

CHAPTER 1

The Transition-State Theory Description of Enzyme Catalysis for Classically Activated Reactions

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1.1 Introduction

Two facts about enzymes have probably been primarily responsible for the many thousands of person-years of research devoted to understanding their mechanisms of action: enzymes are ubiquitous catalysts for biochemical transformations, and they are able to achieve rate enhancements and/or substrate selectivities that have rarely been matched by designed catalysts.

Perhaps it is the combination of these two phenomena that has led some researchers to look for a “vital force” that might give enzymes catalytic powers outside of those explicable by the normal models of condensed-phase kinetics.¹ One does not need to be particularly mystically inclined to subscribe to this view, because enzymes could have evolved, at least in principle, to optimise effects that would otherwise play negligible roles in reaction kinetics. Ideally, the existence or otherwise of such special effects could be probed by comparing observed catalytic capabilities of enzymes with those derived from computer simulations based on standard kinetic models. If the observed effect were significantly different

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from the calculated one, the discrepancy might indeed be a sign that some important phenomenon had been omitted from the simulation. Unfortunately, with rare exceptions, our theoretical models are not yet sufficiently reliable to permit such quantitative comparisons. Consequently, the question of the *deus ex machina* in enzyme catalysis must, for now, be classified as unresolved.

For the purposes of this chapter the issue will be set aside. We will assume that, although enzymes are remarkably good catalysts, the mechanisms by which they operate can be fully described by the standard model of condensed-phase kinetics: transition-state theory (TST).² Furthermore, since the focus of most of the rest of this book is on quantum tunnelling, we will begin by factoring that term out of the TST expression. To do so, it is convenient to employ the generalised TST expression (eqn (1.1)) for a rate constant, presented by Truhlar and coworkers.³

$$k(T) = \gamma(T)(C^0)^{1-n} \frac{k_B T}{h} e^{-\frac{\Delta^\ddagger G^0}{RT}} \quad (1.1)$$

Here $k(T)$ is the temperature-dependent rate constant, $\gamma(T)$ is a generalised transmission coefficient, C^0 is the standard-state concentration (or, sometimes, pressure in a gas-phase reaction), n is the molecularity of the reaction, k_B and h are, respectively, the Boltzmann and Planck constants, R is the gas constant, and $\Delta^\ddagger G^0$ is the standard-state free energy of activation. It is the $\gamma(T)$ term that contains any quantum-tunnelling effects,³ but here will be explicitly set equal to unity. Thus, this chapter will seek to lay a foundation for the later discussion of quantum tunnelling by describing the part of enzyme catalysis that can be ascribed to effects of the protein on the magnitude of the free-energy barrier for a classically activated reaction.

1.2 Quantifying the Catalytic Activity of Enzymes

To begin the discussion, it is necessary to define as clearly as possible the phenomenon that we wish to explain. The following analysis is based on the 1996 article of Cannon *et al.*⁴

One starts by considering an irreversible, unimolecular conversion of a substrate, **S**, into a product, **P**. (Special issues associated with enzyme catalysis of bimolecular reactions have been reviewed by Jencks,⁵ but will not be considered explicitly here.) The rate law for such a reaction is, of course, eqn (1.2). The rate constant k_{uncat} is seen to be the proportionality constant



between the rate of product formation and the *substrate* concentration.

The enzyme-catalysed reaction can be represented by the usual Michaelis–Menten formulation,



where \mathbf{E} is the enzyme and \mathbf{ES} is an enzyme–substrate complex. Application of the steady-state approximation to the concentration of \mathbf{ES} yields

$$\frac{d[\mathbf{P}]}{dt} = \frac{k_{\text{cat}}[\mathbf{E}]_{\text{tot}}[\mathbf{S}]}{[\mathbf{S}] + K_{\text{M}}} \quad (1.4)$$

where K_{M} , the Michaelis constant, has units of concentration and is equal to $(k_{\text{off}} + k_{\text{cat}})/k_{\text{on}}$, and $[\mathbf{E}]_{\text{tot}} = [\mathbf{E}] + [\mathbf{ES}]$. The maximum rate of product formation occurs when $[\mathbf{S}] \gg K_{\text{M}}$ – so-called saturation conditions – under which circumstances:

$$\frac{d[\mathbf{P}]}{dt} = k_{\text{cat}}[\mathbf{E}]_{\text{tot}} \quad (1.5)$$

At first sight, everything looks set for a definition of the rate enhancement brought about by the enzyme. In eqns (1.2) and (1.5) we have unimolecular rate constants for both the catalysed and uncatalysed reaction, and so the dimensionless quantity $k_{\text{cat}}/k_{\text{uncat}}$ looks like the obvious way to determine the quantitative effect of the catalyst. Indeed, many papers and books on enzyme catalysis do use $k_{\text{cat}}/k_{\text{uncat}}$ for that purpose. However, closer inspection of eqn (1.5) reveals a potential problem: k_{cat} is the proportionality constant between the rate of product formation and the *enzyme* concentration. It is consequently not measuring the same thing that k_{uncat} did. The proper comparison requires that we rewrite eqn (1.4) as

$$\frac{d[\mathbf{P}]}{dt} = k_{\text{eff}}[\mathbf{S}] \quad (1.6)$$

where

$$k_{\text{eff}} = k_{\text{cat}} \frac{[\mathbf{E}]_{\text{tot}}}{[\mathbf{S}] + K_{\text{M}}} \quad (1.7)$$

The effective “rate constant” k_{eff} is seen not to be a constant at all, since its value depends on the substrate concentration (eqn (1.7)). Furthermore, the quantity $[\mathbf{E}]_{\text{tot}}/([\mathbf{S}] + K_{\text{M}})$ will generally be $\ll 1$, especially under the saturation conditions that lead to the maximum rate of product formation. Hence, one sees that the ratio $k_{\text{cat}}/k_{\text{uncat}}$ will almost always overestimate the catalytic power

of an enzyme. However, the more defensible $k_{\text{eff}}/k_{\text{uncat}}$ varies with substrate concentration and, somewhat confusingly, actually approaches its maximum value of $k_{\text{cat}}[\text{E}]_{\text{tot}}/(k_{\text{uncat}}K_{\text{M}})$ as $[\text{S}] \rightarrow 0$.

For a series of enzymes used at identical concentrations, the quantity $k_{\text{cat}}/(k_{\text{uncat}}K_{\text{M}})$ – Wolfenden’s “catalytic proficiency”⁶ – will provide a reliable estimate of *relative* activities, which are found to cover a range spanning many orders of magnitude, from carbonic anhydrase at 10^8 M^{-1} to arginine decarboxylase at 10^{23} M^{-1} .⁶ At a typical enzyme concentration of 10^{-5} M , the corresponding maximum rate enhancements, $\{k_{\text{eff}}/k_{\text{uncat}}\}_{\text{max}}$ thus vary from 10^3 – 10^{18} , and at real (*i.e.* nonzero) substrate concentrations, the true rate enhancements drop further. Nevertheless, there clearly remains something worthy of explanation, particularly at the high end of the range.

1.3 Free-Energy Analysis of Enzyme Catalysis

Most effects in mechanistic organic chemistry are described in structural and/or energetic terms. It is therefore tempting to take the rate enhancements described in the previous section and, through our reduced TST expression (*i.e.* with the generalised transmission coefficient set to unity, eqn (1.8)) to express them as differences in standard-state activation free energies for the catalysed and uncatalysed reactions.

$$k(T) = (C^0)^{1-n} \frac{k_{\text{B}}T}{h} e^{-\frac{\Delta^\ddagger G^0}{RT}} \quad (1.8)$$

There are a number of potential pitfalls with this approach. The first is one of interpretation. Comparison of activation free energies for two reactions presumably implies that one believes the reactions in question to be similar enough for the energy difference to be physically meaningful. Few people would expect a comparison of activation barriers for a Diels–Alder reaction and an aldol reaction to yield useful insight. In the case of enzyme catalysis, one needs to enquire whether the mechanisms of the catalysed and uncatalysed reactions are the same. Actually, this question easily leads to a philosophical quagmire. Strictly speaking, the mechanisms of the catalysed and uncatalysed reactions can never be the same – after all one involves a catalyst and one does not! In some cases, the mechanism of the enzyme-catalysed reaction is so obviously different from the uncatalysed one that the energetic comparison is clearly meaningless (*e.g.* when the enzymatic reaction involves covalent bond formation to cofactor that is not available to the uncatalysed reaction). However, even when that is not the case, the interpretation of energy differences requires careful thought and clear definition of terms.

One conceptual (and possibly computational) way of tackling the problem is summarised in Figure 1.1. In this figure, and elsewhere in this chapter, the convention employed is that a subscript or superscript following the Δ defines the type of free-energy difference being described. A double dagger following

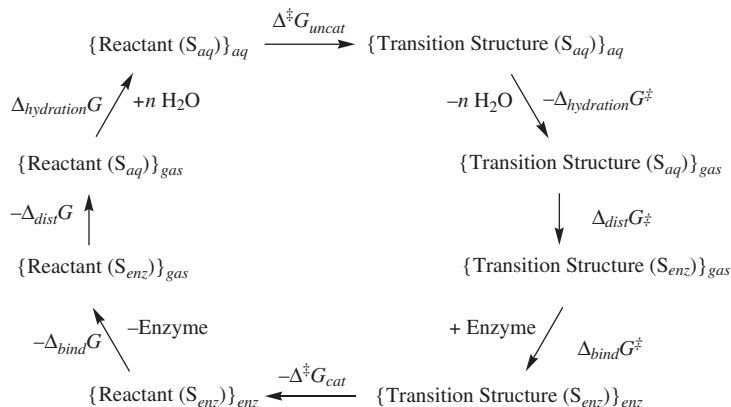


Figure 1.1 A thermodynamic cycle for defining the contributing components to the catalytic effect of an enzyme. The subscript on the S (for structure) defines the medium in which the geometry of the substrate or transition structure is a stationary point, while the subscript on the braces, {}, defines the medium in which the substrate or transition structure finds itself.

the G means that the two free energies being compared are both for the transition structure. Thus, Δ^\ddagger means a difference in free energy between transition state and ground state, whereas, for example, $\Delta_{\text{dist}}G^\ddagger$ means a difference in free energy between two transition structures of different geometry.

The quantity $\Delta^\ddagger G_{\text{uncat}} - \Delta^\ddagger G_{\text{cat}}$ can, in principle, be determined experimentally. It is related, *via* eqn (1.9), to the rate constants k_{uncat} and k_{cat} , discussed above.

$$\Delta^\ddagger G_{\text{uncat}} - \Delta^\ddagger G_{\text{cat}} = RT \ln \left(\frac{k_{\text{cat}}}{k_{\text{uncat}}} \right) \quad (1.9)$$

From the thermodynamic cycle in Figure 1.1, $\Delta^\ddagger G_{\text{uncat}} - \Delta^\ddagger G_{\text{cat}}$ can be decomposed into conceptual components that might provide insight into the origins of enzymic catalysis (although it is perhaps prudent to re-emphasise here that $\Delta^\ddagger G_{\text{uncat}} - \Delta^\ddagger G_{\text{cat}}$ will overestimate the magnitude of the real catalytic effect, for the reasons discussed in the previous section).

$$\begin{aligned} \Delta^\ddagger G_{\text{uncat}} - \Delta^\ddagger G_{\text{cat}} &= \Delta_{\text{hydration}}G^\ddagger - \Delta_{\text{hydration}}G \\ &+ \Delta_{\text{dist}}G - \Delta_{\text{dist}}G^\ddagger + \Delta_{\text{bind}}G - \Delta_{\text{bind}}G^\ddagger \end{aligned} \quad (1.10)$$

The first two terms on the right-hand side of eqn (1.10) evaluate the hydration free energies of the transition structure and reactant, respectively, for the uncatalysed reaction. The third and fourth terms recognise that the minimum-energy

geometries of reactant and transition structure might not be the same for the uncatalysed and enzyme-catalysed reactions, and evaluates the free-energy changes for distortion of one to the other. The final two terms evaluate the binding free energies of, respectively, the transition structure and reactant (at the geometries of each appropriate for the catalysed reaction) to the enzyme-active site. These binding terms might involve important contributions from water molecules that are displaced from the active site by the substrate or the transition structure.⁷

Although the left-hand side of eqn (1.10) can sometimes be determined experimentally, the terms on the right-hand side usually cannot. However, they might be susceptible to calculation. If they were, the magnitudes of the various terms would provide insight into the physical origins of catalysis. Unfortunately, as discussed later, the reduction of this ideal to practice is far from straightforward.

The analysis of eqn (1.10) is further compromised for the many enzyme-catalysed reactions that involve formation of charged intermediates, because then the gas-phase mechanism may not exist at all, or may be so high in energy above alternative charge-neutral pathways that it cannot be reliably calculated. To emphasise this point, it is perhaps worth recalling that the solvolysis of *tert*-butyl bromide, which occurs readily at room temperature in aqueous media, would have a 298 K half-life of $\sim 10^{87}$ years in the gas phase! In fact, without the beneficial effects of solvation on the polar pathway, C-Br *homolysis* is more facile than heterolysis by 340 kJ mol. When an enzyme-catalysed reaction has no gas-phase counterpart, the thermodynamic cycle in Figure 1.1 must be telescoped, so that free-energy changes are calculated directly between aqueous and enzyme-bound structures. However, such a calculation inevitably confounds intrinsic properties of the substrate (*i.e.* the relative free energies of various structures) with solvation and/or binding energies. This blending of effects hampers efforts to decompose the catalytic phenomenon into clearly comprehensible physical components.

A second potential pitfall with free-energy analyses of enzyme catalysis concerns the standard-state concentration term, $(C^0)^{1-n}$, in eqn (1.8).⁸ For a unimolecular reaction, such as that in eqn (1.2), $n = 1$, and so the term becomes unity. Hence, the activation free energy (difference in free energy between the transition state and reactant) is independent of the chosen standard state. However, for the enzyme-catalysed reaction $n = 2$. Consequently the overall free energy of the enzyme-bound transition structure with respect to free substrate and enzyme has a value that does depend on the selected standard state. For solution-phase reactions, the most common standard state is 1 M. However, it is important to recognise that the actual concentrations at which enzymes and substrates exist in biological systems are typically many orders of magnitude lower than that. Figure 1.2 illustrates what happens to the relative standard-state free energies for an uncatalysed unimolecular reaction and its enzyme-catalysed counterpart as the concentration changes.⁸

The left-hand panel of Figure 1.2 illustrates a situation where the enzyme-catalysed reaction is faster than the uncatalysed one. This arises because the

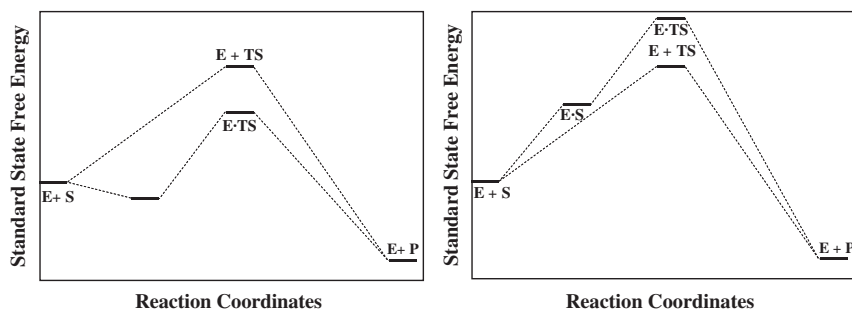


Figure 1.2 Schematic standard-state free-energy profiles for an enzyme-catalysed reaction and its uncatalysed counterpart. The left-hand panel depicts the situation where the components are present in high enough concentration for the enzyme-catalysed process to be preferred. As the concentration is reduced the enzyme-catalysed reaction becomes disfavoured until, in the right hand panel, the uncatalysed reaction is actually faster ($E + TS$ is below $E \bullet TS$).

standard-state free energies of binding for both the substrate and the transition structure are negative, but the latter is more negative. By reducing the concentrations of *all* of the components in solution, one raises the standard-state free energy of the Michaelis complex $E \bullet S$ with respect to the separate components $E + S$, and of the enzyme-bound transition structure, $E \bullet TS$ with respect to $E + TS$. However, the standard-state free-energy differences between $E + TS$ and $E + S$, and between $E \bullet TS$ and $E \bullet S$ are independent of concentration because each step is unimolecular (the enzyme is included in the uncatalysed reaction simply to keep the stoichiometry consistent; it doesn't actually participate in the reaction). If the reduction in concentration is sufficient, the enzyme-catalysed reaction can cease to be the favoured pathway, as illustrated in the right-hand panel of Figure 1.2. Of course, enzymes have evolved to operate under conditions like those in the left-hand diagram, but nonetheless it is important to recognise that the relative favourabilities of enzyme-catalysed and uncatalysed reactions are concentration dependent.

One last point: there may appear to be a contradiction between Figure 1.2, which seems to show that the catalysed reaction becomes less favourable as the concentration decreases, and the earlier analysis (eqn (1.7)) in which we had concluded that the rate enhancement caused by an enzyme reaches a maximum as $[S]$ approached zero. This is actually not a contradiction because the concentration change between the two panels of Figure 1.2 applies to *all* components, whereas the analysis of eqn (1.7) involved reducing *only* the substrate concentration. One can see in eqn (1.7) that reducing the enzyme concentration reduces k_{eff} , whereas reducing the substrate concentration increases it. However, the former effect is more important because of the constant K_M in the denominator. Hence reducing *both* $[E]_{\text{tot}}$ and $[S]$ at the same time will reduce k_{eff} .

1.4 Transition-State Stabilisation or Ground-State Destabilisation?

The recognition that, to be effective catalysts, enzymes must bind the transition structure for a chemical reaction more tightly than they bind the substrate is usually attributed to Linus Pauling.⁹ However, as Wolfenden has pointed out,⁶ essentially the same conclusion, about catalysts in general, had been published by Polanyi over 20 years earlier.¹⁰

This necessary property of catalysts leaves open the question of whether the principal effect arises from the distortion terms^{11–13} or the binding terms^{14,15} in eqn (1.10). Both points of view have been passionately advanced and defended. At one extreme, it could be that the reactant and transition structure in the enzyme-active site have geometries, and therefore free energies, that are very similar to those in aqueous solution. Under such circumstances, the quantity $\Delta_{\text{dist}}G - \Delta_{\text{dist}}G^\ddagger$ would be negligible, and catalysis would have to arise from the sum of the remaining four terms in eqn (1.10) being positive. This would be the case if the difference between transition-structure and reactant binding free energies greatly exceeded the difference in their hydration free energies. In other words, one could view the enzyme to be a protein that had evolved to have a particularly good binding affinity for the reaction transition structure, and a much poorer affinity for the substrate.

At the other extreme, it might be that the difference in binding free energies of the transition structure and reactant to the enzyme is about equal to the difference in their hydration energies. Under such circumstances catalysis could still be expressed, provided that the quantity $\Delta_{\text{dist}}G - \Delta_{\text{dist}}G^\ddagger$ were positive. One way (but not the only way, since, in general, we don't know the signs of the distortion free energy terms) would be to make $\Delta_{\text{dist}}G$ a large positive quantity (or, in any event, more positive than $\Delta_{\text{dist}}G^\ddagger$). In other words, one could view the enzyme to be a protein that had evolved to put the substrate on a rack, and to mechanically force it into a high-energy, and presumably transition-structure-like geometry.¹³

In the judgement of the present author, the dispute between proponents of these extreme points of view has added relatively little to our understanding of enzyme catalysis. In fact, a number of researchers are beginning to conclude that the separation of the catalytic phenomenon into ground-state and transition-state effects is not really meaningful.^{4,16,17} For example, if the enzyme's active site had evolved to be complementary to the reaction transition structure, one consequence should be that the conformations of the reactant bearing the closest resemblance to the transition structure ought to be the most tightly bound to the protein. Under such circumstances, the tight binding of the transition structure and the distortion of the reactant upon binding could simply be viewed as two expressions of the same underlying physical phenomenon.

A case in point is provided by the much-studied group of chorismate mutase enzymes. These enzymes catalyse the conversion of chorismate to prephenate (Figure 1.3) – a step in the shikimate biosynthetic pathway for plants and bacteria.¹⁸ The transformation is apparently a concerted aliphatic Claisen

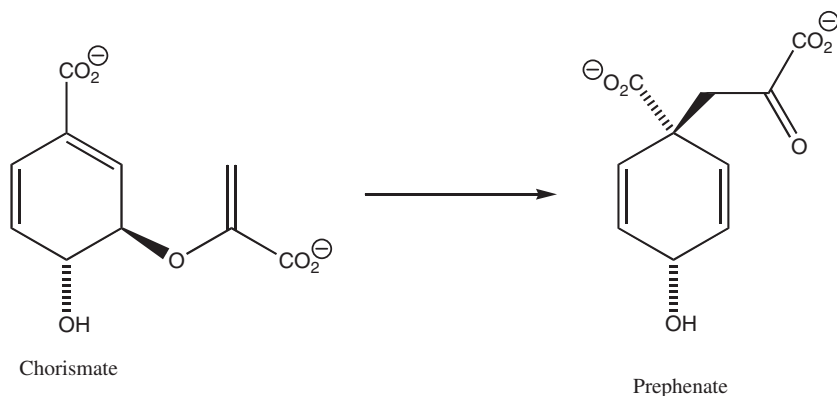


Figure 1.3 The reactant and product in the chorismate rearrangement.

rearrangement, both in the uncatalysed and enzyme-catalysed versions of the reaction.¹⁹ The aqueous-solution reaction occurs with activation parameters²⁰ $\Delta^\ddagger H = 85.8 \pm 1.7 \text{ kJ/mol}$, $\Delta^\ddagger S = -54.0 \pm 1.7 \text{ J/(mol K)}$. In the chorismate mutases from *B. subtilis*, *E. coli*, *K. pneumoniae*, and *S. aureofaciens*, respectively, these parameters are modified to $\Delta^\ddagger H = 53.1 \pm 1.7 \text{ kJ/mol}$, $\Delta^\ddagger S = -38.1 \pm 5.0 \text{ J/(mol K)}$; $\Delta^\ddagger H = 68.2 \pm 2.1 \text{ kJ/mol}$, $\Delta^\ddagger S = -12.6 \pm 6.7 \text{ J/(mol K)}$; $\Delta^\ddagger H = 66.5 \pm 1.7 \text{ kJ/mol}$, $\Delta^\ddagger S = -4.6 \pm 5.0 \text{ J/(mol K)}$; $\Delta^\ddagger H = 60.7 \pm 1.7 \text{ kJ/mol}$, $\Delta^\ddagger S = -6.7 \pm 4.6 \text{ J/(mol K)}$.²⁰ Hence, the enzymes achieve their roughly 10^6 values for $k_{\text{cat}}/k_{\text{uncat}}$ by reducing $\Delta^\ddagger H$ and increasing (making less negative) $\Delta^\ddagger S$. In general, the former effect is larger, although the apportioning of the catalysis between enthalpic and entropic effects seems to be organism specific.

In aqueous solution, NMR studies suggest that chorismate exists in pseudo-diequatorial and pseudodiaxial conformers, with the latter being higher in free energy by only about 5 kJ/mol at 25 °C.²¹ The calculations of Martí *et al.* suggest that there is a substantial solvent contribution to this value.²² They estimate that in the gas phase the pseudodiaxial conformers are significantly less favoured. While it is true that one of the pseudodiaxial conformers is geometrically closer than the other conformers to the transition structure for the Claisen rearrangement, selective binding of this conformer to the enzyme-active site could not, by itself, explain the observed catalysis, since a difference of roughly 35 kJ/mol in $\Delta^\ddagger G^0$ is observed between aqueous and enzyme-catalysed reactions.

A number of hybrid quantum-mechanical and molecular-mechanical (QM/MM) simulations have been conducted on the aqueous and enzyme-catalysed reactions, in an effort to elucidate the principal origins of the catalytic effect.^{17,20,23–33} Although the authors of the calculations agree on some important issues, they differ somewhat on the details, and differ considerably in their descriptions of what is occurring. The principal point of agreement is that a pseudodiaxial conformer of chorismate is favoured upon binding to the active site, and that this conformer is substantially different in geometry from any found as local minima in aqueous solution. In particular, the bound conformer of the substrate seems to have a much

shorter distance between the carbons that are destined to form a bond in the ensuing Claisen rearrangement ($\sim 2.9 \text{ \AA}$ in the enzyme *vs.* $\sim 3.5 \text{ \AA}$ as the closest approach in any of the aqueous-phase local minima). There is clearly an energetic cost to the substrate from this distortion. Furthermore, since the distortion moves the substrate along the reaction coordinate for the Claisen rearrangement, the reactant destabilisation serves to reduce the barrier for the reaction.

The principal points of disagreement among the researchers who have investigated the chorismate mutases are, first, the teleological question of how to characterise what the enzymes are doing, and, second, whether there is any significant component of catalysis arising from factors beyond this substrate distortion. On the matter of teleology, one could argue that the enzymes have evolved to select from solution the rare high-energy conformations (not necessarily local minima) that are closest in structure to the transition state for the Claisen rearrangement, and have provided stabilisation through binding to these privileged “near-attack conformations” (NACs).^{27,33} Alternatively, one could argue that the enzymes have evolved to provide a mechanical stress on the substrate, forcing together the carbons that will become bonded during the Claisen rearrangement,³⁰ and thereby reducing the barrier for the reaction through a “spatiotemporal” effect.^{34,35} Or, seemingly different again, one could argue that the enzymes have evolved to bind the transition structure for the Claisen rearrangement as tightly as possible, and that, as a direct consequence of this strong binding, the substrate will experience a distorting force that alters its geometry in the direction of the transition structure.²⁹ However, it is the present author’s opinion that these questions fall more in the realm of semantics than science, and that they simply represent alternative verbal characterisations of the same underlying physics. If one tried to bind chorismate in its predominant (pseudodiequatorial) aqueous conformation to the active site of a chorismate mutase, there is no doubt that the standard-state binding free energy would be much less negative than that for the reaction transition structure. *That has to be true for catalysis to occur.* However, since the preferred conformation of the substrate bound to the active site is *not* the same as that in solution, one has to decide where to claim that the substrate has paid the energy penalty for this structural change, and how it has been compensated by the enzyme. The various descriptions of the catalytic phenomenon merely choose different accounting schemes to balance these free-energy books.

Where there is truly scientific disagreement among the researchers into chorismate mutases is on the matter of whether there are additional catalytic factors that go beyond the substrate distortion. Evidence has been provided from both experimental^{36–38} and computational²⁶ studies to suggest that the transition structure for the chorismate Claisen rearrangement is more polar than the reactant, and that additional catalytic effect arises from electrostatic²⁹ (including hydrogen bonds^{20,24,31,39}) stabilisation of the transition structure in the active site. However, there is not yet agreement about whether this polar stabilisation is a significant factor.³³ In other enzyme-catalysed reactions, for which charged intermediates are formed, there is no doubt that polar stabilisation plays an important role. It, and other means of stabilising transition structures, are described in the next section.

1.5 Selective Stabilisation of Transition Structures by Enzymes

The point of view of the present author – that the various free-energy analyses of enzyme catalysis differ only in semantics – permits any one of the common descriptions to be selected as the basis for further discussion. In the remainder of this chapter, the language used will be the original Polanyi–Pauling description of selective transition-structure binding.

One of the possible mechanisms for stabilising transition structures selectively is through electrostatic interactions. Warshel has proposed that this effect is the most important in enzyme catalysis (see Chapter 10).⁴⁰ However, he argues that recognition of that fact requires a careful analysis of the *uncatalysed* (*i.e.* aqueous) reaction. As discussed earlier, direct comparison of enzyme-catalysed reactions with their aqueous counterparts corresponds to a telescoping of the thermodynamic cycle in Figure 1.1, with a consequent blending of intrinsic substrate energetics and solvation (hydration) effects. This is important for the following reason. Pairwise interactions of polar functionalities in the reaction transition structure with polar groups in an enzyme-active site are typically not very different in magnitude from interactions of the transition structure with individual water molecules. This fact would, at first sight, seem to argue against selective electrostatic stabilisation of the transition state by the enzyme. However, the water interactions with the transition structure come at a cost of solvent reorganisation energy – *i.e.* partial disruption of the hydrogen bonding structure of pure liquid water. By contrast, the enzyme-active site has preorganised polar groups that have evolved to be complementary to the transition structure, and have paid their organisation energy penalty at the time of folding of the protein. This, Warshel argues, is the principal factor that gives an enzyme its advantage over water.⁴⁰

An obvious factor that enzymes can and do provide for stabilisation of transition structures is a preorganised hydrogen-bond network. Although the proper description of hydrogen bonds is still a matter of debate,^{41,42} it seems clear that a large component of the attractive interaction can be described in electrostatic (*i.e.* ion–dipole or dipole–dipole) terms. Consequently, hydrogen-bond stabilisation could, in general, be viewed as a special case of electrostatic stabilisation. An exception to that conclusion might occur with the so-called low-barrier hydrogen bonds (LBHBs), in which a hydrogen is symmetrically bonded to two heteroatoms (oxygen or nitrogen) that are held at a distance within the sum of their van der Waals radii, and that constitute functional groups of very similar pK_a .^{43,44} It has been suggested that LBHBs are unusually strong, and that they play important roles in enzyme catalysis.⁴³

An example is provided by the classic serine proteases,⁴⁴ which catalyse the hydrolytic cleavage of peptide bonds. They do so by making an acyl–enzyme intermediate, using the hydroxyl group of an active-site serine. This normally poor nucleophile is apparently activated by general-base catalysis, involving a histidine and an aspartate. The three amino acids constitute the so-called catalytic triad (Figure 1.4). Early proposals for the mechanism of the acyl–enzyme

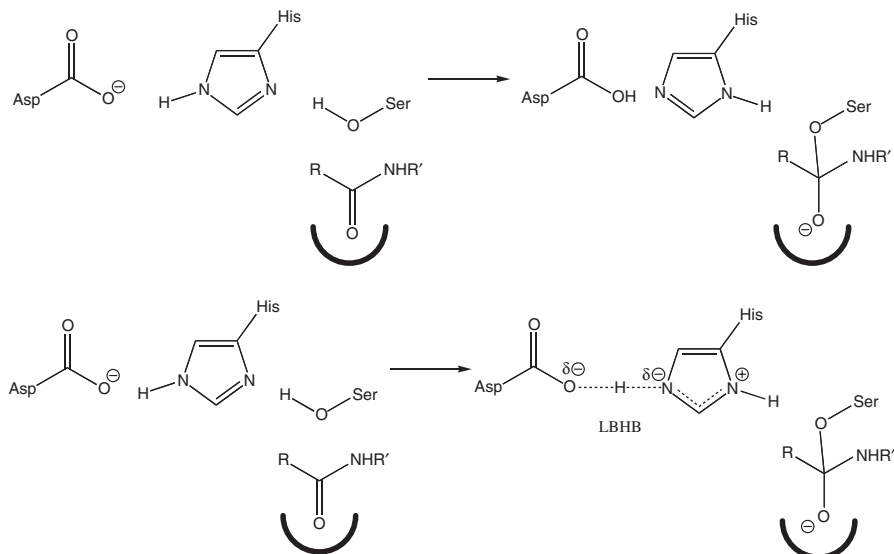


Figure 1.4 Two mechanistic proposals by which the “catalytic triad” of aspartate, histidine, and serine in the active site of the serine protease enzymes could catalyse hydrolysis of a peptide bond. In the upper scheme – the “charge relay” mechanism – two proton transfers take place. In the lower “LBHB” mechanism, the catalysis benefits from the formation of a stable low-barrier hydrogen bond between the aspartate and protonated histidine. The bold curve in each mechanism represents the part of the protein known as the oxyanion hole, in which backbone N–H bonds are oriented correctly to provide H-bond stabilisation of the alkoxide in the tetrahedral intermediate.

formation (generally believed to be the rate-determining step of the overall reaction) involved a so-called charge-relay mechanism,⁴⁵ consisting of two proton transfers. However, subsequent experiments have suggested that only a single proton transfer occurs.⁴⁶ That fact could be accommodated within the LBHB proposal, which posits that the formation of a LBHB between the aspartate and histidine leads to stabilisation of the transition state, and consequent catalysis for the reaction.⁴⁴ Similar effects have been proposed for a variety of other enzyme-catalysed reactions.⁴³

Experimental support for the LBHB proposal was adduced from the ¹H NMR chemical shift of the proton shared between the aspartate and histidine in the protonated state of chymotrypsin, and in its complexes with various analogues of the tetrahedral intermediate. All occurred at ~18 ppm in the spectrum – substantially downfield from hydrogens involved in “normal” hydrogen bonds between oxygen and nitrogen.⁴⁴ This downfield shift has been identified as a characteristic of LBHBs. However, in a recent QM/MM simulation,⁴⁷ Ishida has found that the hydrogen between the aspartate and histidine remains localised on the histidine ring throughout the reaction. Despite

that, the calculated ^1H chemical shift reaches $\delta 20.5$, because of large electrostatic effects. Thus, Ishida's analysis suggests that NMR chemical shift may not be a reliable criterion for the formation of LBHBs. The proposals of important catalytic consequences from LBHB formation in other enzyme-catalysed reactions are awaiting similarly detailed analyses.

Since, in transition-state theory, reaction rates depend on activation free energies, and since free energies, in turn, have enthalpic and entropic components, it is reasonable to enquire whether the selective stabilisation of a transition structure by an enzyme is primarily an effect on $\Delta^\ddagger H$ or $\Delta^\ddagger S$. The idea that an increase in activation entropy (with respect to that for the aqueous reaction), particularly for bimolecular reactions, could be a significant contributor to enzyme catalysis was advanced persuasively by Page and Jencks in 1971.⁴⁸ Although their analysis was probably as careful and detailed as it could be, given the information and capabilities of the time, it may not have been quantitatively correct. In a 2000 paper in which the question was revisited, Villà *et al.* have concluded that some of the approximations used by Page and Jencks were flawed, and that the entropic contribution to enzyme catalysis is, in most cases, quite small.⁴⁹ In any event, direct experimental measurements of activation entropies for enzyme-catalysed reactions are difficult to obtain, and even more difficult to interpret. Although our interest may be in entropy changes associated with the substrate(s) during reaction, experiments determine entropy changes for the whole system (substrate, solvent, and enzyme), and deconvolution into individual contributions from these components can be difficult or impossible.

More recently, Zhang and Houk have paid close attention to the most proficient enzymes, and have concluded that their catalytic effects can only reasonably be explained by (at least partial) covalent bond formation between the enzyme and the reaction transition structure.⁵⁰ The principal basis for this proposal is the calculation of association constants for truly noncovalent complexes of small molecules to biological macromolecules, and comparison with the deduced "association constants" (not directly observable, of course) for enzymes with the transition structures of the reactions that they catalyse. The reference association constants are of antibodies with antigens, averaging $\sim 10^6 M^{-1}$, and of enzymes with transition-structure analogues (*i.e.* inhibitors), averaging $\sim 10^9 M^{-1}$. The kinetically deduced association constants for enzymes with the actual transition structures were found to be much higher, averaging $\sim 10^{16} M^{-1}$, and rising in a few cases to almost $10^{30} M^{-1}$.⁵⁰ In several of the cases included in the Zhang–Houk analysis, there is no doubt that there are covalent bonds formed with enzyme active-site residues, or with cofactors. The problem is that the aqueous-phase, uncatalysed reaction, lacks the capability to follow the same path, and so the mechanisms of catalysed and uncatalysed reactions are really incomparably different. As pointed out in the introductory section of this chapter, the interpretation of a difference in activation free energy for two reactions of unrelated mechanism is problematic. However, some of the enzyme-catalysed reactions included in the Zhang–Houk review do follow mechanisms that are sufficiently similar to those for the

uncatalysed analogues that a free-energy analysis of the kind illustrated in Figure 1.1 is meaningful. The question is then whether these reactions, too, must benefit from covalent bond formation during the enzyme catalysis, if the full magnitude of the catalytic effect is to be explained. At least two commentators^{40,51} think that the “covalent catalysis” proposal is unnecessary, and that the higher association constants of enzymes for the true transition structures than for their inhibitory analogues arises simply from the fact that perfect transition structure analogues are hard to prepare. However, given its relatively recent vintage, the last words of this debate may not have been heard.

Finally, for this section, it is useful to pay attention to the cautionary words of Kraut *et al.* on the difficulty of interpreting data from site-directed mutagenesis in the analysis of enzyme catalysis.⁵² Superficially, the ability to introduce at will new amino acids, even non-natural ones, into selected sites of a protein would seem to give biochemists the freedom to conduct the structure–reactivity analyses that have for so long provided the foundation of mechanistic understanding in physical organic chemistry. Indeed such experiments can and have been done for enzymes.⁵³ The problem, as always, lies in the interpretation of the results. When one makes a change in the chemical constitution of a compound, as usually occurs in a structure–reactivity study, it ceases to be possible to make direct energy comparisons between the original and the modified molecule. Changes in the rates of related chemical reactions of the two molecules, arising from the structural modification, consequently cannot be ascribed unambiguously to ground-state or transition-state effects. If one chooses to set the reactants at the same free energy for the purposes of the rate comparison, then the effect will seem to have occurred in the transition state. However, it would be equally valid to choose the transition states to be at the same free energy, with the resulting appearance of a ground-state effect. This ambiguity has repeatedly led to controversy in physical organic chemistry, for example concerning the origin of substituent effects on bond dissociation enthalpies.^{54,55} Similarly, in site-directed mutagenesis studies of enzymes, one cannot separate catalytic effects from binding effects brought about by the change of an amino acid.⁵² The implication is not that mutation studies are worthless – far from it – but merely that the quantitative effects of an amino-acid substitution on the catalytic activity of an enzyme need to be interpreted with due caution.

1.6 Enzyme Flexibility and Dynamics

The thermodynamic cycle in Figure 1.1 carries a hidden asymmetry. It depicts explicitly the desolvation and distortion of the substrate and reaction transition structure, but does not do the same for the enzyme. However, the vast amounts of structural and dynamic information that have become available in recent years as a result of X-ray diffraction and magnetic resonance studies, and from computer simulations, reveal that changes in hydration state and structure of the enzyme during its catalytic cycle really cannot be ignored.⁵⁶

The rather static, almost architectural picture of organic molecules that was encouraged by the mechanical molecular models in common use in the mid- to late-twentieth century is quickly dispelled as soon as one looks at a movie from a molecular-dynamics simulation. Even relatively small organic molecules are undergoing quite large-scale and complex gyrations at all times. A simple calculation reveals that in a mole of some 10-atom compound at room temperature there is almost no chance that any two molecules would instantaneously have the same structure, if one defines that to be all bond lengths equal to within $\pm 0.001 \text{ \AA}$ and all bond angles and dihedrals equal to within 0.1° . There are 24 degrees of freedom in our molecule, each one of which will have > 100 possible structural values, given a typical room-temperature excursion, and the precision with which we have defined the measurement. This translates to a probability of < 1 in 10^{114} of finding two molecules with the same geometry in a mole. Our bulk measurements and the molecular properties that we derive from them are consequently revealing only average behaviour to which no single molecule is likely to conform!

The possibility of structural heterogeneity obviously becomes vastly greater when one starts to consider molecules as large as enzymes. Now there is not only an incomprehensibly large number of actual geometries within the sample, but there is also a huge range of timescales for interconversions between geometries. Large-scale conformational changes of the protein may take place in milliseconds, whereas the vibrational changes in C–H bond lengths are occurring in femtoseconds.

The first correction to our earlier discussion of enzyme catalysis that should occur as a result of this recognition is a modification of the static and detailed description of binding of substrate and transition structure to the enzyme. Instead, we must recognise that there is a distribution of continuously changing enzyme–substrate complexes each of which will react *via* a different enzyme–transition–structure complex.

An interesting question, and a topic of much current research, is how the dynamical changes in structure of the enzyme are coupled to the changes in the substrate that accompany its passage through the transition state and on to the product. The slow conformational changes of the protein cannot couple directly to the reaction coordinate, but they often do have important roles to play nonetheless, because they can make a deeply buried active site temporarily accessible for binding of the substrate and release of the product.

The more controversial question is whether higher-frequency, more localised motions of the protein could couple to the reaction coordinate in a way that would provide the enzyme with catalytic advantage.^{15,57,58} In the most general terms, the answer to that question is “yes,” because it is guaranteed that there will be protein vibrational motions of the right frequency, and of sufficient proximity to the substrate for coupled motion to occur. The matter of contemporary debate is whether evolution could have selected *particular* dynamical motions of the protein to assist in the catalysis. Discussions of that question in the context of hydrogen tunnelling will appear in several of the later chapters of this book.

References

1. T. D. H. Bugg, *Nat. Prod. Rep.*, 2001, **18**, 465.
2. H. Eyring and A. E. Stearn, *Chem. Rev.*, 1939, **24**, 253.
3. M. Garcia-Viloca, J. Gao, M. Karplus and D. G. Truhlar, *Science*, 2004, **303**, 186.
4. W. R. Cannon, S. F. Singleton and S. J. Benkovic, *Nat. Struct. Biol.*, 1996, **3**, 821.
5. W. P. Jencks, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1975, **43**, 219.
6. R. Wolfenden and M. J. Snider, *Acc. Chem. Res.*, 2001, **34**, 938.
7. J. D. Dunitz, *Science*, 1994, **264**, 670.
8. M. I. Page, *Int. J. Biochem.*, 1980, **11**, 331.
9. L. Pauling, *Chem. Eng. News*, 1946, **24**, 1375.
10. M. Polanyi, *Z. Elektrochem.*, 1921, **27**, 142.
11. F. M. Menger, *Biochemistry*, 1992, **31**, 5368.
12. J. R. Burke and P. A. Frey, *Biochemistry*, 1993, **32**, 13220.
13. W. P. Jencks, *Philos. T. Roy. Soc. A*, 1993, **345**, 3.
14. A. Warshel, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 5250.
15. R. L. Schowen, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 11931.
16. S. Marti, M. Roca, J. Andres, V. Moliner, E. Silla, I. Tunon and J. Bertran, *Chem. Soc. Rev.*, 2004, **33**, 98.
17. J. Giraldo, D. Roche, X. Rovira and J. Serra, *FEBS Lett.*, 2006, **580**, 2170.
18. U. Pindur and G. H. Schneider, *Chem. Soc. Rev.*, 1994, **23**, 409.
19. B. Ganem, *Angew. Chem. Int. Edit.*, 1996, **35**, 937.
20. C. C. Galopin, S. Zhang, D. B. Wilson and B. Ganem, *Tetrahedron Lett.*, 1996, **37**, 8675.
21. S. D. Copley and J. R. Knowles, *J. Am. Chem. Soc.*, 1987, **109**, 5008.
22. S. Marti, J. Andres, V. Moliner, E. Silla, I. Tunon and J. Bertran, *Theochem.*, 2003, **632**, 197.
23. P. D. Lyne, A. J. Mulholland and W. G. Richards, *J. Am. Chem. Soc.*, 1995, **117**, 11345.
24. M. M. Davidson, I. R. Gould and I. H. Hillier, *J. Chem. Soc. Perkin Trans. 2*, 1996, 525.
25. H. Guo, Q. Cui, W. N. Lipscomb and M. Karplus, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 9032.
26. S. E. Worthington, A. E. Roitberg and M. Krauss, *J. Phys. Chem. B*, 2001, **105**, 7087.
27. S. Hur and T. C. Bruice, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 1176.
28. Y. S. Lee, S. E. Worthington, M. Krauss and B. R. Brooks, *J. Phys. Chem. B*, 2002, **106**, 12059.
29. A. Shurki, M. Strajbl, J. Villa and A. Warshel, *J. Am. Chem. Soc.*, 2002, **124**, 4097.
30. C. R. W. Guimaraes, M. P. Repasky, J. Chandrasekhar, J. Tirado-Rives and W. L. Jorgensen, *J. Am. Chem. Soc.*, 2003, **125**, 6892.
31. S. Marti, J. Andres, V. Moliner, E. Silla, I. Tunon and J. Bertran, *J. Am. Chem. Soc.*, 2004, **126**, 311.

32. K. E. Ranaghan and A. J. Mulholland, *Chem. Commun.*, 2004, 1238.
33. X. D. Zhang, X. H. Zhang and T. C. Bruice, *Biochemistry*, 2005, **44**, 10443.
34. F. M. Menger, *Pure Appl. Chem.*, 2005, **77**, 1873.
35. N. A. Khanjin, J. P. Snyder and F. M. Menger, *J. Am. Chem. Soc.*, 1999, **121**, 11831.
36. P. Kast, J. D. Hartgerink, M. AsifUllah and D. Hilvert, *J. Am. Chem. Soc.*, 1996, **118**, 3069.
37. D. Hilvert, *Abstr. Pap. Am. Chem. Soc.*, 1997, **214**, 167.
38. D. J. Gustin, P. Mattei, P. Kast, O. Wiest, L. Lee, W. W. Cleland and D. Hilvert, *J. Am. Chem. Soc.*, 1999, **121**, 1756.
39. A. Kienhofer, P. Kast and D. Hilvert, *J. Am. Chem. Soc.*, 2003, **125**, 3206.
40. A. Warshel, P. K. Sharma, M. Kato, Y. Xiang, H. B. Liu and M. H. M. Olsson, *Chem. Rev.*, 2006, **106**, 3210.
41. I. Rozas, *Phys. Chem. Chem. Phys.*, 2007, **9**, 2782.
42. A. M. Pendas, M. A. Blanco and E. Francisco, *J. Chem. Phys.*, 2006, **125**, #184112.
43. W. W. Cleland and M. M. Kreevoy, *Science*, 1994, **264**, 1887.
44. P. A. Frey, S. A. Whitt and J. B. Tobin, *Science*, 1994, **264**, 1927.
45. W. S. Brinigar and T. L. Chao, *Biochem. Biophys. Res. Commun.*, 1975, **63**, 78.
46. N. Wellner and G. Zundel, *J. Mol. Struct.*, 1994, **317**, 249.
47. T. Ishida, *Biochemistry*, 2006, **45**, 5413.
48. M. I. Page and W. P. Jencks, *Proc. Natl. Acad. Sci. USA*, 1971, **68**, 1678.
49. J. Villa, M. Strajbl, T. M. Glennon, Y. Y. Sham, Z. T. Chu and A. Warshel, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 11899.
50. X. Y. Zhang and K. N. Houk, *Acc. Chem. Res.*, 2005, **38**, 379.
51. T. C. Bruice and P. Y. Bruice, *J. Am. Chem. Soc.*, 2005, **127**, 12478.
52. D. A. Kraut, K. S. Carroll and D. Herschlag, *Annu. Rev. Biochem.*, 2003, **72**, 517.
53. G. Winter, A. R. Fersht, A. J. Wilkinson, M. Zoller and M. Smith, *Nature*, 1982, **299**, 756.
54. S. Gronert, *J. Org. Chem.*, 2006, **71**, 1209.
55. K. U. Ingold and G. A. Dilabio, *Org. Lett.*, 2006, **8**, 5923.
56. H. G. Nagendra, N. Sukumar and M. Vijayan, *Proteins*, 1998, **32**, 229.
57. D. Antoniou and S. D. Schwartz, *J. Phys. Chem. B*, 2001, **105**, 5553.
58. V. L. Schramm, *Arch. Biochem. Biophys.*, 2005, **433**, 13.