CHALLENGES IN ENZYME MECHANISM AND ENERGETICS

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■ **Abstract**   Since the discovery of enzymes as biological catalysts, study of their enormous catalytic power and exquisite specificity has been central to biochemistry. Nevertheless, there is no universally accepted comprehensive description. Rather, numerous proposals have been presented over the past half century. The difficulty in developing a comprehensive description for the catalytic power of enzymes derives from the highly cooperative nature of their energetics, which renders impossible a simple division of mechanistic features and an absolute partitioning of catalytic contributions into independent and energetically additive components. Site-directed mutagenesis has emerged as an enormously powerful approach to probe enzymatic catalysis, illuminating many basic features of enzyme function and behavior. The emphasis of site-directed mutagenesis on the role of individual residues has also, inadvertently, limited experimental and conceptual attention to the fundamentally cooperative nature of enzyme function and energetics. The first part of this review highlights the structural and functional interconnectivity central to enzymatic catalysis. In the second part we ask: What are the features of enzymes that distinguish them from simple chemical catalysts? The answers are presented in conceptual models that, while simplified, help illustrate the vast amount known about how enzymes achieve catalysis. In the last section, we highlight the molecular and energetic questions that remain for future investigation and describe experimental approaches that will be necessary to answer these questions. The promise of advancing and integrating cutting edge conceptual, experimental, and computational tools brings mechanistic enzymology to a new era, one poised for novel fundamental insights into biological catalysis.

CONTENTS

INTRODUCTION

Much of the focus of biochemical investigations throughout the last half of the twentieth century was on the mechanism by which enzymes achieve their enormous rate enhancements and exquisite specificity. Following the identification of proteins as the primary catalysts in biology by Sumner in 1926 (1), progress unraveling the chemical pathways underlying enzyme action was rapid and extensive. Enzymatic cofactors and coenzymes were identified, their chemical properties uncovered, and by a combination of nonenzymatic and enzymatic studies, their roles in facilitating distinct classes of reactions were elucidated (2–6). Although fascinating mysteries remain concerning the chemical mechanism of numerous enzymes, especially those involving oxidation-reduction and free radical chemistry, a reasonably sophisticated student confronted with an unfamiliar enzymatic transformation can, in most cases, identify what coenzymes or cofactors are likely to be involved, determine whether energy input such as ATP hydrolysis is utilized, and write a likely chemical reaction mechanism.

But enzymes are considerably better catalysts than isolated cofactors, general acids and bases, and other simple, small molecule catalysts. Enzymatic rate enhancements of $10^{10}-10^{23}$, relative to the uncatalyzed transformations in aqueous solution, are common, as is exquisite specificity (7–10). And enzymes accomplish these enormous rate accelerations using amino acid side chains and cofactors that have limited intrinsic reactivity, relative to catalysts employed in

organic synthesis. Beyond determination of the chemical mechanisms by which these side chains and cofactors operate, much attention has been paid to the energetic properties of enzymes that lead to this enhanced catalysis and to ways to describe these features (2, 3, 11–42). In this case, however, the central lessons are less clear from a casual inspection of the literature.

Why does the origin of enzymatic catalysis remain unsettled? Part of the answer is that enzymes use multiple mechanisms for catalysis. For example, some active sites take advantage of charge accumulation in the transition state to give strengthened electrostatic interactions, whereas others take advantage of charge dispersal and stabilize the transition state relative to the ground state within a relatively nonpolar pocket (43–45); some use general acids and bases, and others use metal ions. Furthermore, each enzyme uses a combination of strategies to achieve its prodigious catalysis (46–53).
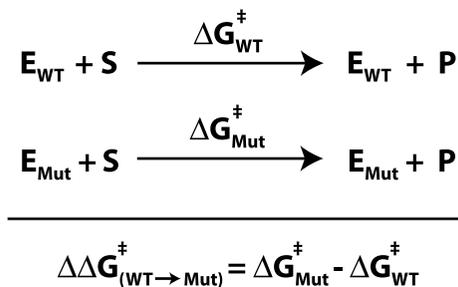
But appreciation of the multiplicity of catalytic strategies is not sufficient to understand the difficulty in comprehending and describing enzymatic catalysis. It is necessary to recognize and appreciate the complexity of enzyme energetics. Catalytic mechanisms and contributions cannot be separated and summed to provide a quantitative accounting of catalysis. This is not a limitation of our experimental abilities, but rather, energetic nonadditivity is a fundamental property of enzymes.

Site-directed mutagenesis has emerged as a powerful tool to probe individual amino acids within an enzyme. The ability to change a specific amino acid and thereby modulate catalysis has been invaluable in determining which groups are directly involved in a reaction. Further, site-directed mutagenesis has allowed the consequences from a wide array of side chain substitutions to be assessed and has been instrumental, in conjunction with other techniques, in unraveling energetic, functional, structural, and dynamic properties of the protein matrix. Nevertheless, site-directed mutagenesis focuses attention on individual residues, which tempts us to ignore the interconnectivity and nonadditivity inherent to enzymatic energetics.

First, we describe why a quantitative breakdown of catalysis into independent and energetically additive factors is not possible and how this complicates the standard scientific reductionist tendency to understand via a divide and conquer approach. We then describe a series of conceptual models that address the question: What are the features of enzymes that distinguish them from simple chemical catalysts? Finally, we formulate questions and describe experimental approaches that will be key in bringing us to the next level of understanding of enzyme catalysis.

THE COMPLEX ENERGETICS OF ENZYMATIC CATALYSIS

As scientists, we search for underlying patterns in Nature. This leads to the reductionist pursuit to find simple principles and commonalities that provide satisfying explanations for complex and seemingly disparate behaviors. Follow-

$$E_{WT} + S \xrightarrow{\Delta G^{\ddagger}_{WT}} E_{WT} + P$$

$$E_{Mut} + S \xrightarrow{\Delta G^{\ddagger}_{Mut}} E_{Mut} + P$$

$$\Delta\Delta G^{\ddagger}_{(WT \rightarrow Mut)} = \Delta G^{\ddagger}_{Mut} - \Delta G^{\ddagger}_{WT}$$

Scheme 1.

ing a reductionist path, one might want to interrogate each enzymatic residue, especially those in the active site, by site-directed mutagenesis to quantitatively determine its contribution to binding and to catalysis. One might also want to identify catalytic strategies and determine how much of the rate enhancement arises from general base catalysis, general acid catalysis, electrostatic interactions with a substrate group that has an increased charge in the transition state, or other mechanisms.

Unfortunately, the fully reductionist approaches outlined above for enzymatic catalysis are incomplete and even misleading. By understanding these approaches and their flaws, we can appropriately evaluate specific experimental data and conclusions, develop a more general description of enzymatic catalysis, and, most importantly, define approaches that will substantially advance our appreciation for how enzymes are able to achieve their enormous rate enhancements and exquisite specificity. In this section, the limits of reductionism applied to enzyme catalysis are described and the interconnectivity of enzyme energetics is highlighted.

Assigning Specific Energetic Contributions to Catalysis: the Limits of Energetic Additivity

It is commonly stated, following a site-directed mutagenesis experiment, that a particular residue or hydrogen bond contributes a certain amount of free energy to binding or to catalysis (or stability in the case of protein folding). There are two problems with such statements. The first is that the energetic value is derived from $\Delta\Delta G$, not ΔG (Scheme 1). The reaction of a mutant enzyme is compared to that of the wild type: Each is characterized by a free energy of activation ($\Delta G^{\ddagger}$), which represents the free energy difference between each ground state and transition state; thus, the difference between the mutant and wild-type reactions is a four way comparison—a difference of differences, or a $\Delta\Delta G$ value. As four different states are being compared, a single ΔG value that represents the contribution of one residue to catalysis in the wild-type enzyme cannot be extracted. Nor is it possible to devise some other scheme to do this—all such

values used to assess the contribution of a residue rely on some comparison state, whether explicitly stated or not, and are thus inherently relative; the relative nature of thermodynamic values is introduced in general terms in physical chemistry texts (54). The second problem deals with energetic nonadditivity, discussed below.

If it were possible to quantitatively assign an energetic value that describes the contribution from one residue, then one ought to be able to do this for each residue and, ultimately, sum the energetic contributions to obtain a quantitative description of catalysis. Stated another way, implicit in assignments of specific energetic values is an assumption that the groups involved are independent of one another; this renders their energetic effects additive. Energetic additivity has been observed in many site-directed mutagenesis experiments probing more than one mutation simultaneously (55). There is, however, no fundamental expectation of energetic additivity in chemical systems; additivity holds as an approximation only in special cases in which local factors dominate (55–59). Below basic experimental and conceptual examples are reviewed to illustrate the limitations of energetic additivity. Recognizing the energetic and functional interconnectedness of chemical systems is a key step in developing a deeper understanding of enzyme catalysis.

The most common example of energetic additivity, taught in introductory college chemistry courses, pertains to enthalpies of formation of organic molecules. In the 1950s and 1960s, Benson and colleagues derived group additivity principles, which have proven remarkably powerful for predicting heats of formation (ΔH_f) for organic molecules (60, 61). These rules work well because local factors dominate bond enthalpies and are hardly perturbed by the remainder of the molecule. The classic exception to simple group additivity rules for ΔH_f is benzene, which is $\sim$30 kcal mol^{-1} more stable than predicted based on adding together the single and double bond energies of "cyclohexatriene" (62). Consider the thought experiment of Figure 1. Building benzene from hexane one bond at a time, a six-membered ring can first be formed to give cyclohexane, and then the double bonds can be added. (Figure 1, path a); the final double bond (DB$_3$) contributes this extra $\sim$30 kcal mol^{-1} of energy. However, if instead the three double bonds are first added to give hexatriene, and then the ring is closed with the addition of a single bond (SB$_6$), it is this final single bond that provides the extra energy of $\sim$30 kcal mol^{-1} (Figure 1, path b). The final bond formed appears to be extraordinarily stable, although it is a different bond in each path. This paradox arises because the enthalpy is not a local property of the new bond alone, but rather a property of the system, and this distributed property is not introduced until the system is fully formed—until the last bond is in place. Aromaticity and resonance stabilization provide ad hoc explanations for the unexpected stability of benzene and conjugated compounds, i.e., the observed nonadditivity. We now know that this extra stability arises from electron delocalization throughout the benzene ring or conjugated system. The properties,
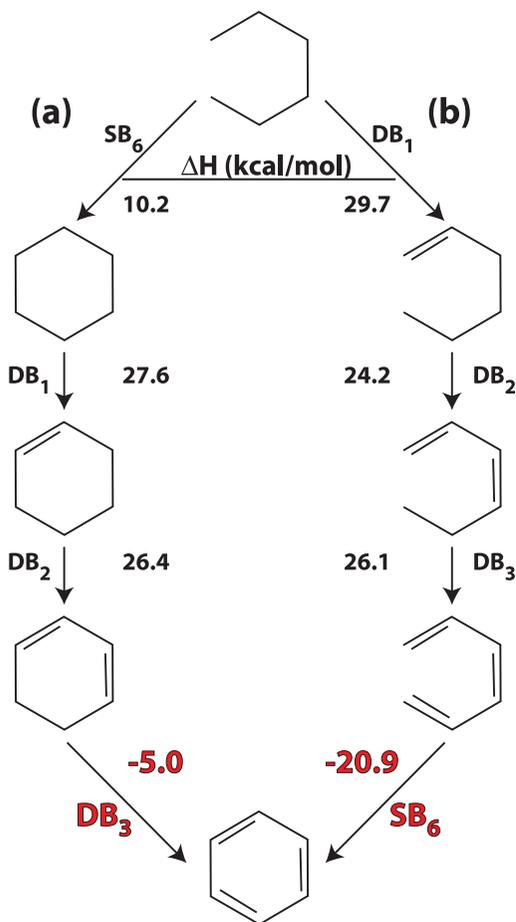
Figure 1   The bonds in benzene do not make independent, additive contributions to the molecule's stability. In pathway *a*, benzene is constructed from hexane by first forming a sixth carbon-carbon single bond (SB_6) to close the ring (with concomitant breakage of two C-H bonds and formation of H_2 gas; this occurs in each step but is omitted for clarity), followed by formation of three carbon-carbon double bonds (DB_1, DB_2, DB_3). Although the first two double bonds cost approximately the same amount of energy, the formation of the final double bond (DB_3) is more favorable by ~30 kcal mol^{-1} (-5.0 versus 27.6 and 26.4 kcal mol^{-1}). In pathway *b*, the double bonds are first added to hexane, followed by the single bond closure of the hexatriene ring. Now the three double bonds are all of about the same energy (29.7, 24.2, and 26.1 kcal mol^{-1}) while the formation of the single bond is more favorable by ~30 kcal mol^{-1} (SB_6 is 10.2 and -20.9 kcal mol^{-1} in pathway *a* and *b* respectively) (62a). The 30 kcal mol^{-1} of resonance energy present in benzene can be expressed in a single bond or a double bond, depending on how the molecule is constructed, which indicates that the bond energies depend on one another.
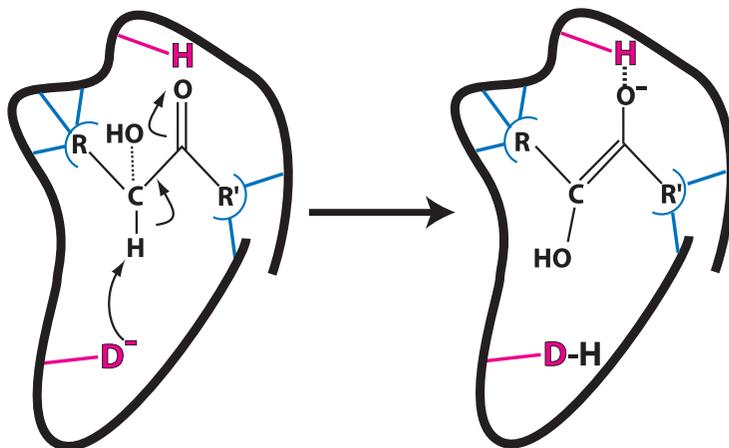
Figure 2   A hypothetical ketose isomerase enzyme. The first reaction step is shown, in which glutamate (D) is used as a general base to remove the proton alpha to the substrate's carbonyl group and histidine (H) donates a hydrogen bond to stabilize developing negative charge on the carbonyl oxygen atom. The enzyme loses essentially all catalytic activity if the His and Glu residues (shown by the magenta H and D) are both mutated to alanine leading to their designation as "catalytic residues." "Binding residues" (i.e., other residues contacting the substrate) are depicted as blue lines, and the remaining structural residues are depicted by the black outline.

and thus energetics, of benzene are not simply the sum of nearly independent local bonding interactions.

An analogous thought experiment conducted on an enzyme demonstrates that nonlocal factors are also critical for enzyme function, so enzymes cannot be considered additive systems. We start with a wild-type enzyme that catalyzes a ketose isomerization (similar to the classic enzyme triosephosphate isomerase) and contains as "catalytic residues" a base to remove a proton (glutamate) and a hydrogen bonding group that stabilizes the negative charge that develops on a carbonyl oxygen atom (histidine) (Figure 2). When we replace these "catalytic residues" with alanine, the enzyme loses all catalytic activity, as all of the other residues are considered binding or structural residues in this model. For the purposes of illustration, imagine that we continue replacing residues until the result is an unstructured poly-alanine of the same length as the starting enzyme. We now add back the wild-type residues one at a time (Figure 3). Three paths are considered. If we first add back the residues required for structure, then the "binding residues," and only at the end add back the "catalytic residues," the addition of the last residues will cause a large increase in catalytic activity, as expected for "catalytic residues" (Figure 3, pathway *a*). However, if after restoring the structural residues the "catalytic residues" are added next, there will be little to no catalytic activity. Without binding interactions to hold the substrate
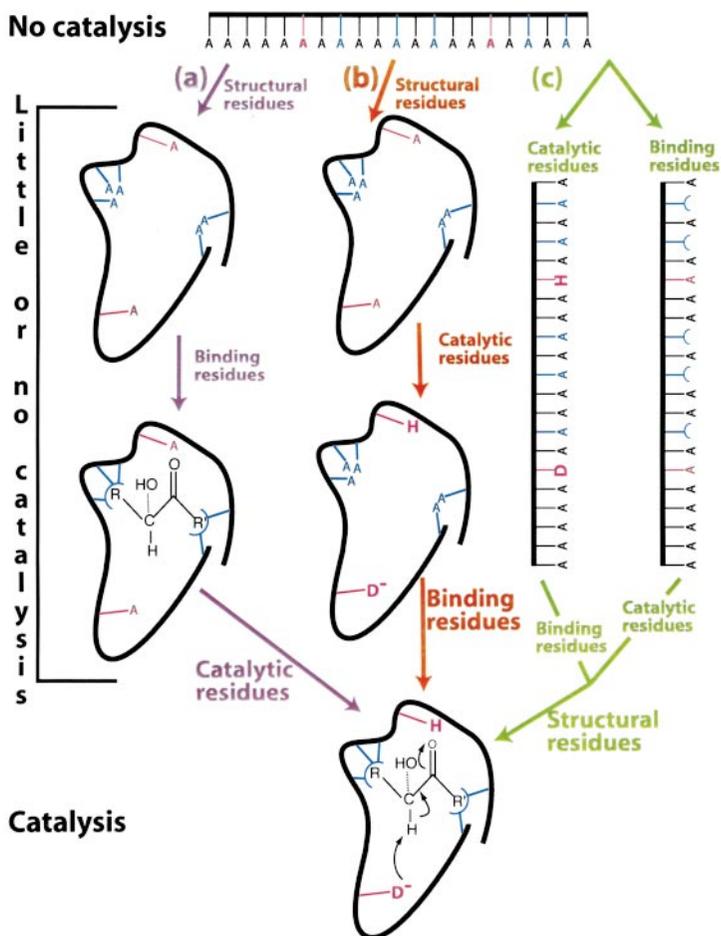
Figure 3   The interdependence of so-called catalytic, binding and structural residues. The enzyme from Figure 2 has been mutated to polyalanine, and three different pathways for conversion back to the functional enzyme are explored (*a*, *b*, and *c*). The pathway taken determines which residues appear to be important for catalysis, demonstrating that the functions of the individual residues are interdependent. The "catalytic" histidine and glutamate are shown in magenta (either as alanine (A) before mutation or as H and D after mutation). "Binding residues" are shown as blue alanines that are converted to blue lines upon mutagenesis to their wild-type identity, and upon formation of a binding site, the substrate is shown bound. "Structural residues" are shown either as black alanines or by the black outline. (Because enzymes typically have >100 residues, not all residues are depicted.)

in place, the "catalytic residues" cannot perform. (The relationship between binding and catalysis is discussed below.) Now addition of the "binding residues" brings the enzyme across the threshold to catalytic activity (pathway *b*). Finally, if the "catalytic residues" and the "binding residues" are restored in either order, no catalysis is realized in the poly-alanine background (pathway *c*). It is only upon addition of sufficient "structural residues" to stabilize the overall fold and position of the "binding" and "catalytic" residues that function is restored. Thus each of the structural residues will exhibit the phenotype of a catalytic residue when it is the one that tips the balance to allow formation of the active structure.

The thought experiment of Figure 3 demonstrates that binding and catalytic residues do not act in isolation—they are not independent of the other residues. Rather, all of the residues contribute to binding and catalysis by the definition typically applied in simple site-directed mutagenesis experiments, i.e., which residue when removed causes a loss in the particular function of interest. The only difference in Figure 3 is that the enzyme system is probed more deeply by carrying out more extensive mutagenesis than is typical (or practical). The resulting distributive assignment of function is equivalent to stating that the enzyme is a cooperative system, a statement we are perfectly comfortable with in other contexts. Thus, independent energetic contributions to catalysis cannot be assigned on a residue-by-residue basis. Similarly for benzene, the C-H groups contribute together to the extraordinary stability.

EXPERIMENTAL EXAMPLES AND CONCEPTUAL ANALOGIES TO FURTHER ELUCIDATE THE LIMITS OF ADDITIVITY   The conserved sequences of the self-cleaving RNAs referred to as the hammerhead and hairpin ribozymes are depicted in Figure 4. Both ribozymes catalyze strand scission to give a 5′-hydroxyl and a 2′,3′-cyclic phosphate, and each self-cleaving RNA has been converted into a multiple turnover ribozyme by separating a catalytic core (outlined letters) from a substrate strand (63–66). The hammerhead ribozyme was subjected to a systematic subtractive mutagenesis approach, akin to alanine scanning for protein enzymes, in which each of the thirteen conserved bases was individually replaced with a hydrogen atom (to give an abasic residue). Despite the modest catalysis by this ribozyme of $\sim 10^8$ fold, nearly all of these residues gave enormous rate decreases, typically $10^4 - 10^6$ fold (67, 68). These large and widespread effects are distinct from observations with protein enzymes in which mutation of only a few residues typically give large effects on catalysis (57, 68).

It is of course highly unlikely that each of these ribozyme residues plays a direct role in catalysis; nor do the modifications significantly affect substrate binding (68, 69). What then is the origin of the large and distributed mutational effects in the hammerhead core? There is evidence that the resting state of the hammerhead ribozyme is a non-catalytic conformation, so that the core must assemble with each catalytic event (70, 71); this is akin to the situation with the hypothetical enzyme when the "structural residues" added last gave energetic signatures of "catalytic residues" (Figure 3, path *c*). Indeed, one form of the

Hammerhead ribozyme
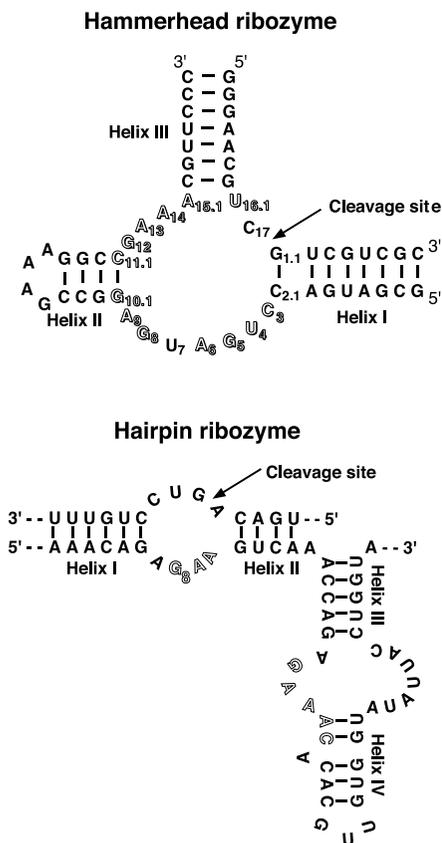

Hairpin ribozyme

Figure 4   The secondary structure of a hammerhead (*top*) and a hairpin (*bottom*) ribozyme with bound substrates. The conserved catalytic core residues are shown in outline, and the cleavage site in the oligonucleotide substrate is shown with an arrow (63–66).

hairpin ribozyme behaves similarly to the hammerhead, with energetic signatures from mutation of many core residues, whereas addition of a remote structural element to aid proper folding removes the large effects of all but one of these conserved residues (64, 72, 73). The remaining susceptible residue presumably plays a more direct role in the chemical process. We emphasize that the discovery of a less mutationally sensitive form of the hairpin ribozyme does not mean that the other residues are unimportant—their importance is merely masked in experiments when residues are mutated individually.

Consider, by analogy, the two houses shown in Figure 5. The one on the left is a minimal unit, and the one on the right is well-built, with many reinforcing beams. The primitive house, lacking structural redundancy, represents the hammerhead ribozyme. In this primitive house removal of any board, i.e., any
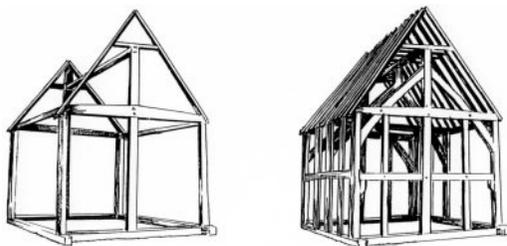
Figure 5   Two houses as metaphors for the role of structure in function and the ability to ascertain function from site-directed mutagenesis. The house on the left represents a primitive enzyme, and the house on the right represents a highly evolved enzyme with structural redundancy. For the house on the left, removal of any beam will cause collapse. In contrast, removing any individual beam will leave the house on the right standing, just as removing any individual structural residue will leave the evolved enzyme functioning. Thus, depending on context, i.e., what other beams are present in a hosue or what other residues are present in an enzyme, site-directed mutagenesis may or may not reveal components involved in overall function. From drawing by A. Peracchi.

mutation, can lead to structural collapse, thereby obliterating overall function. The well-built house is meant to represent a typical protein enzyme or the stabilized hairpin ribozyme. Removal of individual boards still leaves the structure standing in its functional form. [See (68) for a more extensive discussion of structural redundancy.]

In site-directed mutagenesis, a residue is typically defined as important (or not) based on the response of the structure or function to removal of that residue's side chain—or a particular plank in the houses of Figure 5. Residues that give a catastrophic effect in response to a single change are deemed important. Nevertheless, the rest of the enzyme is still important—as elucidated in the thought experiment of Figure 3 and the house analogy of Figure 5: Although removal of any single vertical support beam in a house will leave it standing and still strong, one would not conclude that vertical support beams are unimportant, and few would acquiesce to their removal!

Can Quantitative Energetic Contributions Be Assigned to Specific Catalytic Strategies?

The preceding section demonstrates that additivity does not underlie the energetics of complex, cooperative systems such as enzymes, so a numerical energetic contribution cannot be assigned on a residue by residue basis. As part of a reductionist approach, might one still be able to determine which residues comprise a catalytic strategy (e.g., general acid or base catalysis, electrostatic complementarity to the transition state, and ground state destabilization) and

thereby quantitate the energetic contribution from this strategy? For numerous enzymes, it is known, for example, which residues donate or abstract a proton in general acid or base catalysis. There are nevertheless limitations in our ability to assign catalytic function to specific residues, and our ability to assign energetic contributions to specific catalytic strategies is even more limited. These limitations are illustrated in the following examples.

Experiments with a PI-specific phospholipase C are instructive with respect to functional interconnections between residues (74). Tsai and coworkers examined the catalytic histidines (H32, the general base, and H82, the general acid) as well as two aspartate residues, D274 and D33, thought to hydrogen bond with the histidines. Mutation of either "catalytic" histidine to alanine lead to a rate decrease of 10^5, which lends support to the idea that the histidines are the catalytic residues. However, mutating D274 to alanine caused a 10^4 fold drop in rate, and the D33A mutant had a 10^3 fold drop in rate. These data indicate that mutation of the residues adjacent to the general acid and general base abrogate general acid and base catalysis, even though these are not the residues directly involved in proton donation to or removal from the substrate. The catalysis associated with proton removal or donation is not just a function of the residue that accepts or donates the proton; it is also connected to properties of the surrounding enzyme environment, i.e., the residues around the proton donor and acceptor that determine positioning, electrostatic potentials, and solvation. Although a catalytic value to acid/base catalysis or to a specific residue cannot be assigned from the above experiments, they, along with many other experiments, demonstrate the interconnectedness of the active site and the power of site-directed mutagenesis in uncovering these connections.[1,2]

Because multiple interactions influence a given catalytic strategy, it is impossible to separate the contribution of a given residue in the strategy. Furthermore, enzymes use multiple sets of catalytic strategies, and these strategies are also interconnected, preventing assignment of stabilization energies to a specific strategy. This is illustrated by a hypothetical serine esterase. The active site contains a general base to remove the proton from the attacking serine residue, an oxyanion hole that stabilizes the development of negative charge on the incipient oxyanion, and several groups that bind and position the attacking serine with respect to the ester carbon. It would be desirable to determine the amount

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[1]In some cases there may be significant amounts of shared covalent character in hydrogen bonds, and additional proton rearrangements may accompany proton abstraction from or donation to substrates. These possibilities further illustrate the limitations in discrete assignment of catalytic function. Elucidation of the nature of bonding in these situations as the functional, structural, and energetic origins and consequences of the bonding represents an exciting challenge (75–77).

[2]Other elegant studies have combined site-directed mutagenesis with isotope effects and variation in the identity of the leaving group to reveal roles of residues in general acid catalysis (78–81).

of catalysis provided by each strategy, but a problem arises. The hydrogen bond donors in the oxyanion hole help position the ester carbon with respect to the serine nucleophile in addition to stabilizing charge buildup on the transition state oxyanion. Similarly, the residue that acts as a general base, because of its placement in the active site, helps position the incipient oxyanion with respect to the residues that make up the oxyanion hole, aiding this catalytic function in addition to directly facilitating proton removal. Thus, the catalytic strategies are interconnected: Mutating a group involved in one type of catalysis can adversely affect another catalytic strategy as well. The energetic contributions of each catalytic strategy are not cleanly separable.

In summary, while it is often possible to assign direct chemical participation in catalysis to a particular residue, the residue's capability to act depends on its neighbors and surroundings. Thus, responsibility for a catalytic strategy cannot be assigned to a single residue. Furthermore, the catalytic strategies that an enzyme uses to facilitate reaction are not independent of one another. This functional and energetic interdependency prevents a quantitative dissection of enzymatic catalysis into types of stabilization.

Assigning the Signature of a Residue: Dissecting Binding and Catalytic Contributions

The above examples demonstrate the limitations in assigning energetic contributions to individual residues and to individual catalytic strategies. One might also like to know which reaction step or steps a particular residue facilitates: Does the residue contribute to the binding or chemical step, or both? Such a partitioning of function into neat categories would be highly appealing from a reductionist standpoint, distinguishing the residues (or functionalities) responsible for getting the substrate localized to the active site from those that actually carry out the chemical transformation. Indeed, in the literature this sort of assignment often follows site-directed mutagenesis experiments; residues that, when mutated, give increases in K_M are typically ascribed roles in binding, and residues that give decreases in k_{cat} are typically ascribed roles in the chemical step. (We assume for simplicity that $K_M = K_d$ and $k_{cat} = k_{chemical\ step}$.)

Albery & Knowles, following their classic determination of the triose phosphate isomerase (TIM) kinetic mechanism in the late 1970s, formalized this classification and suggested potential evolutionary ramifications (Figure 6) (31, 32). They noted that the addition of a residue in the course of evolution could stabilize the ground state and transition state equally (Figure 6a, uniform binding) or could stabilize the transition state without affecting the ground state (Figure 6b, specific transition state stabilization); a residue could also give a mixed effect, stabilizing both the ground and transition states but providing more stabilization to the transition state (Figure 6c, differential binding).

As noted above, these categories correspond to what are commonly considered binding and catalytic residues. This mechanistic distinction however, was at odds with the perspective articulated by Jencks. In 1973 Jencks stated "on closer
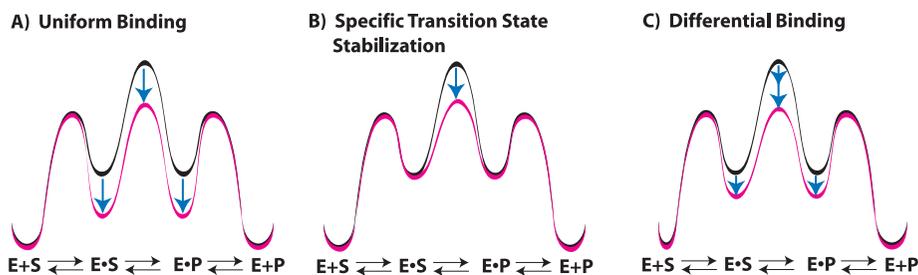
Figure 6 Free energy-reaction profiles demonstrating potential catalytic effects of mutants, as described by Albery & Knowles (31, 32). The profile for a primitive enzyme is shown in black, and possible results from introduction of potentially advantageous mutations are shown in magenta. In typical site-directed mutagenesis experiments, the effects would be in the opposite direction, starting with the magenta profile and going to the black one. (*A*) Uniform binding. All enzyme-bound species are stabilized equally by the mutation, accelerating a reaction with subsaturating concentrations of substrate, but not with saturating substrate (see also Figure 9). (*B*) Specific transition state stabilization. The mutation causes stabilization of the transition state without stabilization of the ground state. This transition state interaction leads directly to enhanced reaction of the bound substrate, and it also increases the rate of reaction of unbound substrate. (*C*) Differential binding. There is a continuum of possible energetic effects between the extremes shown in *a* and *b* in which mutations can stabilize both transition state and the ground state but provide greater stabilization to the transition state.

examination. . . the classical separation of considerations of enzymatic catalysis into the specific binding of substrates and chemical catalysis breaks down completely (15)."

Although site-directed mutagenesis had not yet been applied to enzymes, experiments with substrate analogs had clearly established the interconnection of binding and catalysis. For example, addition of amino acid residues remote from the site of chemical transformation for elastase substrates increased the rate of transformation of the already bound substrate (k_{cat}); similarly, the binding of transition state analogs but not substrates was enhanced (82, 83). The most basic point emphasized by Jencks was that binding interactions (through the intrinsic binding energy provided) can facilitate the chemical transformation (16). The binding interactions can help by positioning substrates with respect to one another, by positioning substrates with respect to functional groups on the enzyme, and by enforcing electrostatically or sterically destabilizing ground state interactions that are relieved as the substrate undergoes electronic and geometric rearrangement in the transition state (16).

DIRECT DEMONSTRATION OF THE USE OF BINDING INTERACTIONS FOR CATALYSIS More recent experiments have directly demonstrated this interconnection between binding interactions and the chemical transformation of bound sub-

(a)

(b)

Figure 7 Substrate binding to the *Tetrahymena* group I RNA enzyme. (*a*) Binding occurs in two steps. First the oligonucleotide substrate (S) binds to the internal guide sequence (IGS) of the ribozyme via base-pairing to form the open complex (K_d^{IGS}). The resulting helix then docks into the active site of the enzyme to form the closed complex (K_{dock}). (*b*) Schematic diagram showing functional groups in the substrate helix that make tertiary interactions with the active site. Only the groups varied in the works referred to in the text are shown, including the -3 (three residues 5′ of the substrate's cleavage site) 2′-hydroxyl group and the exocyclic amino group of G22 on the enzyme strand (84). The energetic cost (in kcal mol^{-1}) of replacement of each group by a hydrogen atom is also shown (84 and references therein).

strates (84). The RNA enzyme derived from the *Tetrahymena thermophila* group I intron catalyzes a reaction analogous to the first step in intron self-splicing (Equation 1). The catalytic mechanism of this RNA enzyme has been studied extensively, using presteady state kinetics to establish a complete kinetic and thermodynamic framework for individual reaction steps and a plethora of substrate analogs with single functional group substitutions to probe interactions with the RNA enzyme and their energetic consequences (85–88).

$$\text{CCCUCU}_p\text{AAAAA} + \text{G}_{OH} \rightarrow \text{CCCUCU}_{OH} + \text{GpAAAAA} \qquad 1.$$
$$(\text{S}) \qquad\qquad\qquad (\text{P})$$

Binding of an oligonucleotide 5′-splice site analog (S) occurs in two distinct steps (Figure 7*a*). In the first step, S forms a duplex with a complementary sequence of the RNA enzyme to form the open complex. In the second step, this duplex docks into the core of the enzyme and makes tertiary interactions; Figure 7*b* shows the functional groups of the duplex involved in these binding interactions, which include several 2′-hydroxyl groups, and the exocyclic amino group of G22, which forms a G·U wobble pair to specify the cleavage site. The two binding states are key to the analysis described below, because the bound oligonucleotide substrate is either positioned for reaction (closed complex) or localized to the enzyme only by base pairing and thus bound but not positioned for reaction (open complex).

In the course of probing the roles of individual functional groups (and attempting to assign discrete roles in binding and catalysis), it was discovered that addition of a single functional group to the substrate, the 2′-hydroxyl group of U(-3), could give either uniform binding or specific transition state stabilization, depending on the context, i.e., depending on the other groups and interactions present (Figure 8a). [U(-3) refers to the substrate position three residues 5′ of the cleavage site (Figure 7b).] With the wild-type enzyme, addition of this 2′-hydroxyl gave uniform binding, as witnessed by a decreased dissociation constant without any change in the rate of the chemical step (Figure 8a, wild type). In a mutant with the 2′-hydroxyl and exocyclic amino group of G22 absent to give deoxyinosine (dI22), addition of the 2′-hydroxyl at U(-3) gave the same total energetic contribution of 1 kcal mol^{-1} as the wild-type, but the energy was expressed only in increasing the rate of the chemical step, thereby giving specific transition state stabilization (Figure 8a, dI22). A group remote from the site of chemical transformation giving uniform binding in the context of the wild-type enzyme, and thus typically thought of as providing binding interactions, can instead specifically stabilize the transition state in a mutant context.

The explanation for these results provides insight into the interconnection between binding and catalysis. Recall the two binding states of S, open and closed (Figure 7a). In the otherwise wild-type context, the duplex with S forms enough tertiary interactions such that it docks and remains in the closed complex whether or not the 2′-hydroxyl at U(-3) is present (Figure 8b). Thus, even without the U(-3) 2′-hydroxyl group, the substrate is positioned for reaction. The docked substrate makes the same remote interactions in the transition state as in the ground state, so addition of the U(-3) 2′-hydroxyl group gives the same 1 kcal mol^{-1} stabilization to both states and uniform binding is observed. In the mutant context, removal of two additional tertiary contacts from the docked complex has tipped the energetic balance so that now the open or unpositioned complex is the stable ground state conformation (Figure 8c). Addition of the 2′-hydroxyl at U(-3) has no effect on binding—it is not sufficient to tip the balance back to the closed complex where an interaction with the enzyme core can be made. Nevertheless, the reaction must occur through the docked complex. Thus, the

Figure 8   The same functional group in different enzyme contexts can provide uniform binding or specific transition state stabilization. Free energy profiles (a) show that adding the same 2′-hydroxyl group (going from red to black) at the -3 position of the oligonucleotide substrate (Figure 7) of the *Tetrahymena* RNA enzyme results in uniform binding in the wild-type enzyme and specific transition state stabilization in the dI22 mutant (missing the 2′-hydroxyl group and the exocyclic amino group at position 22). Models of the significantly populated bound states of the substrate for the wild type (b) and mutant dI22 (c) that explain the respective uniform binding and specific transition state stabilization phenotypes upon addition of the 2′-hydroxyl group at position -3 (shown in red) (84).
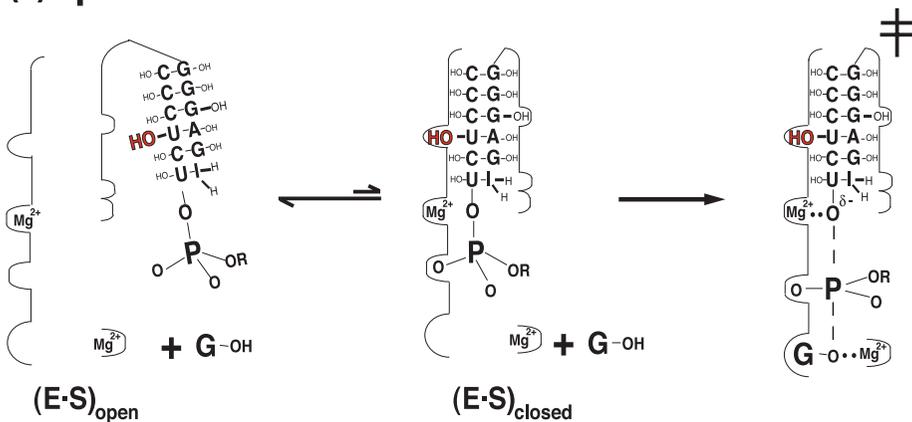
(a)
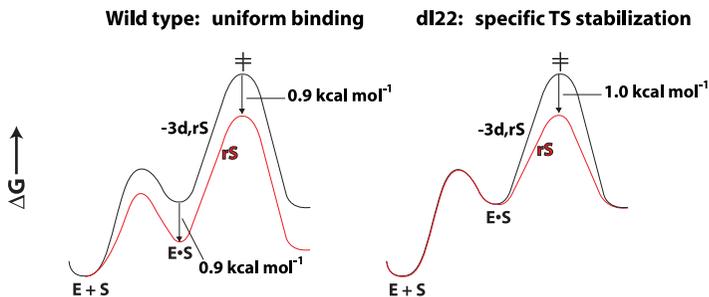
(b) Uniform binding
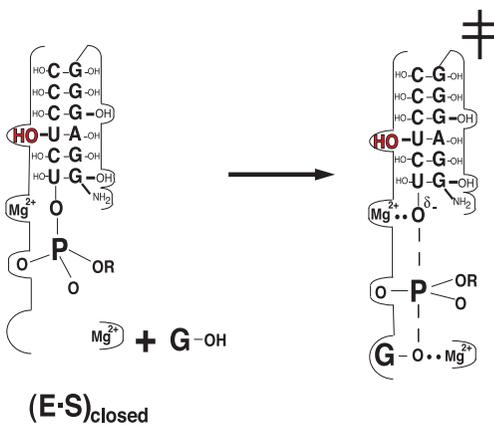
(c) Specific transition state stabilization

U(-3) 2′-hydroxyl interaction is made in the transition state, thereby providing specific transition state stabilization.

Indeed it was shown that each of the functional groups contributing binding energy to docking can contribute to binding or catalysis; this results in a uniform binding or a specific transition state stabilization phenotype dependent only on the total binding energy available from the other functional groups present. If few are present, the duplex is undocked and specific transition state stabilization is observed; if sufficient groups are already present to favor the docked complex, then uniform binding is observed. Functional groups that make binding interactions remote from the site of chemical transformation can contribute specific transition state stabilization instead of uniform binding.

The fundamental contribution of binding interactions to catalysis are masked by the presence of other binding and positioning interactions. Only when a sufficient number of these are removed (and an energetic threshold is crossed such that the unpositioned state is more stable than the positioned state) is the underlying contribution to catalysis with bound substrate revealed; what appears to be a uniform binding interaction is shown to provide transition state stabilization in a different context.

As Albery & Knowles noted, a uniform binding contribution such as that seen in the wild-type ribozyme does not provide catalysis for the enzyme/substrate complex, because the barrier for the chemical step is not lowered (Figure 9) (31). Nevertheless, uniform binding contributions can increase reaction from free enzyme and substrate (k_{cat}/K_M), and a "binding" residue that gives a uniform binding phenotype when mutated in the context of a modern day enzyme could have been selected early in evolution on the basis of a contribution to transition state stabilization via substrate positioning. The later selection for residues that provide additional binding interactions would mask this early and continuing role in catalysis.

These possible changes in phenotype over the course of evolution reveal a basic limitation of site-directed mutagenesis. Removal of one or two residues may be insufficient to unmask the catalytic contributions of "binding" residues, and removal of more residues almost invariably leads to rearrangement of bound complexes to a variety of non-productive and partially productive complexes that obscure straightforward energetic interpretation [e.g., (89, 90)].

In summary, residues involved in positioning, as in the example above, play critical roles in catalysis. Their importance, however, is not readily uncovered by site-directed mutagenesis. The Tetrahymena RNA enzyme has properties, such as simple two-state binding in a positioned or an unpositioned complex, that provided an opportunity to directly demonstrate the inextricable link between binding and catalysis, a link that is at the heart of enzymatic catalysis as further elaborated in the following section.

(a) Uncatalyzed reaction   **(b) Equal GS and TS stabilization**   **(c) Preferential TS stabilization**
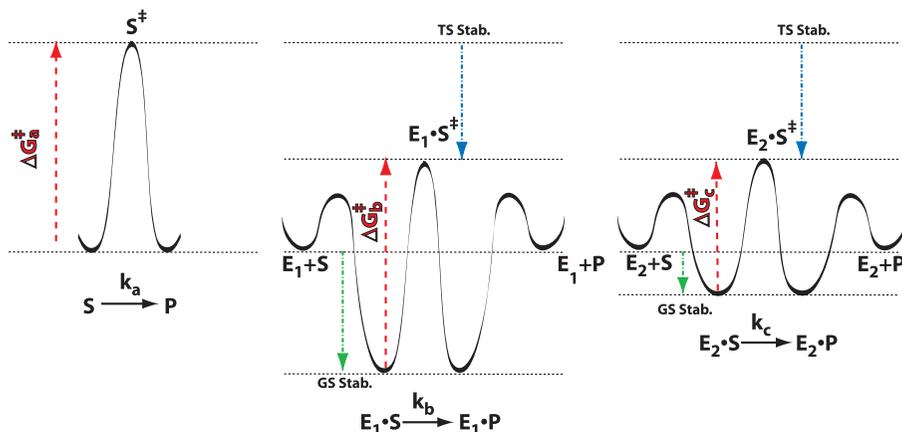

Figure 9 Free energy reaction profiles demonstrating the requirement for preferential transition state stabilization relative to ground state stabilization for catalysis. For simplicity, assume that the chemical step is rate-limiting, in this figure and throughout the review. (*a*) The uncatalyzed reaction of S, with an activation barrier of $\Delta G_a^{\ddagger}$ and a rate constant of k_a. (*b*) Enzyme 1 (E_1) stabilizes the ground state (GS) and transition state (TS) equally, leaving the reaction barrier $\Delta G_b^{\ddagger}$ equal to the reaction barrier $\Delta G_a^{\ddagger}$ for the uncatalyzed reaction; this enzyme is not a catalyst as $k_b = k_a$. (*c*) Enzyme 2 (E_2) stabilizes the transition state more than it stabilizes the transition state such that $\Delta G_c^{\ddagger} < \Delta G_a^{\ddagger}$ so that the rate constant k_c is larger than k_a. Thus, this enzyme is a catalyst.

Summary

We have considered three ways to subdivide enzyme function: assigning quantitative energetic contributions to individual residues; assigning quantitative energetic contributions to the catalytic strategies used by the enzyme; and assigning energetic contributions to residues in binding versus chemical reaction steps. Each is not possible.

Correspondingly, limitations of site-directed mutagenesis have been revealed. This approach, while providing many important insights, cannot provide a unique energetic signature for a residue or a catalytic strategy. Site-directed mutagenesis can reveal the importance of an active site residue for catalysis; for example, replacement of a Glu residue acting as a general base catalyst by Ala will greatly compromise catalysis. However, roles of "binding" residues in catalysis can be masked by the presence of multiple positioning interactions, and structural rearrangements upon removal of the interacting groups can obscure or amplify their underlying contributions.

In all cases the context, i.e., the properties of the surrounding residues, matters quantitatively and/or qualitatively and prevents a unique breakdown of enzyme

function into independent, energetically additive components. This complexity arises from the cooperative nature of enzyme structure and function. As described in the final section of this review, multiple comparisons of the energetics and physical properties of wild-type and mutant enzymes with cognate and modified substrates are essential tools in revealing the behavior and properties of these cooperative systems.

THE DISTINGUISHING PROPERTIES OF ENZYMES: COMPARISON TO SMALL MOLECULE CHEMICAL CATALYSTS

The task of understanding enzyme catalysis is rendered more difficult by the absence of a single answer attainable by quantitative dissection into individual contributions. In the absence of an absolute answer, understanding must be sought through relativistic analysis. Multiple comparisons and multiple types of comparisons are needed to provide different perspectives and insights. In this section, we define a general comparison with the question: What distinguishes enzymes from simple, small molecule chemical catalysts? To address this question, we consider a series of model enzymes that successively build in features, that are associated with enzymatic catalysis, and that ask for each model: Can it account for the known properties of enzyme catalysis? The models highlight catalytic properties of enzymes that are understood and also illuminate areas that remain to be elucidated. This sets the stage for the final section of this review, where the questions, comparisons, and approaches that will be required to further our understanding of enzymatic catalysis are considered.

A Hypothetical Enzyme and Reaction for Comparison

The enzyme models are used to recreate the hypothetical enzyme shown schematically in Figure 10. This enzyme catalyzes an enolization reaction, analogous to triose phosphate isomerase, ketosteriod isomerase, and many other well-studied enzymes (6, 47, 91). Structural work on this hypothetical enzyme identified a His residue at the active site acting as a general base to deprotonate the methylene group and a Gln residue hydrogen bonding to and thereby stabilizing the incipient anionic enolate oxygen atom. In addition, site-directed mutagenesis of these so-called "catalytic" residues to Ala gave dramatic reductions in catalysis, 10^6-fold for His and 10^4-fold for Gln upon side chain removal. As the overall rate enhancement observed by this enzyme is 10^{10}, it is tempting to conclude that all of the catalytic power of this enzyme has been identified. There is a basic flaw with this logic, however. The catalysis does not come solely from what are referred to as the "catalytic groups." This point was emphasized in the previous section, and its fundamental relationship to enzymatic catalysis is highlighted in the models below.
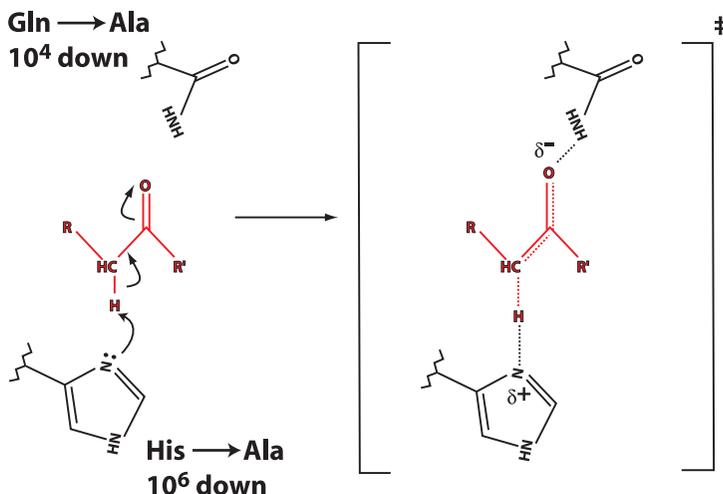
Figure 10 Hypothetical enzyme used as a starting point to develop models explaining enzymatic activity. The enzyme catalyzes an enolization reaction using histidine as a general base to remove the proton alpha to the carbonyl group and glutamine as a hydrogen bond donor to stabilize developing negative charge on the carbonyl oxygen atom. Removal of these two residues leads to complete loss of catalysis.

Model I. "Catalytic" Residues

Taking literally the notion that His and Gln are the catalytic residues of the hypothetical enzyme (Figure 10), it follows that adding both histidine and glutamine to solution should provide catalysis that matches that of the enzyme (Figure 11). Of course this is not the case. The "catalytic" residues only allow enormous rate enhancements when placed within the context of the folded enzyme, as shown also by the Ala mutagenesis example above (Figure 3). This realization leads to a second model.

Model II. Positioned "Catalytic" Residues

Clearly the "catalytic" groups of enzymes need to be positioned with respect to one another. This is accomplished in Model II (Figure 12). The second model enzyme has a hypothetical framework with covalent interconnections that give precise positioning of the His and Gln residues without the large size necessary to form tertiary structure in a protein. This allows us to separately address the potential special effects of the protein framework later and thus to conceptually distinguish these effects. Imagine that advances in synthetic chemistry allows construction of a nano-scaffold that provides precise positioning of the "catalytic" residues in this model.

How does the Model II enzyme rate as a catalyst? Although the His and Gln residues are properly positioned, there is nothing to position the substrate with
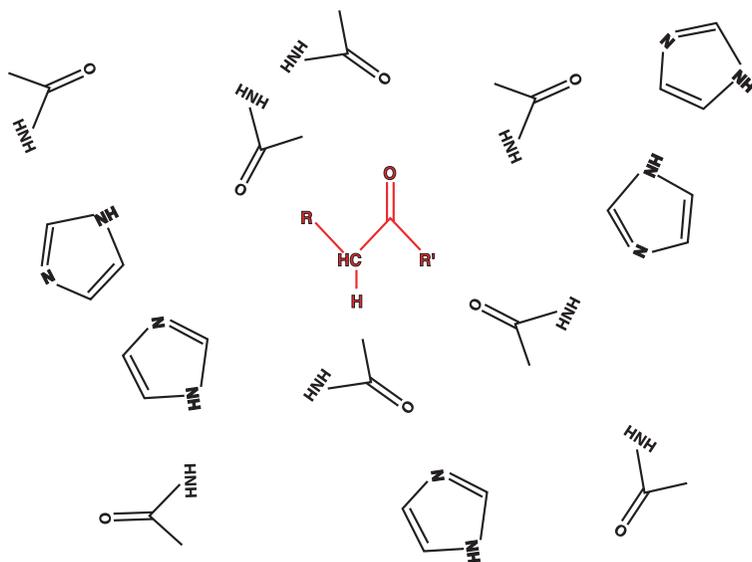
Model I

Figure 11   Model I: "catalytic residues" in solution. Histidine and glutamine (only their side chains are shown) are added to a solution containing the substrate.

respect to these catalytic groups. While reaction may be very efficient when the substrate is appropriately positioned within the active site, such positioning is highly improbable for the Model II enzyme. This problem is corrected in Model III.

Model III. Positioned "Binding" and "Catalytic" Residues

Model III maintains the His and Gln positioning from Figure 12 but introduces binding interactions with the groups that flank the enolization site (Figure 13). Two successive versions of Model III are shown. In the first (Figure 13a), these binding interactions are sufficient to localize the substrate to the active site under the hypothetical physiological conditions. However, even though the substrate is in the active site most of the time, it is not ordinarily positioned for reaction, as represented by its misalignment and mobility in the ground state (Figure 13a). This model illustrates that optimal catalysis requires positioning beyond the loss of translational entropy, as has been described previously in several different ways [e.g., (22, 92, 93–95)].

The situation of Model IIIa is precisely analogous to the Tetrahymena RNA enzyme example above: In a mutant context the substrate was localized to the enzyme in the open complex but not positioned for reaction (Figure 8). As for the
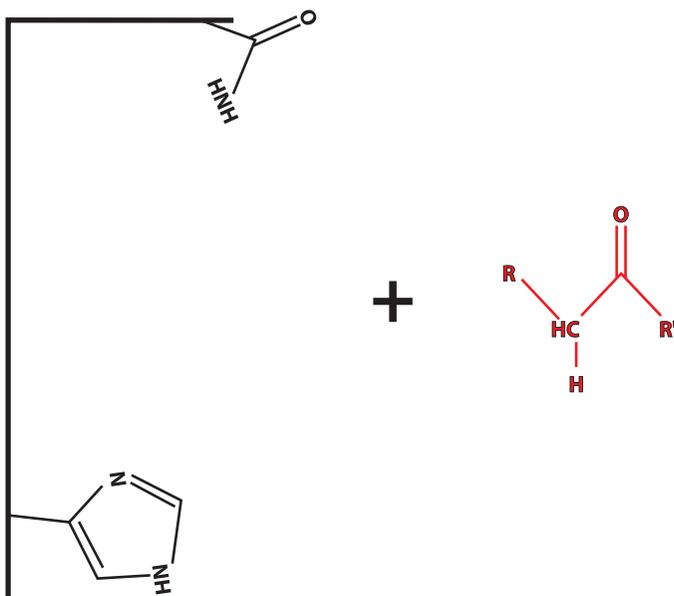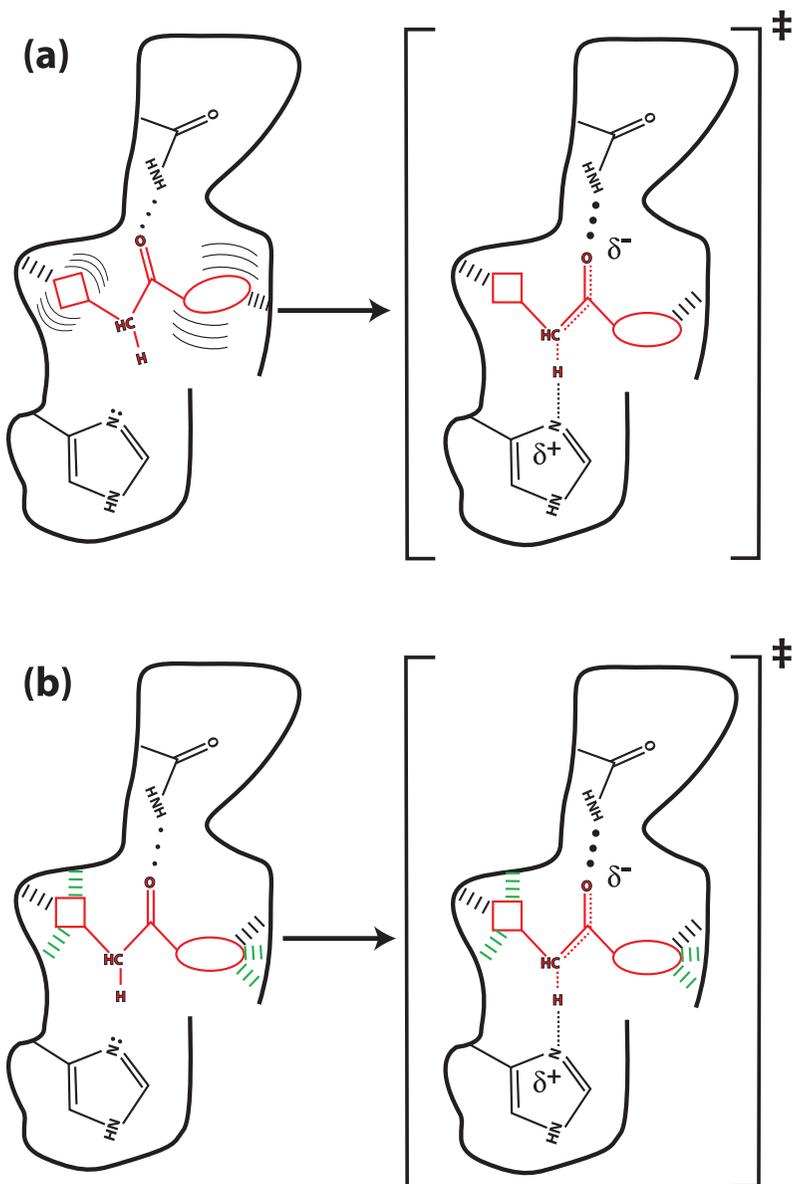
Model II

Figure 12   Model II: positioned "catalytic residues." The histidine and glutamine residues are positioned on a rigid low molecular weight framework such that they are correctly oriented for reaction. However, the model cannot position the substrate relative to the so-called catalytic groups.

RNA enzyme, the solution is to add more binding interactions (i.e., interactions remote from the site of chemical transformation) to provide the necessary fixation of the substrate within the active site (Figure 13*b*). These additional binding interactions, illustrated by the green lines in Model IIIb, position the bound substrate correctly for reaction and thereby speed the chemical transformation of the bound substrate.[3]

In Model III the groups that contribute to binding and reaction of the bound complex are interconnected energetically and functionally. The "binding" residues contribute to catalysis, and the so-called "catalytic" Gln helps in positioning by providing an additional attachment point for the substrate. This functional

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[3]Binding interactions and binding energy in actual enzymes can be added not only by introducing residues that directly contact the substrate but also by adding more distant residues that help position the directly interacting residues for binding and catalysis. This later indirect mechanism was elegantly demonstrated by sequence and structural changes of a catalytic antibody through its maturation (96, 97).

Model III

interconnectedness would not be revealed by single site-directed mutants, but it is nevertheless at the heart of enzyme function.

Model IV: Tuning Interactions and Binding Energy

Would the last model perform catalysis as efficiently as an actual enzyme? There are two likely inadequacies, both related to the strength of binding interactions, addressed and corrected below. (Additional features lacking in these models may or may not prove to be important for catalysis; these features at the frontier of scientific understanding are introduced and discussed in the last section of this review.)

Enzymes need to catalyze reactions relative to reactions in aqueous solution, and preferential stabilization of the transition state over the ground state is required to accomplish this (Figure 9). In solution, reactions for amide and ester hydrolysis and proton abstraction α to carbonyl groups (as in Figure 14) occur with oxyanion-like transition states. There is likely to be hydrogen bond donation from at least two water molecules to the oxyanion; presumably a penalty in electrostatic interaction energy would be paid, relative to aqueous solution, if only a single hydrogen bond were donated by the enzyme. Indeed, in active sites carbonyl oxygen atoms that develop oxyanion character typically accept two hydrogen bonds from the enzyme (98, 99). Therefore a second hydrogen bond donor to the oxyanion is added in Model IVa (Figure 15a).[4] Related issues of electrostatic stabilization within the active site are treated in greater depth in the final section.

The second inadequacy derives, paradoxically, from binding interactions being too strong. Speaking anthropomorphically, an enzyme wants to maximize binding interactions with its substrates to maximize the energy potentially

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[4]In the case of ketosteroid isomerase, mutation of the catalytic base (Asp38) and a hydrogen bonding group (Tyr14) seemed to account for all of the catalytic enhancement of the enzyme, but it was later found that another group (Asp99) also donated a hydrogen bond to the enolate oxygen atom and that mutation of this residue was detrimental to catalysis (100, 101).

Figure 13 Model III: substrate positioning and catalysis. (*a*) This framework provides a limited number of interactions with nonreactive portions of the substrate. These interactions are sufficient to localize the substrate to the active site, but considerable motion remains (depicted by motion lines). Thus, the substrate will be properly aligned for catalysis only a fraction of the time. (*b*) Groups making additional substrate interactions are introduced. This allows the substrate to be bound in a conformation that is aligned for reaction, with the proton that will be removed positioned with respect to the histidine lone pair and the carbonyl oxygen atom positioned with respect to the active site glutamine (compare to Figure 8).
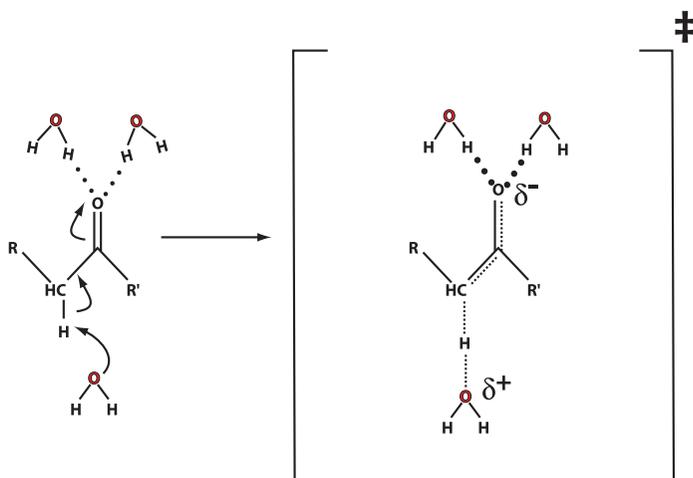
Figure 14   Multiple hydrogen bonds to anionic oxygen atoms. A solution enolization reaction comparable to that used to develop the enzyme-like models (Figure 10). Hydrogen bonds to the carbonyl oxygen atom in the ground state get stronger in the transition state (as depicted by the larger dots).

available for catalysis and specificity (16, 29). In Model IIIb binding interactions were strengthened to position the substrate in a reactive conformation. Superficially one would expect increased precision in the alignment of active site residues to provide precise complementarity for the desired substrate and an ability to provide maximal preferential stabilization of the transition state relative to the ground state, thereby increasing specificity and catalysis. However, the highly precise positioning of the enzyme models creates problems, paradoxically due to their limited flexibility.

Maximal binding interactions will require, in general, interactions of the enzyme on all sides of the substrate. Enzyme flexibility is then needed to allow substrate ingress and product egress. Indeed, enzymatic flap closures and hinge motions to accomplish this are extraordinarily common (102–107). A joint is therefore introduced in the otherwise rigid enzyme to give open and closed states that allow both ready access to the binding pocket and recognition of all elements of the substrate (Figure 15b, Model IVb).

The precise positioning of binding groups in the new model enzyme now seems optimal to position the substrate for reaction in the closed conformation. Nevertheless, problems derived from binding interactions persist for both catalytic turnover and specificity. Precise positioning can cause binding to be too strong, because the modest structural rearrangements that mitigate against very strong binding in real situations are eliminated in the more rigid model enzyme. With very strong binding, product dissociation is slowed and the maximal turnover rate can be lowered [e.g., (85, 108, 109)]. Indeed, it has been suggested
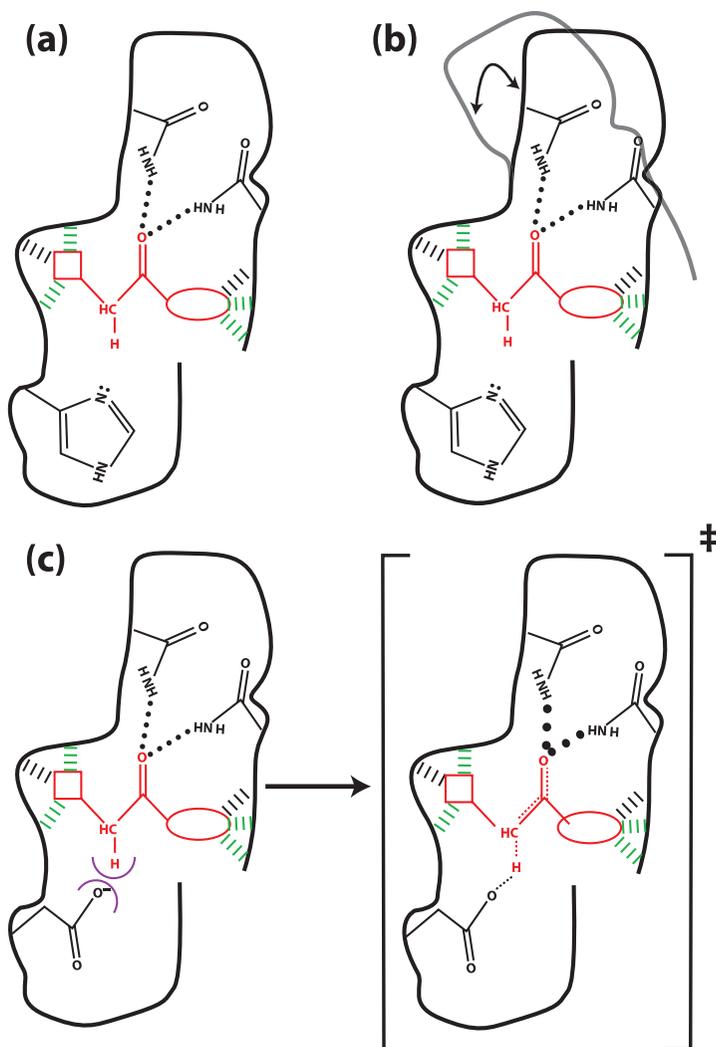
Model IV

Figure 15 Model IV: tuning interactions and binding energy. (*a*) A second hydrogen bonding interaction is added to the negative charge forming on the carbonyl oxygen atom. (*b*) A hinge point is added (arrow); this allows the enzyme to open so that substrate and product can enter and exit the active site while maintaining the specific interactions made on all sides of the substrate. (*c*) The general base is changed to aspartate, which has a full negative charge. Burial of this negative charge in the ground state, upon substrate binding, is destabilizing relative to a neutral group or to interactions in solution; this is represented by the purple lines. This interaction preferentially weakens ground state binding, which allows for rapid turnover and high specificity.

that limitations of antibodies as catalysts may arise from a too-rigid structural template that leads to slow product dissociation (110; see also 111). Too-strong binding also decreases specificity, because even substrates that only make a subset of the binding interactions will react faster than they dissociate, and both desirable and undesirable substrates can therefore react at the rate of diffusional binding (104, 112). To remedy these problems, motion is introduced into the binding residues in Model IVb, thereby weakening binding sufficiently to allow undesirable substrates to dissociate in preference to reacting (104, 113).

Another way to weaken binding interactions without compromising stabilization of the transition state is to replace the His general base with a negatively charged Asp residue (Figure 15*c*, Model IVc). Ground state binding is weakened because the negatively charged Asp is positioned adjacent to a proton of the hydrophobic substrate methylene group by the combination of the model's structural scaffold and its binding interactions. In the transition state, this destabilizing interaction is relieved as proton transfer from the methylene group neutralizes the Asp residue.[5] Ground-state destabilization has been proposed in numerous enzyme systems [e.g., (114–116)] and can contribute to an enzyme's ability to provide preferential stabilization of a reaction's transition state relative to its ground state (Figure 9) [see (16) for a more detailed discussion of the relevant energetics].

Summary

A progression of enzyme models has been presented to help identify and understand the features of enzyme catalysts that are special, or distinct, relative to simple chemical catalysts. Most fundamental is the energetic and functional interconnection of binding and catalysis. This property of enzymes intimately links specificity and catalysis, because only the correct substrates make the interactions that lead to efficient catalysis. The inextricable linkage between binding and catalysis was noted early on by Polanyi, Pauling, and Jencks (16, 27, 28, 117) and has been highlighted by many subsequent enzymologists [e.g., (13, 29, 35, 93)].

Returning to the question of whether our most advanced enzyme models would rival natural enzymes in specificity and catalysis, the answer is not known. Instead, a different question is posed below: What features of actual enzymes are not accounted for by the simplified enzyme models? This comparison helps identify and frame central questions that remain about enzymatic catalysis and the properties and behavior of enzymes.

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[5]Assessment of ground state destabilization requires comparison to some standard condition (e.g., aqueous solution or gas phase), and one can imagine making different comparisons in the same system, some of which give ground state destabilization and others of which do not. Further, whether or not ground state destabilization occurs in any specific case will depend upon what other interactions are made with the Asp residue and upon the environment surrounding the active site.

MEETING THE CHALLENGES OF UNDERSTANDING ENZYME MECHANISM: A MODERN PERSPECTIVE

The face of mechanistic enzymology has changed dramatically over the past two decades. Researchers have gone from speculation and indirect assays for residues involved in the chemical catalysis to direct tests via site-directed mutagenesis [e.g., (33, 118, 119)]. Mechanistic schemes have progressed from cartoon representations of enzymes to atomic resolution supported by X-ray crystallography. Presteady state kinetics has supplanted steady state kinetics for in-depth enzymological investigations, allowing reactions to be defined in terms of microscopic steps. These advances leave us poised to attain an understanding of enzyme mechanism at unprecedented depth.

There are, nevertheless, critical limitations of even these contemporary approaches. Site-directed mutagenesis allows a residue to be removed and effects on function the be probed, but this response is read out in energetic terms (from rate and equilibrium effects), which must then be given molecular interpretations. The structures of wild-type and mutant enzymes can be obtained, but these are static pictures that do not capture the catalytic process.

How can we do this better? A bridge between structure and energetics must be created through both an understanding of dynamics and an understanding of the physical properties of enzymes that goes beyond a description of the differences between mutants and the wild-type (Scheme 1). In this section, questions at the boundaries of the understanding of enzyme mechanism and energetics are raised. These questions are framed both by the intellectual history of mechanistic enzymology and also by the enzyme-like models developed in the last section (Figures 11, 12, 13, 15). The primary differences between actual enzymes and these enzyme-like models lie in what surrounds the groups directly interacting with substrates and the rigidity or flexibility of the interconnections between these groups. Thus, these comparisons may be particularly adept at revealing the functional and behavioral consequences from the enzyme's molecular architecture beyond the active site.

In addition to formulating the right questions to provide insights into the function and behavior of enzymes, a deep understanding of enzyme mechanism and energetics will require developing existing and new experimental and computational tools, and then bringing these tools to bear on these and other questions. Below, we first describe tools that are sufficiently developed to recognize their power in this pursuit and then turn to the pressing questions in mechanistic enzymology that will require our attention, imagination, and inventiveness in the coming years.

## Twenty-First Century Technology For Enzymology

X-RAY CRYSTALLOGRAPHY   As noted above, high resolution X-ray crystallographic structures of enzymes, often with substrate or transition state analogs

bound, are now common. More structures of individual enzymes that are under intense mechanistic scrutiny are needed: structures of one enzyme with substrates, products, and a variety of analogs bound. No transition state analog is perfect, so rearrangements of the enzyme structure often occur to accommodate these imperfections.[6] Thus, structures with multiple analogs should be sought to help unravel the types of rearrangements likely to occur within the enzyme [e.g., (120)]. And these structures are needed at ultrahigh resolution, resolution higher than 1 Å [e.g., (121, 122)]. Because many of the interactions of interest are hydrogen bonds and most enzymatic reactions involve transfer of one or more protons, the inability to directly observe the hydrogen's electron density in lower resolution structures limits mechanistic interpretation. At 0.9 Å resolution, about half of the hydrogens have assignable electron density (G.A. Petsko, personal communication); also, neutron diffraction, while technically challenging, allows observation of the hydrogen atoms (123). Another value of ultrahigh resolution X-ray structures is a reduction of the error in measuring interatomic distances, as precise distances can be of great value in understanding hydrogen bonding interactions and the positioning of groups for bond formation (G.A. Petsko, personal communication; 76, 77, 124).

ENZYME DYNAMICS AND NMR   Energy cannot be discerned even from the highest resolution structure. One fundamental reason for this is that structure is not static, and free energy is a function of enthalpy and entropy, i.e., the number of substates accessible and the energy of each of these states. It is clear that enzymes are highly dynamic entities, held together by noncovalent interactions that are weak compared to covalent bonds. Thus, to understand the basic functioning of enzymes in terms of energetics and the conformational states utilized in function, the dynamic behavior of enzymes must be interrogated. Currently the most powerful approach to study dynamics is NMR, which provides (in principle) the ability to probe motions on timescales ranging from picosecond to millisecond and greater for each residue (125–129). Nevertheless, bringing such extensive data together into dynamic molecular models is a formidable challenge (see Computation of Enzyme Behavior and Function below).

SITE-DIRECTED MUTAGENESIS WITH UNNATURAL AMINO ACIDS   Limitations of site-directed mutagenesis have been highlighted throughout this review. Site-directed mutagenesis cannot provide a reductionist dissection and understanding of enzymatic catalysis (as is sometimes claimed or implied), and the functional importance of certain enzymatic features are masked from discovery by site-

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[6]Rearrangements of noncovalent connections are typically easier than rearrangement of covalent bonds. Thus rearrangements of the packing and positioning within the enzyme is more likely than substantial rearrangement of the covalent bonds of imperfect transition state analogs.

(a) Natural amino acids substituting for Glu

Glu   Gln   Asp   Ala
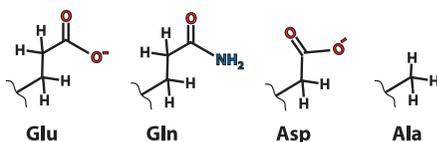
(b) Unnatural amino acids substituting for Glu
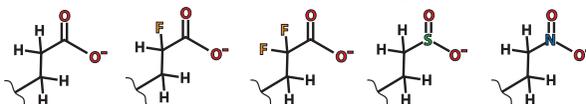
Scheme 2.

directed mutagenesis. Nevertheless, site-directed mutagenesis has proven enormously powerful in identifying residues with direct roles in catalysis. Further, assessing the functional, energetic, and structural response to a vast number of mutations has provided vital information about the behavior of the protein scaffold [e.g., (130–132)].

But despite the usefulness of generalities from site-directed mutagenesis, the scale of the changes introduced often renders interpretation difficult. For example, a Glu residue thought to act as a general base is often replaced by Ala. In this mutant the ability of the group to accept a proton is not altered; rather, it is obliterated. Further, residues around the active site can rearrange in response to removal of the two hydrogen-bond-accepting oxygens of Glu (**Scheme 2***a*). Even if an apparently more conservative change is made of Glu to Gln, two (or three) hydrogen bond acceptors are lost, and two hydrogen bond donors are added; changes that are also likely to lead to substantial destabilization and local rearrangement. If the carboxylate functionality is instead maintained by mutating to Asp, the situation is no better. Now a conformational rearrangement or intervening solvent is required to allow the Asp to act as a general base. Indeed, there is ample evidence for active site rearrangement in response to mutation, and remaining active site functional groups have even been known to rearrange to take the place of the removed group (133).

What we would like to be able to do is to alter, in an incisive and systematic fashion, the properties of a particular enzymatic functional group. Instead of removing the general base, its electronic properties could be varied without varying [or with minimal variation in (134)] its size and geometry (**Scheme 2***b*). The response to this variation can be compared to that observed in related solution reactions. The behavior of this series of analogs can also be compared with different substrates and with enzyme variants at other positions. These comparisons can help reveal the interplay of general base catalysis with other catalytic and structural features of the enzyme and substrate. This approach mirrors that of physical organic chemistry, which has uncovered much about

solution reactions through careful and systematic variation of reactants and conditions (62, 135).

Accomplishing these goals will require ready access to series of unnatural amino acids (and even nonamino acids incorporated into the peptide backbone). And large quantities of protein will be needed so that structural and spectroscopic work can be carried out alongside functional assays. Fortunately, solid phase synthesis and intein ligation methods are emerging that promise to provide ready access to the large amounts of unnaturally substituted proteins that will be required for this next level of mechanistic analysis [(136–139); see also (140)].

VIBRATIONAL PROBES: SPECTROSCOPY AND ISOTOPE EFFECTS   Changes in NMR chemical shifts for residues extending well beyond the site of mutation suggest that mutagenesis changes interactions throughout the protein, even in cases in which no structural perturbation is evident from crystallography [e.g., (141)]. And even the most conservative unnatural amino acid substitutions still leave one comparing properties of a wild-type and mutant enzyme (Scheme 1).

Vibrational probes have the special quality of allowing direct measurement of bonding properties without recourse to mutation. Because bond vibrations are highly sensitive to the molecular surroundings, information is presented about the environment felt within the enzyme. This may allow one of the most vexing questions in enzymology to be addressed: What is the solvation behavior in an enzyme active site? This question is raised below.

PRESTEADY STATE KINETICS AND SINGLE MOLECULE MEASUREMENTS   In the past decades, presteady state methods, which allow the examination of individual reaction steps, have continued to improve in terms of equipment availability, background literature, lower sample requirements, and data fitting approaches (142, 143). Most recently single molecule fluorescence approaches have begun to emerge in enzymology (144–147). Single molecule approaches provide a potential window to intermediate and transient states that do not accumulate and allow dissection of population averages into the behavior of the individual constituents.

COMPUTATION OF ENZYME BEHAVIOR AND FUNCTION   Enzymes are complex cooperative systems. Measured rate and equilibrium constants and even thermo-dynamic parameters (ΔH, ΔS, ΔC_p) provide miniscule information relative to this complexity. Although NMR relaxation has the potential to assay the dynamics of every residue, it does not provide information about the correlation of these motions. Computation will be essential to integrate information from multiple experimental approaches and to provide a thorough and detailed characterization of enzyme behavior and function. Computation may also provide suggestions for new experiments and predictions of their outcomes. Indeed, computation can in principle probe the response of enzymes to systematic controlled variations with higher precision than even the unnatural amino acid approach outlined above.

The charge or size of any group can be varied in silico and the effects assessed [e.g., (148)].

It is encouraging that there has been a considerable increase in the number of investigators applying computational approaches to problems in enzymology over the past few years. Nevertheless, enormous limitations remain: Simulations cannot be carried out on the timescales of enzymatic reactions; the potentials used are limited, typically lacking group polarizability; only part of the protein can be treated quantum mechanically; and not all configurations can be sampled. Computations typically start from structural coordinates determined crystallographically. But several ensemble kinetic studies and more recent single molecule studies have suggested that the activity of individual enzyme molecules can vary on the millisecond and longer timescales (149–153); i.e., slow conformational transitions may have profound effects on catalysis. The resulting uncertainties about the catalytic competence of a given structure, if widespread, will limit current computational approaches, but these uncertainties may also provide opportunities to more incisively test computational models and, subsequently, predict functional behaviors.

Computational power will certainly continue to grow in the coming years as technical challenges are met. But this alone will not render computation a powerful tool in enzymology. Theoreticians and experimentalists will need to join forces to develop an extensive interface that provides experimental constraints for computational development and experimental tests of detailed predictions from computational models. This is a critical challenge for both communities; without this partnership, computation will remain ungrounded by experiment, and experimental observations will not be placed in the most advantageous context of comprehensive and predictive models.

Questions at the Frontier of Enzymology

As described in the first section of this review, there is no absolute or universal breakdown of energy and function into component parts. Instead, comparisons are needed to understand enzymes and their catalysis. Most basically, the enzyme-catalyzed is compared to the uncatalyzed reaction in solution. But even here numerous comparisons are possible, and different comparisons can lead to different insights. The enzymatic reaction can be compared to reaction in the gas phase, to general acid, to general base, or to metal ion catalyzed reactions in aqueous or other solutions, for example. And for each comparison a standard state must be defined: What concentrations will be used to compare reactions that have different molecularities (154)?

There has been considerable discussion in the enzymological literature about the choice of standard state. One idea is to use physiological concentrations of substrates. Physiological concentrations are often near enzymes' K_M values, an observation that can be rationalized in terms of an evolutionary driving force to increase catalytic throughput. Throughput can be increased by increasing K_M while maintaining k_{cat}/K_M at a constant value; this frees enzyme that would

otherwise be sequestered in enzyme/substrate, intermediate and product complexes and thus inaccessible for reaction with new substrate. Once k_{cat}/K_M is near the diffusional limit and K_M is near the physiological substrate concentration, there is little further advantage from increased K_M or k_{cat} – values; most of the enzyme is free to react with substrate, and this reaction occurs at a rate near the diffusional limit (155).

But a set of comparisons that provides insight into the evolution of enzyme function may not be the most powerful tool to derive mechanistic and energetic insights. Indeed, the same interaction can help, not affect, or hinder catalysis, depending on the standard state used. Consider a residue that when added to an evolving enzyme contributes uniform binding (Figure 6a). With subsaturating substrate, this residue speeds the enzymatic reaction by increasing the probability that the substrate will be bound at the enzyme's active site. With saturating substrate, there is no effect for the enzyme because the ground and transition states are stabilized to the same extent; this leaves an identical barrier to overcome. For an enzyme (say more evolutionarily advanced) that already binds substrates (and products) strongly, addition of this same residue can hinder catalysis by slowing rate-limiting product release. Combining these comparisons provides a richer understanding of the enzyme than any individual comparison, but the comparisons must be defined explicitly. Often disagreements in the literature arise because two groups are asking different questions and making different comparisons, and therefore getting different answers. Spelling out models, assumptions, and comparisons as explicitly as possible fosters communication of ideas and sharing of perspectives within and beyond the enzymological community.

Comparing our small model enzymes with real enzymes, we start with two basic questions stemming from their differences: Why are most enzymes proteins, and why are enzymes so big? Three sections of questions follow; each deals with different aspects of the consequences of the large size and proteinaceous nature of enzymes. Throughout, comparisons highlight the limitations imposed by and opportunities created by the molecular properties of enzymes.

WHY ARE MOST ENZYMES PROTEINS?   Modern-day biological catalysts arose from accidents of evolution, tempered by the capabilities and limitations of their molecular constituents. Currently the vast majority of biological catalysts are proteins, but several RNA enzymes remain from what many believe to have been an RNA world early in evolution (156–158).

Why did the RNAs, like the dinosaurs, lose their preeminence? The simplest explanation is that RNA side chains lack the functional groups that tend to be directly involved in chemical transformations within protein enzyme active sites, such as the carboxylate group of Asp and Glu, the amine cation of Lys, the imidazole of His, and the sulfhydryl of Cys (159). This then begs the question: Why did these side chains evolve for proteins but not RNA? There are many possible (and not mutually exclusive) answers. Perhaps the metabolic machinery

could not readily explore and adapt to the synthesis of nucleic acid side chains with a wide array of functional groups, or perhaps RNA was constrained evolutionarily by simultaneous requirements to act as an information store and a functional macromolecule. A newly emerging macromolecule such as a peptide might have been freer to evolve, akin to gene duplication freeing one copy of a required gene to explore sequence space and new functions. There may be an additional constraint for RNA that is more fundamental to its molecular character: a limited ability to pack tightly, exclude water, and thereby manipulate the active site environment (see 159).

No single experiment or approach could explain why proteins are the dominant biological catalyst, given the complexity of this question and its intimate connection with evolution. But an ability to assess the different classes of macromolecules as catalysts, deconvolved from their evolutionary history, would speak to their catalytic potential. Early mechanistic work explored the catalytic potential of lipid micelles and carbohydrate templates (160, 161), and developed and developing in vitro selection methods will provide more powerful approaches to assess catalytic potential. In vitro selection of RNA (and DNA) enzymes, the emerging ability to carry out in vitro selections with proteins, and future tools to allow encoded selection of unnatural polymers will allow mechanistic comparisons of enzymes that carry out the same molecular transformation but are made from different building blocks (159, 162–164). Protein-like side chains and functionality can be introduced into RNA, and conversely, the number and type of protein side chains can be limited (165, 166). It will also be interesting to explore whether protein or other templates can effectively use the more reactive functionalities exploited in organic and inorganic synthesis. In general, these approaches should allow isolation of catalytic consequences from different aspects of the structure of each polymer family.

WHY ARE ENZYMES SO BIG?   Only a small fraction of the residues of an enzyme directly participate in the chemical transformation or contact the substrates. It is generally recognized that the remaining residues are involved in forming the overall structure necessary to position the residues that make these direct interactions [e.g., (167)]. The enzyme-like models of the last section (Figures 11, 12, 13, 15) use covalent bonds to arrange the groups directly contacting the substrates, whereas noncovalent forces accomplish this positioning for proteins. Noncovalent forces are weaker than covalent bonds, and the hydrophobic effect requires gathering a quorum of nonpolar groups. Thus, a polypeptide of substantial length is required to allow folding to a functional structure, whereas the models with covalent architecture can be quite small.

It would be wonderful to separate the catalytic consequences from the large size of protein enzymes by creating low molecular weight catalysts akin to the models of the previous section. Interestingly, several smaller structured peptides, especially those acting as neurotoxins, contain multiple disulfide bridges (168). Could disulfide fortification be used to obtain smaller polypeptide catalysts? Not

all attempts to engineer disulfides into preexisting protein templates have increased stability, and many have been destabilizing [e.g., (169–171)]. Advances in computational prediction of structure and stability will be required to successfully design such a potential catalyst.

Even if much smaller polypeptides could be designed to rival enzymes in catalytic efficiency, it does not necessarily follow that Nature would have done so. In some cases, the larger size is used to recognize larger substrates or to carry out complex reactions (like the passage of DNA strands by topoisomerase). But there are likely additional causes of this variation (see 159). It is possible that the evolutionary driving force to conserve energy by making smaller proteins is not strong or not strong enough relative to the difficulty of this task through natural selection. From a functional standpoint, enzymes of course do more than catalyze reactions; many bind other proteins and non-substrate ligands as part of control mechanisms and the integration of cellular signals and responses.

CONSEQUENCES OF THE PROTEINACEOUS NATURE OF ENZYMES   The low molecular weight enzyme-like models (Figures 11, 12, 13, 15) were designed to have the same groups as actual enzymes directly interacting with the substrates and directly participating in the chemical transformation. The difference lies in what surrounds these interacting groups and the rigidity or flexibility of the interconnections between these groups. These molecular properties impose limitations on what enzymes can do and also create opportunities. For example, the less rigid but closely packed protein scaffold allows residues directly interacting with substrates to be energetically and structurally interconnected with the residues that appear to constitute the enzyme's framework for the binding and catalytic residues. This linkage in turn allows communication of binding and chemical events at the active site to other sites on the enzyme. The coupling that results is the raw material for allostery, signaling, and complex coupled reactions such as the proton pumping and ATP synthesis carried out by the F1/F0 ATPase. In the discussions that follow, we emphasize the consequences for basic catalytic events rather than these more complex conformational events.

How does the solvation environment differ between enzymes and solution?   The most obvious difference between the reaction at an enzyme active site versus our low molecular weight model is that there is a lot more "stuff" that surrounds the interacting residues in the protein enzyme. The proteinaceous material excludes solvent, thereby introducing both difficulties and opportunities for the protein enzyme.

Difficulties arise because most transition states have greater charge separation than ground states. Imagine that there is only grease (hydrophobic groups) in the area surrounding the substrate. Even with direct polar and ionic interactions at the active site, the enzyme-bound transition state may be less stable than that in aqueous solution because of the large energetic cost of full desolvation of the inner and outer water layers; this unfavorable effect could then cancel other
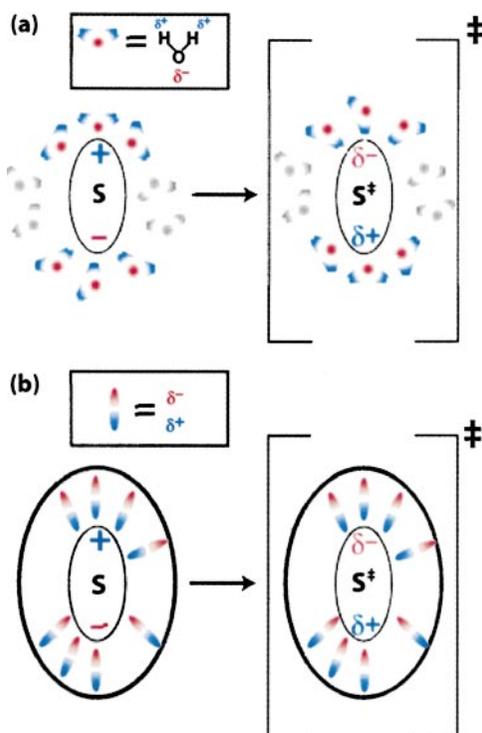
Scheme 3.

catalytic contributions, thereby rendering the enzyme ineffectual (172; K.S. Carroll & D. Herschlag, unpublished calculations). But enzymes do have dipoles, minimally in each backbone unit, and dipolar and charged side chains can be introduced that are not in direct contact with the substrate. The structural task would then be to align these more remote groups to stabilize the transition state via longer range electrostatic interactions in a manner somewhat akin to solvation (**Scheme 3**b, S‡). Indeed, several examples of helix dipoles oriented to make favorable transition state interactions have been noted (173–176). It is possible that enzymes transform the potential liability of solvent exclusion into an asset. This could be accomplished by having more numerous oriented dipoles than water, by prepaying the cost to orient the stabilizing dipoles during folding of the enzyme, and by increasing the electrostatic discrimination between transition and ground states, as described below.

It remains to be determined, experimentally and computationally, to what extent such alignments exist and what the energetic and catalytic consequences of such alignment are. Computational methods are improving in this area but cannot yet be considered reliable or predictive, and thus these methods must be bolstered by recourse to experiment. Vibrational spectroscopy provides a pow-

erful opportunity to assess the electrostatic environment at different places within an enzyme, because bond vibrations are highly sensitive to the environment (177–182).

Any interaction that can discriminate between the ground state and transition state can be beneficial to catalysis. The solvent-excluding protein matrix may be able to maximize the difference in electrostatic energy between the ground and transition states. In bulk solution, for a given change in charge (Δq_1), the energetic response in the presence of second charge (q_2) is determined by the dielectric constant (ϵ) of the medium (Equation 2). The concept of a dielectric constant loses meaning on the molecular scale,[7] but the response of the local environment to changes in charge distribution must still be considered. This is because electrostatic changes occur along a reaction coordinate, in going from a ground state to a transition state. Our model catalyst, by virtue of its small size, does not prevent water from closely approaching the bound reactants. Thus, water's strong dipole, small size, and ability to readily rearrange to stabilize either the ground state or the transition state can mitigate against large electrostatic discrimination between these states. In contrast, the larger size of protein enzymes and their tight packing could increase the electrostatic discrimination by excluding water and by placing the enzyme dipoles and charges within a structural matrix that minimizes their ability to rearrange in response to changes in the charge distribution at the active site (Scheme 3*ab*) (36, 37, 185–187).

$$\Delta E \propto \frac{\Delta q_1 q_2}{\varepsilon r} \qquad 2.$$

We are aware of two experiments that provide initial tests of whether protein enzymes do indeed provide environments that allow greater electrostatic discrimination. One utilized a series of ligands with varying hydrogen bond accepting ability and compared the change in binding affinity observed on the enzyme with changes in hydrogen bonding equilibria in solution (188, 189); the other used unnatural amino acids, a series of fluorotyrosines, and determined folding stability (190).[8]

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[7]The response to a change in charge within a molecular environment can vary depending on which individual site is substituted and can even vary for different substitutions at the same site. In addition, actual experiments typically involve more than a simple change in charge, e.g., pK_a measurements have been used to assess the electrostatic environment of an enzyme, but the observed pK_a value will be affected by the ability to rearrange to accommodate the change in size and change in hydrogen bond donors and acceptors present as a result of protonation. In other words, the observed response is a function of the local surroundings and their properties, not simply some global property of the protein. Thus, a dielectric constant or even a single effective dielectric constant cannot be defined for a protein interior (178, 183, 184).

[8]It is important to recognize that these hydrogen bonding experiments give results in terms of affinities or stabilities, not individual bond strengths, and so rely critically on an ability

One of these experiments suggested hydrogen bonding energetics similar to what is observed in aqueous solution, whereas the other suggested a substantially greater sensitivity in the enzyme environment (185). Much additional work will be required to determine whether different enzyme sites give different behaviors and to more completely characterize these interactions. For example, it will be of great value to combine these energetic analyses with controls to monitor possible structural effects and vibrational spectroscopy to provide complementary information about the hydrogen bonds and their environment.

Can enzymes distort substrate conformations to facilitate reaction?   As noted above, catalysis relies on preferential stabilization of a transition state relative to the corresponding ground state (Figure 9). Enzymes can pick out certain substrate conformers, and this can favor the desired reaction and also disfavor undesirable side reactions. For example, enzyme-bound Schiff base conjugates to pyridoxal phosphate position the hydrogen destined for removal perpendicular to the pyridoxal ring, favoring removal of that proton and disfavoring removal of other protons (191, 192); triose phosphate isomerase binds its substrate in such a way as to disfavor elimination of the phosphate from the enediolate intermediate (193–195).

Enzymes can select conformers from solution, but can enzymes actually distort substrates to the desired conformer, akin to the older idea that enzymes might act like the ancient torture device referred to as the rack, which stretched victims to uncomfortable and painful lengths (39)? Do enzymes cause analogous discomfort and pain for chemical bonds? On the one hand, the enzyme structure is enforced by noncovalent interactions, which tend to be weaker than the covalent bonds of substrates; thus distortions of these covalent bonds might be difficult to accomplish at an enzyme active site. Steel bars can distort rubber bands, but rubber bands are unlikely to bend steel! It is easier to imagine our model enzyme, with its rigid template enforced by a network of covalent bonds, distorting substrates. On the other hand, the cooperative formation and tight packing of protein native structure might enhance rigidity along the coordinate needed for bond distortion, and the enzyme's electrostatic environment might also promote distortion. The ability to distort substrates and the extent of this distortion will be a function of the sensitivity of a particular substrate motion to applied physical and electrostatic forces and the magnitude of these forces in the active site.

X-ray structures have suggested that glycosidases can distort sugar conformations toward the half chair, the conformation adopted in the oxycarbonium-like transition state (196–198). Distortions of bound substrates of serine

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to vary the property of a functional group without removing it and with minimal geometrical side effects; traditional site-directed mutagenesis cannot effectively address this question.

proteases and other enzymes have been detected by changes in vibrational states, vibrational spectroscopy, and isotope effects on binding [e.g., (199, 200)].

How can these distortions be understood? Most substrate distortions may arise simply from placing substrates in an environment designed to stabilize a different species, the transition state, rather than a design by the enzyme to physically distort the ground state (178). This suggestion is in keeping with the idea that enzymes evolved active sites to complement transition states and not ground states as discussed previously (27, 28, 117).

Knowledge of the physical environment of enzyme active sites and how this environment can affect bound ligands and transition states is in its early stages (see below). Higher resolution structures with bound ligands will help address this question and so will additional vibrational spectroscopy and binding isotope effect data. These complementary approaches will be most powerful carried out with extensive series of substrates, substrate and transition state analogs, and subtly and systematically modified enzymes supplemented with kinetic and thermodynamic analysis.

Do enzymes change the nature of transition states from those observed in solution?   This question parallels that concerning ground state distortions, but here the focus is on the transition state. In strict energetic terms, the least amount of transition state stabilization is required if the enzyme stabilizes the most stable solution transition state. However it may be easier for an enzyme to provide more stabilization along an alternative reaction path or with a modified transition state.

Modest changes in transition state structures have been surmised for glycosyl transfer and hydrolase enzymes based on extensive isotope effect analysis and on calculated vibrational modes for series of potential transition state geometries; nevertheless, all of the transition states obtained from these analyses retain considerable oxycarbonium ion character as observed in solution (201). There have been many suggestions of altered transition states in enzymatic reactions, most prevalently for phosphoryl transfer reactions. These suggestions have typically been based on inspection of structures with bound substrate, substrate analogs, or transition state analogs, whereas functional data that can assess aspects of transition state structure—isotope effects and linear free energy relationships—do not support these proposals [e.g., (202–204)]. In general, transition state analogs cannot be assumed to faithfully mimic transition states, and resting ground-state structures do not neccesarily represent reactive conformations. Further, the extent of transition state distortion will be a function of the relative energies of the different potential transition state structures, the electronic and geometrical properties of these potential transition states, and the forces at the enzyme active site, precisely analogous to the situation for ground state distortions. Experimentally the challenge is greater for transition state distortions, because transition states can only be probed indirectly. Characterization of enzymatic (and solution) transition states will remain a major chal-

lenge, one that will require a synthesis of energetic, structural, spectroscopic, and computational approaches.

DISTINGUISHING BETWEEN THE TRANSITION STATE AND GROUND STATE TO PROVIDE CATALYSIS

As demonstrated schematically in Figure 9, catalysis requires preferential stabilization of a transition state relative to a ground state. The term "transition state stabilization" is often used in the literature to refer to specific electrostatic interactions that are strengthened in the transition state. However, any effect that leads to greater catalysis gives, by definition, transition state stabilization; i.e., stabilization of the transition state relative to the ground state is not a mechanism of catalysis but rather an energetic truism that follows from transition state theory (16, 21, 29). The imprecision in the usage of this term has led to unnecessary confusion and disagreement in the literature. Transition state stabilization does not provide an explanation for catalysis. Instead, it specifies the rate advantage relative to a comparison reaction, and thus it encompasses all possible catalytic factors, which range from general acid and base catalysis to direct electrostatic interactions, to indirect effects from the protein matrix that affect electrostatic interaction energies (185), and to changes in the number of accessible states.

Preferential stabilization of the transition state is a remarkably difficult task. Most of a substrate such as ATP or NADH is unchanged in going to the transition state, and the enzyme must focus its attention on the region(s) of bond making and breaking. The differences between ground and transition states can be divided, at least conceptually, into three areas: electrostatic changes, geometrical changes, and changes in the freedom of motion of substrates. Each is dealt with in the following questions. The rigidity versus flexibility of the active site and surrounding protein is key in all cases for determining the capabilities and limitations of enzymes to take advantage of each of these differences. The issue of enzyme dynamics is addressed in a separate question at the end of this section, because this basic property of enzymes has relevance for nearly all of the other questions posed and arguably represents the largest challenge to deepening our understanding of enzyme function and energetics.

How do enzymes discriminate between the electrostatic properties of transition and ground states, and how well do they do this?   This topic has been covered in previous sections of this review, so here only a brief recap is provided. Most generally, charge build-up in transition states is stabilized by well-positioned hydrogen bonds and metal ions and by the presence of general acid and base residues to donate and accept protons. Indeed, the majority of biological reactions involve some shuttling of protons; for in-depth discussions of this topic, see (2, 205, 206). Hydrogen bond donors and acceptors are also common, and an example was emphasized in the models of Figures 11, 12, 13, and 15. Of course,

(a)   **(b)**

Oxycarbonium-like transition state   **Lactone transition-state analog**
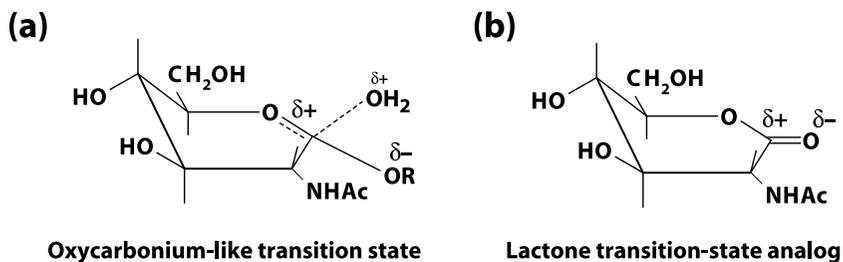
Scheme 4.

there are also hydrogen bonds to and from water for the corresponding solution reactions. Enzymes can provide catalysis via these interactions by (a) using stronger hydrogen bond donors and acceptors than water (185, 207, 208); (b) prepositioning the donors and acceptors to avoid a reorganization penalty from solution; and (c) creating an enzyme environment that allows a greater change in electrostatic stabilization energy than in solution (Equation 2; Scheme 3; Figure 15c). For reactions such as decarboxylations in which charge is more delocalized in the transition state than in the ground state, the active site may provide electrostatic interactions that are weaker or more repulsive than in solution (44). Nevertheless, some electrostatic interactions between the enzyme and the substrate will typically be required to position substrates for reaction (209).

Can enzymes discriminate between ground and transition states on the basis of geometrical differences?   This question is highly related to those above concerning the ability of an enzyme to distort or change ground state and transition states. To underscore this, the same reaction classes, glycosidases and proteases, are used. Geometrical changes typically occur in the process of bond formation and breaking, such as planar ground states going to tetrahedral-like transition states (e.g., sp^2 carbons going to sp^3).

It has long been suggested that glycosidases can bind preferentially to compounds in the half chair geometry, the geometry adopted in the oxycarbonium ion-like transition state that has sp^2 character at C1 (198) (Scheme 4a). However, the compounds used also have electrostatic differences. The carbonyl group, introduced in the transition state analog of Scheme 4b to give an sp^2 center at C1 carbon and to favor the half chair conformation, also introduces a dipole. We cannot distinguish whether this analog binds strongly to the enzyme because of the partial positive charge ($\delta+$) at C1 (which can interact with a neighboring active site Asp residue), because the half chair geometry fits better sterically into the active site, or because of a combination of these factors.

Similarly, tetrahedral transition state analogs of proteases often bind strongly. But the charge density on the oxygen atom of these analogs is greater than on the carbonyl oxygen atom of substrates so that stronger electrostatic interactions with

the protease's oxyanion hole could be responsible for the observed affinity increase instead of geometrical factors.

The motion of atoms at the reaction center, such as the carbonyl oxygen atom in a peptide bond or the non-bridging phosphoryl oxygen atoms in a phosphate ester, will be on the scale of $\sim$0.1–0.2 Å, very small changes (see also 210, 211). On the other hand, the distances between groups forming new single bonds will change on the order of an angstrom. Alternatively or in addition, the geometrical changes at the reaction center might be propagated to regions of the substrate that are more distal to give larger changes, somewhat akin to allosteric changes in enzyme structure. Such a lever arm effect would require limited conformational flexibility in the substrate, which could be intrinsic to the substrate or could be imposed by interactions with the enzyme.

The length scale that enzymes can sense is not known, and it will be necessary to understand much more about the flexibility of proteins to address this question at its most general level. For example, it has been suggested that enzymes precisely position the nucleophile and leaving group in phosphoryl and glycosyl transfer reactions, such that the predominant reaction coordinate motion is that of the phosphorus atom of the transferred phosphoryl group or the C1′ carbon of the transferred sugar as it migrates from the leaving group to the nucleophile (W.W. Cleland, personal communication; 124, 212, 213).

Testing possibilities for geometrical discrimination will require development of substrate and amino acid series that allow electrostatic and geometrical effects to be distinguished. Indeed, the desired variations to test for geometrical versus electrostatic discrimination can, in principle, best be carried out computationally, where features can be varied individually and systematically. This underscores the need to use experiment to guide the development of more powerful computational approaches.

How well can enzymes position substrates and catalytic groups for reaction? The role of positioning of substrates and catalytic groups with respect to one another has received an enormous amount of attention over the years, and may have been described with more different terms than any other physical phenomena [e.g., (35, 40, 42, 95, 214–217)]. Here the intellectual history of this subject is briefly reviewed, with the idea of laying out basic concepts and emphasizing the important challenges that remain. Additional perspectives can be found in a number of treatments in the literature [e.g., (218–223)].

Early on it was thought that the largest rate enhancement obtainable from positioning was $\sim$55 M (2, 19). The units of molarity come from comparing a unimolecular process with a bimolecular process (Equation 3), and the enhancement is often referred to as an effective concentration or effective molarity (154). The value 55 M is the concentration of pure water and represents an approximate limit for physically attainable concentrations. However, comparisons of intra-

and inter-molecular nonenzymatic reactions gave effective concentrations far exceeding 55 M. For example, rate enhancements corresponding to effective concentrations of up to $\sim 10^8$ M for intramolecular formation of anhydrides were observed by Bruice & Pandit, and many other examples with large effective concentrations were also observed (224, 225). Koshland recognized that 55 M should not be a limit because even if two substrates are next to one another, they can be situated in a nonreactive orientation (42). He then surmised that enzymes and model systems could do better by orienting or steering the orbitals that needed to react. This insight allowed the limit of 55 M to be surpassed conceptually and remains the most intuitive teaching tool. While the quantitative theory of orbital steering that grew out of this insight is inconsistent with determinations of orientational restrictions in actual chemical bonds (94, 226, 227), the underlying insight is correct and instructive.

$$A + B \xrightarrow{k_2(M^{-1}s^{-1})} A\text{-}B \qquad A + B \xrightarrow{k_1(s^{-1})} A\text{-}B \qquad\qquad 3.$$
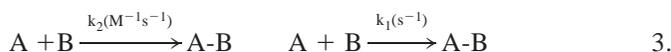
$$\frac{k_1}{k_2} = \frac{s^{-1}}{M^{-1}s^{-1}} = M$$

Page & Jencks considered this problem from a thermodynamic perspective (94). They recognized that the process of bond formation is accompanied by an entropic transformation. Basically, two molecules that together have six degrees of translational entropy and six degrees of rotational entropy are converted into one molecule with three degrees of translational entropy, three degrees of rotational entropy, and six extra degrees of vibrational entropy. Because there is typically much greater entropy in translational and (free) rotational modes than in vibrational modes, there can be a large loss in entropy upon formation of a new bond (or in the transition state for new bond formation, as this has partial covalent bonds). If an enzyme positions substrates such that this entropy has already been lost on binding, the entropy loss can result in a rate enhancement.

Entropic values for this process can be estimated reasonably well in the gas phase. Page & Jencks then used simplifying assumptions to convert estimated gas phase values to solution. The value obtained of $\sim 10^8$-10^{10} M represents an estimated maximum rate enhancement for a bimolecular reaction from such positioning effects.

In more recent computational work, Bruice revisited a series of compounds that gave very large effective concentrations for intramolecular anhydride formation (228). The results suggest that as the effective concentration increases, the enthalpic rather than the entropic barrier to anhydride formation decreases. Apparently, cyclization of the unconstrained parent compounds results in the formation of unfavorable eclipsed geometry of methylene groups; constraining the starting material in ring structures allows this strain energy to be paid by the

introduced covalent bonds. The results are consistent with the treatment of Page & Jencks, because the rate enhancements observed in this series are larger than predicted from simply freezing the rotational entropy of the molecule (94) and thus imply that an additional factor is involved in the rate enhancement. Although the intramolecular series investigated by Bruice and colleagues does not have direct biological relevance, as there are a limited number of biological cyclization reactions and thus few requirements for directly analogous eclipsed geometries, the result makes the general and important point that constraining molecules can overcome entropic and enthalpic barriers to reaction.

Effective molarities on the order of 10^4-10^5 M and larger have been obtained for enzyme ligands (229–232). These observations and the well-ordered binding sites and well-positioned ligands observed in X-ray crystallography and NMR dynamics are consistent with an ability of enzymes to effectively minimize substrate entropy and position groups for reaction [e.g., (127, 129, 233)]. But effective molarities, as noted above, can contain nonentropic contributions, and disorder and relaxation values from crystallography and NMR cannot be reliably transformed into quantitative measures of entropy.

The obvious solution would then seem to be direct measurements of entropies of binding and activation. Unfortunately, such measures are generally uninformative. The discussion above has focused on the configurational entropy of the reactants in the ground and transition states. However, thermodynamic parameters such as entropy changes are properties of the entire system. Indeed, most of the degrees of freedom are associated with solvent molecules, and many solvent molecules are displaced when a substrate binds to an enzyme. And even if one could focus only on the enzyme-ligand complex, most of these vibrational modes predominantly involve the enzyme. Thus, there is a disconnect between what we think of conceptually as entropy (related to the number of accessible configurations of the substrates) and the actual thermodynamic observable.

In principle, with sufficiently accurate potentials and advanced computational skills, one could model the system and fully describe its thermodynamics. But even if this were possible, it is not clear what would be gained conceptually. The entropy change is one number, and it represents an enormously complex sum of terms. Although these brute force approaches would certainly be useful, there is a more immediate need of conceptual advances to solve this central but vexing problem. Is it possible to understand configurational entropy and/or a positioning term that describes how preorganization of the various enzymatic components provide a rate advantage? Can the mixing of thermodynamic terms in enthalpy/ entropy compensation that occurs in practice be meaningfully deconvoluted? Careful comparative work focusing on revealing thermodynamic trends may provide the best route to such advances. For example, obtaining $\Delta\Delta S$ and $\Delta\Delta H$ values for a series of enzymes and ligands varied systematically at a particular location, in conjunction with structural and dynamic data, may lead to new energetic insights [e.g., (234)].
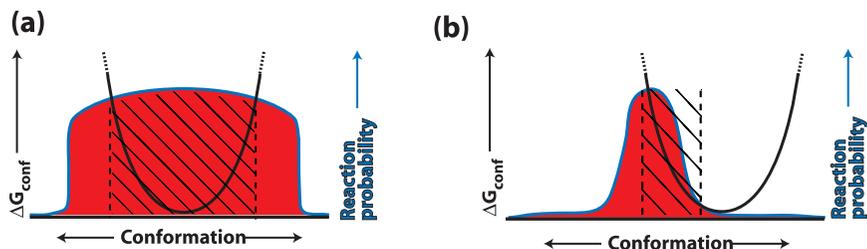
Figure 16   Potential interplay between enzymatic conformational states and reactivity. The possible conformations of an enzyme are represented by the black energy well (ΔG_{conf}); the probability of reacting from a given conformation is shown by the blue line, which is accentuated by the red under the curve. The product of these two probabilities in which 95% of the reaction will occur is shown with the hatch marks. Two possibilities are shown. (*a*) Reaction occurs from the most stable enzyme state, and fluctuations are not important for reactivity. (*b*) Most reaction occurs from enzyme conformations away from the most stable states.

WHAT IS THE DYNAMIC BEHAVIOR OF ENZYMES, AND WHAT ARE THE ENERGETIC AND FUNCTIONAL CONSEQUENCES OF THESE MOTIONS?

As noted above in the section "Consequences of the Proteinaceous Nature of Enzymes," the noncovalent forces holding proteins together result in dynamic behavior, and this behavior can be important for functions such as product release, complex multi-step reactions, allostery, and signal transduction. Here we discuss the role of dynamics in the catalytic event and the importance of understanding dynamics to understand this event. Two extreme views of the importance of dynamics can be considered. Both extremes can be ruled out but are instructive.

The first view is that dynamics are essentially inconsequential to catalysis—that we need only consider the average or most stable enzyme structure to account for catalysis. This model is depicted schematically in Figure 16*a*; the reaction rate or probability of reaction is a shallow function of enzyme motion, which drops off only for extreme conformational fluctuations. In-depth studies of isotope effects for enzymes that react via hydrogen tunneling events pioneered by Klinman and coworkers have provided an unexpected window into dynamics (235–238). Although the details of these systems are beyond the scope of this review, several features of these reactions appear only to be explained by models in which the majority of reaction occurs from an enzyme state that represents a conformational excursion from the most stable structure (represented schematically in Figure 16*b*). An oversimplified but conceptually useful view of this model comes from recognizing that tunneling events are extremely sensitive to

barrier widths. It is therefore easy to imagine rare conformational excursions that bring the hydrogen donor and acceptor close to one another providing such a large increase in reaction probability that most of the reaction occurs through these rare states. More generally, dynamics, or conformational excursions, can lead to distance changes between reacting groups and to rearrangements of enzyme dipoles that facilitate reaction.

The opposite extreme view to the illustration in Figure 16*a* maintains that the overall dynamics of the enzyme are exquisitely tuned to facilitate the reaction. There are at least two types of arguments against this extreme view. The first comes from recognition that the barrier crossing for breaking of a covalent bond occurs on the subpicosecond timescale. Most motions and rearrangements of the protein matrix, because of its large size, are expected to occur on much slower timescales. Thus models invoking overall enzyme vibrational modes that directly correlate with and guide the bond breaking process have been strongly discounted (12, 149). Even motions of water molecules in solution reactions do not appear to be directly coupling to or in resonance with subpicosecond bond making and breaking events (239). Second, if the overall vibrations of the enzyme were exquisitely tuned, one might expect modifications that alter these vibrational modes to have major catalytic consequences. This experiment has been done many times, although not with this question in mind. Many enzymes have been extensively mutated in their cores for structural and folding studies with little effect on activity (see references in 68).[9]

In summary, fully choreographed enzyme dynamics that mirror bond formation and cleavage events appear to be highly unlikely, but the opposite extreme, reaction probabilities that are insensitive to dynamic fluctuations (Figure 16*a*), is, at a minimum, not universal. The questions then arise: Is reaction through conformational excursions a special property of enzymes with large tunneling contributions or is it a more general phenomenon? Are the motions that lead to reaction favored on the enzyme's energy landscape, having been tuned through evolution, or are these dynamics random events that are inherent properties of the protein scaffold? For example, it is easier to imagine evolutionarily choreographed motions for enzymes that recognize substrates with high specificity, as opposed to enzymes that work more broadly on classes of substrates.

We do not yet have the tools to describe or dissect the dynamic behavior of enzymes. As noted above, NMR can provide dynamic information on a variety of timescales, but the trajectories and correlations of motions are not revealed.

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[9]Similar results have been obtained from solvent isotope effect measurements. The large number of solvent isotope experiments carried out provide no indication of larger than expected rate effects that could arise if the exchange of deuterium for protium in backbone amide bonds throughout the enzyme disrupted vibrational coupling, and primary isotope effect experiments with unique substitutions at nonexchangeable sites provide no indication of smaller than expected rate effects that could be attributed to a large reduced mass from coupled motions that involve portions of the protein (13).

Computational approaches for these problems are in their infancy and will certainly be required. But even if the dynamics of an enzyme could be completely described, how would these motions be understood and related to function? If computational power were sufficiently strong, it might be possible to predict what motions are integral to catalysis and what substitutions would disrupt those motions. But as always, functional disruptions must be interpreted with extreme care: Residues that are candidates for important dynamic processes may also (or instead) contribute to catalysis in other ways.

Presumably it will still be necessary to use comparisons in an effort to identify and, if possible, isolate features of the enzyme dynamics linked to function. But what comparisons are likely to be fruitful? One idea is to compare the dynamics and catalysis of a series of homologous enzymes that catalyze the same reaction but are derived from organisms that grow at different temperatures and to carry out these comparisons as a function of temperature [e.g., (240–242)]. An additional step would be to carry out in vivo selections using this series of enzymes to replace the wild-type enzyme in organisms that grow over a range of temperatures and then to analyze the dynamics and function of the resultant enzymes, again as a function of temperature.

In summary, protein dynamics are clearly integral to some biological mechanisms. One often sees claims that a specific set of dynamic behaviors probed in a particular protein are then important to that protein's function. But consider the following analogies. A weight lifter is straining under a heavy weight, with her arms and even her entire body shaking; the shaking is a consequence of the strain on her muscles, not the causative feature allowing her to lift the weight! A magician waves a wand and would have the audience believe that these movements are the cause of the astounding acts he performs; these dynamics are not causative and, indeed, are meant to distract and mislead the audience. Although we assume no analogous intent on the part of enzymes, the same danger nevertheless lurks. There are many different timescales, directions, distances, and degrees of coupling of enzyme motions; it would be astounding if all of these dynamic events were finely choreographed by evolution to accomplish function. There are simply too many degrees of freedom to expect any but a miniscule subset of motions to be directly linked to function. But, these few motions may be critical to a complete description of catalysis.

Finally, understanding enzyme dynamics is critical for understanding catalysis of individual reaction steps, because a complete energetic description of an enzyme/substrate system requires an accounting of the number of accessible states as well as the energy (or enthalpy) of those states. Although we often refer to the transition state for a reaction and use two-dimensional reaction coordinates and transition state theory in discussing nonenzymatic and enzymatic reactions, a single transition state structure, a single reaction coordinate, and an exclusively classical view of the transition state will be inadequate to fully describe and understand enzymatic reactions, their mechanisms, and their energetics (12, 236,

237, 243, 244). These represent daunting, yet fascinating challenges in the quest to unravel the secrets of enzyme action.

PERSPECTIVE

The enormous rate accelerations and exquisite specificity of enzymes have captured the imaginations of generations of scientists. These features, rate acceleration and specificity, are inextricably linked, and in this linkage lies a key to enzyme function. Over the past decades, the level of atomic detail, kinetic description, and energetic insight obtained has revolutionized our understanding, and clarified our view, of the most basic behavior and function of biological macromolecules.

Enzymology now lies poised at the interstices of chemistry, physics, biology, and evolution. From this new perspective and with tools of unprecedented power available, we have the capacity to ask still more probing questions and to apply novel and more powerful approaches. But even with this enormous power at our hands, we cannot expect breakthroughs to come easily. The systems are complex and irreducible to independent component parts; new experimental and computational tools are needed; and conceptual insights will be required to phrase questions and to parlay these questions into understanding. We look forward to these enormous challenges in enzymology and to the next era of mechanistic understanding that lies ahead.

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