Enzyme catalysis: over-the-barrier or through-the-barrier?

Early in our undergraduate careers we learn that enzymes achieve high catalytic rates by evolving complementarity with the transition state of the substrate. The simplest textbook description presents a 'static' potential energy barrier to the progress of an enzyme-catalysed reaction and posits the idea that enzymes facilitate catalysis by lowering the activation energy through preferential binding of the transition state[1,2]. This elementary view of biological catalysis – so much the driving force for the *de novo* synthesis of biological catalysts (for example, as 'abzymes' or catalytic antibodies[3,4]) – finds its roots in transition state theory (TST), which was popularized by Eyring and co-workers for reactions in the gas phase[5]. Pauling was responsible for the formal application of TST to enzymatic reactions[6]. Of course, enzymologists now recognize (see below) that there is more to catalysis than the binding energy realized on moving from the ground state to the transition state, but, in general, catalysis is envisaged to occur over the potential energy barrier via the transient population of a transition state. And so, at the opening of the 21st century – following 100 years of research effort, which, en route, embraced Fischer's idea of 'lock and key', and Haldane's notion of 'the imperfect key' – the mysteries of biological catalysis are rooted in classical, over-the-barrier depictions. This picture is now being questioned as roles for through-the-barrier (i.e. quantum tunnelling) mechanisms facilitated by protein dynamics are becoming established alternatives (at least for some enzymes) as a result of recent experimental findings.

Over-the-barrier catalysis

The TST presents a 'static' potential energy surface to the course of a reaction[2,7]. The treatment invokes the existence of a ground state (e.g. an enzyme–substrate complex, ES) and a transition state (e.g. $ES^{\ddagger}$) (Fig. 1a). Strong binding interactions in the ground state are seen as being catalytically wasteful, although it is argued that formation of the ES complex orientates the reactants for catalysis. In TST, binding interactions realized in the transition state ($ES^{\ddagger}$) favour formation of the transition state over that formed in free solution, and thus facilitate passage over the potential energy barrier (the height of which is known as the 'activation energy'). This view of enzyme catalysis has fuelled studies of transition-state complementarity by structural methods. These studies have attempted to identify those noncovalent interactions realized in the transition state that can account for observed rate enhancements. A common and complementary approach has been to 'edit out' key interactions conjectured to form in the transition state and to investigate the consequences of these changes on catalysis[8].

So is the binding of the transition state sufficient to account for rate enhancements achieved by enzyme molecules? The answer is a resounding 'no'. The catalytic antibody field was founded on the concept of transition-state stabilization, but our inability to isolate efficient catalysts by challenging the immune system with transition-state analogues argues that binding of the transition state alone is not sufficient for enzymes to achieve high catalytic rates. For catalytic antibodies, additional factors are important, for example, the positioning of critical catalytic acids and bases and the orientation of the substrate. Rate enhancements by enzymes can be of the order of 10^8–10^{18}-fold (c.f. $<10^6$ fold for catalytic antibodies), but, as argued by others (e.g. Ref. 7), there is insufficient binding energy realized in the transition state to account for these dramatic rate enhancements. Recent reviews on biological catalysis have addressed the issue of the additional factors needed to satisfy observed rate enhancements[9]. Popular ideas invoke the use of low-barrier hydrogen bonds (LBHBs)[10], preorganizational and orientational effects[11,12], and the role of the enzyme in protecting the substrates from solvent[11]. A melding of theoretical and experimental approaches will assess the importance of these aspects, but should we also look to alternative and additional explanations of the catalytic power of enzymes?

So far, we have considered enzymes as static structures, thus overlooking their intrinsic flexibility. Do these dynamic changes contribute significantly to the catalytic process? Although coupling of dynamic processes to the reaction coordinate is not new[13], the idea has been popularized in recent years with reference to classical 'over-the-barrier' models of catalysis[7]. Here, motion along the reaction coordinate (for example, the motion involved in the making or breaking of a chemical bond) is assumed (incorrectly, in at least some cases) to be independent of all other motions. The time scales of these motions need to be as fast, or faster, than the rate of reaction. Clearly, any dynamical treatment of enzyme catalysis diverges from TST, from the viewpoint that the potential energy barrier is 'fluctuating' rather than 'static'. Given the highly dynamic properties of protein molecules, a fluctuating barrier is realistic, but a detailed appreciation of dynamics is rarely gained from structural studies (e.g. crystallography), and this presents an additional hurdle to our understanding of enzyme action. The importance of protein dynamics in catalysis has been reinforced recently in experimental studies of H-transfer in selected enzyme systems, for example methylamine dehydrogenase, sarcosine oxidase and thermophilic alcohol dehydrogenase (see below). These studies have highlighted an apparent shortcoming of TST for these enzymes. TST predicts that the rates of C–H bond cleavage for different isotopes of hydrogen will have different temperature dependencies. However, experimental observations with the above enzymes show that the change in rate as a function of temperature is identical for hydrogen and deuterium, and thus the activation energy for both nuclei is identical, contrary to the predictions of TST. These observations have been interpreted as indicating, first, passage through, rather than over, the barrier by invoking quantum tunnelling and, second, a role for protein dynamics.

Tunnelling through-the-barrier

Matter is usually treated as a particle. However, it can also be treated as a wave (wave-particle duality). These wave-like properties, which move our conceptual framework into the realm of quantum mechanics, enable matter to pass through regions that would be

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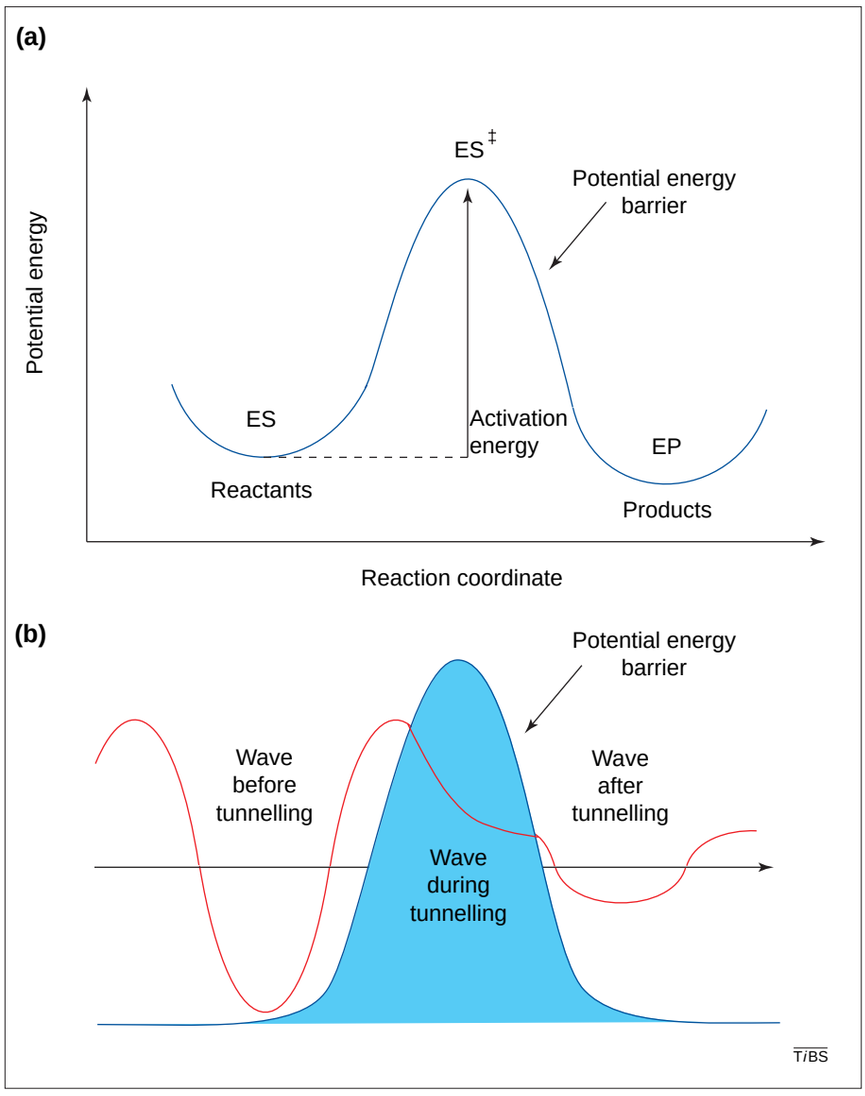

Figure 1

(a) The textbook approach to modelling enzyme catalysis has been to visualize an energy barrier that must be surmounted to proceed from reactants to products. This process is shown schematically. For the reaction to proceed, the reactants (the enzyme–substrate complex, ES) must pass over the potential energy barrier to the product (EP) side via the so-called transition state (denoted by $ES^{\ddagger}$) at the top of the energy barrier. This transition state is energetically unstable. The greater the height of this energy barrier, the slower the rate of reaction. Enzymes reduce the energy required to pass over this barrier, thereby increasing reaction rate. This classical over-the-barrier treatment – known as 'transition state theory' – has been used to picture enzyme-catalysed reactions over the past 50 years. However, recent developments indicate that this representation is, at least in some circumstances, fundamentally flawed and should instead be considered in terms of quantum tunnelling through the barrier. **(b)** Tunnelling of a wave through a potential energy barrier. The narrower the barrier, the smaller the mass of the particle or the smaller the area under the barrier, or both, the greater the tunnelling probability. The amplitude decays within the barrier, because the barrier 'resists' passage of the wave. If the amplitude of the wave 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).

inaccessible if it were treated as a particle (Fig. 1b). In the quantum world, the pathway from reactants to products might not need to pass over the barrier but could pass through the barrier by quantum tunnelling. Quantum tunnelling is more pronounced for light particles (e.g. electrons), because the wavelength of a particle is inversely proportional to the square root of the mass of the particle. Quantum tunnelling is now firmly established for biological electron transfer[14] and can take place over distances as large as 25–30 Å. But what about other particles? The mass of the hydrogen (i.e. protium) nucleus is 1840 times that of the electron. Protium can therefore tunnel over a distance of 0.58 Å with the same probability as an electron tunnelling over 25 Å. This distance is small, but it is similar in length to a reaction coordinate (i.e. width of a potential energy barrier). This suggests that tunnelling might be important in enzymatic H-transfer. Isotopes of hydrogen [i.e. deuterium (D) and tritium (T)] have increased mass and tunnel with the same probability over 0.41 Å and 0.34 Å, respectively, as an electron does over 25 Å (c.f. 0.58 Å for protium). The decreased probability of D- and T-transfer compared with H-transfer makes isotopic substitution within a substrate molecule an attractive means of detecting H-tunnelling in chemical and biological reactions[15].

Klinman and co-workers were the first to obtain experimental evidence consistent with H-tunnelling in an enzyme-catalysed reaction on the basis of deviations in kinetic isotope effect from that expected for classical behaviour. Since their proposal of H-tunnelling at physiological temperatures in yeast alcohol dehydrogenase[16], they have also demonstrated similar effects in bovine serum amine oxidase[17], monoamine oxidase[18] and glucose oxidase[19]. Tunnelling in these systems was described in terms of the 'static' barrier depictions of TST, but, rather than passing over the barrier, H-transfer was envisaged to occur just below the saddlepoint of the potential energy surface by quantum tunnelling. More recently, Klinman has suggested that 'pure' tunnelling (i.e. the substrate does not ascend the barrier) of H and D occurs in the reaction catalysed by lipooxygenase, and this too was interpreted using the 'static' barrier depictions of TST[20]. In this latter case, the reaction rate was found to be essentially independent of temperature, giving rise to the assumption that the energy barrier is static rather than dynamic. However, as discussed for classical over-the-barrier transfers, protein dynamics, which give rise to a 'fluctuating' potential energy surface, are envisaged to play a major role in biological catalysis. The possibility of a link between protein dynamics and quantum tunnelling (termed vibrationally assisted tunnelling) therefore needs to be explored.

Quantum tunnelling facilitated by protein dynamics

Until very recently, experimental evidence suggesting a role for protein dynamics in enabling H-tunnelling has

been lacking, but several theoretical treatments have recognized a role for both substrate[21] and protein[22] dynamics. Kinetic studies of a bacterial methylamine dehydrogenase using methylamine and perdeuterated methylamine as substrate have highlighted a likely role for protein dynamics in driving enzymatic H-tunnelling reactions[23]. In this case, tunnelling occurs from the ground state of the substrate. This was demonstrated by analysis of the temperature dependence of the rate of reaction with methylamine and perdeuterated methylamine. Pure quantum tunnelling reactions are temperature independent (because thermal activation of the substrate is not required to ascend the potential energy surface). The rate of C–H and C–D bond cleavage by methylamine dehydrogenase was found to be strongly dependent on temperature, indicating that thermal activation or 'breathing' of the protein molecule is required to facilitate the tunnelling reaction (Fig. 2). Moreover, the temperature dependence of the reaction is independent of isotope, reinforcing the idea that protein (and not substrate) dynamics drive the reaction and that tunnelling is from the ground state. These observations counter some of the alternative explanations for anomalous behaviour in those systems where tunnelling is inferred to occur just below the saddlepoint, but in the absence of protein dynamics. Additionally, good evidence is now available for vibrationally assisted tunnelling from studies of the effects of pressure on deuterium isotope effects in yeast alcohol dehydrogenase[24]. Combining the experimental evidence, our view is that the argument for vibrationally assisted tunnelling is now compelling. The dynamic fluctuations in the protein molecule are likely to compress (transiently) the width of the potential energy barrier and equalize the vibrational energy levels on the reactant and product sides of the barrier[25–27]. Compression of the barrier reduces the tunnelling distance (thus increasing the probability of transfer), and equalization of vibrational energy states is a prerequisite for tunnelling to proceed. Following transfer to the product side of the barrier, relaxation from the geometry required for tunnelling 'traps' the H nucleus by preventing quantum 'leakage' to the reactant side of the barrier.

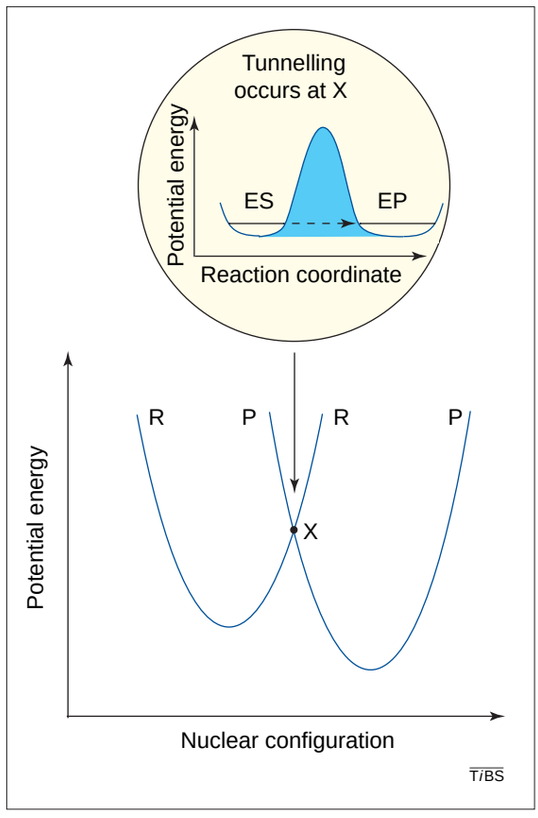

Figure 2

The dynamic barrier model of enzyme catalysed reactions. Reactant (R = ES) and product (P = EP) energy curves for distortion of the protein structure. The tunnelling (i.e. H-transfer) reaction (inset) does not occur until the geometry of the protein is distorted, so that the R and P curves intersect – the intersection point (X) is the optimum geometry required for the reaction to occur. Thus, thermally induced conformational change in the protein is a prerequisite for the tunnelling reaction.

Since the demonstration of kinetic behaviour consistent with vibrationally enhanced tunnelling in methylamine dehydrogenase, similar behaviour has been observed in other enzyme systems. For example, Klinman and co-workers have demonstrated similar behaviour in a thermophilic alcohol dehydrogenase[28], and we have made similar observations during C–H bond cleavage in the complex flavoprotein, the heterotetrameric sarcosine oxidase[29]. By invoking 'pure' tunnelling, an enzyme avoids the need to activate a substrate thermally to populate a transition state. The latter process could be energetically expensive if the bond to be broken is stable (e.g. a substrate C–H bond). In our view, vibrationally enhanced tunnelling mechanisms for energetically difficult transformations (e.g. cleavage of stable C–H bonds) might be more commonplace than currently seen. Similar vibrationally assisted tunnelling mechanisms have been recognized for some time in other areas of science (e.g. the diffusion of hydrogen gas in metals and semiconductors[30]). It is perhaps surprising that the biological community has taken so long to realize the potential of tunnelling mechanisms in enzyme catalysis, especially for energetically difficult transformations.

Challenges ahead

The challenge for the future is to identify which vibrational modes in the enzyme facilitate the tunnelling process and discern whether enzymes have evolved to favour those motions that increase tunnelling probability by (i) equalizing the reactant and product energy states and (ii) transiently compressing the potential energy barrier. These dynamical aspects of enzyme structure need to be incorporated into rational programmes of enzyme design and redesign if we are to exploit enzymes effectively.

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Protein–protein interactions mapped by artificial proteases: where σ factors bind to RNA polymerase

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Interactions between proteins are important to understand but difficult to study. Conjugating a protein to a small artificial protease endows it with the ability to cut other proteins where it binds to them. Analysing the sites cut on the target proteins leads to new understanding of the structure of each complex. The binding of σ factors to a common region on RNA polymerase provides an example.

THE BINDING OF one protein to another has been studied by techniques such as X-ray crystallography, various microscopy techniques, cross-linking, fluorescence spectroscopy and a host of molecular biological methods such as site-directed mutagenesis, alanine scanning and two-hybrid experiments[1–5]. A complementary approach has recently emerged that involves cleaving the polypeptide backbone of a target protein using a cutting protein that binds to the target. The cutting protein is prepared by conjugating a protein to a small chemical reagent that acts as an artificial protease. Using tools available in most biochemistry laboratories, the sites on the target protein within reach of the cutting protein can be readily identified.

This technology has evolved from roots in inorganic chemistry[6–8] through the attachment of cutting reagents at unique cysteine sites on complex proteins[9,10] to mapping where *Escherichia coli* σ^{70} binds to core RNA polymerase (RNAP)[11] and to DNA in transcription complexes[12]. A new development involves the random labeling of lysine residues with cutting reagents to yield a library of probe molecules[13,14].

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Protein footprinting

To gain a perspective on cutting proteins, we can compare this technique to DNA footprinting, pioneered by Galas and Schmitz in 1978 (Ref. 15), which involves randomly cutting the polynucleotide backbone of DNA and analysing the fragments by gel electrophoresis. Binding of proteins to DNA prevents cutting at those sites in the region of DNA bound by the protein, which are easily detected by the absence of bands on the gel. Inorganic reagents such as iron–EDTA chelates have been developed to cut nucleic acids, outlining ligand-binding sites at single-nucleotide resolution[16,17]. Similar reagents also cut the polypeptide backbones of proteins[6,18,19] and small proteins can be successfully footprinted by iron–EDTA[20].

When cut by iron–EDTA, large proteins (>100 kDa) produce many fragments, because their solvent-exposed surfaces are so extensive[21]. This makes it experimentally difficult to study the limited part of the surface where the interacting protein binds. Also, protein gel electrophoresis does not provide the single-residue resolution characteristic of DNA gels, so interfering bands on a protein gel can easily obscure changes in cut sites. Tethering the cutting reagent to the binding protein provides a way forward: under appropriate conditions, only those proteins actually bound to the target will cut it, and the cuts are limited to locations near the binding site. This produces only a small number of bands on a protein gel, allowing simple analysis.

Tethered cutting reagents

Progress has been made by designing unique sites in proteins at which a small reagent with cleavage activity can be tethered[11]. This approach (Fig. 1) uses molecular cloning to prepare a set of mutant proteins, each with a unique cysteine residue at a chosen location

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