrate and product have the same energy; the total free energy change, $\Delta G = 0$), the transition state is predicted to be symmetrical and vibrations in the reactive C–H bond are lost at the top of the barrier. In this scenario, the maximum KIE is realized. However, when the transition state resembles much more closely the enzyme–substrate complex ($\Delta G < 0$) or the enzyme–product complex ($\Delta G > 0$), the presence of vibrational frequencies in the transition state cancel with ground-state vibrational frequencies, and the KIE is reduced. This dependence of transition-state structure on the KIE has become known as the Westheimer effect (Westheimer 1961).

3. A role for protein dynamics in classical transfers

The TST is probably an oversimplification when applied to enzyme catalysis; it was originally developed to account for gas-phase reactions. Solvent dynamics and the natural 'breathing' of the enzyme molecule need to be included for a more complete picture of enzymatic reactions. Kramers put forward a theory that explicitly recognizes the role of solvent dynamics in catalysis (Gavish 1986; Kramers 1940). For the reaction S → P, Kramers (1940) suggested that this proceeds by a process of diffusion over a potential-energy barrier. The driving force for the reaction is derived from random thermally induced structural fluctuations in the protein, and these 'energize' the motion of the substrate. This kinetic motion in the substrate is subsequently dissipated because of friction with the surroundings, and enables the substrate to reach a degree of strain that is consistent with it progressing from S to P (along the reaction pathway): the so-called 'transient-strain' model of enzyme catalysis. The rate constant, k, is related to the height of the potential-energy barrier, ΔU, by

$$k = (1/\tau)\, e^{(-\Delta U/RT)}, \tag{3.1}$$

where τ is the time constant of structural fluctuations (and is proportional to the local viscosity). Equation (3.1) takes the same form as the phenomenological Arrhenius equation (equation (3.2)), which has been used to describe classical transfers over a static potential-energy barrier,

$$k = A\mathrm{e}^{(-\Delta E_a/RT)}, \tag{3.2}$$

where A is the so-called pre-exponential factor, and ΔE_a is the activation energy. However, the dynamic nature of molecules is incorporated into ΔU, but not into ΔE_a. By acknowledging the dynamic nature of protein molecules, Kramers' (1940) theory (but not TST) for classical transfers provides us with a platform from which to develop new theories of quantum tunnelling in enzyme molecules.

4. Wave—particle duality and the concept of tunnelling

Tunnelling is a phenomenon that arises as a result of the wave properties of matter. The (de Broglie) wavelength, λ, of a particle can be calculated from its mass, m, and its kinetic energy, E, using the de Broglie equation:

$$\lambda = h/(2mE)^{1/2}. \tag{4.1}$$

Thus, the lighter the particle, the longer its de Broglie wavelength, and, as the particle mass is reduced, there is higher uncertainty in its position. Quantum tunnelling is the penetration of a particle into a region that is excluded in classical mechanics (due to it having insuffcient energy to overcome the potential-energy barrier). An important feature of quantum mechanics is that details of a particle's location and motion are defined by a wave function. The wave function is a quantity which, when squared, gives the probability of finding a particle in a given region of space. Thus, a non-zero wave function for a given region means that there is a finite probability of the particle being found there. A non-zero wave function on one side of the barrier will decay inside the barrier where its kinetic energy, E, is less than the potential-energy of the barrier, V (i.e. $E < V$; if $E > V$, it can pass over the barrier). On emerging at the other side of the barrier, the wave function amplitude is non-zero, and there is a finite probability that the particle is found on the other side of the barrier, i.e. the particle has tunnelled (figure 2).

Quantum tunnelling in chemical reactions can be visualized in terms of a reaction coordinate diagram (figure 3). As we have seen, classical transitions are achieved by thermal activation: nuclear (i.e. atomic position) displacement along the R curve distorts the geometry so that the intersection of the R and P curves is reached (the so-called transition state). Quantum mechanics is based on the premise that energy is quantized (i.e. can have only specific, discrete values). Thus, in the reaction coordinate diagram, the quantized vibrational energy states of the reactant and product can be depicted (figure 3). At ambient temperatures, it is almost exclusively the ground-state vibrational energy levels that are populated.

Factors that enhance tunnelling are a small particle mass (increased de Broglie wavelength) and a narrow potential-energy barrier. To illustrate the effect of particle mass, consider the somewhat idealized rectangular potential-energy barrier (height V, width ℓ), where the tunnelling probability, P, is related to the mass of the tunnelling particle by the following relationship:

$$P \propto \exp\left[\left(-2\ell\sqrt{2mV}\right)/\hbar\right], \tag{4.2}$$

where $\hbar$ is $h/2\pi$. In biology, electron transfer is known to occur over large distances (up to *ca*. 25 Å). Using the above expression, and given that the mass of protium is 1840 times that of the electron, the same probability for protium tunnelling gives a transfer distance of 0.58 Å. This distance is similar to the length of a reaction coordinate, and is thus suggestive of high tunnelling probability. The larger masses of deuterium and tritium lead to corresponding transfer distances of 0.41 Å and 0.34 Å, respectively, thus making KIE studies attractive for the detection of H-tunnelling in enzymes.

The effect of barrier width is readily illustrated by using a more realistic potential-energy barrier shape (e.g. a truncated parabola). The temperature dependence of a unimolecular (i.e. single-molecule) rate constant k may be represented by

$$k(T) = AQ(T)\,\mathrm{e}^{-V/RT}, \tag{4.3}$$

where V is the height of the barrier, and A is the temperature-independent frequency of collisions with the barrier. $Q(T)$, the tunnelling correction factor, is defined as the ratio of the quantum mechanical to the classical barrier transmission rates, which approaches unity at high temperatures (i.e. the apparent activation energy approaches V). For a truncated parabolic energy barrier

$$Q(T) = \frac{h\nu}{2k_{\mathrm{B}}T \quad \sin\,(h\nu/2k_{\mathrm{B}}T)}, \tag{4.4}$$

where v is the reaction coordinate frequency (e.g. the frequency of a vibrating C–H bond). Treatment of the vibrating bond as a simple harmonic oscillator allows the reaction coordinate frequency to be formulated in terms of Hooke's law:

$$\nu = \frac{1}{2\pi}\left(\frac{k_{\mathrm{bond}}}{m}\right)^{1/2}. \tag{4.5}$$

v is, therefore, related to the mass of the particle m and the force constant k_{bond} of the vibrating bond. Large force constants produce high and narrow barrier shapes and, therefore, v is large. Consequently, in this regime, $Q(T)$ is large and transfer is by quantum tunnelling. For low and wide barrier shapes (i.e. small values of the force constant), $Q(T)$ is small, and transfer is dominated by the classical route.

Different strategies are required for optimizing enzyme structure for reactions to proceed by quantum tunnelling rather than classical transfer. For classical transfers, the enzyme has evolved to reduce the height of the potential-energy barrier and to stabilize the transition state (rather than ground state). In the quantum regime, it is reduction of barrier width and not height that optimizes rate. Quantum tunnelling from the ground state requires little or no structural reorganization of the substrate, and the need to stabilize a transition state is thus eliminated. Exclusion of water from the active sites of enzymes prevents coupling of solvent motion to the transfer reaction, and this leads to a reduction of mass for the transferred particle. In the following sections, we review the evidence for quantum tunnelling in biological catalysis and discuss the strategies employed by enzymes to optimize the transfer process. Surprisingly, and unlike for biological electron transfers, reports of H-tunnelling in enzymatic reactions have been restricted to only a small number of enzyme molecules. The realization that H-tunnelling occurs in enzymes has been relatively recent (Basran *et al*. 1999; Cha *et al*. 1989; Grant & Klinman 1989; Jonsson *et al*. 1994, 1996; Kohen *et al*. 1997). This may, in part, be due to

i. the misconception that the much larger mass of the H nucleus is inconsistent with tunnelling; and

ii. the erroneous assumption that measured KIEs less than 7 are always indicative of classical H-transfer (Bruno & Bialek 1992).

Our recent work has demonstrated that H-tunnelling in proteins is inextricably coupled to protein dynamics (Basran *et al*. 1999). This provides a link to the established theories for electron tunnelling in proteins. To provide a framework for the discussion of H-tunnelling in enzymes, protein-mediated electron transfer is discussed below.

5. Electron tunnelling in proteins

The transfer of electrons in proteins by a quantum mechanical tunnelling mechanism is now firmly established (DeVault & Chance 1966; Marcus & Sutin 1985). Electron transfer within proteins occurs between two ‘centres’ (known as redox centres, since one reduces the other, and in so doing is itself oxidized): the ‘electron donor’ (which is thereby oxidized) supplies an electron to the ‘electron acceptor’ (which is thereby reduced). This can be modelled using quantum mechanics. If the wave function of the reactant state is denoted $\Psi(R)$ and the wave function of the product state denoted $\Psi(P)$, then electron transfer between two redox centres A and B is viewed as a transition in state from $\Psi(R)$ = (a*, b) to $\Psi(P)$ = (a, b*). Here *a* and *b* are functions that describe the nuclear and electronic motions of their respective redox centres. The electron, denoted by an asterisk, is transferred from *a* to *b* during the course of the reaction. This results in a change in charge distribution (since the location of the electron changes). In turn, this alters the position of polar groups around the redox centres. Thus, electron transfer alters the nuclear states (i.e. atomic positions) as well as electronic states of the protein.

It is well established that electron transfer in proteins is driven by distortion in the nuclear (protein) geometry of the reactant state. This is facilitated by the natural, thermally activated, breathing of the protein molecule. Thermal activation of the reactant state leads to overlap with the potential-energy curve for the product state; the point of overlap is the nuclear geometry that is compatible with electron tunnelling. At this intersection point, there is an energy barrier through which the electron must tunnel to arrive on the product side. The theory for protein-mediated electron transfer reactions illustrates an important role for protein dynamics in driving the tunnelling process. The importance of dynamic fluctuations in the protein can be appreciated by considering those reactions that have a non-zero thermodynamic driving force for the electron transfer reaction. Since tunnelling is significant only between states of nearly equal energy, tunnelling is unlikely in such instances. However, dynamic fluctuations in the protein overcome this problem. These equalize the energy between the reactant and product at the intersection point of the R and P curves (i.e. their configurations are identical), thus enabling transfer by quantum tunnelling. The term ‘vibrationally assisted tunnelling’ is, therefore, appropriate for protein electron transfer reactions. As described below, our recent work has also demonstrated a similar role for dynamic fluctuations of the protein during enzyme-catalysed H-tunnelling. Electron transfer theory therefore provides a useful framework for understanding enzymatic H-tunnelling. Despite this, until very recently, tunnelling derivatives of TST (Bell 1980)—which do not take into account the fluctuating nature of the enzyme—have been used to account fully for enzymatic H-tunnelling. As a backdrop to the very recent dynamic treatments of H-tunnelling in enzymes, we describe below static barrier approaches, i.e. tunnelling correction theories of TST, that have been applied to some enzyme systems.

6. TST and corrections for H-tunnelling

In TST, a static barrier is used to represent the reaction. The temperature dependence of such a reaction can be understood in terms of the Arrhenius equation (equation (3.2)). Deviations

from classical behaviour are usefully probed via the kinetic isotope effect (§ 2). For non-enzymatic reactions, several factors—in addition to inflated KIEs (i.e. KIEs above 7)—have been used to indicate quantum tunnelling of hydrogen. A particularly striking indication of quantum tunnelling is the observation of curvature in the Arrhenius plot (i.e. ln (rate) versus $1/T$) over an extensive temperature range. Interestingly, this has been observed in non-enzymatic radical reactions (Bromberg *et al*. 1972; Brunton *et al*. 1976; Wang & Williams 1972). However, curvature in Arrhenius plots is not a useful indicator of quantum tunnelling because the limited experimental temperature range available in studies using enzymes makes it impossible to detect any curvature. An alternative approach is to estimate, from the Arrhenius plot, the activation energy for the reaction and the pre-exponential factors. Large differences in the activation energies for protium and deuterium transfer ($\Delta\Delta E_a > 5{:}4$ kJ mol^{-1}) (Bell 1980), and values deviating from unity for the ratio of Arrhenius pre-exponential factors ($A^H{:}A^D \neq 1$) (More O'Ferrall 1975; Schneider & Stern 1972), can indicate non-classical behaviour. In conjunction with inflated KIEs, these parameters have been used to demonstrate quantum tunnelling in enzyme molecules.

Small deviations from classical behaviour have been reported for the enzymes yeast alcohol dehydrogenase (YADH) (Cha *et al*. 1989), bovine serum amine oxidase (BSAO) (Grant & Klinman 1989), monoamine oxidase (MAO) (Jonsson *et al*. 1994) and glucose oxidase (GO) (Kohen *et al*. 1997). More recently, the enzyme lipoxygenase has been shown to catalyse H-transfer by a more extreme quantum-tunnelling process (Jonsson *et al*. 1996). In this case, the apparent activation energy was found to be much smaller than for reactions catalysed by YADH, BSAO, MAO or GO, suggesting a correlation between apparent activation energy and the extent of tunnelling. Use of a static (TST-like) barrier in the treatment of H-tunnelling in enzymes has allowed the construction of (hypothetical) relationships between the reaction rate and temperature (Jonsson *et al*. 1996). These relationships are readily visualized in the context of a temperature-dependence plot[†] (a plot of ln k/T versus $1/T$) and are observed in studies that employ isotope (i.e. H, D and T) substitution within the reactive bond. The plot can be divided into four regimes (figure 4): regime I describes classical (TST) behaviour, conforms to the unimolecular rate law and is characterized by large values of $\Delta H^{\ddagger}$ and a $A'^H{:}A'^D$ ratio of approximately 1 (the prime is used to distinguish this ratio from the $A^H{:}A^D$ ratio calculated from the classical Arrhenius plot). Regimes II—IV reveal the effects of quantum tunnelling on the temperature dependence of the reaction rate; the extent of quantum tunnelling increases from regime II to regime IV. In regime II, protium tunnels more extensively than deuterium, thus giving rise to inflated values for the KIE, and an $A'^H{:}A'^D$ ratio less than 1. Regime III is characterized by extensive tunnelling of both protium and deuterium, and the $A'^H{:}A'^D$ ratios are diffcult to predict. Finally, regime IV is the predicted regime for transfer solely by ground-state tunnelling. In this case, the $A'^H{:}A'^D$ ratio equals the KIE and the reaction rate is not dependent on temperature (the reaction passes through, and not over, the barrier, thus there is no temperature-dependent term).

Relationships between reaction rate and temperature can, therefore, be used to detect non-classical behaviour in enzymes. Non-classical values of the $A'^H{:}A'^D$ ratio ($A'^H{:}A'^D \neq 1$) and

[†]Previous studies of H-tunnelling in enzymes have been analysed in terms of the phenomenological Arrhenius plot (e.g. Jonsson *et al*. (1996), i.e. a plot of ln(k) versus $1/T$). Although the Arrhenius plot appears linear in the accessible temperature range, it is in fact curved and asymptotically approaches infinity at high temperatures. Apparent linearity in the accessible temperature range does not compromise data analysis in this regime, but temperature-dependence studies should strictly be analysed in terms of the equation describing a unimolecular reaction, which can be conveniently written as:

$$\ln\,(k/T) = \ln\,(k_B/h) + \Delta S^{\ddagger}/R - \Delta H^{\ddagger}/RT.$$

The activation parameter $\Delta H^{\ddagger}$ is calculated from the slope of the plot.

$\Delta\Delta H^{\ddagger}$ (greater than 5.4 kJ mol^{-1}, i.e. greater than the difference in zero-point vibrational energies of the C–H and C–D bonds) have been the criteria used to demonstrate H-tunnelling in the enzymes mentioned above. A major prediction from this static-barrier (TST-like) plot is that tunnelling becomes more prominent as the apparent $\Delta H^{\ddagger}$ decreases. This holds for the enzymes listed above, but the correlation breaks down for enzymes catalysing the breakage of C–H bonds (Basran *et al*. 1999): a direct result of the type of potential-energy barrier that is used. Temperature-independent tunnelling is a direct result of invoking a static (Eyring-like) potential-energy barrier. However, an alternative approach comes from invoking a fluctuating (Kramers-like) potential-energy barrier (Chandrasekhar 1943; Kramers 1940). This is conceptually more realistic as it takes into account the dynamic motion of the protein. These dynamic effects will give rise to more complex temperature dependencies for rates of H-transfer than those illustrated in figure 4. The role of protein dynamics in driving enzymatic H-tunnelling is discussed below.

7. H-tunnelling driven by protein dynamics

Vibrational enhancement (i.e. thermal activation) in non-enzymatic H-tunnelling reactions has also been considered, but these thermal fluctuations are usually restricted to the reacting species (Borgis & Hynes 1991,1996;Suarez & Silbey 1991). In recent years, attempts have been made to model, theoretically, enzymatic H-tunnelling by incorporating thermal vibrations. However, and importantly, none of these approaches have been verified experimentally. Recently, the kinetic data for BSAO have been re-evaluated for thermally activated substrate vibrations, but with the protein molecule treated as rigid (Antoniou & Schwartz 1997). Computational molecular-dynamics simulation studies have also suggested a dynamic role for the protein molecule in enzymatic H-tunnelling (Bala *et al*. 1996; Hwang *et al*. 1991; Hwang & Warshel 1996). Indeed, some theoretical treatments have recognized the role of thermal motion in the protein in H-tunnelling (Dogonadze *et al*. 1977; Sumi & Ulstrop 1988), but have failed to predict the experimentally observed KIE; again, experimental verification of these theories is lacking.

The only (to the best of our knowledge) theoretical treatment of H-transfer by tunnelling to explicitly recognize the role of protein dynamics, and relate this in turn to the observed KIE, was described by Bruno & Bialek (1992). This approach has been termed vibrationally enhanced ground-state tunnelling theory (VEGST theory). A key feature of this theory—and one that sets it apart from many other theoretical approaches—is that tunnelling occurs from the ground-state vibrational energy levels of the substrate, i.e. there is no thermal activation of the substrate. The temperature dependence of the reaction is, therefore, attributed to the natural thermally induced breathing of the enzyme molecule, thus shortening the distance the hydrogen must tunnel. Thus, the natural breathing of the enzyme molecule can be visualized in the context of the familiar R and P potential-energy curve depiction encountered in discussions of electron transfer in proteins (§ 5). H-tunnelling does not occur until the geometry of the protein is distorted so that the R and P curves intersect (figure 5). At the intersection point (X) of the two curves, the nucleus tunnels; the average tunnelling probability is decreased when heavier isotopes (e.g. deuterium) are transferred, thus giving rise to a KIE greater than 1. At the intersection point, tunnelling is from the vibrational ground states, since vibrational quanta are comparable to barrier height (Bruno & Bialek 1992), and, therefore, vibrational excitation would lead to a classical ‘over-the-barrier’ transfer.

Clearly, protein dynamics is hypothesized to play a major role in driving H-tunnelling in enzymes. However, like all hypotheses, this requires experimental verification. The activation energy of the reaction is associated with distortion of the protein molecule. Following the tunnelling event, rapid movement away from the intersection point along the P curve prevents coherent oscillations of the H nucleus between the R and P curves. As such, the reaction is

modelled in much the same way as electron transfer in proteins (i.e. Fermi's golden rule applies and the non-adiabatic regime operates). A key prediction of this theory is that H-tunnelling can occur even when the value of the KIE is less than 7, thus suggesting that (contrary to current dogma) KIEs may be poor indicators of quantum tunnelling in enzymes. This is an important point, since static barrier models of H-tunnelling suggest that H-tunnelling does not occur when the KIE is less than 7. This indicates that detailed temperature-dependence studies are required to demonstrate, unequivocally, that tunnelling is a feature of an enzyme-catalysed reaction.

The fluctuating enzyme model of H-tunnelling can be divided into two reaction components: (i) a thermally activated nuclear reorganization step, and (ii) the H-tunnelling event at the intersection point of the potential-energy curves. This leads to three possible rate-limiting regimes in which either (i) nuclear reorganization is rate-limiting, (ii) quantum tunnelling is rate-limiting, or (iii) both factors contribute to the observed rate. The value of the KIE is affected directly by these steps. When nuclear reorganization is rate limiting, the KIE is unity (since this is independent of isotope) and reaction rates are dependent on solvent viscosity (i.e. the ease with which the protein structure can reorganize). In the quantum-tunnelling limiting regime, the KIE is not dependent on solvent viscosity and is not unity (since tunnelling rate is a function of isotope). However, when both nuclear reorganization and quantum tunnelling contribute to the observed rate, the KIE is viscosity dependent, as viscosity increases the nuclear reorganization step becomes rate limiting, and, thus, the KIE tends to unity. In experimental studies, measurements of (i) increased viscosity or (ii) decreased temperature effects on the KIE may be used to discriminate between these possible regimes, since both would be expected to selectively perturb geometrical distortion of the protein.

VEGST assumes that H-transfer occurs entirely by quantum mechanical tunnelling. The model is, therefore, appropriate for those enzymes catalysing ground-state tunnelling (see below). The model is likely to be incomplete for those enzymes in which tunnelling occurs just below the saddle point of the energy surface (i.e. the reactant passes up the energy barrier before tunnelling); in these situations, H-transfer is likely to occur by a combination of classical and quantum mechanical behaviour (Garrett & Truhlar 1980; Truhlar & Gordon 1990). In the case where H-transfer is by a combination of classical and quantum mechanical effects, the activation energy will reflect partitioning of energy into a wide range of modes within the protein, e.g. changes in protein geometry, bond angles of reacting substrate, etc., as well as thermal excitation of the reactive C–H bond. However, experimental verification of VEGST would demonstrate the importance of protein dynamics in enzymatic H-tunnelling. By analogy, therefore, protein dynamics would also be expected to play a major role in those enzymes where H-tunnelling is not from the ground state, but from an excited state of the substrate molecule. Experimental verification of a role for protein dynamics is, thus, a key milestone in developing theories for enzymatic H-tunnelling. This verification is described below.

8. Experimental demonstration of VEGST

Kinetic data for BSAO were originally analysed in terms of the tunnelling correction derivatives of TST (Grant & Klinman 1989), but the data are also consistent with—although not verification of—VEGST theory (Bruno & Bialek 1992). Alternatively, the BSAO data can also be interpreted in terms of an H-tunnelling reaction driven by substrate oscillations (Antoniou & Schwartz 1997). Thus, ambiguity remains concerning the correct theoretical treatment of the BSAO kinetic data. This ambiguity arises because the complex temperature dependence of the reaction can be modelled in a variety of ways. Our recent studies on enzymatic C–H bond cleavage have, however, provided verification of VEGST theory, and also, for the first time, proved the existence of a ground-state H- and D-tunnelling regime in an enzyme molecule (Basran *et al*. 1999).

Our KIE and temperature-dependent studies of the reaction catalysed by the bacterial enzyme methylamine dehydrogenase (MADH) have revealed that the rate of reduction of the enzyme redox centre (tryptophan tryptophylquinone (TTQ)) by substrate has a large, temperature-independent KIE (Basran *et al*. 1999). Reduction of this redox centre is a convenient way of following C–H bond breakage in this enzyme, since breakage of the bond and reduction of the cofactor occur simultaneously. A plot of ln k/T versus $1/T$ reveals that ground-state quantum tunnelling is responsible for the transfer of the hydrogen nucleus. This is indicated by the linear and parallel nature of the plots for C–H and C–D bond breakage, which should be compared with regime IV of the corresponding hypothetical plot for a static potential-energy barrier (figure 6). However, contrary to the static potential-energy barrier model for H-tunnelling, reaction rates are strongly dependent on temperature (apparent activation $\Delta H^{\ddagger} \sim 45$ kJ mol^{-1}) and, importantly, $\Delta H^{\ddagger}$ was found to be independent of isotope. These observations indicate that thermal distortion of the protein scaffold—but not vibrational excitation of the substrate—are required to drive H-transfer. Thus, a fluctuating energy surface is a feature of the tunnelling process. The VEGST equivalent of regime IV of the static barrier plot (figure 6) recognizes that thermal motions of the protein molecule are required to distort the protein scaffold into conformations compatible with H-tunnelling. Regime IV of the VEGST plot, therefore, has a non-zero value for the slope, the value of which is the energy required to distort the protein into the geometry compatible with H-tunnelling. With MADH, it has thus been possible to quantify the energy term associated with structural distortion of the protein during an enzyme-catalysed reaction.

The temperature dependence in regime IV (ground-state tunnelling) for VEGST contrasts markedly with that for the static barrier model. Although there is a sizeable energy term in this regime for the VEGST model ($\Delta H^{\ddagger} \sim 45$ kJ mol^{-1}), the apparent linearity seen in the accessible temperature range for MADH probably does not extend to lower temperatures. At low temperatures, nuclear vibrations will be frozen, thus preventing distortion of the nuclear scaffold into geometries compatible with hydrogen tunnelling. Thus, over a large temperature range, complex temperature dependencies of the reaction rate are predicted.

Ground-state tunnelling driven by protein dynamics (VEGST) is the only theoretical treatment consistent with our work on MADH. As indicated above, a prediction of VEGST is that ground-state tunnelling may occur even when the KIE is less than 7; a regime interpreted previously as indicating classical behaviour. The KIE with MADH is large (approximately 18), and thus the presence of tunnelling is predicted by current dogma. However, our recent analysis of H-tunnelling in trimethylamine dehydrogenase (TMADH) has indicated that, under certain conditions (and contrary to current dogma), ground-state tunnelling occurs even when the KIE is less than 7 (Basran, Sutcliffe & Scrutton, unpublished results). This observation lends support to the validity of VEGST in describing enzymatic H-tunnelling.

9. Significance of H-tunnelling in enzymes

Both MADH and TMADH catalyse the breakage of stable C–H bonds. These are difficult reactions if viewed in terms of the classical TST approach to catalysis, but the structural plasticity of MADH and TMADH (in common with other enzymes) provides a means of circumventing this problem by facilitating ground-state tunnelling. Vibration-driven ground-state tunnelling may, therefore, be a common mechanism for the breakage of C–H bonds by enzymes, and this may extend to other types of H-transfer reactions.

The dynamic barrier approach to catalysis has major implications for how H-transfer reactions —and, indeed, other reactions—are modelled theoretically. Given the dynamic nature of protein molecules, it is perhaps surprising that the indiscriminate use of TST has persisted for so long. For classical transfers, Kramers's theory seems appropriate, and this is an excellent

platform from which to develop theories of quantum tunnelling in enzymes. For those reactions that proceed by quantum tunnelling, it is the energy barrier width that is important in determining reaction rate. Tunnelling probability depends on the mass of the transferred particle, the net driving force and the height and width of the reaction barrier. Proteins can facilitate this by (i) reduction of mass (e.g. exclusion of water), (ii) an equalization of energy states for reactants and products (Albery & Knowles 1976; Nambiar *et al*. 1983), and, most importantly, (iii) a reduction in barrier width (Rodgers *et al*. 1982). Exclusion of water from enzyme active sites is achieved readily and documented amply in the literature. The exploitation of protein dynamics to equalize energy states and shorten tunnelling distance is, however, less well appreciated, but, nevertheless, pivotal.

10. Enzymology into the new millennium

An in-depth understanding of biological catalysis is central to the successful exploitation of enzymes by mankind. At the end of the last century, the ‘lock-and-key’ mechanism propounded by Emil Fischer—in which the enzyme accommodates a specific substrate like a lock does a key—opened the door to our understanding of enzyme catalysis. This has evolved to take account of protein motion in the ‘induced-fit’ model of catalysis (Bennet & Syeitz 1978; Koshland 1973), in which the enzyme has one conformation in the absence, and another conformation in the presence, of substrate. The induced-fit model of catalysis recognizes preferred complementarity to the transition state and has provided a conceptual framework for TST (Kraut 1988). Now, as we move into the new millennium, our understanding has progressed yet further by highlighting the role of (i) protein dynamics and (ii) quantum tunnelling in enzyme catalysis. Thus, the rules underpinning our design and understanding of enzymes have changed significantly. Important areas in which these rules apply include enzyme redesign, the production of catalytic antibodies, design of enzyme inhibitors (drugs and pesticides), enzymatic fine chemical synthesis, and use of enzymes in bulk processing (e.g. paper manufacture, food industry and detergents).

Enzyme redesign strategies currently attempt to reduce the activation energy (i.e. the barrier height) by seeking maximum complementarity with the transition state and destabilization of the ground state. This is the approach adopted in producing catalytic antibodies. Here, an animal’s immune system is exposed to a transition state analogue, thus inducing antibodies with surface complementarity to the transition state. Although, in principle, this is an elegant approach to producing novel catalysts, in practice it is usual for catalytic antibodies to have poor catalytic rates. These studies imply that knowledge of the transition state alone is not suffcient to develop a good catalyst. Insight into additional factors required for effcient catalysis has come from recent work. An important determinant of catalytic effciency is the role of protein dynamics. The structural plasticity of protein molecules is important in driving both classical and quantum mechanical transfers. As we have seen, in quantum mechanical transfers, distortion of the enzyme molecule transiently compresses barrier width and equalizes reactant and product energy states. In contrast to classical models of catalysis, for vibrationally driven ground-state tunnelling, maximum complementarity with the ground state should be sought. Additionally, the exclusion of water will reduce the mass of the transferred particle (thus increasing tunnelling probability). The challenge will therefore be to incorporate these new aspects into programmes of rational enzyme redesign and to provide a unified theory for enzyme-catalysed reactions. Over the last century, our understanding of catalysis has been based primarily on static pictures of enzymes and enzyme—ligand complexes. As we look to the new millennium, our quest for a better understanding will be driven by an appreciation of a role for protein dynamics—both experimental and computational—in driving enzyme-catalysed reactions.

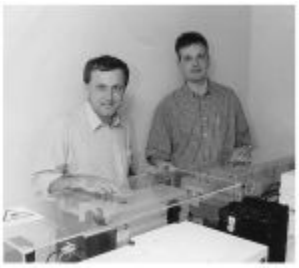
Biography

M. J. Sutcliffe

Born in Rochdale, Lancashire, Michael Sutcliffe (left) studied at Bristol, where he graduated with first class honours in chemical physics in 1985, and at Birkbeck College, London, where he obtained his PhD in protein modelling in 1988. Aged 35, he was a SERC/NATO Fellow at Oxford University and Junior Research Fellow at Linacre College, a Royal Society University Research Fellow at Leicester University, and joined Leicester University as a Lecturer in 1998, where he is currently Reader. His research involves the development and use of computational methods to address one of the major challenges in the biomolecular sciences, understanding the relationship between protein structure and function. He has over 60 publications, including specialized reviews, and was elected a Fellow of the Royal Society of Chemistry in 1999. His recreational interests include hill walking, cycling and canoeing.

N. S. Scrutton

Nigel Scrutton (right) was born in Cleckheaton, Yorkshire. He graduated from King's College, London with first class honours in 1985 and was awarded the Sir William Robson prize. Nigel obtained his PhD at Cambridge as a Benefactors' Scholar. In 1988 he was elected Research Fellow at St John's College and was awarded the Humphreys Research prize. At Cambridge, Nigel was a Research Fellow of the Royal Commission for the Exhibition of 1851 and Royal Society University Research Fellow. He was elected a Fellow of the Royal Society of Chemistry in 1997. Aged 35, Nigel is now a Professor at Leicester University and Lister Institute Research Fellow. He is a recipient of the Colworth Medal of the Biochemical Society. His scientific interests include mechanistic and quantum enzymology: recreational interests include Victorian and College philately.



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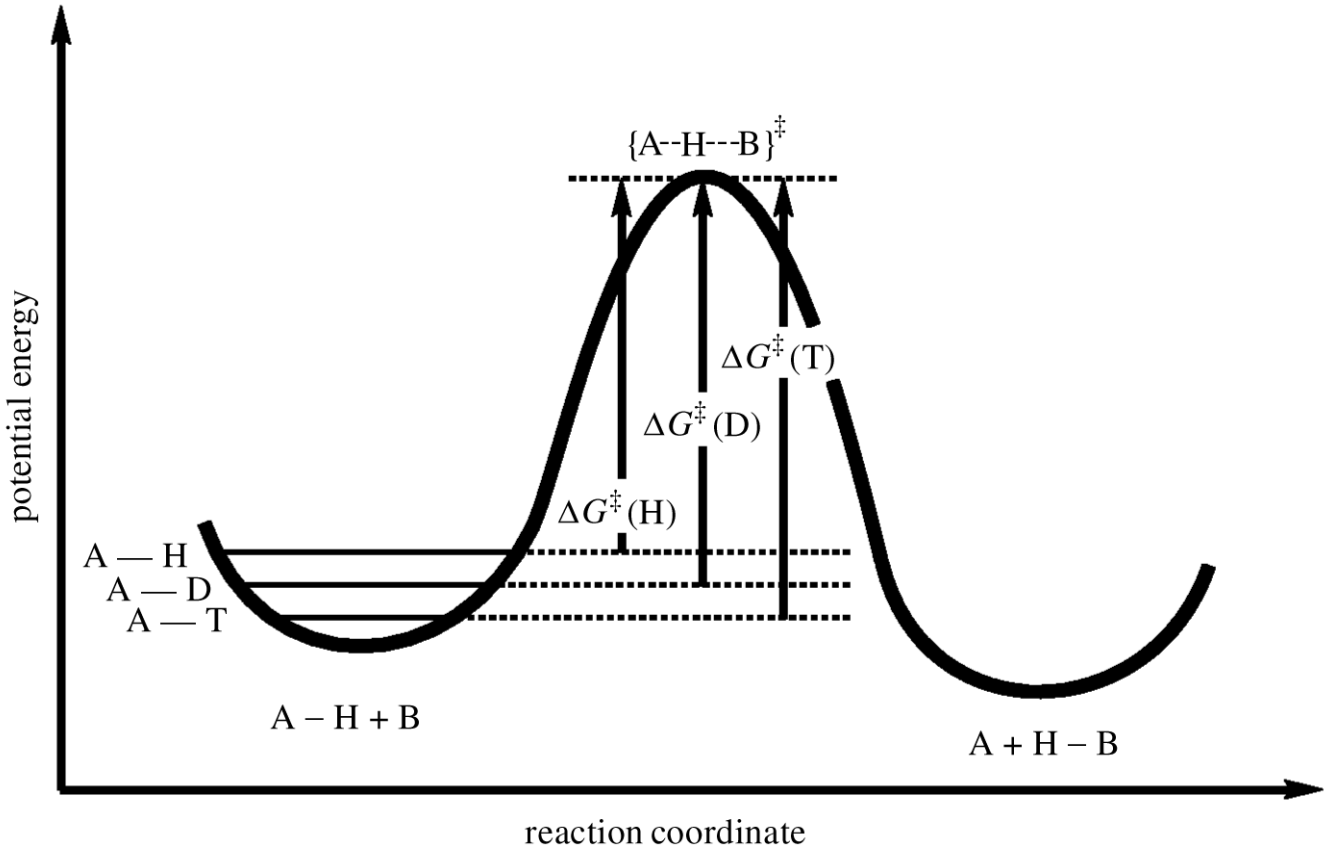

Figure 1.
Schematic representation of the energetics for an enzyme-catalysed reaction. For the reaction (A–H + B → A + H–B) to proceed, reactants must pass over the potential-energy barrier to the product side via the so-called transition state (denoted by ‡) at the top of the energy profile. The molecular vibrations of the reactive bond are superimposed on the energy profile for the reaction. The ground-state vibration energy levels with different isotopic substitution are shown. Each isotope gives rise to a different value for the activation energy ($\Delta G^{\ddagger}$) required to surmount the barrier.

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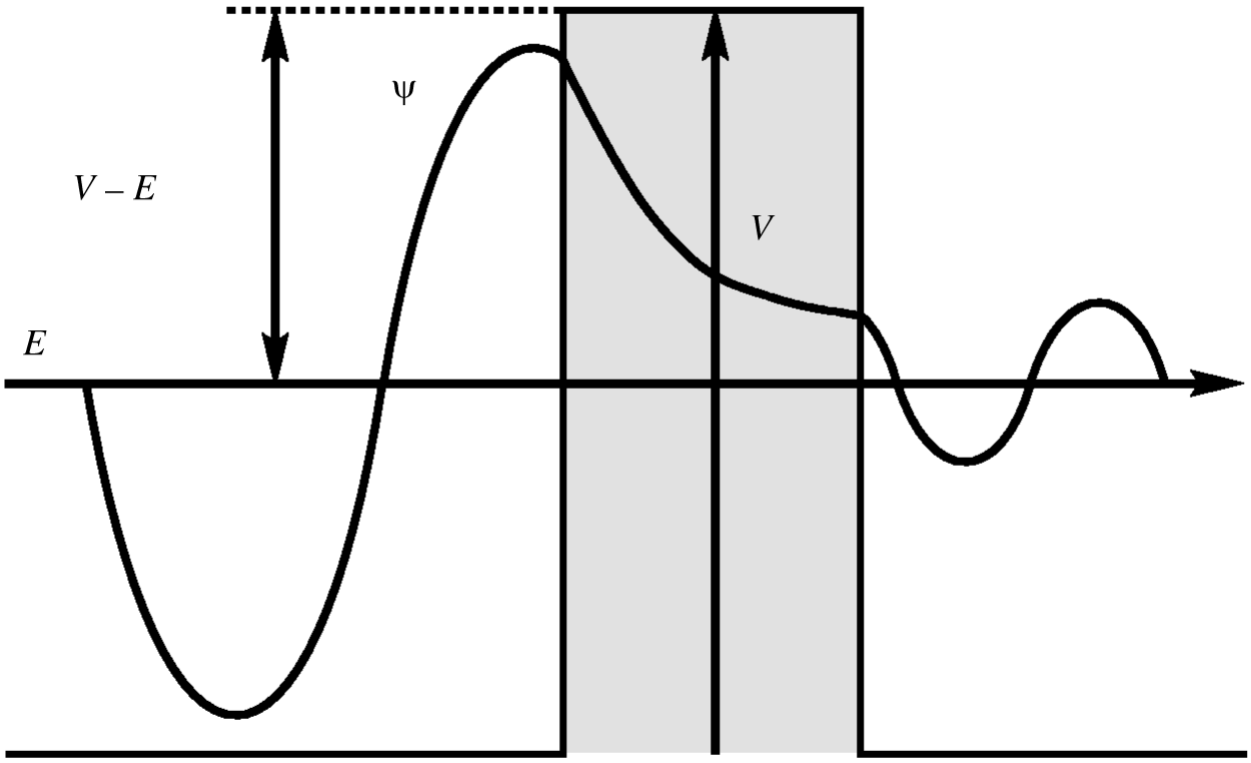

Figure 2.
Tunnelling of wave function, Ψ, with kinetic energy E through a rectangular potential-energy barrier with height V. The narrower the barrier, the smaller the mass of the particle, and the smaller the difference between V and E, the greater the tunnelling probability. If the amplitude of Ψ has not reached zero at the far side of the barrier, it will stop decaying and resume the oscillation it had on entering the barrier (but with smaller amplitude).

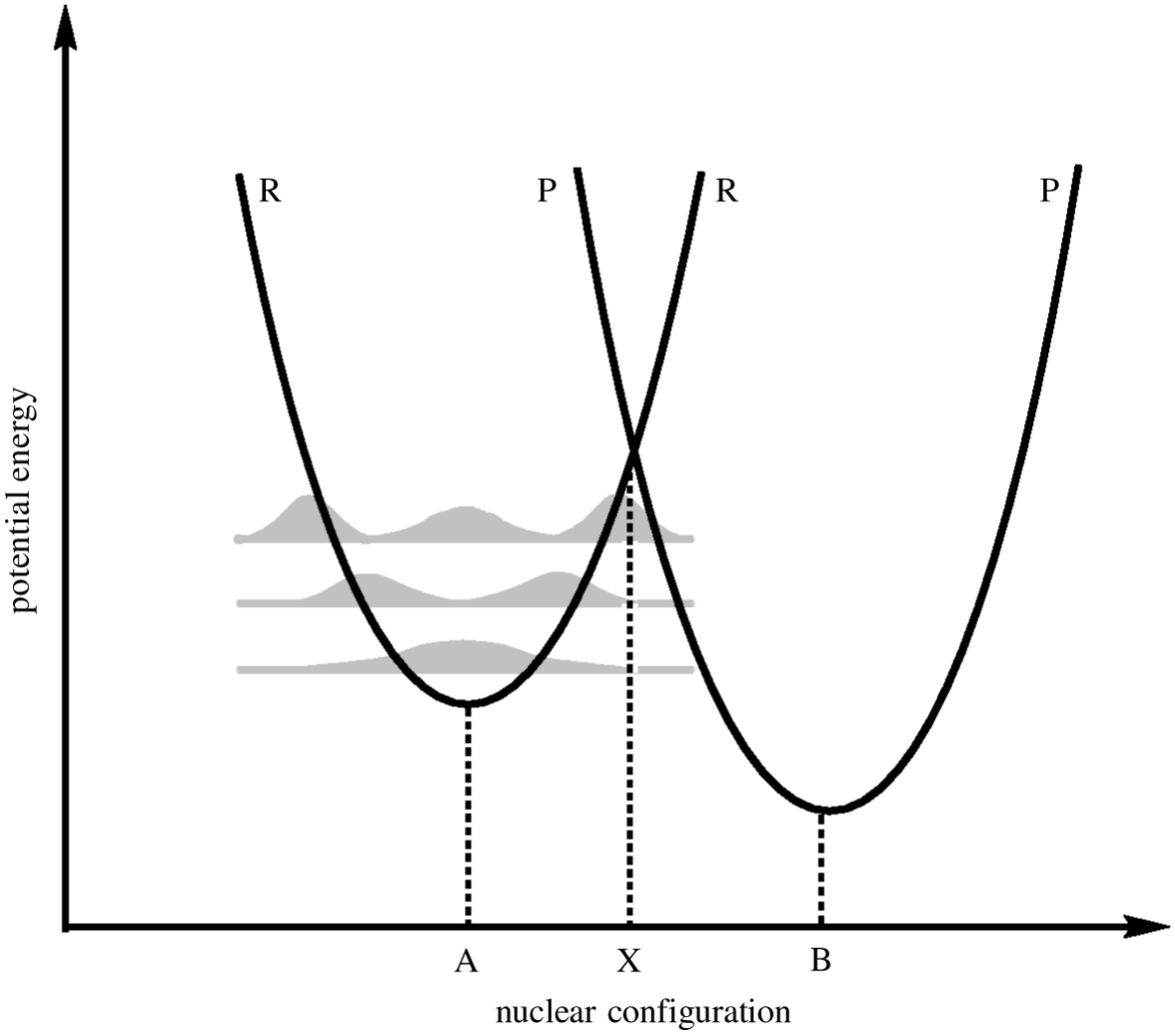

Figure 3.
Reaction coordinate diagram for a simple chemical reaction. The reactant A is converted to product B. The R curve represents the potential surface of the reactant, and the P curve the potential-energy surface of the product. Thermal activation leads to an over-the-barrier process at transition state X. The vibrational states have been shown for the reactant A. As temperature increases, the higher energy vibrational states are occupied leading to increased penetration of the P curve below the classical transition state.

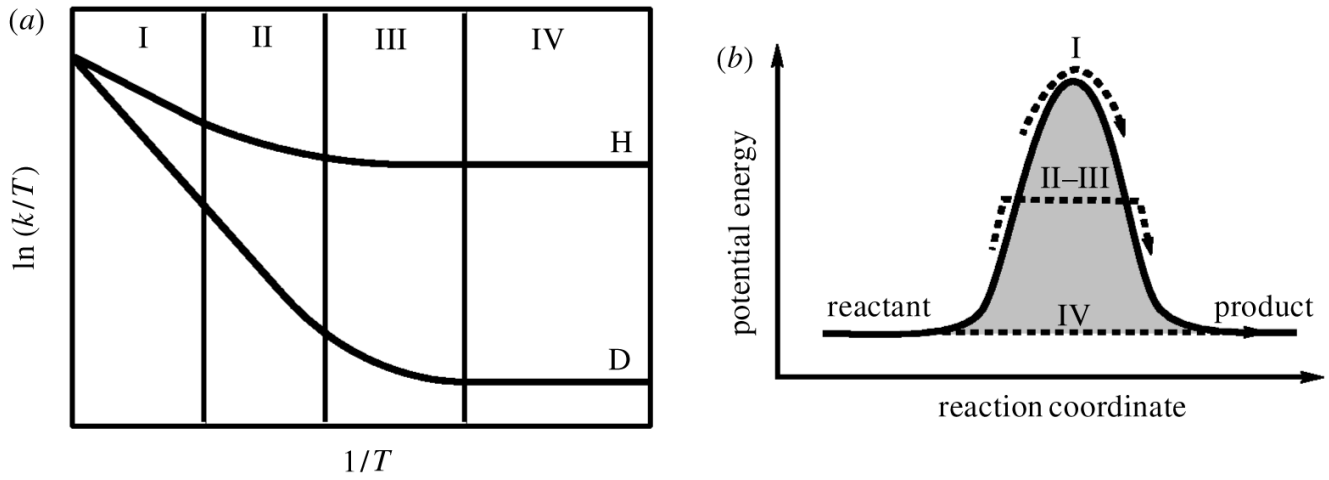

Figure 4.
The static barrier (TST-derived) model of H-tunnelling and definition of tunnelling regimes. (*a*) H-tunnelling regimes. (*b*) A static barrier indicating transfer to the product side in each of the regimes shown in (*a*). In regimes II and III, thermal activation may be required to populate higher vibrational energy states of the reactive C–H bond.

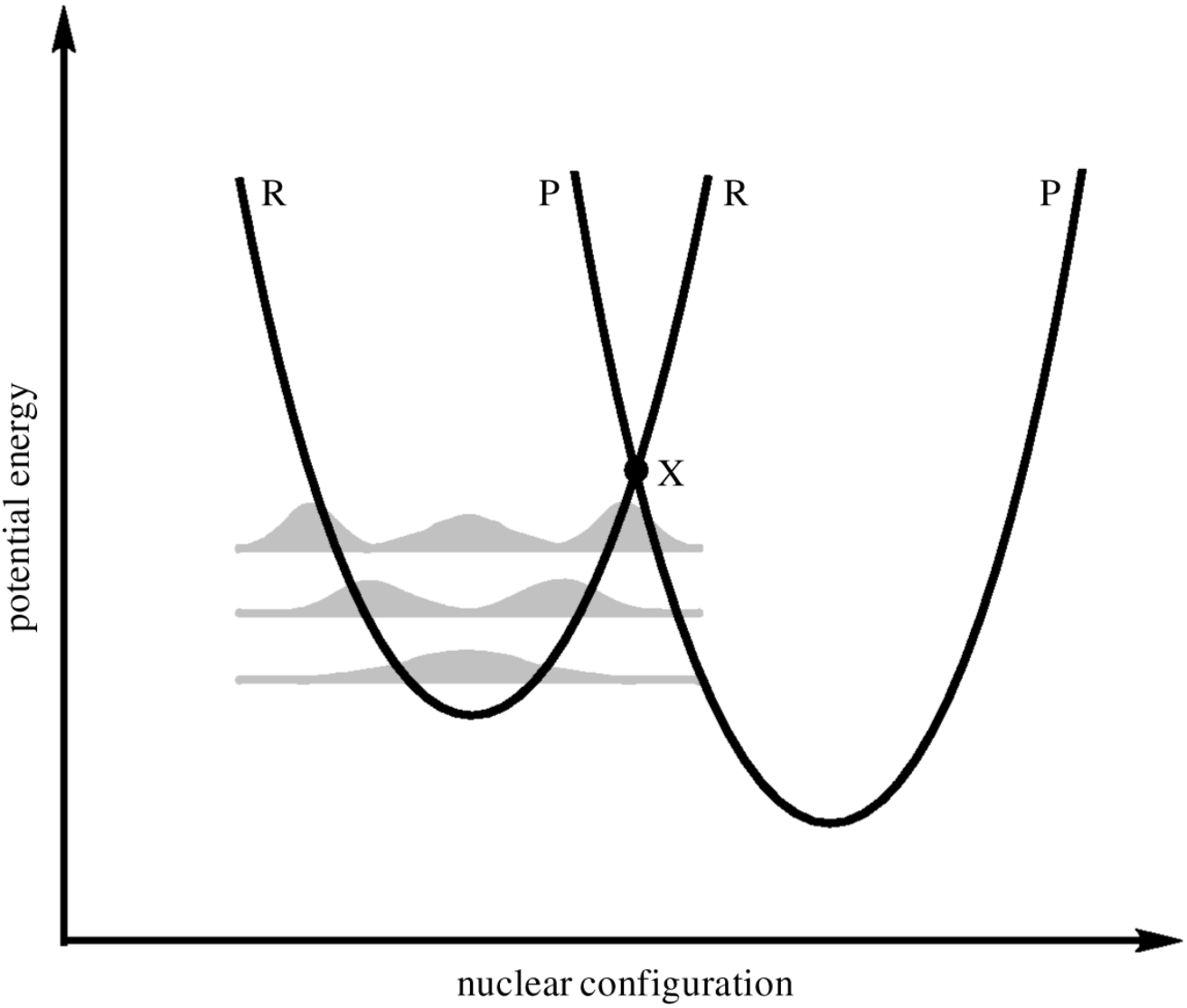

Figure 5.
The dynamic barrier model of H-tunnelling. Reactant (R) and product (P) energy curves for distortion of the protein scaffold. The intersection point (X) is the optimum geometry required for H-transfer. At the intersection point, transfer can be by the classical (I), ground-state tunnelling (IV) or intermediate regimes (II) and (III). In regimes II and III, additional thermal activation (other than that required to distort the protein scaffold to the optimum geometry for transfer, i.e. the R–P intersection) may reflect (i) partition into higher vibrational levels of the reactive C–H bond; and/or (ii) transfer via a combination of classical (over-the-barrier) and quantum mechanical routes.

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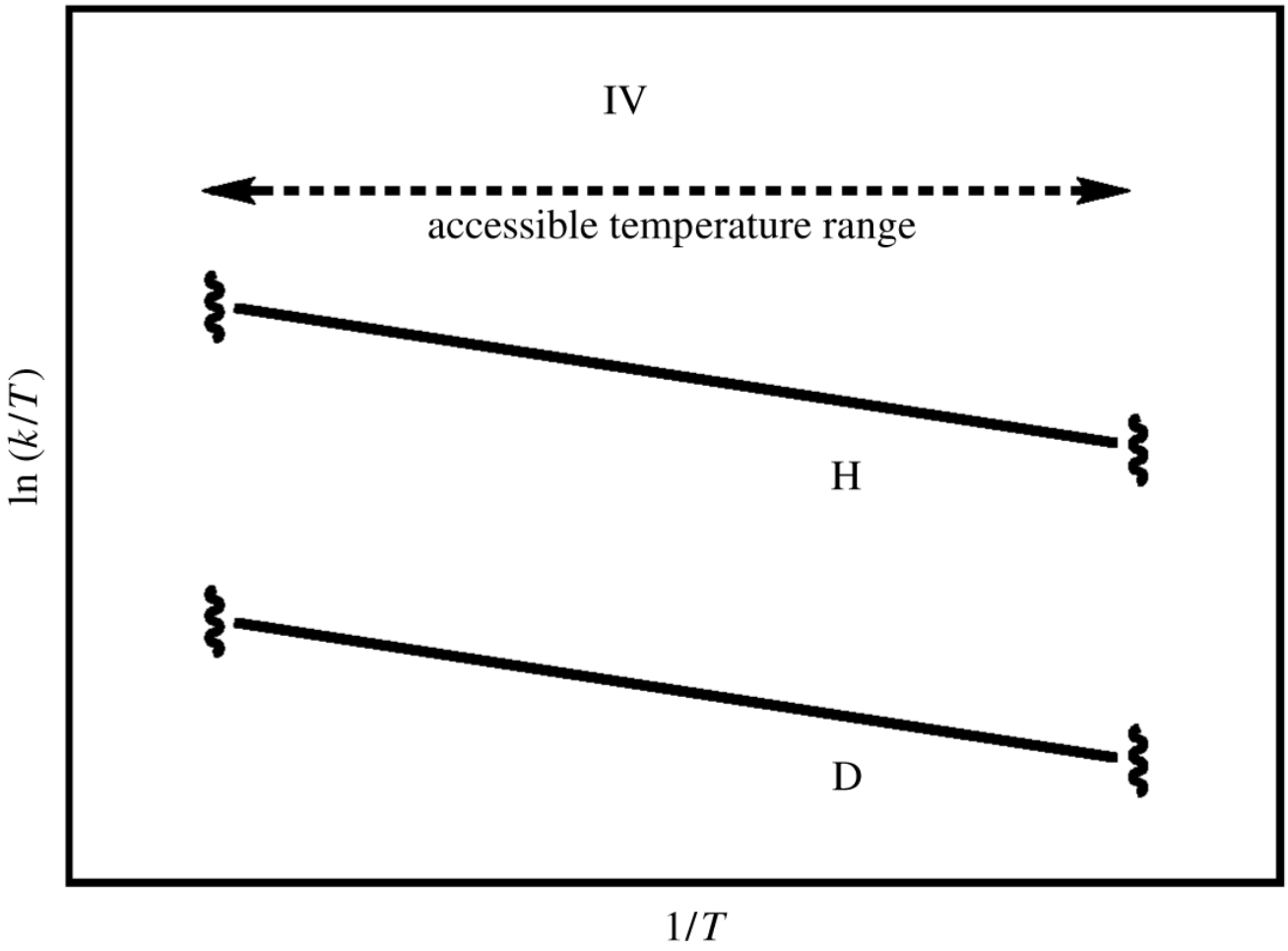

Figure 6.
Expected temperature dependence (in the accessible temperature range) in regime IV in the context of figure 5. Ground-state tunnelling occurs in regime IV. The experimental data for MADH are apparently linear in regime IV, but, as noted in the text, this linearity will probably not extend to cryogenic temperatures.