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Understanding Enzymic Reactivity – New Directions and Approaches

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ABSTRACT

New approaches towards understanding the reactivity of enzymes–central to chemical biology and a key to comprehending life itself–are discussed herein. The approach overall is based on the idea that structural and reactivity features uniquely characteristic of enzymes–in being absent in normal catalysts–are likely to hold the key to the catalytic powers of enzymes. The quintessentially physical-organic problem is addressed from several angles, both kinetic and phenomenological. (Generally, the Pauling theory of transition state stabilization is adopted as the rigorous basis for understanding enzyme action).

The kinetic approach focuses on the inadequacies of the Michaelis-Menten equation, and proposes an alternative model based on additional substrate binding at high concentrations, which satisfactorily explains experimental observations. The phenomenological approaches focus on the inadequacies of the intramolecularity criterion, thus leading to alternative strategies adopted by nature in the design of these mild yet powerful catalysts, characterized by exquisite selectivity.

Preferential transition state binding at the active site, via both hydrophobic and van der Waals forces, appears to be the major thermodynamic driver of enzymic reactivity. In operational terms, however, multifunctional catalysis–practically unique to the highly ordered enzyme interior–is likely the key to enzymic reactivity. A new concept, 'strain delocalization', possibly plays an important role in orchestrating these various effects, and indeed justifies the need for a large proteinic molecule for achieving the enormous rate enhancements generally observed with enzymes.

Thus, this renewed approach to understanding enzymic reactivity departs significantly from currently held views: radically, in abandoning the Michaelis-Menten and intramolecularity models; but also

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commandeering existing ideas and concepts, although with a shift in emphasis towards transition state effects (including the entirely novel idea of 'strain delocalization').The coverage is not exhaustive, but aims to introduce new ideas along with fresh insights into previous works.

Keywords: Active site; catalysis; hydrophobic; intramolecularity; Michaelis-Menten; multifunctional catalysis; strain delocalization; van der Waals.

1. INTRODUCTION

1.1 General Considerations and Historical Background

Enzymes are the ubiquitous and versatile catalysts of the biological world, a key molecular species that enables the sustenance and propagation of life. Nature employs enzymes variously and exquisitely, drawing upon their large proteinic frameworks to accomplish remarkable feats of metabolic regulation and control. Enzymes thus represent a critical nodal point in the flow of biological information, which manifests the genetic blueprint as the plethora of molecular phenomena that constitute life [1].

The broad features of enzyme activity, regulation and control are now fairly well understood [2-4]. Thus, enzymes are almost always relatively large proteins with well-defined conformations and tertiary structures. This leads to an active site cleft, as also a well-ordered interior with pendent catalytic groups: although these are the general acids and bases of conventional organic chemistry, it is the manner of their deployment that is critical to understanding enzymic reactivity.

Historically, enzymes have been employed since antiquity in fermentations (although unbeknownst as such). The turn of the 19th century witnessed the first faltering steps towards reaching a scientific understanding of enzymes, with the use of plant and other extracts for the hydrolysis of starch. These studies ultimately led up to Buchner's landmark demonstration of fermentation with cell-free extracts of yeast (1897). The isolation, purification and crystallization of enzymes were accomplished in the ensuing decades of the 20th century.

Enzymes are also the key to understanding biological evolution in molecular terms [5], because genetic changes manifest as alterations in the coded amino acid sequences of the various enzymes that regulate metabolism. Evolutionary selection pressure thus leads to improvements in enzyme function, essentially as an increase in the catalytic rates. It has been hypothesized that a perfectly evolved enzyme functions under diffusion control (essentially implying a negligible free energy of activation).

1.2 The Era of Mechanistic Enzymology

The realization that enzymes were large proteinic catalysts with defined molecular structures, paved the way for exacting chemical studies directed towards unravelling their likely modes of action. Their extraordinary catalytic powers were beginning to be increasingly recognized, leading to intriguing theoretical questions and practical possibilities. These developments allowed Pauling (1948) to explain enzyme reactivity in terms of transition state theory [6], the emerging paradigm of chemical kinetics and reactivity [7]. Indeed, Pauling's landmark theory of transition state stabilization marked the beginning of the physical organic study of enzyme action, and remains a leitmotif in chemical biology to this day.

The idea that enzyme reactivity was based in the stabilization of the transition state by the enzyme was, of course, a very generalized proposition [8]. This clearly assumed that enzymes employed the same general reaction mechanisms as the (corresponding) uncatalyzed in vitro reactions. However, the fine details of the stabilizing mechanisms employed by enzymes–perhaps idiosyncratically in each case–were yet to be explored. Advances in protein crystallography were clearly critical to these efforts, the early work on chymotrypsin (1967) serving as a pioneering landmark therein [2,9].

The succeeding decades witnessed attempts to model the key reaction sequences believed to be employed by enzymes, in smaller non-enzymic molecules. Studies on these intramolecular models apparently led to a belief that they also held the key to the reactivity of the enzymes [10,11]. Thus, intramolecular reactivity was gradually conflated with enzyme reactivity, in a clear–though unstated–departure from the prevailing dogma of transition state stabilization.

It is possible that these developments were aided (perhaps subliminally) by the prevailing kinetic theory of enzyme catalysis [2,3,7,12]. This employed the Michaelis-Menten equation, which was based in the idea of the pre-equilibrium formation of an enzyme-substrate complex that was "turned over" to product in a rate-limiting step. Michaelis-Menten kinetics thus gained wide acceptance, particularly as it apparently explained the characteristic "saturation kinetics" displayed by enzymes. However, it is likely that this also led to the belief that, as the critical turnover step was per se unimolecular, intramolecularity held the key to enzymic reactivity.

The problem with this view is that the enzyme reaction is overall bimolecular, hence the Pauling theory must apply. Importantly, however, this is not so much about conforming to dogma as to adhering to the fundamental principles of chemical reactivity. As has been argued previously [11], intramolecular reactivity arises from ground state effects that are absent in the enzyme case, hence cannot serve as a basis for enzymic reactivity.

A more fundamental problem, in fact, concerns the Michaelis-Menten equation itself. Thus, this leads to the curious result that the overall equilibrium constant at "saturation" is a ratio of the forward and reverse turnover numbers! As has been previously argued [12], this implies that the Michaelis-Menten scheme is manifestly flawed and needs to be abandoned. Indeed, an alternative scheme of enzyme kinetics has been proposed, which explains "saturation kinetics" as essentially the result of additional binding of substrate at high concentrations, leading to inhibition of product release.

1.3 Departures and New Proposals

This paper represents a status report of the physical organic chemistry of enzyme catalysis, based on the background ambiguities as mentioned above. The essential challenge of enzyme catalysis lies in explaining how enzymes achieve their phenomenal rate accelerations that cannot be replicated in non-enzymic model systems. The approach adopted herein is based on the idea that features uniquely characteristic of enzymes–in being absent in non-enzymic systems–hold the key to enzymic reactivity. New possibilities are explored in reasonable detail, an attempt being made to reach a coherent and cogent picture of enzyme reactivity in conformity with accepted principles of chemical reactivity in general.

The conventional theory of enzyme catalysis is covered by any number of standard textbooks, monographs and reviews [1-3,10], and it would make little sense to include it here. However, it is used as a comparative backdrop to the new proposals discussed herein. Recent developments leading to a fundamental reassessment of the theory of enzymic reactivity–essentially initiated by the author–are discussed clearly but succinctly, the reader being referred to earlier papers for the details [11,12]. A formal derivation of the proposed alternative to the Michaelis-Menten equation is included in the Appendix.

This is by no means an exhaustive review of previous work, but rather a selective collation of the key recent developments that point to a new direction, which is urgently necessary for both theoretical and practical reasons. An attempt is also made to provide critical insights into previously established ideas and concepts. Mechanistic enzymology lies at the borderline between the chemical and biological sciences, and represents a key to the reductionist view of life. Enzymic reactivity raises fundamental questions about the physico-chemical origins of the phenomenon, leading to the basis of chemical reactivity itself. It behooves one and all to rise to this challenge and seek honest answers in the true spirit of scientific inquiry.

2. DISCUSSION

2.1 Previous Approaches and Inadequacies

2.1.1 The Michaelis-Menten equation

The Michaelis-Menten equation was introduced in 1913 and has since served as the accepted paradigm of enzyme kinetics [1-3,12]. The equation is based on the idea of a rapid pre-equilibrium formation of an enzyme-substrate complex, which is converted to products in a slow "turnover" step (Fig. 1). The derivation of the equation apparently follows standard protocols of chemical kinetics, but ends up with a fundamentally invalid result, as discussed below.

Confidence in the Michaelis-Menten equation was apparently bolstered by the fact that it reproduces the observed change in kinetics with increasing substrate concentration. However, an enigmatic problem is that applying the Michaelis-

Menten equation at overall equilibrium under "saturation" conditions, leads to the result that the equilibrium constant (K) is a constant ratio of the forward and reverse turnover numbers (k_f and k_r respectively, *cf.* Equation 1).

$$K = (k_f/k_r) \quad (1)$$

This is clearly thermodynamically invalid (and intriguingly led to the idea of "one-way enzymes"). A fairly detailed analysis of this problem has been presented previously [12], but may be summarized as follows: The derivation of the Michaelis-Menten equation relates the overall rate to the concentration of the enzyme-substrate complex at a certain time (t), whereas the overall rate should be related to the concentrations of enzyme and substrate at time *t.*

Fig. 1. The reaction of an enzyme (E) with a substrate (S) to form the product (P), via the enzyme-substrate complex (ES)

The enzyme-product complex and the release of free enzyme are not shown

Also, the purported "saturation kinetics" was previously explained as arising from the saturation of the pre-equilibrium between enzyme and substrate. However, this manifestly contravenes transition state theory, by which the overall rate depends only on the free energy difference between ground and transition states. (In other words, the concentration of the intermediate is inconsequential.)

In fact, the observed "saturation kinetics" represents an apparent decrease in the overall rate constant at high substrate concentrations. As previously argued, this can be explained as arising from the binding of a second molecule of substrate at the active site. This relatively weak binding would prevent the release of the product molecule–and the free enzyme–from the enzyme-substrate complex. The resulting relative deceleration would become manifest at higher substrate concentrations as the second molecule of substrate binds weakly at the active site (likely at the periphery). In other words, an increasing fraction of the original enzyme concentration is "locked up" with increasing substrate concentrations, leading to corresponding levels of inhibition of the overall reaction.

These qualitative arguments can be supported by a modified kinetic scheme, a formal derivation of the resulting equation being presented in the Appendix section herein (which is based on a rigorous approach to the overall kinetics). It is particularly noteworthy that this avoids the anomaly represented by Equation 1, instead leading to the overall equilibrium constant that fully conforms to thermodynamic principles.

The collapse of the Michaelis-Menten equation clearly demands a fundamental reappraisal of the theory and practice of enzyme catalysis. In particular, the view that the purported saturation stage leads to the turnover number (k_{cat}) is now unviable, so the existing collection of k_{cat} and K_M (Michaelis constant) values for innumerable enzymes has become irrelevant.(Note that only the Michaelis-Menten equation is invalid, not the reaction scheme itself; hence, k_{cat} and K_M retain their original significance, *cf.* Appendix.)

Thus, renewed attempts need to be made to obtain the real k_{cat} and K_M values for all known enzymes. Apparently, it would be best to first obtain the K_M value via the concentration of the enzyme-substrate complex: an obvious strategy would be by inhibiting the turnover step, so that a measurable concentration of the complex builds up. The overall rate constant, obtained from the kinetics, will yield the k_{cat} upon being divided by the K_M. This is the only rigorous way of obtaining these kinetic parameters (*cf.* Appendix).

2.1.2 The intramolecularity problem

As mentioned above, the high rate constants of intramolecular reactions (relative to their intermolecular analogs) led to the view that these can serve as models for the rate accelerations observed in the case of enzymes [10]. This view was apparently bolstered by the fact that, in the enzymic reaction, the turnover step is unimolecular. However, as was argued at length previously [11], this seems unviable as the enzyme catalyzed reaction is overall bimolecular (involving enzyme and substrate).

Thus, although intramolecular reactions can model the reactions occurring during the turnover step, the origin of the rate accelerations would be different in the enzymic case. Intramolecular reactions essentially derive their accelerations from a raised ground state, often resulting from conformational restrictions. In the enzymic case, however, the ground state would be the free enzyme and substrate: these, and the rate determining transition state, together determine the overall free energy of activation. Clearly, the enzymic acceleration must derive from a stabilized transition state (the Pauling theory) [6].

These arguments imply that the source of the enzymic accelerations must be sought in the enzyme active site or even the interior, rather than in the enzyme-substrate complex (as represented in intramolecular models). This idea is explored further in some detail below, involving several possible effects and modes, generally within the ambit of known effects of structure and medium on reactivity.

2.1.3 Conflict with transition state theory

Interestingly, the above problems involving both the Michaelis-Menten equation and the intramolecularity model [11,12], apparently arise from a fundamental conflict with transition state theory [7]. A key principle of transition state theory is that the overall reaction rate constant depends only on the free energy difference between the ground and transition states. Thus, the nature and stability of intermediate species are irrelevant to the overall rate constant.

The Michaelis-Menten equation is derived on the basis of the concentration of the enzyme-substrate complex, the "saturation" of this concentration being attributed to the observed kinetics at high substrate concentrations. However, this ignores the fact that the pre-equilibrium is linked to the turnover step, which would also be accelerated with increasing substrate concentrations, so saturation of the pre-equilibrium is a tenuous assumption. By transition state theory, again, the saturation–or not–of the pre-equilibrium is irrelevant to the overall rate.

This is solely dependent on the overall free energy of activation and the concentrations of enzyme and substrate at a given time, the overall rate constant being given by (k_{cat}/K_M). Thus, the only acceptable rate equation for enzyme kinetics is as given in Equation 2 (ν_t is the overall rate, $[E]_t$ and $[S]_t$ being the enzyme and substrate concentrations respectively, all at time t):

$$\nu_t = (k_{cat}/K_M)[E]_t[S]_t \quad (2)$$

The intramolecularity idea also represents a similar conflict with transition state theory, as the idea assumes that the overall rate can be related to the stability of the enzyme-substrate complex, which is modeled in the intramolecular analog of the enzymic reaction. Thus, the Pauling theory of transition state stabilization remains rigorous and paramount [6].

2.1.4 Beyond the Pauling hypothesis?

In fact, the original Pauling proposal has been criticized as not being able to account for very large rate enhancements by enzymes (> 10^{11}) [8]. It is believed that the formation of covalent intermediates between substrate and enzyme is responsible for these accelerations. The idea appears to be that, if a covalent bond is formed prior to the rate determining transition state (resulting in an enzyme-bound intermediate), the binding can be carried over to the said transition state. There are two problems with this proposal, however.

Firstly, the formation of the enzyme-bound covalent intermediate needs to be much faster than the overall reaction, else the release of free enzyme will become rate limiting. (Of course, the formation of the intermediate itself can be catalyzed by the same enzyme, which only raises the same questions about the source of the acceleration!)

Secondly, a new (stronger) covalent bond would be worth tens of kcals mol^{-1}, whereas free energies of activation are typically ~ 25 kcals mol^{-1} (for the uncatalyzed case) [13]. This not only represents a considerable overkill, but also implies that the enzyme-bound intermediate will be more stable than the starting substrate! Of course, the enzyme-bound intermediate can be destabilized by (say) steric crowding in the active site pocket, perhaps orienting the bound substrate for subsequent catalytic reactions.

The marginally stronger covalent bond would–if carried over to the rate determining transition state–extend the binding stabilization beyond that of the hydrogen bond. The stability of such a covalent intermediate may be easier to fine tune (than in the case of hydrogen bonds). Indeed, data indicates that the majority of enzymes function via covalent intermediates [8]. However, it is doubtful whether this can be considered as an alternative to transition state stabilization, as it is only a variant of other interactions in the enzyme-substrate complex that are also carried over to the transition state. (This proposal also seems to be related to the intramolecularity model in an oblique manner).

2.2 New Proposals

In the following sections, the key causative effects and phenomena that are the basis of enzyme catalysis are briefly reviewed. Although

they may appear well established principles, they have been contentious at times, hence a critical review would hopefully offer fresh insights. It is noteworthy that enzymic reactivity remains enigmatic, despite sustained and ingenious efforts over several decades.

2.2.1 General considerations

Enzyme catalysis works by lowering the free energy of activation of a reaction [2,3,7]. By the Pauling theory [6], this is accomplished by stabilizing the transition state: this is not so much dogma, as the only realistic and rigorous possibility. Applying the Pauling theory, however, leads to interesting conceptual challenges and dilemmas that constitute a fascinating intellectual exercise in itself. This intimately involves transition state theory, but also subtle extensions of key ideas drawn from the principles of in vitro chemical reactivity and catalysis.

Firstly, the stabilization of the reaction transition state by the enzyme implies prior binding of the substrate in its ground state (Fig. 1). This indeed must lead to the stabilization of the substrate itself (Fig. 2a). These elementary protocols, however, have been the subject of much discussion and speculation [2], essentially because the stabilization of the substrate ground state, in certain cases, could result in lowered reactivity (or even loss of catalytic activity). Thus, enzymes have evolved to strike a balance between these two modes of binding, apparently with exquisite perfection. (Also, the binding of the substrate is accompanied by a substantial loss of entropy, which must be overcome by the binding energy.).

Thus, enzyme catalysis relies on “moderate” binding of the substrate ground state but strong binding of the transition state (Fig. 2a). Here “moderate” is used in both a relative and an absolute sense. The binding of the substrate ground state may well be carried over to the reaction transition state, although this is not a requirement. Indeed, the substrate ground state and the reaction transition state may, in principle, be stabilized by entirely different modes of binding. An extreme possibility is that the enzyme binds only the transition state and not at all the substrate ground state. However, this is clearly impractical, considering the fleeting existence of the transition state.

In fact, eliminating the enzyme-substrate complex altogether is unnecessary for efficient catalysis, on thermodynamic grounds. In practice, this can be achieved by closely matching the increase in free energy (via entropic loss, etc.) upon binding, with the free energy of the binding. Whilst this would mostly smooth out the free energy profile (Fig. 2b), it does not increase the efficiency of catalysis, hence would waste evolutionary efforts.

It is, of course, true that the enzyme should not bind the substrate ground state so strongly that it increases the activation energy of the reaction (Fig. 2c). To reiterate, the key to enzyme catalysis lies in weak binding of the substrate ground state and strong binding of the reaction transition state. Again, both cases lead to considerable loss of entropy relative to the uncatalyzed case, so the binding needs to overcome the corresponding increase in the Gibbs free energy.

A particular problem is that too strong a binding of the substrate ground state can lead to the release of the free enzyme becoming slow and rate determining (Fig. 2d). Thus, even if the overall activation energy is lowered, slow release of free enzyme from the enzyme-product complex (not shown) will partly negate the overall acceleration.

It would appear, therefore, that enzymes evolve by striking a balance between the above effects, but most importantly, by stabilizing the transition state by selective binding. This must employ a combination of weak forces–notably, hydrophobic, van der Waals and hydrogen bonding–in such a manner as not to affect the substrate ground state (lest release of free enzyme become slow).

2.2.2 Quantitative considerations

A measure of the lowering of the Gibbs free energy of activation in enzyme catalysis can be obtained from the Eyring equation (Equation 3) [7], relating the rate constant (k) to the Gibbs free energy of activation ($\Delta G^{\ddagger}$) of a reaction:

$$k = (k_B T/h)\exp(-\Delta G^{\ddagger}/RT) \quad (3)$$

(T is the absolute temperature, while k_B, h and R are Boltzmann’s, Planck’s and gas constants, respectively.) The ratio of the enzyme catalyzed rate constant (k_{EC}) to the uncatalyzed rate constant (k_{UC}) is then given by Equation 4:

$$(k_{EC}/k_{UC}) = \exp(-\Delta' G^{\ddagger}/RT) \quad (4)$$

Here, $\Delta' G^{\ddagger} = [(\Delta G^{\ddagger})_{EC} - (\Delta G^{\ddagger})_{UC}]$, with $(\Delta G^{\ddagger})_{EC}$ and $(\Delta G^{\ddagger})_{UC}$ being the Gibbs free energy of

activation of the enzyme catalyzed and uncatalyzed reactions, respectively. Thus, Equation 4 leads to a thermodynamic measure of the efficiency of enzyme catalysis in terms of the Gibbs free energy of activation.

A reduction in the Gibbs free energy of activation in the enzyme catalyzed reaction ($\Delta' G^{\ddagger}$) by 12 kcal mol^{-1}, would thus lead to a rate acceleration ((k_{EC}/k_{UC}) of ~ 10^9 (a billion-fold). This typical value gives a quantitative idea of the efficiency of enzyme catalysis.

The corresponding stabilization of the transition state is achieved by a combination of weak forces, with the stabilization of the substrate ground state being avoided at all costs. (The "excessive" binding of the transition state is nearly impossible as transition states are high energy species!)

In fact, recent computational methods have led to the development of molecular docking studies of enzyme-substrate binding, that provide detailed insight into the above discussed interactions [14,15], as elaborated below.

2.2.3 The hydrophobic effect

The hydrophobic effect has its origins in the greater solubility of an organic molecule in a non-polar solvent relative to water [16]. In the context of enzyme catalysis, the hydrophobic effect arises as the active site cleft provides a hydrophobic environment. This is essentially a consequence of protein folding, which places hydrophobic amino acids deep in the interior of the protein, thus avoiding contact with the external water solvent [9]. (The active site exists within the interior of the enzyme protein.)

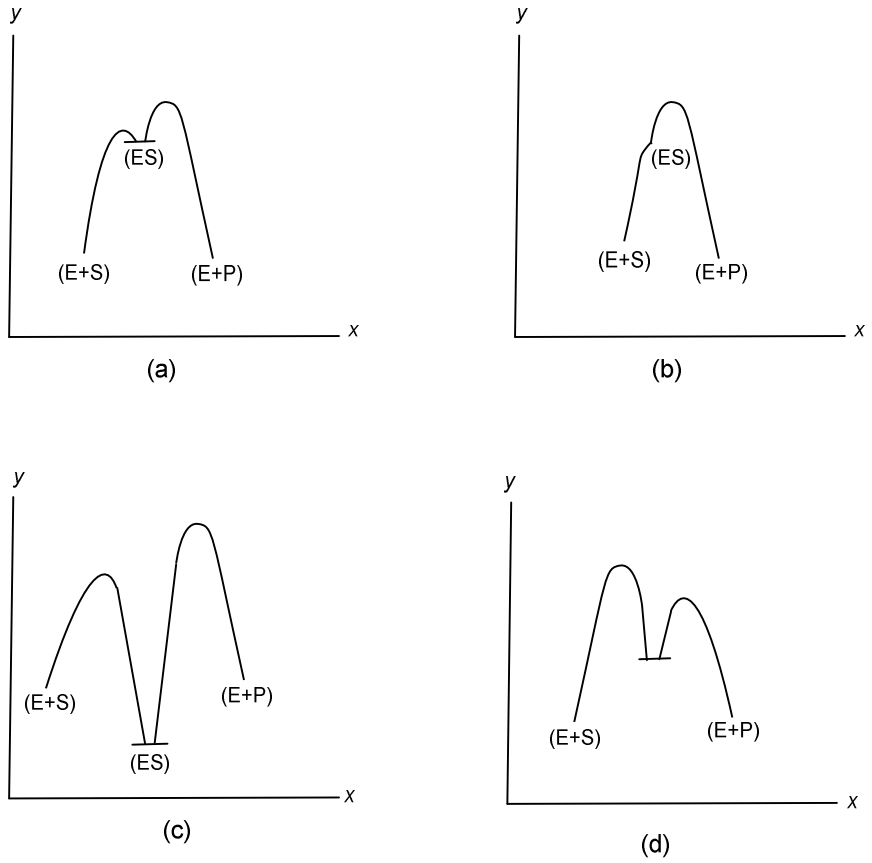

Fig. 2. Energy profiles for enzyme catalyzed reactions: the importance of weak interactions and selective transition state binding

The x axis represents the reaction coordinate and the y axis the Gibbs energy, in all cases. E, ES and P refer to enzyme, enzyme-substrate complex and product, respectively

It has long been known that the hydrophobic effect plays an important role in enzyme catalysis [2,3], in "luring" the substrate into the active site cavity. The resulting binding is accompanied by the loss of considerable entropy (particularly translational), which must be more than offset by the hydrophobic effect for any rate acceleration to occur. For a maximum acceleration the hydrophobic pocket needs to be complementary to the rate determining transition state, else steric effects (in particular) will decrease the binding and the acceleration.

In fact, the role of the hydrophobic effect in non-enzymic reactions is complex and controversial [16]. This is essentially because the hydrophobic effect is largely nullified by the low solubility of the substrate in water. However, this problem would not apply in the enzymic case as the solubility of the substrate is inconsequential, the comparison being between the enzymic and non-enzymic reaction in the same water solvent. Interestingly, therefore, enzyme catalysis apparently represents a clear-cut example of the hydrophobic effect on reactivity!

2.2.4 van der Waals and related forces

The need for weak binding of the substrate ground state leads to the critical role of van der Waals and other dispersion forces [13,17]. Although the hydrophobic effect (*vide supra*) is weak, it is essentially a relative effect (between water and non-polar media), that is also geometrically ill-defined. The van der Waals force, however, is valid in itself, apparently arising from attractive interactions between the fluctuating dipoles of atoms in close contact (although at an ideal distance).

The van der Waals force is also notoriously weak (1–2 kcal mol^{-1}). At an enzyme active site, however, it can be additively amplified by multiple contacts between the enzyme and the bound substrate. This leads to the possibility that an active site complementary to the transition state–although not so much to the substrate ground state–would be effective.

Apparently, therefore, whereas the hydrophobic effect acts on the substrate ground state, the binding of the transition state would be dominated by the van der Waals force. Of course, the hydrophobic effect would also act at the transition state, although the van der Waals force would provide the major binding that is also spatially well-defined to match the contours of the transition state.

2.2.5 Hydrogen bonding and other polar binding modes

Hydrogen bonding interactions between enzyme and substrate offer interesting possibilities for binding and stabilization [17,18]. Indeed, because of their moderate strengths (typically ~ 5 kcals mol^{-1}) [13], hydrogen bonds would add substantially to other weak binding modes (*vide supra*). In fact, hydrogen bonds to negatively charged centers can be enormous (~ 30 kcals mol^{-1} involving F^-), which indicates that they likely play a major role in stabilizing polar transition states.

However, being the strongest of the weak forces, hydrogen bonds carry the risk that release of free enzyme may become slow and rate determining. In fact, hydrogen bonding interactions may not easily distinguish between the substrate ground state and the reaction transition state [18]. This is because hydrogen bonding has rather precise spatial requirements, and the shapes of the substrate ground state and the reaction transition state may not be very different. (Transition states often resemble the ground state of the substrate.)

Interestingly, this would be less of a problem with the van der Waals force, which acts via multipoint interactions spread over a wider three-dimensional space, that includes the substrate and other reactants.

All the same, an intriguing type of hydrogen-bonded relay may play a critical role in enzyme catalysis [18], by involving the α-helix and β-sheet subunits of the protein backbone of the enzyme molecule. A critical problem in enzyme catalysis is that the transition state is generated within a hydrophobic active site pocket, so the stabilization of the charges in the transition state by solvation is essentially ruled out. However, it has been suggested that this can be overcome if the α-helix and β-sheet subunits around the active site can hydrogen bond with the charges on the transition state, thereby relaying the charges away to the aqueous exterior [18]. The proposal apparently enjoys modest support from both experimental and theoretical studies.

2.2.6 Multifunctional catalysis

Multifunctional catalysis is particularly unique to enzymes [2,3], and is likely to be the key to their exceptional reactivity as also being impossible to replicate in simpler (catalytic) systems.

(Intermolecular analogs of multifunctional catalysis in free solution would, of course, be ruled out by their high entropic demands.) This is because multifunctional catalysis requires the precise location and ordering of a complex set of functional groups in three-dimensional space, that can only be achieved in the proteinic interior of an enzyme.

A well-known case is the catalytic triad in serine proteases (Fig. 3A), involving an aspartate carboxylate group, a histidine imidazole moiety and a serine hydroxyl group [2,3]. Apparently, this hydrogen-bonded assembly creates a super-nucleophilic serine hydroxyl group, which functions as a nucleophile catalyst. Intriguingly, though, the pK_a order of the involved groups in this charge relay system is absurdly inverted [10], the overall effect being the deprotonation of an alcohol by a carboxylate group!

However, this could imply that pK_a orders *per se* are inverted in the hydrophobic interior of an enzyme. In fact, it is known that pK_a's are essentially determined by solvation effects, as the pK_a orders are very different in the gas phase relative to the solution phase [19].This possibly suggests that the above carboxylate group is largely undissociated, so charge dispersal in the hydrogen-bonded assembly is the key to its reactivity.

In fact, charge dispersal as the basis of "anchimeric assistance" is well-established in the dissociation of organic substrates (including the non-classical ion case) [13]. The above case of the catalytic triad thus indicates that this could be a key rate enhancing effect within hydrophobic enzyme interiors, wherein normal solvation effects are obviated.

Another case of multifunctional catalysis is found in the glycosidases, in which dissociation at the anomeric center is assisted by concerted general acid-base catalysis (Fig. 3B) [2,3]. This is a particularly revealing example, for essentially two reasons. Firstly, the almost concatenated arrangement of reactive functionality around the reaction center, would be nearly impossible to achieve in simpler catalytic systems. Secondly, the presence of strong general acids and bases together is noteworthy, as this is impossible in free solution (as they would neutralize each other). The relatively rigid protein framework of the enzyme keeps the acidic and basic groups apart, although positioned optimally for their catalytic functioning.

Thus, multifunctional catalysis is widespread in all enzymes as a key component of the overall catalytic mechanism: it would be no exaggeration to state that enzyme catalysis is essentially characterized by multifunctional catalysis as a dominant effect in the overall sequence of reactions.

2.2.7 Strain delocalization

An intriguing and novel concept that likely plays a role in enzyme catalysis, is based on the possibility that bond angle strain can be "delocalized", leading to the enhanced stability of the molecule as a whole [20].The idea is based in the principles of molecular mechanics [21], by which bond angle strain is proportional to the square of the deviation of the angle from an idealized value. Thus, the total strain (S_{tot}) spread over n bond angles each carrying a deviation of m° would be given by Equation 5 (A is a proportionality constant):

$$S_{tot} = Anm^2 \quad (5)$$

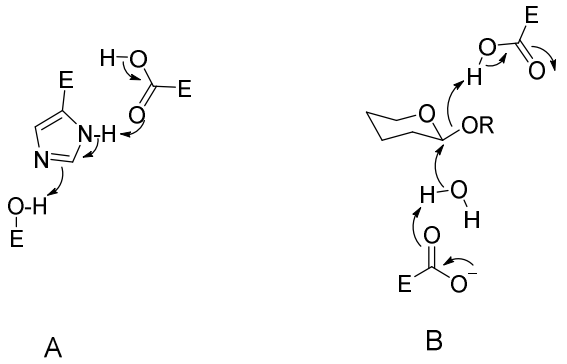

Fig. 3. Multifunctional catalysis: (A) catalytic triad in proteases; (B) general acid-base catalysis in glycosidases

E refers to the enzyme backbone

However, if all the strain is localized in a single bond angle, the total strain would be given by Equation 6, noting that the bond angle deviation is now (*nm*):

$$S_{tot} = An^2m^2 \quad (6)$$

Clearly, the former possibility leads to greater stability ($nm^2 < n^2m^2$).

The significance of these ideas lies in the fact that large molecules would normally tend to be less strained, as the same bond angle strain can be spread over a greater number of angles (other considerations being equal). This would apply to macrocycles, in particular, but also large acyclic molecules (especially coiled ones).

In the case of enzymes, which are generally large non-linear proteins, this implies greater flexibility of the overall framework. Intriguingly, this would lead to ideal reaction trajectories being realized more easily, relative to the case of a smaller molecule. This implies a lower free energy of activation for reactions occurring within the enzyme interior and involving the bound substrate.

In fact, torsional strain behaves rather similarly [20,21] and can also be "delocalized". These considerations indicate that a large proteinic enzyme molecule has the right balance of rigidity and flexibility, for conducting and orchestrating the complex series of mechanistic steps that need to be executed in its interior. This is likely to contribute significantly to the overall catalytic efficiency of the enzyme. This also indicates that a large protein molecule is required in order to achieve the extraordinary catalytic powers the enzymes are renowned for.

3. CONCLUSION

The above discussion has reviewed the current state of understanding of enzyme catalysis. The discussion includes both novel proposals that depart radically from current ideas, and existing ideas although with fresh insights. The coverage is topical and brief rather than exhaustive, although key references to previous works have been provided.

The most significant conclusion of the review is the urgent need to reassess the Michaelis-Menten equation, thus indicating its abandonment as the basis of enzyme kinetics. This is because the Michaelis-Menten equation leads to results that are contrary to the principles of thermodynamics, in particular the idea of "one-way enzymes" at high substrate concentrations. An alternative formulation of enzyme kinetics is proposed that avoids these problems and is also in accord with transition state theory. Thus, enzyme reactions are generally best viewed as bimolecular reactions following conventional second order kinetics at low substrate levels. At higher substrate levels, however, competitive weak binding of a second molecule of substrate leads to the inhibition of the enzyme, with a drastic reduction in the apparent rate constant. (This led to the illusion of "saturation" in the earlier scheme.)

Furthermore, the Pauling theory of transition state stabilization indicates the abandonment of the view that intramolecular reactivity holds the key to enzymic reactivity. This follows from the fact that intramolecular reactivity is derived from ground state effects (although intramolecular models can be employed to validate a proposed enzyme mechanism).

Although enzymic reactivity is based on the preferential net stabilization of the transition state, this is achieved via a complex skein of effects involving known concepts and their subtle extensions to the enzymic case. Thus, the inevitable formation of the enzyme-substrate complex requires the weak binding of the substrate ground state that is likely driven by the hydrophobic effect, followed by the stronger binding of the transition state by a variety of forces. These are primarily cumulated dispersion forces, apart from hydrogen bonding and polar forces. These latter, however, may lead to the slow release of the free enzyme and possible inhibition of the overall reaction. Enzyme-bound covalent intermediates extend the strengths of these stronger forces, and with similar risks.

In fact, multifunctional catalysis almost certainly holds the key to enzymic reactivity, noting that it is unique to the enzyme interior and impossible to replicate in simpler catalytic systems. It is noteworthy therein that it is possible for strong general acids and bases to coexist only within the enzyme interior. Generally, charge dispersal seems to be the key to reactivity, in the absence of normal solvation effects in the hydrophobic environment (as apparent in the catalytic triad of the serine proteases). An intriguing charge relay mechanism for stabilizing the transition state likely employs α-helix and β-sheet subunits in the protein framework, thus overcoming a serious limitation of the hydrophobic interior of the enzyme molecule.

An entirely novel proposal, however, is based on the idea of "strain delocalization", which is likely to play a significant role in enzymic reactivity. This is based in molecular mechanics, by which the bond angle strain is proportional to the square of the deviation of a bond angle from its ideal value. Thus, a collection of smaller angle deviations generates less strain than a single large deviation. This implies that a large proteinic enzyme molecule possesses a measure of flexibility, which allows ideal reaction trajectories to be attained with negligible overall strain.

In summary, the theory of enzymic reactivity has apparently arrived at an exciting cusp, when the existing conceptual framework can be propelled in a new direction.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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APPENDIX

This section includes a new kinetic approach to enzyme catalysis, which departs from the conventional Michaelis-Menten equation. However, the reaction mechanism and scheme remain the same as previously assumed (Fig. 1).

In this proposal (Fig. 4), the enzyme is inhibited by secondary binding of the substrate (or product, for the reverse reaction), at high concentrations of substrate (or product) [12]. The change in the slope of the reaction rate, and the onset of a regime with a lower rate of increase in the apparent rate constant, at higher substrate levels, can be explained as a consequence of the secondary binding. Most importantly, the overall equilibrium constant remains unaltered at high substrate (or product) levels, hence there is no violation of thermodynamic principles.

Let E_F represent free enzyme, E_o the starting enzyme, ES_2 and EP_2 the enzyme complexes with two molecules of substrate and product respectively, υ_f and υ_r the forward and reverse rates of the enzyme catalyzed reaction, with k_f and k_r their respective rate constants. S and P represent substrate and product respectively, the subscript "eq" referring to their equilibrium concentrations.

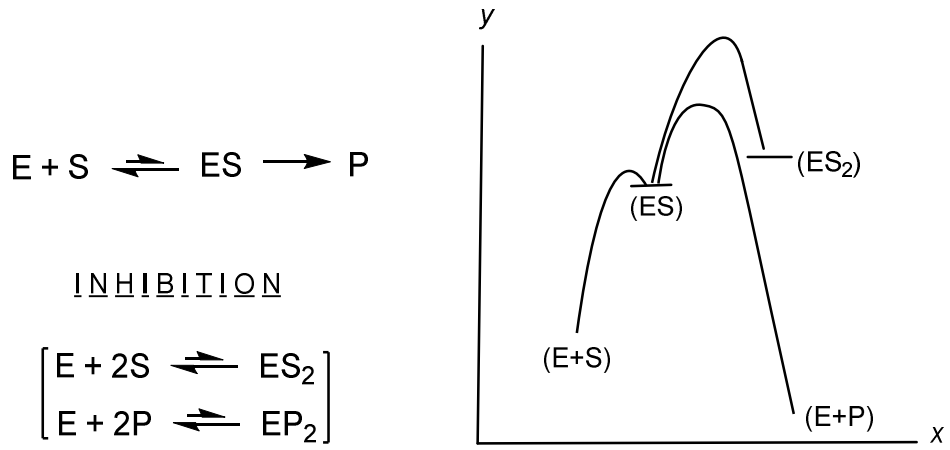

Fig. 4. Enzyme inhibition at high substrate levels via secondary binding to form ES_2 (alternative to Michaelis-Menten kinetics)

Reaction scheme at left; energy profile at right only displays forward reaction (cf. Fig. 1)

$$[E_F] = [E_o] - ([ES_2] + EP_2]) \quad (1A)$$

$$\upsilon_f = k_f[[E_F][S] \quad (2A)$$

$$\upsilon_r = k_r[[E_F][P] \quad (3A)$$

At equilibrium, $\upsilon_f = \upsilon_r$:

$$k_f[[E_F][S_{eq}] = k_r[[E_F][P_{eq}] \quad (4A)$$

$$(k_f/k_r) = [P_{eq}]/[S_{eq}] \quad (5A)$$

Eq. 5A is valid in the "saturation regime" and differs entirely from the MME result, and implies that all species will reach thermodynamic equilibrium.

INHIBITION

$$[ES_2] = K_S[E_F][S]^2 \quad (6A)$$

$$[EP_2] = K_P[E_F][P]^2 \quad (7A)$$

(K_S and K_P are equilibrium constants for the formation of ES_2 and EP_2 respectively, *cf.* Fig. 4.) For equilibrium, the following changes occur:

$$[E_o] \rightarrow [E_F];\ [S_o] \rightarrow [S_{eq}];\ [P_o] \rightarrow [P_{eq}]$$

"SATURATION"

From (1A) and (6A):

$$[E_F] = [E_o] - ([ES_2])$$

$$= [E_o] - (K_S[E_F][S]^2) \quad (8A)$$

From (2A) and (8A):

$$\upsilon_f = k_f[[E_F][S] \quad (2A)$$

$$= k_f\{[E_o] - (K_S[E_F][S]^2)\}[S] \quad (9A)$$

$$= k_f[E_o][S] - k_fK_S[E_F][S]^3 \quad (10A)$$

From (8A) and (9A): as [S] increases, $(K_S[E_F][S]^2$ increases exponentially, so $[E_F]$ decreases correspondingly drastically. Because K_S is relatively small, this regime picks up only at relatively high [S].

In (10A), $k_f[E_o][S]$ refers to the rate in the absence of secondary binding, and $k_fK_S[E_F][S]^3$ to the "rate" involving secondary binding itself. This latter term increases exponentially, thus reining in the overall rate drastically, as [S] increases. Note, however, that by (6A) $[E_F]$ also decreases substantially with increasing [S], so this would moderate the exponential effect.

In fact, by (6A), the ratio $([ES_2]/[E_F])$ increases exponentially with [S], so $[E_F]$ decreases less than $[S]^2$ increases (as $[ES_2]$ must also increase), hence even less than $[S]^3$. The overall effect is a steep increase in the $k_fK_S[E_F][S]^3$ term with increasing [S].

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