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The Moderately Efficient Enzyme: Futile Encounters and Enzyme Floppiness

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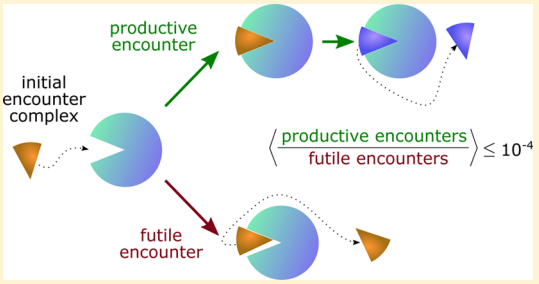
ABSTRACT: The pioneering model of Henri, Michaelis, and Menten was based on the fast equilibrium assumption: the substrate binds its enzyme reversibly, and substrate dissociation is much faster than product formation. Here, we examine this assumption from a somewhat different point of view, asking what fraction of enzyme–substrate complexes are futile, i.e., result in dissociation rather than product formation. In Knowles' notion of a "perfect" enzyme, all encounters of the enzyme with its substrate result in conversion to product. Thus, the perfect enzyme's catalytic efficiency, k_{cat}/K_M, is constrained by only the diffusion on-rate, and the fraction of futile encounters (defined as φ) approaches zero. The available data on >1000 different enzymes suggest that for ≥90% of enzymes $\varphi > 0.99$ and for the "average enzyme" $\varphi \geq 0.9999$; namely, <1 of 10^4 encounters is productive. Thus, the "fast equilibrium" assumption holds for the vast majority of enzymes. We discuss possible molecular origins for the dominance of futile encounters, including the coexistence of multiple sub-states of an enzyme's active site (enzyme floppiness) and/or its substrate. Floppiness relates to the inherent flexibility of proteins, but also to conflicting demands, or trade-offs, between rate acceleration (the rate-determining chemical step) and catalytic turnover, or between turnover rate and accuracy. The study of futile encounters and active-site floppiness may contribute to a better understanding of enzyme catalysis, enzyme evolution, and improved enzyme design.



Michaelis and Menten formalized Henri's model in what has become known as the Michaelis–Menten equation, or model.[1] Their model assumed a simplified scenario whereby the product concentration is essentially zero. Under such conditions, product inhibition and the reversible reaction could both be ignored, resulting in the following mechanism:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P \quad (1)$$

The model was also based on the assumption of a fast enzyme–substrate equilibrium, namely of $k_2 \gg k_3$, and thereby the Michaelis constant $K_M \approx k_2/k_1 = K_S$. Later, Briggs and Haldane used a steady-state model, which assumes only that the concentration of ES does not change over time (i.e., with no assumptions regarding the relation of k_3 to k_2, or of K_M to K_S), and obtained the same rate equation in which $K_M = (k_2 + k_3)/k_1$.[2] An interesting implication of the "fast equilibrium" assumption ($k_2 \gg k_3$) is that the majority of enzyme–substrate complexes dissociate rather than go forward to yield the product. In other words, most enzyme–substrate encounters are futile.

Shortly after the Michaelis–Menten model was devised, the macroscopic kinetic parameters (K_M and k_{cat}) of a few enzymes became known, but their microscopic kinetic constants remained unknown. To date, more than a century later, while the macroscopic kinetic parameters of many hundreds of enzymes have been measured, the data regarding the microscopic parameters remain limited. It is thus impossible to directly assess how widely valid the Henri–Michaelis–Menten assumption of $k_2 \gg k_3$ was. Here, we apply a simple model that compares the enzyme catalytic efficiency, k_{cat}/K_M, to the diffusion-limited collision rate and thereby allows the macroscopic-based assessment of the fraction of futile substrate–enzyme encounters. We found that for the average enzyme, <1 of 10^4 encounters is productive. We subsequently address the molecular origins of futile encounters and their relation to the coexistence of multiple enzyme sub-states, or enzyme floppiness.

THE APPLIED MODEL

In the Michaelis–Menten equation, k_1 represents the association rate leading to the enzyme–substrate complex.[3] However, the experimentally measured k_1 values may refer to different steps along the trajectory leading eventually to the product, and also to enzymes whose mechanisms and rate-determining steps widely differ. In some cases, k_1 is measured

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by coupling to catalysis, and in others, it is measured directly, e.g., by fluorescence quenching upon substrate binding. In the latter case, k_1 may represent the rate constant for the formation of an encounter complex (including nonproductive ones) or more advanced complexes leading to catalysis. Thus, to assess what fraction of substrate−enzyme encounters result in product formation, we use a generic definition that is derived from the perfect enzyme notion and is independent of the experimental determination of k_1. We therefore consider a modified "black box" formalism of the Michaelis−Menten mechanism in which the k parameters are modified such that $\widetilde{k}_1$ corresponds to the diffusion-limited enzyme−substrate collision rate and ÉS represents the initial substrate−enzyme encounter complex:

$$\text{É} + \text{S} \underset{\widetilde{k}_2}{\overset{\widetilde{k}_1}{\rightleftarrows}} \text{ÉS} \xrightarrow{\widetilde{k}_3} \text{É} + \text{P} \tag{2}$$

In a so-called "perfect enzyme", the catalytic efficiency, k_{cat}/K_M, is equal to the diffusion on-rate, i.e., to the rate constant for the occurrence of substrate−enzyme encounters.[4,5] Drawing from this notion, in our "black box" formalism, we indicate every initial encounter of a substrate with any enzyme molecule regardless of its state is counted, and those that do not lead to product are defined as futile encounters. Thus, $\widetilde{k}_1$ corresponds to the diffusion-limited enzyme−substrate collision rate, and thus, ÉS represents the initial substrate−enzyme encounter complex. We thus refer here to the initial encounter complex as the complex whose rate of occurrence is limited only by diffusion (what is considered as the "initial encounter complex", and the corresponding models for estimating the diffusion rate, is discussed below). We also denote the enzyme by É, instead of E, as the former corresponds to the total enzyme concentration rather than the free enzyme: a random collision by a substrate can occur with the enzyme molecule, including those with occupied active sites (such a case will also count as a futile encounter because there is already an ÉS formed). $\widetilde{k}_3$ encompasses all downstream steps leading to product formation, including the formation of a substrate−enzyme complex (denoted ES, which may be a more advanced stage than the initial ÉS encounter complex) in which the substrate is productively bound within the active site. Similarly, $\widetilde{k}_2$ encompasses all dissociation rates that result in substrate release, including the dissociation of the initial encounter complex and of any other downstream complex (ES, etc.).

It therefore follows that the fraction of futile encounters between an enzyme and its substrate can be defined as

$$\varphi = 1 - (k_{cat}/K_M)/\widetilde{k}_1 \tag{3}$$

where $\widetilde{k}_1$ represents the diffusion limit rate, as discussed below.

THE DIFFUSION RATE LIMIT

What is the rate constant of encounters between an enzyme and its substrate? Calculating the so-called diffusion limit of enzyme rates has attracted much attention over the past century (e.g., refs 6−14). For our purposes, we focus on two extreme cases. First, let us consider the rate of collisions with a sphere the size of which corresponds to the whole protein. This model assumes absorption of every colliding substrate molecule, and that the colliding substrates are kept on the protein's surface until they reach the active site, by van der Waals and/or electrostatic forces acting between the enzyme and the substrate.[15] Considering all collisions, the diffusion limit rate constant is $\sim 10^{10}$ s^{-1} M^{-1}.[6,11,12] This value can be derived from the diffusion equation using characteristic parameters of the diffusion constants of metabolites ($D \sim 10^{-5}$ s^{-1} cm^2)[16,17] and the average physical radius of proteins (r = 2 nm)[18] being substituted in the expression for the diffusion limit on rate, $4\pi Dr$.[16]

By a more conservative estimate, only collisions with the enzyme's active-site patch are considered as being potentially productive, and the rest of the protein surface is taken as being inert toward the substrate. Using characteristic patch sizes, diffusion on-rates of $\sim 10^9$ s^{-1} M^{-1} have been calculated.[6,11,12]

Overall, as a benchmark for evaluating enzymes, we consider $\widetilde{k}_1$ to be the range defined by the two cases mentioned above, namely between 10^9 and 10^{10} s^{-1} M^{-1}.

THE PERFECT ENZYME

The title of "perfect" has been applied to many enzymes, but in fact, only few so-called perfect enzymes, having k_{cat}/K_M values well within the range of the diffusion limit mentioned above (10^9−10^{10} s^{-1} M^{-1}), have been described. For example, the k_{cat}/K_M of carbonic anhydrase is 10^{10} s^{-1} M^{-1} or possibly higher,[19] and the k_{cat}/K_M values of fumarase[6] and of superoxide dismutase are very close to 10^{10} s^{-1} M^{-1}.[20] The value of a mutant of the latter has been shown to exceed 10^{10} s^{-1} M^{-1}[20] (k_{cat}/K_M may exceed the diffusion rate limit because of electrostatic steering, as discussed below). Other reported examples include a prolyl 4-hydroxylase with -X-Pro-Gly- substrates,[21] acyl-CoA dehydrogenase (and its F105L mutant in particular) accepting 2-methylbutanoyl-CoA,[22] and 3-phosphoglycerate kinase accepting 1,3-bisphosphoglycerate.[23] Triosephosphate isomerase, the first enzyme proclaimed "perfect",[4] was reported to have a k_{cat}/K_M of 4×10^8 s^{-1} M^{-1}, less than half of our lower limit for the diffusion rate.

What does a k_{cat}/K_M that equals the diffusion rate constant mean?

(1) All steps following the formation of the initial encounter complex, namely, formation of the enzyme−substrate complex (the classic ES complex) from the initial encounter complex (ÉS), chemical transformation, product release, etc., occur at rates much faster than the diffusion rate.

(2) The rates of dissociation of the ÉS encounter complex, as well as of the subsequent complexes, are significantly lower than the rate of product formation. In other words, in a perfect enzyme, every substrate−enzyme collision results in conversion to product. Hence, the fast equilibrium assumption does not hold for the perfect enzyme.

THE AVERAGE ENZYME

How far are most enzymes, or how far is the "average enzyme", from perfection? Namely, how does the average enzyme perform in terms of the criteria mentioned above? What is the average fraction of futile encounters (φ)?

To address these questions, we took a simplistic approach that draws from the model described above (eq 3) and uses a data set of the published kinetic parameters of thousands of different enzymes.[24,25] We have previously found that the average k_{cat}/K_M value for this data set is $\sim 10^5$ s^{-1} M^{-1}.[24] Thus, for the vast majority of enzymes, the overwhelming majority of encounters with substrates are futile. Figure 1 presents the k_{cat} and K_M values for ~1400 enzyme−substrate pairs, where the green lines correspond to the diffusion limits of 10^9 and 10^{10}

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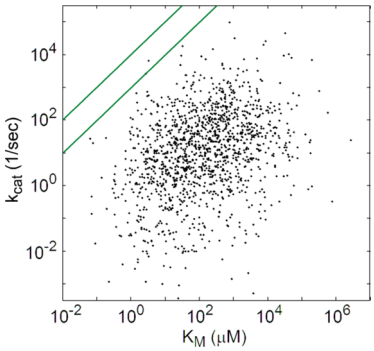

Figure 1. Nearly all enzymes (≥98%) are at least 1 order of magnitude slower than the diffusion on-rate limit. Each dot corresponds to a k_{cat}/K_M value measured for a given enzyme–substrate pair[24] and reported in the BRENDA database.[25] The top green line corresponds to a k_{cat}/K_M of 10^{10} s^{-1} M^{-1} and the bottom one to a k_{cat}/K_M of 10^9 s^{-1} M^{-1}, representing the range discussed in the paper for the diffusion rate. The presented estimate should be taken as a ballpark. For example, some of the enzymes with especially high k_{cat}/K_M values (discussed in the text) are not included in this set because their individual values of k_{cat} and K_M were not measured or these values are not recorded in the BRENDA database and hence are not shown here.

s^{-1} M^{-1}. More than 90% of enzyme–substrate pairs exhibit k_{cat}/K_M values that are at least 100-fold slower than the lower estimate of the diffusion rate; i.e., $k_{cat}/K_M < 10^7$ s^{-1} M^{-1}, and thus, $\varphi > 0.99$. Approximately 2% of enzyme–substrate pairs show k_{cat}/K_M values within an order of magnitude of the lower diffusion rate, namely with k_{cat}/K_M values of $\geq 10^8$ s^{-1} M^{-1} ($\varphi < 0.90$), and can thus be termed "nearly perfect" (in the diffusion limit sense). As noted above, perfect enzymes are extremely rare: fewer than 10 enzymes have values that fall within the range of diffusion rates (10^9–10^{10} s^{-1} M^{-1}).

The fact that the k_{cat}/K_M of the average enzyme is 4–5 orders of magnitude below the diffusion rate constant limit is interpreted by our model as having a φ of ≥0.9999. In other words, for every 10^4–10^5 initial encounters, only one results in product formation.

THE MODERATELY EFFICIENT ENZYME

Some, or possibly many, of the moderately efficient enzymes are suboptimal in the sense that given enough selection pressure and/or evolutionary time, their catalytic efficiency could significantly increase.[26] Other enzymes might have reached the limit of the catalytic efficiency because of various chemophysical constraints (e.g., ref 27). Regardless of whether an enzyme is suboptimal, what might be the mechanistic reasons for a high fraction of futile encounters?

As schematically depicted in Figure 2, several non-mutually exclusive factors can be considered.

(1) Most ÉS encounter complexes dissociate before the ES complex is established. This can result either from a lack of strong interaction between the enzyme's active site, or surface in general, and the substrate or from inefficient steering of the substrate into the active site.

(2) Most ES complexes dissociate before the occurrence of the downstream steps, because of a weak affinity of the ES complex and/or a slow rate of conversion to product.

(3) Enzymes and substrates usually coexist in multiple structural conformations, tautomers, and protonation states, which are identified here as sub-states. Although all these sub-states closely resemble the native state, only some have the capacity to yield a productive complex. An encounter in which either the enzyme or substrate is in the "wrong" state, the formation of an advanced encounter complex, or even of an ES complex with the wrong sub-state will be futile.

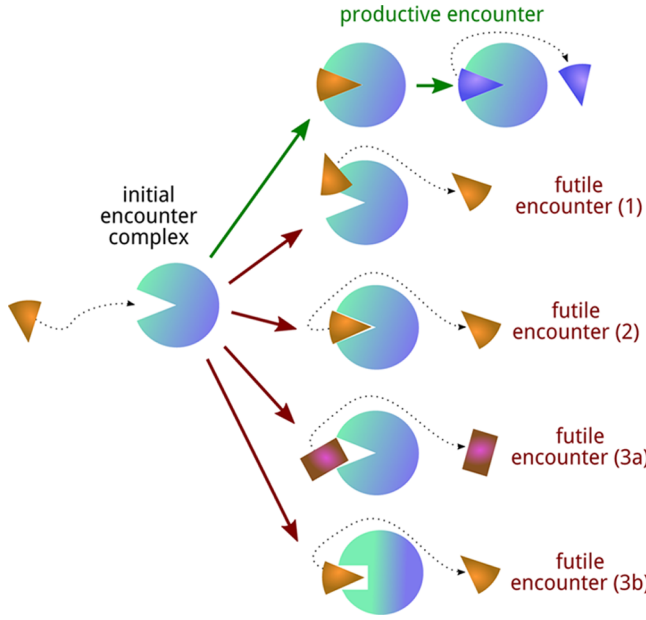

Figure 2. Cartoon representation of various scenarios leading to futile encounters. In a productive encounter, the initial encounter complex leads to the ES complex and product release. In a "perfect enzyme", the forward rates of the last two steps (green arrows) are faster than the diffusion limit, and thus, k_{cat}/K_M is equal to the diffusion rate and all encounters are productive ($\varphi = 0$). Futile encounters may occur by various scenarios. (1) The initial encounter complex dissociates before an ES complex is established. (2) The ES complex is established, but the substrate dissociates rather than being converted to product. (3) The encounter involves either a substrate molecule (3a) or an enzyme molecule (3b) that is in a nonreactive sub-state (e.g., protonation states, conformations, rotamers, or tautomers that differ from the reactive ones).

The second factor is generally accepted and has been extensively analyzed: in many if not most enzymes, the chemical step is highly challenging and comprises the primary constraint on the optimization of catalytic efficiency (see, for example, refs 3 and 24). We therefore elaborate on the first and third factors.

FROM ENCOUNTER TO ENZYME–SUBSTRATE COMPLEX

As most cellular metabolites are charged,[28] it is likely that electrostatic interactions play a major role in attracting the substrate into the enzyme surface and steering it into the active site (e.g., ref 14). As shown for protein–protein interactions that proceed at very high rates, electrostatic steering decreases the fraction of futile encounters.[29] Substrate steering similarly applies to enzymes, and particularly to nearly perfect or perfect enzymes such as acetylcholinesterase[30] or superoxide dismutase.[20] Indeed, the patterns of surface electrostatics seem to be highly conserved throughout certain enzyme families and superfamilies.[31] As indicated in the case of acetylcholinesterase, the primary effects of active-site electrostatics relate to transition-state stabilization and thus directly affect the catalytic chemistry; facilitating substrate encounters may relate to only 10–20% of the overall reduction in activation energy.[32]

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Accordingly, conserved electrostatic patterns are related primarily to the catalytic residues,[33] but they also extend to the active-site periphery and beyond.[31] In addition, as the van der Waals binding energy between proteins and small ligands can be as high as 20 kJ/mol,[15] short-range forces may also contribute to the adherence of substrates to enzyme surfaces and hence to higher catalytic efficiencies.[11,12]

However, the surface electrostatics of enzymes, let alone surface hydrophobicity, cannot be freely optimized with the sole aim of attracting the substrate. Maintaining protein stability and physiologically relevant interactions with certain cellular proteins and preventing unwanted interactions with others (including aggregation) impose severe constraints on the evolution of protein surfaces.[34,35]

Additional constraints related to enzyme action are at play. The case of superoxide dismutase provides an example for a trade-off between the rate of formation of the initial encounter complex and the rate of the substrate entering the active site.[20] This enzyme actually repels its substrate because both the enzyme's surface and the substrate have a net negative charge. Simulation of an enzyme variant with more neutral surface electrostatics suggests that while the rate of enzyme−substrate encounters increases, the probability that encounters result in the formation of a productive enzyme−substrate complex decreases from ∼35 to <2%.[20] While superoxide dismutase is still able to achieve catalytic perfection, in other enzymes, trade-offs restricting surface optimization (e.g., selection against undesired interactions with other cellular proteins and/or against aggregation) may more severely limit their catalytic efficiency.

NONPRODUCTIVE ENZYME−SUBSTRATE COMPLEXES

Another factor that leads to futile encounters is nonproductive binding events. Substrates entering the active site might be released without conversion because their orientation with respect to the active-site residues, and foremost with respect to the catalytic residues, is incorrect. Nonproductive binding has been recorded in many enzymes.[36−40] It is most evident when initial rates decline with increasing substrate concentrations beyond a certain value, suggesting that the substrate also binds with an alternative, lower-affinity, nonproductive mode that competes with the productive one. Admittedly, the evidence often relates to enzymes with non-native substrates (or promiscuous substrates, as discussed below). However, nonproductive binding is likely to be ubiquitous in forms that are not readily detected. For example, nonproductive binding occurring with K_M values that are similar to or lower than those for productive binding might be manifested only in a lower apparent k_{cat} value with no deviation from the Michaelis−Menten model.

Nonproductive complexes may also relate to the binding of substrate forms, or sub-states, e.g., protonation states, tautomers, or rotamers that are nonreactive. The substrate of chorismate mutase, for example, comprises two coexisting conformers, a pseudodiequatorial conformer and a pseudodiaxial conformer, whereby the less favored one (diaxial) comprises the actual substrate.[41] Another example includes oxidoreductases that utilize formaldehyde: some can accept only in the aldehyde form, while others can use only the gem-diol (acetal) form.[42] Similarly, oxaloacetate coexists in three forms (keto, enol, and gem-diol), only some of which might be accepted by oxaloacetate-utilizing enzymes.[43] Note that, regardless of the microscopic mechanism (the ones mentioned above and the ones discussed further below), the macroscopic manifestation would be a higher fraction of encounters that result in dissociation rather than conversion to product.

NONPRODUCTIVE ENZYME SUB-STATES

A substrate colliding with an enzyme may encounter a nonproductive active-site sub-state. Briefly, enzymes exist as an ensemble of conformers, and/or sub-states, with very similar free energies separated by low activation energy barriers. These sub-states may include different backbone configurations (e.g., of active-site loops) and/or side-chain rotamers. Sub-states, however, may also refer to subtle single-atom variations, e.g., protonated versus nonprotonated forms of various side chains. Many of these sub-states are "on-pathway", i.e., relate to the enzyme's configuration during one of the steps of the catalytic cycle. Other sub-states, possibly most, are "off-pathway"; they are a mere outcome of protein structure being held together by weak forces (for a discussion of how the degree of structural order may affect catalytic efficiency, see ref 44). The on-pathway sub-states are assumed to occur in a concerted ordered manner along the reaction coordinate. However, on-pathway conformational sub-states may also coexist, and be "selected", rather than induced, by ligand binding (e.g., refs 45−47). By definition, this means that only a fraction of enzyme molecules are in the right sub-state at the right time. Indeed, many if not most enzymatic reactions go through more than one transition state, each of which may be stabilized by a different enzyme substrate.

As indicated in several cases, evolution toward higher catalytic efficiency resulted in a concerted sequence of sub-states that occur along the enzymatic reaction coordinates (e.g., refs 47 and 48), but it is also the case that our experimental tools (and minds) are biased toward the detection of structural changes that are correlated with one specific step of the catalytic cycle. However, given the very small energy differences between sub-states, and that most sub-states are very transiently and marginally populated (particularly the most activated ones),[46] it is highly unlikely that the "on-pathway" sequence is perfectly concerted, i.e., deterministic. A certain if not a considerable element of stochasticity is inevitable. Given many possible sub-states and several catalytic steps, a significant fraction of enzyme molecules is likely to be "out of phase", i.e., in the wrong sub-state, at any given moment. Substrates encountering an inadequate enzyme sub-state would dissociate, thus resulting in a futile encounter. Macroscopically, encounters with an inadequate enzyme sub-state may be observed in single-molecule measurements in cycles with a lower rate.[49,50]

The energetic profile of the catalytic cycle plays a major role in determining the relative representation of the on-pathway sub-states. Specifically, if the enzyme's complex with one of the reaction's components (substrate, product, or any of the intermediates connecting the two) is too stable, it will be kinetically trapped. Hence, the perfect enzyme is expected to destabilize all reaction intermediates while stabilizing all transition states.[3,4,51] The energetics of many intermediates and transition states may be coupled such that the same enzyme interaction may favorably affect both. However, in some cases, there exists an inevitable coupling and consequently a trade-off between these demands: stabilizing a transition state may lead to overstabilization of an intermediate whose structure is similar to that of this transition state; similarly, destabilizing a reaction intermediate might result in

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the destabilization of the transition state leading to, or from, that intermediate. The more complex the energy landscape (i.e., the more reaction intermediates and transition states), the more pronounced these trade-offs and the higher the potential for futile encounters are expected to be.

The abundance of nonproductive sub-states also relates to structural dynamics [and, more broadly, to the configurational stability of enzymes (see refs 52 and 53)]. Conformational transitions between sub-states often occur on a time scale slower than that of chemistry.[54,55] In this context, it is noted that catalysis, by definition, has two components: (1) rate acceleration, reducing the reaction's activation energy barrier, and hence having the reaction occur faster in the presence of the catalyst, and (2) by the end of the reaction, the catalyst should remain intact. The latter relates to enzyme turnover, namely, to product release and the recycling of the enzyme. The former, the "chemistry", occurs most efficiently when the substrate meets a highly preorganized and rigid (single-sub-state) active site.[56−59] Turnover, however, may demand multiple sub-states, and typically, structural dynamics promotes product release.[60−62] Futile binding events may therefore relate to the substrate meeting an enzyme sub-state that differs from the one that is optimally preorganized to perform the rate-determining chemistry. Thus, rate acceleration and enzyme turnover trade off: efficient turnover demands alternating sub-states, but the higher the number of coexisting sub-states, the higher the likelihood of encountering the wrong sub-state is. We note, however, that the existence of conformational changes along the reaction coordinate does not exclude "perfection"; rather, the structural and hence evolutionary constraints on the optimization of catalytic efficiency become more demanding.

It is also clear that dissociation of the substrate may occur much faster than certain conformational exchanges. The outcome of such rate differences would be a large fraction of futile encounters. This has been observed, for example, in a ligand-binding antibody whereby an initial low-affinity complex ($K_d \approx 40\ \mu M$; a value in the range of the median K_M value, 130 μM[24]) is followed by a loop rearrangement and formation of the final complex with >1000-fold higher affinity. The measured on-rate of formation of the initial complex is $\sim 10^7\ s^{-1}\ M^{-1}$. However, the rate constant for dissociation of the initial complex ($\sim 100\ s^{-1}$) is much faster than that forward rate constant that is determined by a loop rearrangement ($\sim 2\ s^{-1}$). Overall, the kinetics indicate that, on average, the ligand is "trapped" only once in 150 ligand binding events.[63] If diffusion-limited encounters are considered by our model's definition ($\widetilde{k}_1 \sim 10^9\ s^{-1}\ M^{-1}$), then only one in $\sim 10^4$ encounters results in the ligand being trapped in the high-affinity complex.

Floppiness, the coexistence of multiple sub-states, affects catalytic efficiency as also seen in the influence of temperature on enzymatic rates. Given the rate temperature dependency of nonenzymatic reactions, all enzymes, including thermophilic enzymes, become less effective catalysts as the temperature increases even well within their stability range (~2.5-fold per 10 °C change). This loss of rate acceleration relates to several factors, but the role of thermally induced vibrations in compromising the active site's catalytic configuration seems evident. Thus, many enzymes, including thermophilic ones, may be insufficiently rigid, but not too rigid.[53]

Overall, it appears that the existence of nonproductive enzyme and substrate sub-states may considerably reduce the rate of catalysis. As noted by Mattei and Hilvert regarding chorismate mutase,[41] "the relatively low value of the 'on' rates for chorismate (the substrate) and prephenate (the product), $2 \times 10^6\ M^{-1}\ s^{-1}$, probably reflects the need for a rare conformation of the enzyme, the ligand, or both for successful binding." Notably, as indicated by the dependency of chorismate mutases on solvent viscosity, the enzyme's second-order rate constant is in fact diffusion-limited. However, only very few encounters (possibly fewer than 1 in 1000) result in productive binding and product formation, thus accounting for the k_{cat}/K_M value of chorismate mutase being 3 orders of magnitude lower than the diffusion rate constant limit. However, given a certain fraction of productive encounters, having more encounters per unit time results in a faster rate of product formation. Thus, the rate of diffusion substrate−enzyme encounters affects the rate of every enzyme even though the rate of product formation may be much slower than the diffusion limit.[64]

PROMISCUITY MANIFESTS IN A HIGH FREQUENCY OF FUTILE ENCOUNTERS

Promiscuity [enzymes catalyzing reactions other than the one(s) for which they evolved] or substrate ambiguity (enzymes catalyzing the native reaction with non-native substrates) provides an opportunity to examine how enzymes act in a suboptimal manner. Indeed, futile encounters are readily observed with promiscuous, and generally poor, substrates (e.g., ref 65). This can be illustrated with PON1, an enzyme whose promiscuous mode of action has been investigated in detail. This mammalian calcium-dependent hydrolase, dubbed serum paraoxonase-1 (PON1), was originally characterized with what turned out to be promiscuous activities, hydrolysis of organophosphates such as the pesticide paraoxon, or aryl esters, e.g., phenyl acetate. Only much later was it discovered that the enzyme's native function is lactonase, with δ-lactones, and lactones with lipophilic side chains being the preferred substrates.[66,67] With these lactones, and when the enzyme is bound to its natural carrier, high-density lipoprotein particles, k_{cat} can reach $600\ s^{-1}$ and k_{cat}/K_M exceeds $10^7\ s^{-1}\ M^{-1}$.[68] Thus, with its presumed native substrates, PON1 is reasonably efficient, with a k_{cat}/K_M in the top decile of catalytic efficiencies.[28]

The hydrolysis of the promiscuous substrates, and of phosphotriesters especially, is characterized by much lower k_{cat}/K_M values, e.g., $6 \times 10^3\ s^{-1}\ M^{-1}$ with paraoxon.[67] These values suggest that it takes more than 10^5 encounters between PON1 and these substrates to achieve one catalytic event ($\varphi > 0.99999$). Notably, all substrates, native and promiscuous, are hydrophobic, dipolar, and uncharged, and thus, it is reasonable to assume that their encounter rates with the enzyme are similar. Further, all PON1's promiscuously catalyzed substrates tested (>40 different substrates altogether, including aryl esters) exhibit K_M values within the range of 0.8−4 mM (ref 69 and references cited therein). However, the promiscuous substrates differ in their k_{cat} values by nearly 6 orders of magnitude: from $700\ s^{-1}$ (phenyl acetate) to $0.001\ s^{-1}$ (3-fluorophenyl-diethyl phosphate).[69] Thus, the fraction of fruitful encounters for these promiscuous substrates is exceedingly small as well as highly variable, in the range of 10^{-3} to 10^{-10}.

What factors might be responsible for the dominance of futile encounters with promiscuous substrates? Take, for example, paraoxon: its hydrolysis is mediated by a rarely populated calcium mode, a mode that differs in the location of the calcium ion (a 1.8 Å displacement relative to the native,

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lactonase mode) and in the rotamers of some of its ligating residues. The most populated mode is seen in the crystal structure of wild-type PON1 and applies to the catalysis of lactone hydrolysis.[70] In addition, binding of paraoxon in a productive mode occurs within an enzyme conformation that differs from that observed with lactones. This change involves a large motion of a flexible active-site loop as well as rotameric switches of surface residues.[71] Nonproductive binding of paraoxon is seen experimentally as well as in simulations in which only a small fraction of binding events ($\leq 10^{-4}$) overlap the catalytic mode.[70,71] Thus, paraoxon hydrolysis makes use of an enzyme sub-state that is rarely represented, explaining, at least in part, the low k_{cat}/K_M, and the accordingly small fraction of fruitful encounters of $<10^{-5}$.

Overall, it appears that promiscuous activities make use of secondary, less abundant enzyme sub-states, and the inefficiency of catalysis can be attributed to the rarity of these sub-states. PON1 is no exception, and multispecificity and promiscuity generally correlate with structural flexibility[72] (for another example, see ref 73).

FLOPPINESS AND ENZYME DESIGN AND EVOLUTION

In natural enzymes, elaborate interaction networks that maintain the key catalytic residues of enzymes can be seen. These interactions do not directly affect substrate binding or catalysis. Rather, these second- and third-shell interactions restrict the configurational freedom of the catalytic residues. Their crucial contribution to catalytic efficiency is seen, for example, in membrane-associated enzymes, whereby membrane binding dramatically stimulates catalysis. A recent structural analysis of such an enzyme indicated a network of hydrogen bonds that connects the membrane-binding residues with a key catalytic residue that resides >15 Å away.[65] It appears that, in general, membrane binding immobilizes long-range interactions via second- and third-shell residues that reduce the active site's floppiness and preorganize the catalytic residues.[74,75]

The crucial role of second- and third-shell interactions in restraining active-site floppiness is also demonstrated by their absence in human-made enzymes. Floppy active sites are commonly observed with computationally designed enzymes whose catalytic efficiencies are far lower than those of natural enzymes. The backbone configurations of designed enzymes may perfectly overlap the computational model, but these enzymes are still catalytically inefficient.[76] In some cases, the side-chain rotamers of active residues sometimes differ from those expected. For example, an active-site tryptophan designed to bind an aromatic substrate was found to adopt a flipped rotamer in the free enzyme, although in complex with a transition-state analogue, its position was more similar to that of the designed rotamer.[77] A similar tryptophan rotamer flip was observed in a catalytic antibody that catalyzes the same reaction and was shown to limit the turnover rate.[78] Similarly, molecular dynamics simulations suggest that a glutamate side chain that acts as the catalytic base in another computationally designed enzyme (a Kemp eliminase) is mostly populated in conformations that differ from the designed one and are less reactive or nonreactive. In the course of the evolutionary optimization of the computationally designed enzyme, these alternative configurations were minimized but not completely eliminated.[77,79] In another designed enzyme from the same series that had also been subjected to directed evolution, simulations indicate an increasingly rigid and more precisely preorganized active site.[80] Similarly, in the directed evolution of a computationally designed retro-aldolase, the designed catalytic lysine was replaced with an adjacent lysine within the designed pocket. The rearranged pocket, and substrate realignment within the new pocket, were facilitated by the rearrangement of a flexible active-site loop.[81]

Altogether, it seems that floppiness, as manifested in the ubiquity of nonproductive sub-states, and hence in an extremely large fraction of futile encounters, underlies inefficient catalysis, be it in designed enzymes or in the promiscuous action of natural enzymes.

Floppiness may be costly in terms of catalytic efficiency, but on the other hand, it provides the basis for evolutionary innovations. Alternative binding modes of substrates and alternative active-site conformations and sub-states facilitate the promiscuous catalysis of alternative substrates and reactions as discussed above. Adaptive mutations do not create something from nothing. Rather, they shift the distribution of molecular sub-states such that a marginal sub-state becomes the dominant one.[82] Accordingly, evolution reshapes active sites to reinforce such latent, alternative sub-states, thus turning weak, haphazard activities into new enzymatic functions.[26,81,83,84] Likewise, designed enzymes may be amenable to evolutionary optimization for the same reason that they are catalytically inefficient, namely, because their active sites are floppy.[77]

While conformational diversity and the coexistence of different sub-states (floppiness) underlie the evolutionary potential of new enzymatic functions, the dominance of catalytically nonproductive sub-states may lead to an evolutionary dead end. In a recent study, we aimed to understand why two adaptive mutations in the antibiotic resistance enzyme TEM-1 b-lactamase that individually increase the enzyme's catalytic efficiency with a third-generation antibiotic lead to a loss of activity when combined (negative epistasis). It appears that both these mutations allow TEM-1's active site to adopt alternative conformations and accommodate the new antibiotic. However, when combined, critical second- and third-shell contacts are broken, resulting in the predominance of nonproductive active-site configurations. Specifically, Asn170 that coordinates the deacylating water molecule adopts a noncatalytic configuration, even when a covalent inhibitor occupies the active site. The entropic cost of selecting from multiple sub-states, and foremost, the dominance of an impaired conformation of N170 underlie the very low catalytic efficiency of the double mutant.[85]

CONCLUDING REMARKS

By applying a simplified model that extends Knowles' notion of the "perfect enzyme", we derived a measure for enzyme efficiency, i.e., the fraction of fruitful encounters, φ. We show that, by a conservative estimate, in >90% of enzymes, $\varphi > 0.99$, i.e., fewer than 1 in a 100 substrate−enzyme encounters results in product formation; in the average enzyme, fewer than 1 of 10000 initial encounters is fruitful. We hypothesize that in addition to the widely recognized factor of a slow, rate-determining chemical step, "floppiness", the coexistence of multiple substrate binding modes, and of multiple enzyme sub-states, is a primary reason for low frequencies of productive encounters. Floppiness is an inevitable feature of proteins but also the result of conflicting demands (trade-offs): the need to stabilize different intermediates along the catalytic cycle and to promote rate acceleration (chemistry) as well as turnover (e.g., product release).

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A slow, rate-determining chemical step may well be the factor that limits the rate of many enzymes, but other factors may be equally dominant, as suggested by the very wide range of catalytic efficiencies observed between enzymes belonging to the same class, or even the same enzyme in different organisms.[24] Thus, how much natural enzymes can be improved for their native substrates is unknown, let alone, whether in those that can be improved, higher catalytic efficiency will relate to a lower degree of floppiness. More refined kinetic models, and direct measurements of the fraction of productive encounters, and possibly of futile ones, may become available in the future and thus provide better hypotheses regarding the molecular origins of futile encounters.

The quantitative contribution of each factor discussed here for the large fraction of futile encounters remains an open challenge. Are all factors similarly important, or does one dominate in a category of all reaction types? Understanding the molecular origins of futile encounters may also improve our ability to design new enzymes. For example, the structural features underlying futile encounters can be analyzed by molecular dynamics simulations whereby, typically, only a small fraction of the simulated trajectories result in product formation (for examples, see refs 84 and 86) and the remaining trajectories therefore simulate futile encounters. Thus, in principle, minimization of futile encounters can be used as an optimization parameter in enzyme design protocols. However, to this end, simulation methodologies that analyze the energetics of sub-state ensembles in relation to the energy barriers of the chemical step(s) are needed (for a more detailed discussion, see ref 87). Futile encounters could be minimized by selecting not only for the optimal transition-state stabilizing conformation but also by selecting, in parallel, against alternative, nonproductive substrate binding modes and active-site sub-states (negative design[88]). Another, so far unexploited, contribution to the laboratory optimization of enzymes could be maximizing the on-rate of enzyme−substrate encounters by promoting electrostatic attraction of the substrate to the enzyme's surface as well as substrate steering into the active site. As demonstrated in protein−protein interactions, such optimization might be relatively straightforward.[89]

Overall, in the same manner that the study of enzyme promiscuity has become an insightful endeavor alongside the traditional study of enzyme specificity, understanding the physicochemical and evolutionary constraints that limit the catalytic efficiency of enzymes, and of the molecular origins of enzyme inefficiency, may promote our understanding of enzymes and our ability to engineer new ones.

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