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Fundamental Challenges in Mechanistic Enzymology: Progress toward Understanding the Rate Enhancements of Enzymes

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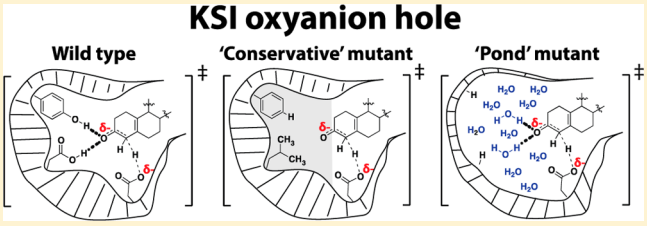
ABSTRACT: Enzymes are remarkable catalysts that lie at the heart of biology, accelerating chemical reactions to an astounding extent with extraordinary specificity. Enormous progress in understanding the chemical basis of enzymatic transformations and the basic mechanisms underlying rate enhancements over the past decades is apparent. Nevertheless, it has been difficult to achieve a quantitative understanding of how the underlying mechanisms account for the energetics of catalysis, because of the complexity of enzyme systems and the absence of underlying energetic additivity. We review case studies from our own work that illustrate the power of precisely defined and clearly articulated questions when dealing with such complex and multifaceted systems, and we also use this approach to evaluate our current ability to design enzymes. We close by highlighting a series of questions that help frame some of what remains to be understood, and we encourage the reader to define additional questions and directions that will deepen and broaden our understanding of enzymes and their catalysis.



Enzymes are amazing in their ability to accomplish enormous rate enhancements with extraordinary specificity for reactions conducted under mild conditions. These chemical transformations are at the heart of biology because reactions, and just the right reactions, must be accelerated to outpace natural dissipative forces so that living systems can create and maintain their requisite order and organization. Competition between organisms, both within and across species, provides a further selective pressure to evolve faster (and more specific) enzymes that allow an organism to garner more nutrients, to grow and reproduce faster, to respond faster to changing conditions, and even to outswim, outcrawl, or outrun a predator.

Given the centrality of enzymes in nearly all biological processes, and their prevalence as drug targets, it is no wonder that enzymes have been intensely scrutinized—conceptually, experimentally, and theoretically—over many decades. We have learned an enormous amount over these decades, including the centuries-old discovery of the existence of biological catalysis,[1−3] the seminal finding that catalysis can occur outside of a living system,[4] and the identification of proteins as the primary catalysts in biology.[5]

The mid-20th Century saw the rapid elaboration of the identity of the chemical transformations that are performed by enzymes, as well as the identification of coenzymes and cofactors that help facilitate these reactions.[6] Practitioners of physical organic and bioorganic chemistry elucidated viable mechanisms for these chemical transformations outside of the enzyme environment,[7,8] and many clever kinetic and chemical tests were developed by enzymologists to indirectly (but powerfully) derive information about the transformations taking place within the active site.[9−16] These tests provided information about reaction intermediates, covalently bound enzyme species, and the enzyme groups involved in forming these species and catalyzing these reactions. In the past two decades, structural studies have proliferated, providing a context for prior and ongoing functional and mechanistic studies, and even outstripping those studies to provide early information about the reaction environment from which mechanistic models and catalytic hypotheses can be generated.

So, where are we now? What do we understand about how enzymes work, and what is left to be understood? Remarkably, after taking a (good) course in enzymatic reaction mechanisms, a reasonably sophisticated undergraduate or beginning graduate student can, when presented with new biochemical reactions, propose plausible reaction mechanisms and identify coenzymes or cofactors that are likely to be utilized, and be correct a vast majority of the time. So, at the level of "arrow pushing" or bioorganic chemistry, our advances have been truly remarkable.[17−19,a]

But beyond understanding the reaction pathways of biochemical transformations in solution and within enzyme active sites, where are we with the parallel goal of understanding the enormous rate enhancements that enzymes achieve, and where are we with the ambitious goal of engineering new enzymes that catalyze new reactions? After a brief review of our current overall understanding, we present recent results that push the boundaries of our understanding and we discuss and evaluate attempts to engineer new enzymes. We close by presenting examples of remaining challenges.

General Historical Overview of Understanding Enzymatic Rate Enhancements. Very early ideas about how

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enzymes worked invoked complementarity between a reaction's transition state and the binding surface of the enzyme, what we now term the enzyme's active site. Remarkably, these ideas predated knowledge of the atomic and molecular nature of enzymes.[20−22] This complementarity can now be visualized from the myriad of enzyme X-ray structures, many with bound substrates, substrate analogues, or transition state analogues.

Functional work, much of it predating the structural determinations, supports the same overall picture in which active sites are structurally optimized to discriminate even subtle differences between ground states and transition states. Modest changes in a substrate remote from the site of bond formation and cleavage, even the atomic-level change of an oxygen to a sulfur, can enormously alter the ability of an enzyme to utilize that substrate (e.g., refs 23−34). Further, ligand binding can be strong when the shape and charge at a position are complementary to the active site shape and charge (i.e., positive vs negative or hydrophobic vs hydrophobic) but is weakened when these features are not matched. Indeed, the features of strong-binding ligands generally mimic those thought to be present in the transition state, and such ligands are often referred to as "transition state analogues".[35,36]

These structural, functional, and binding observations support a common picture in which active sites are complementary in shape and electrostatic properties to transition states of reactions (Figure 1). A formal description of this complementarity can be derived from transition state theory, which posits that the highest-energy species on a reaction coordinate can be thought of as if it is in equilibrium with the ground state reactants, allowing a straightforward consideration and comparison of interactions in the ground state and "in" the transition state, such that an enzyme "binds" the transition state more strongly than the ground state.[37−39,b]

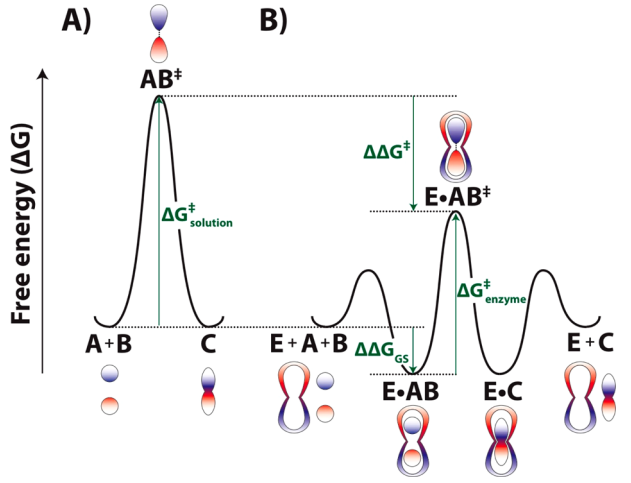

Figure 1. Hypothetical free energy profiles illustrating enzyme–transition state complementarity and selective stabilization of the transition state by an enzyme relative to the ground state. (A) Free energy profile for an uncatalyzed reaction. Changes in electrostatics and geometry as the reaction proceeds are shown with different colors and shapes. (B) Free energy profile for the same reaction but now catalyzed by an enzyme. The enzyme positions the substrates with respect to one another, and electrostatic and shape complementarity of the enzyme to the transition state, shown here schematically, renders interactions between the enzyme and the transition state more favorable than those between the enzyme and the ground state ($\Delta\Delta G^{\ddagger} > \Delta\Delta G_{GS}$). Thus, the barrier to reaction on the enzyme is smaller than in solution ($\Delta G^{\ddagger}_{enzyme} < \Delta G^{\ddagger}_{solution}$).

INSIGHTS FROM SELECTED CASE STUDIES

Extreme Mutagenesis with Ketosteroid Isomerase. Most modern enzymology and functional research has focused on specific interactions within active sites and recognition regions, in part because of the ease and power of identifying interactions via X-ray crystallographic structures and of probing them via site-directed mutagenesis. We would like to be able to use site-directed mutagenesis to fully account for enzymatic catalysis from the contribution from each residue (or functional group), adding those contributions to fully account for the observed rate enhancement, i.e., the enzymatic reaction rate relative to the rate of the corresponding nonenzymatic reaction. However, this accounting is not possible, because the effects of individual groups on catalysis are neither additive nor independent. The sum of individual effects, from mutations, or deletion or truncation of substrate moieties, far exceeds the overall catalysis;[40] the size of the effect of mutating a residue can depend on what other residues are present; and whether an effect is manifest in binding or catalysis can also depend on what other residues are present.[40,41,c]

In this section, we focus on the enzyme ketosteroid isomerase and its so-called "oxyanion hole". This and the following sections highlight the need for clearly defined questions and comparisons to make meaningful inroads in understanding contributions to catalysis, and we present below a novel strategy that enables one such comparison.

Ketosteroid Isomerase. Bacterial ketosteroid isomerase (KSI) catalyzes a straightforward chemical transformation that is similar to many biological reactions (Figure 2A), and it falls into the common reaction classes for which reasonable mechanisms can be inferred from chemical intuition.[42] A proton α to a carbonyl group is abstracted to give a carbanion equivalent that is stabilized by its ability to form an enolate intermediate; for KSI, there is still further stabilization because a dienolate intermediate can form. Reprotonation, but at the other end of the conjugated system, as shown in Figure 2A, gives the isomerized product.

Considering the expected transition states for this reaction, there is partial proton abstraction and charge accumulation on the incipient oxyanion (Figure 2A).[d] Thus, even if we did not have years of functional work on this enzyme and numerous X-ray structures with bound transition state analogues, we would posit the following features: a general base to abstract the proton, positively charged groups or hydrogen bond donors situated around the incipient oxyanion, and a binding pocket that helps orient the substrate with respect to these catalytic features (Figure 2B). Indeed, NMR and X-ray structures "confirm" these expectations, while also identifying the groups involved in these interactions and revealing their structural context (Figure 2C).[43−45]

KSI Oxyanion Hole. Many enzymes have oxyanion intermediates and oxyanion-like transition states. The oxyanion hole was first named in serine proteases,[46,47] and other enzymes that stabilize oxyanions provide hydrogen bonding or electrostatic stabilization that is analogous to the serine protease oxyanion hole.[48−57] We use KSI to evaluate the energetics of these interactions because its oxyanion hole consists of two side chain hydrogen bond donors, Tyr16 and protonated Asp103 (Figure 2C), whereas protease oxyanion holes consist of at least

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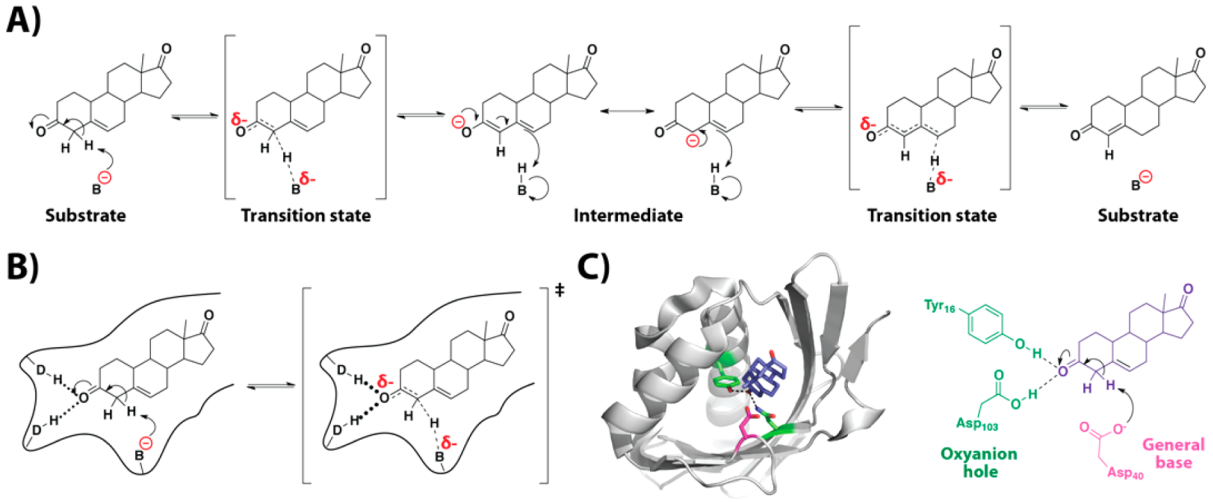

Figure 2. Chemical mechanism of the reaction catalyzed by ketosteroid isomerase (KSI). (A) Reaction scheme. (B) Expected active site features of an enzyme that catalyzes this reaction, including a general base (B), hydrogen bond donors (D-H), and a binding pocket (black outline). (C) Structure of KSI bound to a product (4-androstene-3,17-dione, purple) and a schematic representation with a general base Asp40 (magenta) and an oxyanion hole formed by the side chains of Tyr16 and Asp103 (green) (Protein Data Bank entry 3NHX).

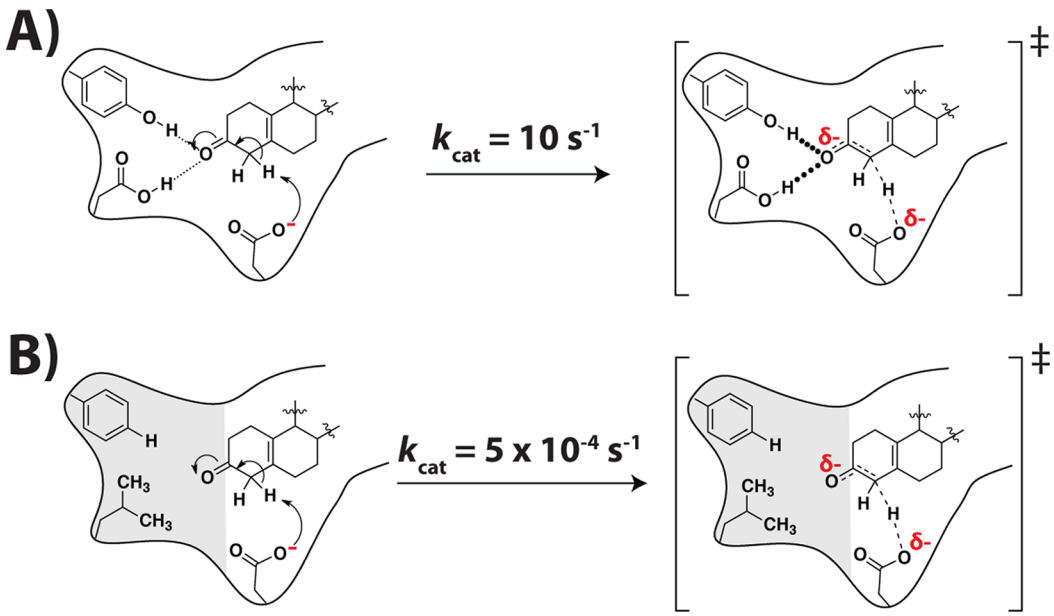

Figure 3. Schematic of the effect of conservative mutations of the oxyanion hole on KSI catalysis. (A) The wild-type KSI oxyanion hole has two hydrogen bond donors that stabilize the incipient oxyanion. (B) Conservative mutations of the oxyanion hole replace the hydrogen bond donors with hydrophobic groups. Values are from ref 76.

one and usually two backbone amides and so limit the changes that can be introduced.

In principle, the simplest way to identify the energetic contribution from these (and other) hydrogen bonds would be if there were transferable energetic properties of hydrogen bonds. In other words, if the energetic contribution of a hydrogen bond were always the same from one system to another, then we would simply have to count hydrogen bonds (of each type) and add the results. Unfortunately, we know that this is not the case. This lack of transference is most strikingly seen in the ~40 kcal/mol difference in the energy of formation of the $F^{-}\cdots HF$ hydrogen bond in the gas phase versus that in aqueous solution.[58−60] Therefore, the environment in which the hydrogen bond forms matters, and can matter a great deal. So does the degree of positioning of the interacting hydrogen bond partners, as we describe below.

The next simplest way to consider assigning energetic contributions to the oxyanion hydrogen bonds would be if there were empirical hydrogen bond energies that held in (all) active site environments. The observation that mutating away hydrogen bonding groups often gives similar energetic effects[48,50,52,53,61−65] might lead to optimism that this approach could work. Indeed, the oft-used phrase "the energy of the hydrogen bond" implicitly implies that this is a transferable and additive property. But the overall energetics of catalysis cannot be described in terms of independent and additive components, as the sum of energetic consequences of all mutations is greater than the observed catalysis in the systems. The same conclusion, and underlying concepts, hold for individual

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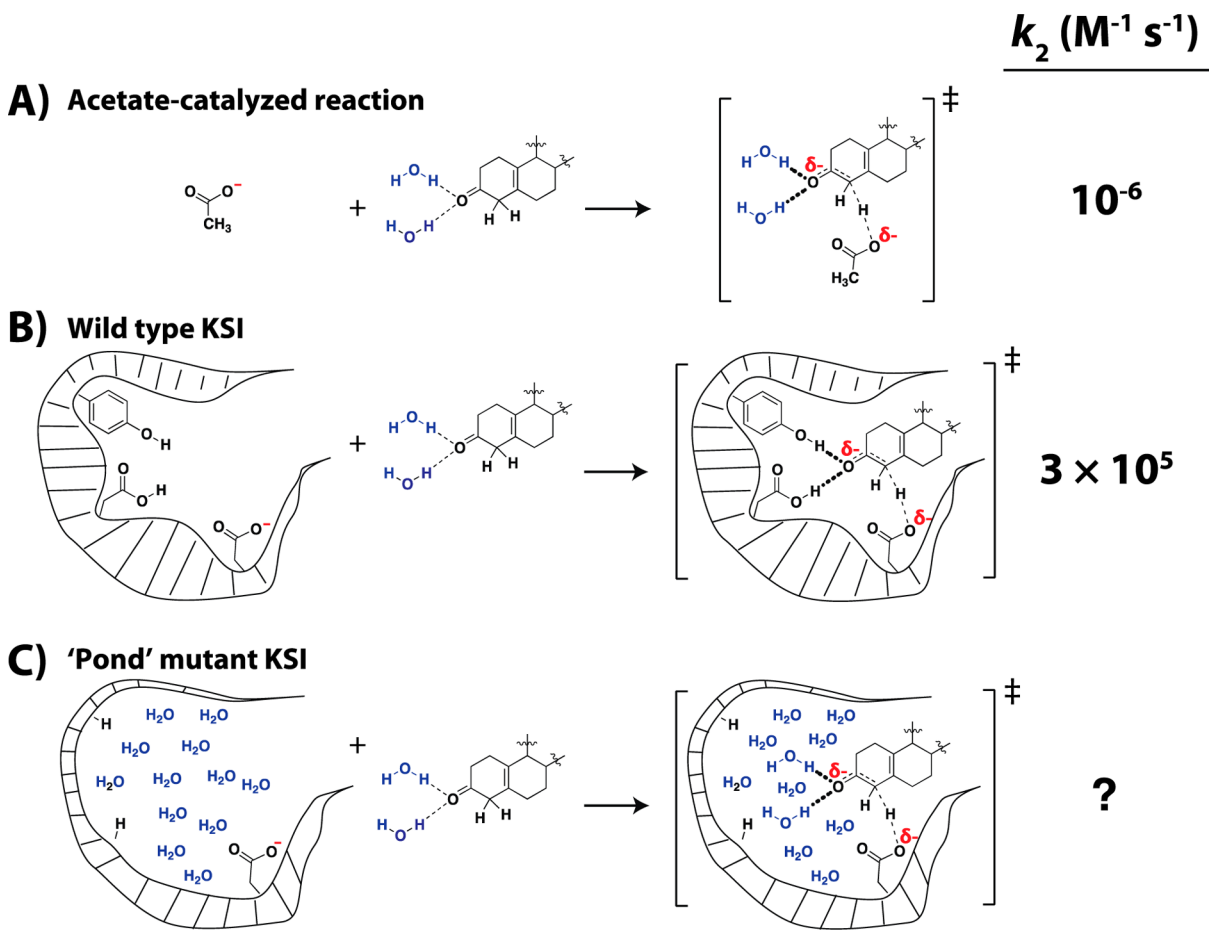

Figure 4. Evaluating the catalytic contribution from the KSI oxyanion hole. (A) Second-order reaction between acetate and substrate in solution.[75] (B) Second-order reaction between wild-type KSI, which also uses a base with the same chemical reactivity as acetate, and substrate. Relative to the acetate-catalyzed reaction, wild-type KSI provides ~10^{12}-fold catalysis.[76] (C) Second-order reaction between a "pond" version of KSI, where the oxyanion hole has been replaced by an aqueous environment.

hydrogen bonding interactions. Consider an enzyme with the residues surrounding the oxyanion hole mutated so that the oxyanion hole residues are present but the oxyanion hole is not formed. In that case, mutation of the oxyanion hole residues themselves will have no effect. In other words, so-called catalytic residues do not act alone; their energetics are synergistic with residues involved in forming the overall and local structure.[c]

The case of KSI below clearly illustrates the fundamental flaws in assuming the existence of an intrinsic or invariant free energy contribution from hydrogen bonds. This case study also illustrates the need for fully defining questions that can be answered by clear and explicit comparisons.

Consider first the simplest experiments, conservative mutagenesis of KSI's oxyanion hole hydrogen bond donors. The effects of the Tyr16Phe and Asp103Ala/Leu mutations are large, 10^4–10^5- and 10^2–10^3-fold, respectively, and mutants with both hydrogen bond donors removed gave very large deleterious effects on catalysis of 10^5–10^8-fold (Figure 3; exact values depend on the substrate used and enzyme source[66−69]). These results led to conclusions that the oxyanion hole provides enormous catalysis and that its hydrogen bonds are particularly strong.[44,70,71]

But what do these rate effects really mean? What do the million-to-billion-fold effects from these mutations tell us about catalytic mechanism and catalytic contributions? We must make explicit comparisons and, before that, ask clear questions. What question is asked when Tyr16 is mutated to Phe? In essence, we are asking about the ability to stabilize the incipient oxyanion with a neighboring hydrogen bond donor versus doing so with a neighboring hydrophobic group. We know that anions are highly destabilized when removed from a hydrogen bonding solvent (water) and placed in a nonpolar solvent, and indeed, ^{19}F NMR data provide strong evidence of hydrophobic character in this mutated site.[72] So we should *expect* a large deleterious effect from mutations such as the Tyr16Phe mutation; indeed, any time a small effect is observed for such a mutation (provided that there is a genuine oxyanion interaction) it likely indicates an ability of the site to rearrange to make alternative interactions with other protein groups or with water that enters the mutated and rearranged site. Thus, two things are clear. (i) These site-directed mutagenesis experiments do not reveal "intrinsic" contributions to catalysis, and (ii) the energetics involves many factors and is extremely complicated.[73]

So what do the results described above have to do with catalysis, other than to demonstrate the obvious, that generating an anion in a hydrophobic environment is not a good thing to do? How can we pose a question about catalysis that is both useful and answerable?

We first note that there are many such questions. There is no single question the answer to which will reveal all of an

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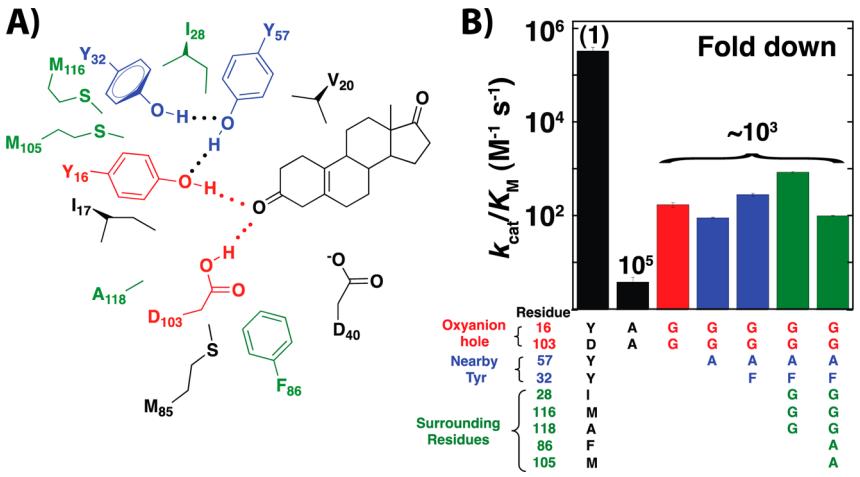

Figure 5. "Excavating" the KSI oxyanion hole to evaluate its catalytic contribution. (A) Schematic of residues in and around the oxyanion hole, with the oxyanion hole residues colored red, nearby tyrosine residues that could potentially replace the oxyanion hole donors colored blue, and other residues surrounding the oxyanion hole colored green. (B) Rate effects from excavating the oxyanion hole, with bars colored as in panel A from ref 76.

enzyme's secrets. Claiming that one question is important while other questions are not has led to unnecessary and unproductive squabbles and has obscured advances in enzymology. Not clearly defining the question or questions being asked has been still more obfuscatory.

We defined the following question about KSI's oxyanion hole. Because enzymes' substrates are (mostly) present in aqueous solution prior to binding and transformation, and these enzymes have evolved against a backdrop of aqueous reactivity, it is reasonable to ask the question of the magnitude of the rate enhancement for the enzymatic reaction relative to the rate of the corresponding reaction in aqueous solution, but even here, we have to be more precise. *What* solution reaction are we using in our comparison? One comparison would be to pure water, say at pH 7. Questions using this comparison might be especially appropriate in considering questions involving reactivity above certain background levels. But for mechanism, one would minimally want to know if the aqueous reaction were acid- or base-catalyzed, and, if so, something about the position and timing of the protonation or deprotonation events, to know how similar or dissimilar the solution process is to the process taking place in the enzyme's active site.

For KSI, on the basis of prior knowledge of the non-enzymatic and enzymatic reactions, we compare the enzymatic reaction, which uses an aspartate side chain as a general base in proton abstraction, to the solution reaction with acetate, which has essentially the same proton affinity and reactivity as the Asp side chain in water (Figure 4A,B). Thus, this comparison allows us to ask what is "special" to the enzyme; i.e., we are looking at the same reaction (or nearly so)[e] and asking how it is made more probable by the presence of the enzyme. We again emphasize that this is one of (many) possible useful questions. For example, we could have used as our reference state the analogous reaction in the gas phase. Indeed, gas phase reactivities are sometimes referred to as intrinsic reactivities.[74] An advantage of the gas phase comparison is that, because our understanding of aqueous solution and solvation is limited, we can avoid consideration of these factors. A related advantage of the gas phase comparison is that, with fewer system components, higher-level theory and more thorough computation can be applied. Disadvantages of using the gas phase comparison state include the experimental difficulty in measuring gas phase reactions, their tendency to follow distinct mechanisms, and the very large electrostatic energetic interactions that can dominate in the gas phase but are greatly dampened in solution and, very likely, in the enzyme environment. On a practical level, large gas phase energetic terms (and uncertainties in them) can make comparisons difficult.

We have chosen, for now, to compare the KSI reaction to the corresponding solution reaction catalyzed by acetate (Figure 4A,B). The ratio of k_{cat}/K_M for the enzymatic reaction (for a substrate with a chemical rate-limiting step) to the second-order reaction with acetate ion, k_{OAc}, is 10^{12}; i.e., by this comparison, KSI provides 10^{12}-fold catalysis.[75] We can now ask a defined and specific question: What is the contribution of the oxyanion hole to KSI catalysis relative to the analogous interactions in aqueous solution? In experimental terms, what is the catalysis of KSI relative to a version of this enzyme in which the oxyanion hole is replaced by an aqueous environment without compromising other catalytic features (Figure 4B,C)?

The above set of questions meets the criteria of being specific and of defining a *comparison*, rather than looking for an unattainable absolute energetic description. But that does not mean the experiment can be conducted and the question answered. Indeed, mutations extensive enough to create a local aqueous environment will likely cause an enzyme to fall apart or greatly rearrange in most cases. For KSI, we appear to have been lucky, and there is even some evidence to suggest that the results obtained are reasonable and may be generalizable.

The comparison described above, reaction of wild-type KSI versus the Tyr16Phe mutant (e.g., Figure 3), gave large deleterious effects. In most systems, more extensive mutagenesis leads to greater rate effects, and this is typically attributed to secondary rearrangements that more broadly disrupt binding and/or catalysis. For KSI though, mutation of Tyr16 to Ala or several other small amino acids had a considerably smaller effect than the Phe mutation, and ^{19}F NMR suggested a more, though not completely, aqueous environment.[72]

We decided to conduct extensive mutagenesis to test whether a new group fortuitously provided partial rescue for

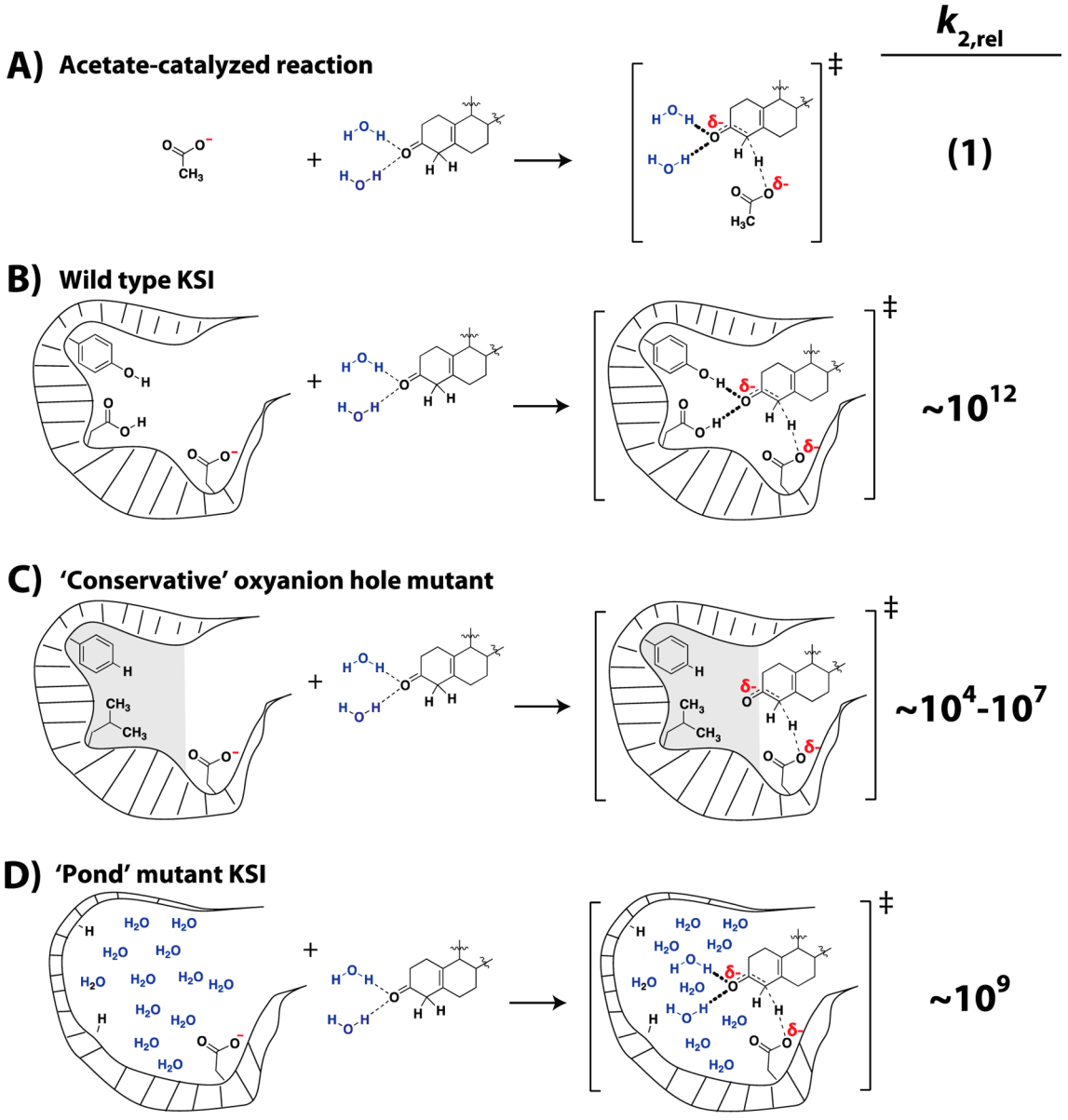

Figure 6. Rate enhancement relative to the rate of the acetate-catalyzed reaction provided by different KSI variants. (A) Second-order reaction between acetate and substrate in solution. (B) Second-order reaction between wild-type KSI and substrate. (C) Second-order reaction between KSI with conservative mutations of the oxyanion hole that replace the hydrogen bond donors with a hydrophobic environment. The rate enhancement provided depends on the particular mutant and substrate used.[67,69,76] (D) Second-order reaction between substrate and a version of KSI in which the oxyanion hole has been replaced with an aqueous environment.[76]

the missing Tyr (and Asp) and to try to create a "pond" in which the oxyanion hole used to be—in other words, to remove sufficient bulk to allow access of the oxyanion to a large number of solvent molecules. Mutations were introduced to remove both hydrogen bond donors, to remove nearby Tyr residues to show that they could not substitute for Tyr16 or Asp103, and to remove surrounding residues to allow access by solvent molecules (Figure 5). The ability of KSI to maintain its overall catalytic structure was supported by the X-ray structure of a mutant with both oxyanion hole hydrogen bond donors removed, by the negligible effects from truncation of more and more oxyanion hole side chains (to a total of nine side chains), and by the overall modest rate effects from this ablation (Figure 5).[76]

Pond mutants with space for ~20 water molecules resulted in a rate decrease of only ~10^3-fold, far smaller than the deleterious effects from so-call "conservative" mutations in either or both of the oxyanion hole hydrogen bond donors (Figure 5B).[76] This means that KSI remains enormously efficient without its oxyanion hole, providing ~10^9-fold catalysis, relative to the nonenzymatic reaction with acetate ion (Figure 6). Had we assumed that all that was necessary to assess the catalytic contribution of the oxyanion hole was to ablate Tyr16 and Asp103 via the most conservative mutations, we would have concluded that the enzyme missing these residues provided only 10^4–10^7-fold catalysis (Figure 6), and that the oxyanion hole plays a far larger role in catalysis than it actually does.

While it is possible that some of the contributions from binding and positioning and from general base catalysis are compromised because of the absence of the oxyanion hole or disruption of the internal electrostatics or dynamics of the

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enzyme, these would render the 10^3-fold effect an *over*-estimate. It is alternatively possible that removal of the oxyanion hole allows other interactions to be independently optimized and provide an increased contribution, a model that seems less probable but remains to be tested.

Thus, the KSI variant missing its oxyanion hole is a surprisingly effective catalyst, exhibiting a rate enhancement of 10^9-fold relative to the rate of the reaction with acetate in solution. We have shown that some of this rate enhancement arises from binding interactions that localize and position the substrate within the active site,[75] and we have begun to dissect the KSI features that render it so effective in general base catalysis.[77]

With subtilisin and related proteases, mutations of one of the two oxyanion hole hydrogen bond donors, the one coming from a side chain group rather than a backbone amide, to a wide range of amino acids give similar $\sim 10^3$-fold effects.[48,61,62,78] Given that this effect is not increased upon introduction of an anionic residue in the oxyanion hole[78] and that serine proteases bind substrates with their carbonyl facing away from the active site and into solution,[79,80] we suggest that the 10^3-fold effect for subtilisin may represent a similar complete loss of oxyanion hole interactions and replacement by an aqueous environment, although clearly additional tests and examples are required.

Assuming that 10^3 represents a rough measure of the contribution of an oxyanion hole to KSI catalysis, how far does that take us toward understanding this contribution? Minimally, it defines an overall constraint; e.g., any model that invokes the oxyanion hole as the dominant catalytic factor or as being necessary for effective general base catalysis is not correct. However, these studies identify the size of an effect, not its *origins*. For example, the observed 10^3-fold effect represents the net of multiple differences that may include the number of hydrogen bond donors (e.g., there may be three on average in solution but only two in the oxyanion hole); the partial positive charge on the hydrogen bond donor (hydrogen bond equilibria are dependent on partial charges, often empirically described by pK_a and ΔpK_a values[81]); the degree to which these groups are restricted when their hydrogen bonds are formed in the transition state, relative to their freedom of motion in the respective ground states (i.e., prepositioning); effects of the extended environment (e.g., dipoles from the second shell and beyond); and whether the factors listed above affect the structural and energetic nature of the hydrogen bonds that are formed (e.g., refs 82 and 83). We have begun to address these questions,[84−86] but much more work is needed.

EVALUATION OF DESIGNED ENZYMES

An ability to predict the behavior or properties of a system is valuable and often informative but is not synonymous with understanding. Similarly, an ability to conduct design *can* provide an excellent test of one's understanding but does not always, and the corollaries that "if you can design something you understand it" and "if you cannot design it, you do not understand it" are not correct. For example, a design can work that is not based on physical laws but rather on correlations derived from empirical observations or empirical computational algorithms (e.g., machine learning algorithms). Conversely, design can fail in the face of substantial understanding if a problem's complexity exceeds computational capabilities. We consider two approaches to engineer or design new enzymes that have received the most attention and evaluate the lessons learned from these exercises.

CATALYTIC ANTIBODIES

Early efforts to "design" enzymes involved the use of transition state analogues and the selection of antibodies that bound that analogue, and the screening of the selected antibodies for catalytic activity.[87,88] Rate enhancements of up to 10^9-fold were observed, although more typical values ranged from 10^3- to 10^5-fold, and there were presumably many antibodies with lower levels of activity that could not be distinguished from background.[89−91] Given the fact that naturally occurring enzymes typically provide much greater levels of catalysis, from $\sim 10^{10}$- to $\sim 10^{29}$-fold,[92,93] design efforts via catalytic antibodies have generally been viewed as limited in their success. What are the reasons for the limited catalytic capacity of these antibodies? Several models can be considered (see also ref 91).

(i) The limited sequence space explored in antibody maturation[94] is likely smaller than that typically explored in the optimization of new enzymes, and this exploration mainly via point mutations may not be as effective in developing new functions as the insertions and deletions that appear to be common in the evolutionary journeys between related enzymes.[95]

(ii) The concentration of the antigen present as an antibody matures limits the affinity that can be selected for. For example, if the concentration is in the nanomolar range, then there is little selective pressure for tighter than nanomolar binding. In addition, concentrations that are too low would result in antibody−antigen binding that is too slow (e.g., a concentration of 1 pM would give association half-times of $\geq$1 h, assuming typical association rate constants of 10^6−10^8 M^{-1} s^{-1}). To put this affinity in context, formal application of transition state theory for enzymes with rate enhancements of 10^{15}- and 10^{25}-fold leads to apparent transition state affinities of $\sim 10^{-17}$ and 10^{-27} M, respectively.[f]

(iii) The antibody scaffold may be ill suited to catalysis. Certain structures have been used in biology to catalyze many different reactions: the eight-fold $\beta\alpha$ or TIM (triose phosphate isomerase) barrel has been discussed most and is found in 21 superfamilies with many distinct catalytic functions.[96] The antibody scaffold may be less amenable to the variations needed for a wide range of catalysts, or as noted above, there may be insufficient exploration of loop and domain additions during antibody maturation to create extensive binding and catalytic interactions needed for efficient catalysis.

(iv) Transition state analogues are imperfect mimics of actual transition states, so that optimal catalytic groups are not selected for. The two following models represent variations on this model.

(v) Because there is no counterselection against strong ground state binding, there may be overly strong binding of the (structurally related) ground states, such that saturation is too easily achieved and product release readily becomes rate-limiting. However, this model could account only for low values of k_{cat}, not low values of k_{cat}/K_M.[39]

(vi) Catalytic antibodies may lack dynamics required for optimal catalysis. Such a limitation, if important, could arise from the static nature of the transition state analogue used in selection and/or the nature of the antibody motif.

None of the above models is particularly profound, yet they are rarely discussed together as a complete set. Of the models

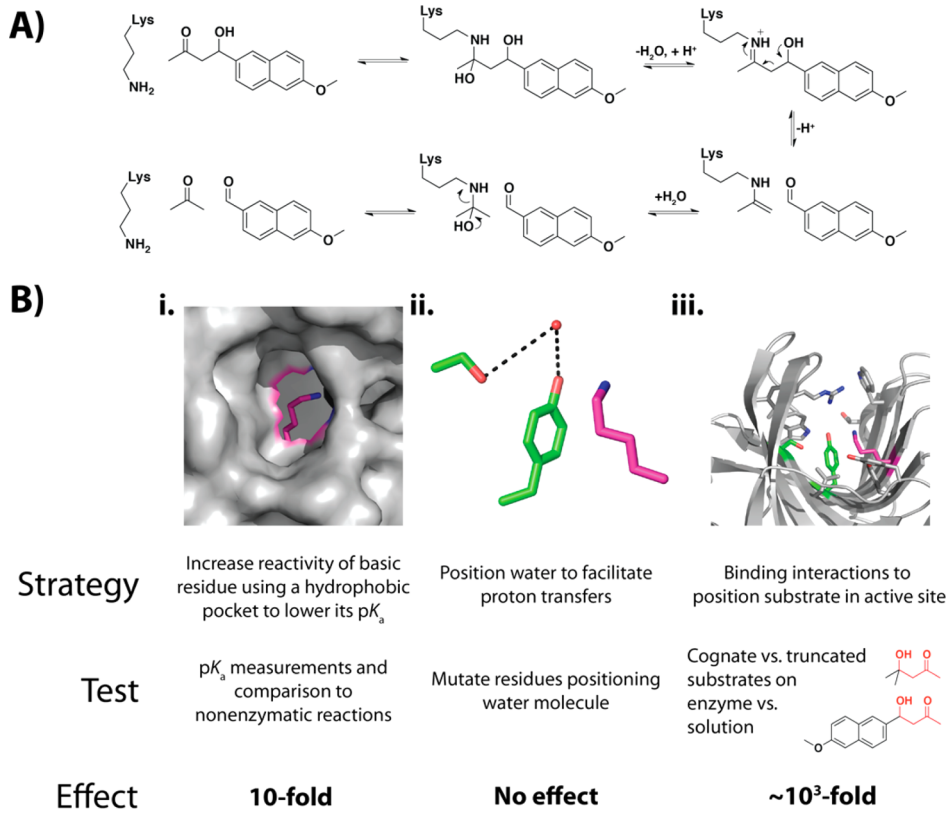

Figure 7. Evaluating a computationally designed retroaldolase. (A) Reaction schematic with some proton transfers omitted for the sake of brevity. (B) Strategies that were a part of the computational design and results from evaluating each of their catalytic contributions. The active site lysine is colored magenta; residues positioning water are colored green, and residues in the binding pocket are colored gray. Results are from ref 120.

listed above, the suggestion of a need for dynamics is the most provocative, and indeed, it has sometimes been argued that the limited capabilities of catalytic antibodies provide evidence of the importance of dynamics in enzymatic catalysis. However, this perspective ignores the other models and potential explanations, which are expected to hold to at least some degree.

Most generally, there is a need in enzymology and all areas of the biological and chemical sciences to go beyond "favorite models". There is a tendency in science to emphasize (and perhaps overstate) new or exotic mechanisms, as these seem most exciting and are likely to draw the most attention, and because we as investigators get most excited about the possibility of a novel explanation (e.g., ref 97).[g]

To curb this temptation and to remain true to the scientific method, we urge a two-step process: drawing out all possible models, as we have attempted to do above for the catalytic antibody case, and testing those models. Further, those tests need to be of a type that can readily falsify a model;[98,99] too often, so-called "tests" of models are in reality a quest for observations that are consistent with a particular favored model. As tests of the models described above with catalytic antibodies have been limited, we turn to recently designed enzymes and mechanistic tests of the design features that were used.

EVALUATION OF A DESIGNED ENZYME

There has been great excitement about the recent design of enzymes catalyzing retroaldol condensation, Kemp elimination, and other reactions.[100−105] This work has been published in the highest-profile journals, accompanied by perspectives,[106,107] and has been the subject of several news and review features (e.g., refs 108−112).

It is remarkable that computational algorithms can be used to determine arrangements of amino acids that often lead to stable, folded proteins,[113−115] and it is more remarkable that some of these folded proteins can be engineered to be catalysts. However, it is also true that some proteins like bovine serum albumin (BSA), which did not evolve to be a catalyst, can catalyze certain reactions[116] and that enzymes can often catalyze reactions in addition to the reactions they were selected to carry out.[117−119,h] Indeed, catalysis by these nondesigned and "accidental" enzymes is often similar to or faster than those of designed enzymes.[111,116]

Given this backdrop, how can we gain perspective on these new findings in the face of the associated excitement? We suggest that rational evaluation of designed enzymes is needed, first by determining the overall rate enhancement of the designed enzyme and then by evaluating the individual designed catalytic features. We conducted such a study for one of the first designed retroaldolase enzymes, termed RA61, as outlined below.[120]

Figure 7A shows the retroaldolase reaction catalyzed by RA61. The substrate is not known in biology, and its leaving group is fluorescent, which simplifies activity assays. RA61 utilizes a primary amine of an active site Lys residue to form a Schiff base and act as an electron sink, as shown in the figure. Figure 7B shows the three design features of RA61: (i) a catalytic Lys residue buried in a hydrophobic pocket to lower its pK_a and thereby increase the amount of reactive neutral amine present at pH 7, (ii) protein side chains to position a

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water molecule and thereby facilitate proton transfers, and (iii) a hydrophobic pocket for binding and positioning the substrate.

We first determined the overall catalysis by this enzyme by comparing the second-order reaction with RA61 (k_{cat}/K_M) with the second-order nonenzymatic reaction with a simple analogous primary amine. The rate enhancement of 10^5-fold is very low relative to those for naturally occurring enzymes, which typically provide rate enhancements of $>10^{10}$-fold and can provide rate enhancements of up to 10^{29}-fold.[92,93] Low catalytic efficiency is a general observation for enzymes designed to date.[111]

We next evaluated each of the catalytic strategies for their contribution to this overall rate enhancement (Figure 7B).[120] The pK_a of the reactive Lys was indeed lowered, by ~3 units to a pK_a of ~7, as envisioned in the design. This lowering gives a higher fraction of the reactive neutral amine at pH 7 but is also expected to reduce its reactivity by rendering it a weaker base. Measuring the nonenzymatic reactivity of a series of primary amines of varying pK_a values allowed us to determine this reduction in reactivity, which partially counterbalances the increase in the fraction of neutral amine, and allowed us to estimate an overall catalytic effect of (only) 10-fold from this first catalytic strategy (Figure 7B, i). Mutation of the residues designed to facilitate proton transfers via a positioned water molecule gave no rate decrease, strongly suggesting that this catalytic design was unsuccessful (Figure 7B, ii).[i] Finally, we utilized a truncated substrate, missing most of the hydrophobic binding surface but maintaining the same reactive center and essentially the same nonenzymatic reactivity, to assess the contribution of binding interactions to catalysis (Figure 7B, iii). The rate enhancement for the truncated substrate was ~10^3-fold less than for the full substrate, and the full binding effect is likely larger because the truncated substrate retained two methyl groups that could interact in the binding pocket. In summary, of the overall 10^5-fold effect, 10-fold arises from lowering the Lys pK_a and all or nearly all of the remaining catalysis arises from binding interactions that presumably localize substrates near this Lys residue.[j]

As noted above, the overall level of catalysis by RA61, and other designed enzymes, is much lower than that typical of evolved enzymes, and the following observations reveal additional properties typical of natural enzymes that are not reproduced by the design.[120] (i) The designed enzyme operates on both substrate enantiomers with equal or near equal efficiency; in other words, stereospecificity, a hallmark of naturally occurring enzymes, is lacking. (ii) The aldehyde reaction product "slides" over in the active site to form a Schiff base with the active site Lys and strongly inhibit the reaction. Evolved enzymes rarely exhibit such promiscuous binding and typically are not subject to strong product inhibition.

The designed enzymes, like nonenzymatic, biomimetic catalysts that preceded them,[121] use positioning, or colocalization, of reactive groups to facilitate reaction, in this case the Lys side chain and the substrate carbonyl group, but the results described above suggest that this positioning is not sophisticated or optimized. Indeed, the designed enzymes can be improved by screening of mutant libraries for more active variants, and these results suggest limitations in packing and binding arrangements in the original design, consistent with the low specificity.[111,122,123] In addition, the designs did not utilize hydrogen bonding for positioning, because of practical computational limitations,[102] whereas natural enzymes typically use extensive networks of hydrogen bonding and hydrophobic packing to position groups; hydrogen bonds generally provide greater precision in positioning than hydrophobic interactions.[124]

Larger rate enhancements will be possible with designs utilizing more hydrophobic surface area for binding and incorporating highly reactive abiological active site cofactors, but such successes will not illuminate the mechanisms underlying the highly efficient and specific catalysis of naturally occurring enzymes. Are the low observed rates in designed enzymes caused by suboptimal use of catalytic strategies or the fact that not enough strategies or interactions were used? For the retroaldolase, it seems likely that both play a role, nonoptimized positioning of the reactive Lys residue and substrate and an absence of facilitating interactions with the reactant's oxygen atoms. Additional complications are introduced by attempting to design a catalyst for a multistep reaction such as retroaldol condensation, although rate enhancements for one-step reactions have been similar.[111]

While we cannot rule out new or exotic mechanisms that are fundamental to enzymatic catalysis but currently beyond our ken, it seems likely that the predominant reasons underlying the engineering shortcomings are limitations in the selection process for catalytic antibodies and limitations in computational power and accuracy for designed enzymes. So it seems that design, while captivating and potentially transformative, for now represents an imperfect use of recognized catalytic strategies.[k]

■ OUTLOOK AND FUTURE QUESTIONS

Overall, despite our limited practical capabilities in enzyme engineering and the lack of a complete and quantitative description of enzymatic catalysis, we have a good sense of the mechanisms employed by enzymes and fundamental principles that underlie their action, built up from many contributions and multiple perspectives over the past half-century, but there are also real limits to our understanding.

Enzymes are complex systems with nonadditive energetics, so by definition simple, fully additive descriptions do not exist. Consider, for example, our dissection of KSI's oxyanion hole described above. We removed the oxyanion hole and replaced it with a "pond", thereby providing a meaningful comparison to the corresponding reaction in aqueous solution. But the Tyr and Asp residues when present in the oxyanion hole depend on neighboring residues and the overall enzyme structure and architecture to provide their stabilizing hydrogen bonds, and the energetics of these hydrogen bonds and the comparison to the hydrogen bonds present for the solution reaction are very complex. A current challenge might be to attempt to engineer or reengineer just a portion of an enzyme such as an oxyanion hole and follow that with in-depth structural, dynamic, and energetic analyses.

A corollary of the nonadditivity noted above, and a complementary way to appreciate the inherent challenges in understanding the energetics of enzyme catalysis, is to recognize that there is no single description or comparison that will yield a full understanding. We introduced this important point in the section above on KSI, prior to comparing the enzymatic reaction to the reaction in aqueous solution following the same overall reaction mechanism, noting that other comparisons are possible and will be instructive.

Specific unresolved (and sometimes ill-defined) issues tend to be the focus of discussion. This is natural for the cutting-edge literature but can also obscure our foundational

understanding and substantial foundational agreement.[1] We have emphasized above the need to clearly define unresolved questions and to clearly lay out comparisons to allow enzymologists to communicate effectively with one another and with those outside the field, and to allow enzymologists to identify important areas that are in need of further investigation.

Below we highlight some current questions that help elucidate outstanding challenges to push our understanding of enzyme catalysis to the next level. These questions will need to be further honed in the context of specific systems and in no way represent the full set of challenges and opportunities for enzymologists, but we hope that they stimulate discussion and new experiments to address these and other fascinating questions that lie ahead in enzymology.

CURRENT QUESTIONS IN MECHANISTIC ENZYMOLOGY

How Good Are Enzymes at Positioning Substrates and Catalytic Groups? The statistical mechanical concept of conformational positioning through reduction in translational and rotational entropy is simple, as initially outlined for enzymologists by Page and Jencks,[125] yet assessing these effects in complex solution and enzyme environments remains an unmet challenge.[40,126−128]

NMR relaxation methods can provide information about dynamics over a wide range of time scales to assess the degree and types of motion that remain in enzyme−substrate and enzyme−transition state analogue complexes, and there are exciting pioneering efforts to use NMR data to learn about conformational ensembles and even conformational entropy.[129−131] In principle, computational approaches can be used to dissect these effects, but computation of conformational ensembles is a highly challenging problem; coupling of such computations to the quantum mechanical approaches required to obtain information about transition states is still more challenging.

Computation has the *potential* to "see" events on the atomic scale, a scale not possible with current experimental approaches, which explains the popularity of such approaches in enzymology. Nevertheless, there is growing recognition that computational results must be directly tied to nontrivial experimental predictions to move this and other fields forward (e.g., refs 132−134). In other words, computational results are models (based on approximate force fields and quantum mechanical treatments), and empirical (experimental) observation is our direct window to reality that we require in testing *all* models, computational and experimental. An enormous future challenge is to bring together conceptual, experimental, and computational approaches to frame and quantitatively address the question of substrate and catalytic group positioning.

What Are the Energetic Consequences of Conducting Reactions in the Special Environment of the Enzyme Interior? It is clear that the energetics of association, such as complex formation involving hydrogen bonds mentioned above, is highly sensitive to the surrounding environment. It is also clear that an enzyme interior is different from an aqueous environment, the gas phase, or any continuous solvent, but it is much harder to understand that unique environment, in part because it is in direct physical contact with the reactants, unlike a homogeneous dielectric material that responds to electric fields through space and can be described by a dielectric constant, and in part because it is not homogeneous. Instead it is an idiosyncratic, heterogeneous medium that responds to charge and charge rearrangement differently at different points within it and it cannot be characterized by any single descriptor.

There have been many interesting proposals about the potential importance of the enzyme environment, beyond its role in positioning groups for reaction. Models include creating an environment in which hydrogen bonds are stronger, positioning dipoles throughout the enzyme to "focus" electrostatic stabilization of the transition state, and excluding water to eliminate its ability to dampen differential electrostatic effects between ground and transition states (e.g., refs 71 and 135−138).

Advances in vibrational and Stark spectroscopy with site-specific probes are providing new windows into local electrostatic fields within enzyme interiors and how these fields respond to ligand binding and charge rearrangements.[139−144] Coupling such measurements to functional studies that correlate changes in electrostatic fields and effects on catalysis, binding of transition state analogues, and proton transfer equilibria provide novel probes of the energetic effects of the enzyme environment.[84,143,145] Our probes of the energetics of KSI catalysis, including the work described above uncovering a smaller-than-expected contribution of its oxyanion hole, suggest that sculpting of the electrostatic environment of proteins beyond the interacting groups may contribute less than is often proposed.[76,84]

Another challenge arises because electrostatic and dynamic properties of the enzyme interior that contribute to and affect transition state interaction energies are also connected to the ability of enzymes to position groups for reaction. This connection underscores the interrelatedness of catalytic features and mechanisms and the ongoing challenge of understanding these fundamental catalytic contributors.

Are Transition States Changed on Enzymes? There are many examples of reaction pathways on enzymes that are distinct from those in solution because of the recruitment of cofactors and the placement of reactive groups in the enzyme active site. Here we consider reactions that, ostensibly, follow the same reaction mechanism in active sites and in solution and ask the question of whether the transition states are similar or different in those different environments.

For group transfer reactions, such as glycosyl and phosphoryl transfer, the preponderance of evidence suggests that transition states on and off enzymes are generally similar in character, with oxocarbenium-like transition states for glycosyl transfer and loose transition states for phosphoryl from monosubstituted species.[36,146,147] Nevertheless, extensive isotope effect data on glycosyl transfer reactions from Schramm and others have revealed differences in isotope effects for reactions of the same or similar substrates conducted in the active sites of different enzymes.[36,148−150] Thus, there are differences in these transition states off and on enzymes, though the scale of these changes may be modest.

There have been more extensive discussions of changes in reaction pathways for reactions that involve proton, hydrogen, and hydride transfer, based on extensive isotope effect studies and substantial modeling work.[151−156] Indeed, primary and secondary isotope effects for KSI, our enzyme highlighted above, are different in the corresponding solution (acetate-catalyzed) and enzyme-catalyzed reactions and consistent with enhanced coupled motion of the substrate along the reaction coordinate in the enzymatic reaction.[156] But it is difficult to ascertain whether the changes in reaction properties in this and

other enzymatic reactions are *causative* for catalysis. Thus, the current challenge is to understand why these changes occur: both their physical origins and their significance for rate enhancement. In other words, are these changes *selected for* because they cause the reaction to be more efficient, or are they simply a *consequence* of placing reactants in an enzymatic environment or in different enzymatic environments?

Considering a more specific current model, it has been suggested that reactions of enzyme-bound species occur preferentially from transient conformers that are higher in energy than the most prominent conformational states but have the reactants more closely positioned.[157,158] An appealing aspect of this model is that tunneling probability (and thus reaction rates) is expected to be increased by transiently bringing reactants closer together and by matching their energy levels with those of the products, as tunneling probability is highly dependent on barrier width and matched energy states.[158] Of course, positioning is also an important catalytic factor in group transfer reactions, and in those reactions, substrates must approach more closely than the sum of the van der Waals radii to allow a new bond to form; therefore, predominant reaction from states other than the preferred ground state may hold in those cases as well. Questions that arise from these models include the following: Does the general tendency to restrict conformational freedom in active sites cause both rate enhancements and altered vibrational properties (and thus observed isotope effects), or are special vibrational modes that increase the probability of forming transient matched reactant−product energy states selected for?[m]

Regardless of the extent of direct catalytic relevance, isotope effects, because they are highly sensitive reaction probes, will likely continue to provide a window into the detailed properties of enzymatic reactions and a strong experimental constraint on computational models.

Recently, enzymes have been made heavy via isotopic substitution, and potential promoting vibrations within enzymes have been explored.[159−162] These exciting and provocative results will be subject to further experimental tests over time, and questions analogous to those listed above will be posed. As all reactions require vibrations to exchange energy and react, are promoting vibrations in enzymes specially *selected for* or are the observed vibrational properties a *consequence* of the system being a molecular system (and especially one that is tightly packed)? And if selected for, what is the rate advantage contributed by this mechanism, distinct from other roles of residues and networks engaged in these vibrations?

Are There Special Dynamic Properties of Enzymes That Contribute to Catalysis? Our understanding of a system can be only as good as the clarity of our fundamental definitions and descriptions of models, as we have emphasized herein, and there are so many facets of dynamics in an enzymatic reaction that it is often difficult to know what aspects are under discussion and what precise comparison is being made.[n]

Consider the question "Are dynamics important for enzyme function?" If the enzyme is myosin, the answer is "of course", because motion is the function of the enzyme. Or if one is asking questions about the most basic process of bond making and bond breaking, then dynamics are "of course" important because bond making and breaking is dynamic by definition. Although the answers to the questions above are both "yes", these answers are not illuminating because our questions are not clear and precise.

Consider then a different question: Does a loop closure event help catalysis? Now we are focused on a particular dynamic event, but we need to hone the question still further and define comparison states. What if we compare the enzyme to one that is locked (hypothetically or via mutagenesis) with its loop in the open state: Does the loop closure help? In this comparison, the loop closure helps because, say, it brings a general base to the substrate that otherwise would not be able to participate in the reaction. We have an answer, and although this answer alone does not provide much enlightenment, it spurs us on to ask additional questions.

For example, let us now make our comparison state the enzyme "locked in" (or favoring) the closed state.[o] We can ask a very simple question: Is the rate of the chemical step the same for the wild-type and locked enzyme? We have a strong expectation that it will be, which helps refine the model of what loop dynamics are doing for the enzyme.

Following this expectation, let us now assume that the overall loop opening and closing event per se does not contribute to reaction probability within the closed ensemble of states, a quite reasonable supposition. We can now move to the next set of questions about why the loop and loop motion exist, and we focus on this question both because loop and domain closures are common in enzymes and because there has not always been clarity surrounding analysis of these phenomena. In the interest of brevity, we list possible models.

(i) Loop closure excludes solvent and thus may alter the electrostatic environment or exclude water that could engage in side hydrolysis reactions.[163,164] These mechanisms are often assumed in the literature and in textbooks, without consideration of other possible models.

(ii) There is no benefit from a flexible loop, relative to the same interactions arising from a fixed conformational element, but evolution took a pathway that introduced these interactions via insertion of a sequence encoding a loop. It is possible that loops are frequent in enzymes because they arise via an evolutionarily probable path.

(iii) Loop and domain closure events often create a bound state from which substrates and products cannot escape and, conversely, states that would be inaccessible for substrates to bind to. So, if the favored catalytic state is "closed", then an "open" state is needed for substrate and product ingress and egress. This model requires coupling with (at least) one of the other models to account for the existence of the closed state. A related model is one in which loop closure slows release of reactive intermediates that could have deleterious side reactions.[165]

(iv) The simplest argument for the advantage of a closed state is that closed states allow an enzyme to maximize interactions with its substrates, forming interactions with all regions and surfaces of the substrates.[35,166] Maximizing interactions with substrate functional groups in turn can provide specificity for the correct substrates and can provide the strongest catalysis via optimal positioning of substrates and active site groups.

(v) Superficially, it would seem that strengthening interactions with substrates would continually increase reaction specificity; however, when binding is too strong, the favored substrate becomes "sticky", such that every time it binds it goes on to react, which means that further increases in the rate of conversion of bound species increase the reactivity for

noncognate substrates alone, thereby lowering rather than raising specificity.[167,168] Thus, having a conformational step that weakens binding can *increase* specificity. It can also increase K_M values and thereby prevent premature saturation.[167,169]

The models described above are fascinating and, through the process of laying them out, provide clarity and direction for current and future research.

In this Current Topic, we have focused mainly on the actual chemical reactions, i.e., the transformations once substrates are bound. Reactions are, by definition, dynamic processes. As introduced above, all reactions, enzymatic and nonenzymatic, require vibrations and energy exchange, so observation of these features alone does not indicate that these are catalytic properties that have been selected through evolution to enhance catalysis. Nevertheless, many enzymes have moving parts that are integral to performing work or assembling the active form of the enzyme−substrate complex, and all proteins are (much) more than the single static structure that we so clearly see in X-ray crystallography. However, regardless of whether these dynamic properties are selected aspects of the enzyme's catalysis, we want to understand the dynamics to improve our understanding of the basic properties of enzymes and active sites and to derive better and more complete energetic models for enzymatic catalysis in terms of microstates of the enzyme−substrate complex, the probability of reaction from each microstate, and the dynamic properties of the reactions from each of those microstates. While an ability to provide such comprehensive and detailed descriptions remains a ways off, this vision helps us focus on a deep understanding of and an in-depth description of enzymatic reactions.

■ CLOSING REMARKS

In closing, we are awed by the insights and advances in enzymology that have been published over the past several decades and even centuries, arising from careful experimental observations, clever analyses, and deep consideration of conceptual issues. We believe that there is much more excitement ahead for enzymology. This excitement builds on a strong foundation and a newly emerging ability to apply multiple experimental and computational approaches focused on "well-described" systems with well-honed questions, and this will lead us to a deeper and more comprehensive understanding of enzymes and how they act. There are many other exciting areas where enzymology will leave its mark that have not been emphasized herein. One important area to note as we traverse the postgenomic era and move from identifying genes to the processes and interactions in which they are involved is the fact that the tools and approaches of enzymology have an enormous amount to offer toward understanding the processes that underlie all of biology, how and why they occur as they do and how they might be manipulated in engineering and medicine.

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■ ABBREVIATIONS

EM, effective molarity; GS, ground state; KSI, ketosteroid isomerase; TS, transition state.

■ ADDITIONAL NOTES

[a]While this statement holds in general, there remain numerous frontiers in enzymatic reaction mechanism in which the reaction intermediates and the basic reaction pathways have yet to be elucidated. Many of these remaining reactions involve radical and redox chemistries, for which nonenzymatic models have been more difficult to develop. There is much vibrant and exciting current research in this area.

[b]For example, if an interaction with an unreactive region of a substrate remains the same throughout the course of a reaction (E·S → E·P), then (in the simplest model) removal of that interaction would not affect the probability of reaction (i.e., the reaction rate). That interaction, though, is made upon binding (E + S → E·S), so that it can increase the probability of reaction from free E and S. One can then describe such an interaction as providing "uniform binding" in the ground and transition states. Consideration of these interactions in the transition state complex as if they were equilibrium interactions is useful conceptually and, further, allows predictions about the catalytic consequences of changes in such interactions to be made (DOI: 10.1021/bi301491r).

[c]The fundamental interdependence of contributions to catalysis, i.e., the nonadditivity, can be seen most generally from a complete mutagenic cycle for a hypothetical enzyme, all the way to converting each residue to Ala.[40] Mutations of the so-called catalytic residues reduce the extent of catalysis, as expected, but if all of the binding residues are gone (mutated) or all of the "structural" residues are gone, then introducing these same "catalytic residues" does not provide catalysis. Similarly, if the binding and catalytic residues are present but the structural residues have been removed, there is no significant catalysis. Now if we add back the structural residues sequentially, we will cross some threshold at which catalysis becomes significant, and catalysis will continue to increase as additional structural residues are added back, until a point is reached at which the structure is sufficiently robust and there is little or no additional catalytic advantage from reintroduction of the final residues. But again, had those residues been added earlier, then they would have been counted among residues that provide catalysis. Thus, structural, binding, and catalytic residues all contribute to catalysis. However, at least some of their contributions are masked in the types of experiments that are typically performed by us and accessible to us.[40,41,170] The energetic description of the catalytic contributions of residues changes in accord with the suite of other residues that are present. Thus, there is no unique or complete description of enzymatic catalysis that can be made in terms of its chemical

components; the system does not consist of additive and independent components.

[d]As the dienolate is a transiently stable species, we expect two transition states, for formation from substrate and for formation of product (or, equivalently, for partitioning of the intermediate to substrate or product). These transition states are expected to have very similar properties, and for the sake of simplicity, we consider only one transition state. There are also geometrical changes that we also do not address here for the sake of simplicity. Some discussions of geometric complementarity can be found in refs 80 and 85, and future work will address geometrical effects in the KSI reaction (unpublished results).

[e]The details of the nonenzymatic and enzymatic reactions can, in principle, vary because of different positioning and environmental effects that bias the nature and properties of the reactions' transition states (see Are Transition States Changed on Enzymes? and refs 36 and 147).

[f]These apparent affinities are obtained by application of transition state theory, which postulates that chemical reactions proceed via the formation of a rare species, the transition state, that exists in equilibrium with the ground state reactants (with equilibrium constant $K^{\ddagger}$) and that upon formation, the transition state decomposes within the lifetime of a bond vibration to generate products (with a rate constant ν). Within this framework, the second-order rate constant for an uncatalyzed reaction between water and a substrate is given by the equation $k_2 = \nu K^{\ddagger} = (\nu[S^{\ddagger}])/([H_2O][S])$. Similarly, the apparent second-order rate constant for product formation in the presence of enzyme and substrate under subsaturating conditions $k_{cat}/K_M = (\nu[ES^{\ddagger}])/([E][S])$. If rate acceleration is defined as $(k_{cat}/K_M)/k_2$, then by standard algebraic manipulation, the formal affinity of the enzyme for the transition state $K_d(ES^{\ddagger}) = ([E][S^{\ddagger}])/[ES^{\ddagger}] = [H_2O](k_{cat}/K_M)/k_2$. See refs 37 and 38 for details.

[g]One of us recalls Bob Abeles saying the following: "If a result seems too good to be true, chances are it is. (But of course you should test it and find out.)" The same advice is often given about buying bridges, other financial investments, and advertising claims for weight loss, hair gain, and other miracle cures.

[h]This property is termed "catalytic promiscuity" and likely has been central in the evolution of new enzymatic activities.[171,172]

[i]This result provides strong evidence that the desired design was not met. In contrast, an observation of a large rate decrease would not show that the designed mechanism was employed; rather, that result would show that removal of a particular side chain and replacement with another has a deleterious effect on some aspect of catalysis. Further studies would then be required to address the mechanistic origins of the observed effect.

[j]We have not addressed the important question of nonadditivity directly, but the Lys pK_a is lowered for free RA61, suggesting that the 10-fold enhancement of amine reactivity at pH 7 is not interdependent with the binding interactions (in the context of the already folded protein).

[k]Indeed, designs to date have used theoretical templates, "theozymes", derived from reasonably sophisticated quantum mechanical models, but there is no indication that designs require this level of sophistication or have this level of discrimination. It would be interesting to conduct blind design trials with different assumed transition states and with crude models of transition state partial charges and a geometry map conjured by knowledgeable organic chemists.

[l]It is also important to recognize that even in the midst of general agreement about catalytic principles, the details of any individual system present real challenges. Even the best-studied enzymes are not fully understood. Ribonuclease A, often touted as the consummate example of an enzyme with an established mechanism, lacks comprehensive and unifying models to account for the detailed roles and the energetics of the active site His and Lys residues and for the effects of substituting fluorohistidine for one or both of the active site His residues.[173−176] It is often uncertainty about these (important) details that obscures broader agreement.

[m]As a simple model, consider two unrestricted reactants in solution. They will occasionally come close, with a rather flat distribution of probabilities versus approach distance (all other factors being ignored in this simple illustrative example). Now imagine, again in an oversimplified illustrative example, that the only thing an enzyme does is to restrict the conformational space explored by reactants. In essence, the reactants are placed in a box, and the better the enzyme, the smaller the box. With a sufficiently small box, or a sufficiently restrictive enzyme, one can imagine that the bias in the conformational landscape could lead to a higher fraction of time that the reactants are very close (even if these are not the most probable states) and thus preferential reaction from the subset of very close states. We do not know if such restrictions might preferentially enhance reactions that can involve tunneling, how difficult (or likely) it is to evolve such a mechanism for rate enhancement, and whether such a mechanism is generally accessible or idiosyncratic to particular reactions and active sites. As a null model, one can imagine that the restricted space could alter the vibrational properties of the reactants and transition state and thereby alter the observed isotope effects (think of our reactants coupled by a simple spring potential; restriction in a "box" would not allow the spring to fully expand but would not hinder spring contraction or close approach of our reactants). We emphasize that this is a purposely oversimplified example to help illustrate general questions, and the null model in which an enzyme evolved to enhance catalysis by positioning reactants could also, as a consequence, alter vibrational properties, reactant distances from which tunneling occurs, and observed isotope effects.

[n]We addressed some aspects of dynamics under the preceding question, but dynamics has been so widely discussed in enzymology over the past decade that it warrants additional attention. Because transition state theory does not account for the dynamic events in chemical transformations, there has been a tendency to argue against the utility of this framework. Nevertheless, all models are approximations, and the utility of a model is dependent on the questions being asked. We hope that the usefulness of the transition state theory approach, its limitations, and when it can and cannot be productively invoked are apparent from this and the accompanying Current Topics.

[o]This comparison currently needs to be to a hypothetical enzyme, but creating actual enzymes to serve as these and other comparison states is a good goal for current selection and design efforts, as such enzymes would serve the double goal of serving as tools for understanding and providing good systems for evaluating and honing our ability to conduct design.

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