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Why Enzymes Are Proficient Catalysts: Beyond the Pauling Paradigm

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ABSTRACT

Pauling proposed that "enzymes are molecules that are complementary in structure to the activated complexes of the reactions that they catalyze, ..., [rather than] entering into reactions". This paradigm has dominated thinking in the field. While complementarity of the type proposed by Pauling can account for acceleration up to 11 orders of magnitude, most enzymes exceed that proficiency. Enzymes with proficiencies $((k_{cat}/K_M)/k_{uncat}) > 10^{11}$ M^{-1} achieve over 15 kcal/mol of "transition state binding" not merely by a concatenation of noncovalent effects but by covalent bond formation between enzyme or cofactor and transition state, involving a change in mechanism from that in aqueous solution. Enzymes enter into reactions with substrates and do not merely complement the transition states of the uncatalyzed reactions.

Enzyme Catalysis and Proficiency

The notion that enzymes provide binding by complementing the shapes and characteristics of transition states is exemplified in the Pauling quote from *Nature*: "enzymes are molecules that are complementary in structure to the activated complexes of the reactions that they catalyze, ..., [rather than] entering into reactions",[1] as well as a famous *Chemical & Engineering News* article,[2] and his brilliant *Silliman Lecture* at Yale.[3] The Pauling paradigm has been the guiding principle behind drug discovery, the development of catalytic antibodies, and the attempt to produce artificial catalysts emulating enzymes. All of these fields have been inspired by the idea that noncovalent molecular recognition is the source of the awesome power of enzyme catalysis. A quantitative measure of enzyme acceleration is the proficiency of enzyme catalysis, defined by Wolfenden as the rate constant of the enzyme-catalyzed process when substrate concentration is low (k_{cat}/K_M), divided by the rate constant for the uncatalyzed reaction in water (k_{uncat}). The proficiency $((k_{cat}/K_M)/k_{uncat})$ is formally the equilibrium constant for conversion of the transition state of the uncatalyzed reaction in water and the enzyme in water into the transition state−enzyme complex.[4] This equilibrium is never achieved in practice because the transition states in enzyme and water may be entirely different from each other.[5]

Enzymatic rates are often diffusion-limited, so $k_{cat}/K_M \approx 10^9$ M^{-1} s^{-1} in water.[6] The enzyme K_M values, which measure approximately the enzyme−substrate dissociation constant, average about 10^{-4} M.[7] Consequently, k_{cat} is 10^5 s^{-1} on average for proficient enzymes. Many uncatalyzed reactions are extremely slow in water, but Wolfenden has measured or estimated k_{uncat} values from 10^{-20} to 10^{-1} s^{-1} for a few dozen of these very slow reactions.[4,8,9] The corresponding proficiencies range from 10^8 to an astonishing 10^{27} M^{-1} for cases studied to date,

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corresponding to free energies of "transition state binding" by enzymes of 11−38 kcal/mol.

Previous Views of the Origins of Enzyme Proficiency

While catalysis by enzymes could arise from lowering the free energy of activation or increasing the transmission coefficient, in general the alteration of the free energy of activation plays the dominant role.[10] The origins of the lowering of the free energy of activation by enzymes have been the subject of ongoing, sometimes contentious, discussions. These considerations have all been framed by Pauling's model of transition state complementarity.

Noncovalent Catalysis and Transition State Binding. A variety of noncovalent factors to explain enzyme efficiencies have been proposed, such as transition state electrostatic stabilization,[11] ground-state destabilization and desolvation,[12] the strong binding of a spectator group accompanied by stress on the reacting part of the molecule ("Circe effect"),[13] a restriction of motion of the reacting fragments of the substrate in the enzyme active site (entropy trap)[14] and related effects—approximation, proximity, propinquity, and togetherness,[15,16] reduction of reorganization energy by binding in near attack conformations (NACs),[17] the spatiotemporal hypothesis,[18] dynamic coupling of protein fluctuations to motions of reactants in the transition state,[19] dynamic enhancement of tunneling,[20] induced fit,[21] noncovalent cooperativity, and enhanced enzyme packing.[22,23] These are all specific physical effects that contribute to the greater complementarity of the enzyme for the transition state than the substrate.

Covalent Catalysis. The direct covalent bonding of the enzyme or cofactor to the reacting substrate is also well-known.[6,24,25] General acid−base catalysis and low-barrier hydrogen bonds that are partially covalent have also been invoked to explain catalysis.[26] Remarkably, on the basis of a survey of 465 enzyme mechanisms available in 1982, Spector proposed that all enzyme catalysis involves covalent linkage to substrates.[27]

However, while covalent mechanisms are well accepted for many enzymes,[6,23,27] most enzymologists reserve the term covalent catalysis for those cases where an actual covalent intermediate is detected with the enzyme, such as an acyl enzyme or a Schiff base intermediate. Except for such cases, explanations of enzyme activity focus on noncovalent factors, the Pauling paradigm.[28]

The Covalent Hypothesis for the Origins of Enzyme Proficiency

Our recent survey of aqueous binding constants for all types of organic and protein hosts suggested, however, that there are limits to the binding possible through noncovalent intermediates.[7] In this Account, we elaborate on an alternative to the Pauling paradigm—the covalent hypothesis—to explain why enzymes are proficient catalysts.

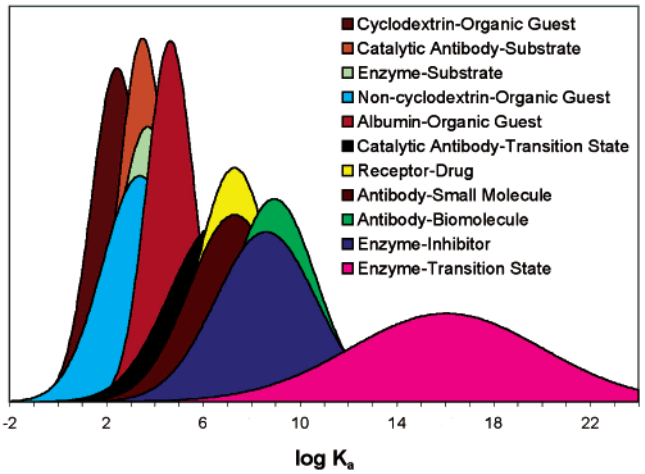

FIGURE 1. Summary of the typical binding constants for host−guest complexes. For each class, the binding distribution is represented by an idealized normal distribution. Each curve is normalized to have the same area, the maximum occurs at the average value of K_a, and the standard deviation is used to set the width of the curve.[7] The receptor−drug binding data have been added to this plot, the original version of which was conceived by Andrew G. Leach.

Survey of Host−Guest Binding Constants. A survey of host−guest association constants is shown in Figure 1. This graph shows the normal distinction of association constants found experimentally for each class of host−guest complexes; the details for each type of complex are given in our recent review.[7]

These association constants fall into three regions. Cyclodextrins and noncyclodextrins (synthetic organic hosts) binding organic guests, catalytic antibodies and enzymes binding their substrates, and albumins binding organic guests belong to the first group. The vast majority of complexes involving such hosts and neutral organic molecules have average dissociation constants in the decimolar (10^{-1} M) to hundred micromolar (10^{-4} M) range, corresponding to log K_a of 1−4 in Figure 1. The second group includes catalytic antibody−transition state complexes, receptor−drug complexes, antibody−antigen complexes (including antibody−small molecule and antibody−biomolecule complexes), and enzyme−inhibitor complexes. Stronger binding constants are reflected in average dissociation constants in the micromolar (10^{-6} M) to nanomolar (10^{-9} M) range (log K_a of 6−9 in Figure 1). The strongest binding is observed for the association of enzymes and transition states. With an average association constant of $10^{16\pm4}$ M^{-1} (log K_a of 16 ± 4 in Figure 1), most enzyme−transition state complexes have dissociation constants in the picomolar (10^{-12} M) to ten zeptomolar (10^{-20} M) range.[7] This generalization is based on the 24 enzymes for which uncatalyzed rates are available or have been estimated from measurements by Wolfenden.[4,8,9]

The Origins of the Limits in Noncovalent Association Constants. Kuntz et al. found that the "maximal affinity of ligands" (drugs and inhibitors) to receptors and enzymes corresponds to an association constant of 10^{11} M^{-1}, or ΔG of binding of about −15 kcal/mol, exceeded by only a very few complexes, such as biotin−streptavidin.[29] Gilli

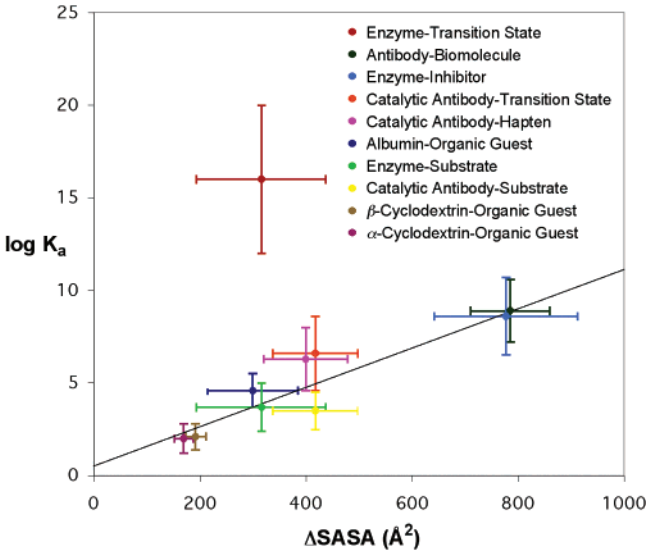

FIGURE 2. Plot of log K_a versus buried solvent accessible surface area (ΔSASA). Three classes, enzyme−substrate, enzyme−transition state, and catalytic antibody−transition state complexes, are added in addition to the original plot in ref 7. ΔSASA values of enzyme−substrate were obtained from the 34 crystal structures of enzyme−substrate complexes available in the PDB database (information on the PDB codes of these X-ray crystal structures and ΔSASA calculated with Grasp is available in Supporting Information). ΔSASA of transition states was obtained by estimation. We assume a transition state has the same ΔSASA as the corresponding substrate.

et al. proposed that the maximum affinity of inhibitors with enzymes or receptors is 10^{11} M^{-1} because of entropy−enthalpy compensation.[30] While up to 21 kcal/mol binding has been observed for a few cases, the vast majority, 92% of cases from the Kuntz and Gilli studies, have binding free energies of 15 kcal/mol or less. The origins of the distribution of association constants observed for the broad range of host−guest systems summarized in Figure 1 have been explored.[7] A correlation of the average association energy of a complex with the surface area of the guest that is buried upon complexation is found for these complexes (Figure 2). These average binding constants in water reflect hydrophobic binding, which is influenced strongly by the buried surface areas of ligands and is both enthalpic and entropic in nature.[31] Specific nonbonded interactions, such as hydrogen bonding and electrostatic interactions, can provide substantial selectivity within a given class of hosts or proteins. However, the strength of binding of antigens to antibodies or inhibitors to enzymes rarely exceeds 15 kcal/mol by noncovalent binding (log K_a < 11).

Extraordinary Binding of Transition States by Enzymes. Enzymes exhibit much higher "transition state binding" as reflected in the notable deviation from the linear correlation in Figure 2. The 10^{16} M^{-1} average binding constant is much greater than expected from the surface areas of transition states. This indicates that the extraordinary binding is the result of covalent bond formation, which may involve the formation of an intermediate covalently bound to the enzyme or cofactor or can be proton transfer (general acid−base catalysis and possibly

Table 1. Catalytic Mechanisms of Twenty-Four Enzymes

−log K_{tx}	enzyme[a]	catalytic mechanism
26.9	FBP	Mg^{2+} binding; general acid−base
26.3	PPA	Mn^{2+} binding; general acid−base
25.2	IMP	Mg^{2+} binding; general acid−base
23.9	ADC	pyridoxal cofactor; general acid−base
23.3	ODC	both covalent (iminium intermediate or general acid−base catalysis) and noncovalent mechanisms have been proposed
22.3	FUM	FADH cofactor; general acid−base
22.2	SPA	general acid−base
19.8	STN	Ca^{2+} binding; general acid−base
18.6	MRA	Mg^{2+} binding; general acid−base
17.5	CPB	Zn^{2+} binding; general acid−base
16.9	ADA	Zn^{2+} binding; general acid−base
16.7	AMN	Ca^{2+} binding; general acid−base
16.0	OSBS	Mg^{2+} binding; general acid−base
16.0	CDA	Zn^{2+} binding; general acid−base
15.8	PTS	Zn^{2+}/Mn^{2+} binding; general acid−base
15.3	CPA(O)	Zn^{2+} binding; general acid−base
15.2	KSI	general acid−base
14.9	CPA(N)	Zn^{2+} binding; general acid−base
14.7	ATD	general acid−base
13.8	TIM	general acid−base
13.7	ACE	Zn^{2+} binding; general acid−base
10.6	CMU	(noncovalent) H-bonding, electrostatic interactions
9.0	CAN	Zn^{2+} binding; general acid−base
8.7	CYC	(noncovalent) H-bonding

[a] FBP, fructose 1,6-bisphosphate; PPA, phosphoryl-phosphorylase A; IPP, inositol-1-phosphate; ADC, arginine decarboxylase; ODC, OMP decarboxylase; FUM, fumarase; SPA, sweet potato *b*-amylase; STN, staphylococcal nuclease; MRA, mandelate racemase; CPB, carboxypeptidase B; ADA, adenosine deaminase; AMN, AMP nucleosidase; OSBS, *o*-succinylbenzoate synthase; CDA, cytidine deaminase; PTS, phosphotriesterase; CPA(O), carboxypeptidase A for ester hydrolysis; KSI, ketosteroid isomerase; CPA(N), carboxypeptidase A for amide hydrolysis; ATD, ascite tumor peptidase; TIM, triosephosphate isomerase; ACE, angiotensin-coverting enzyme; CMU, chorismate mutase; CAN, carbonic anhydrase; CYC, cyclophilin.

low-barrier hydrogen bonds) occurring in the transition state or bonding to metal cations in the transition state.[32] We propose that the apparent binding constants are the result of altered mechanism enabled by enzyme chemistry not just by noncovalent molecular recognition.

Catalytic Mechanisms of the Twenty-Four Wolfenden Enzymes. To test this hypothesis, we surveyed the literature for mechanistic evidence on the catalytic mechanisms for the 24 enzymes with known proficiencies from Wolfenden's work. The results are summarized in Table 1 (information on the reactions catalyzed by the 24 Wolfenden enzymes and the literature cited are available in Supporting Information). The catalytic activity of these enzymes mostly depends on general acid−base catalysis, coordination with metal ions (Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, etc.), or organic cofactors (pyridoxal, FADH, etc.).[23] The mechanisms involve the formation of partial covalent bonds to carbon in the transition state, to protons in flight, or to metal ions.

The one exception involves the highly proficient orotidine decarboxylase (ODC in Table 1). The mechanism of catalysis by this enzyme is still under debate, and both covalent and noncovalent mechanisms have been proposed.[33] We claim that the 10^{23} M^{-1} proficiency of ODC

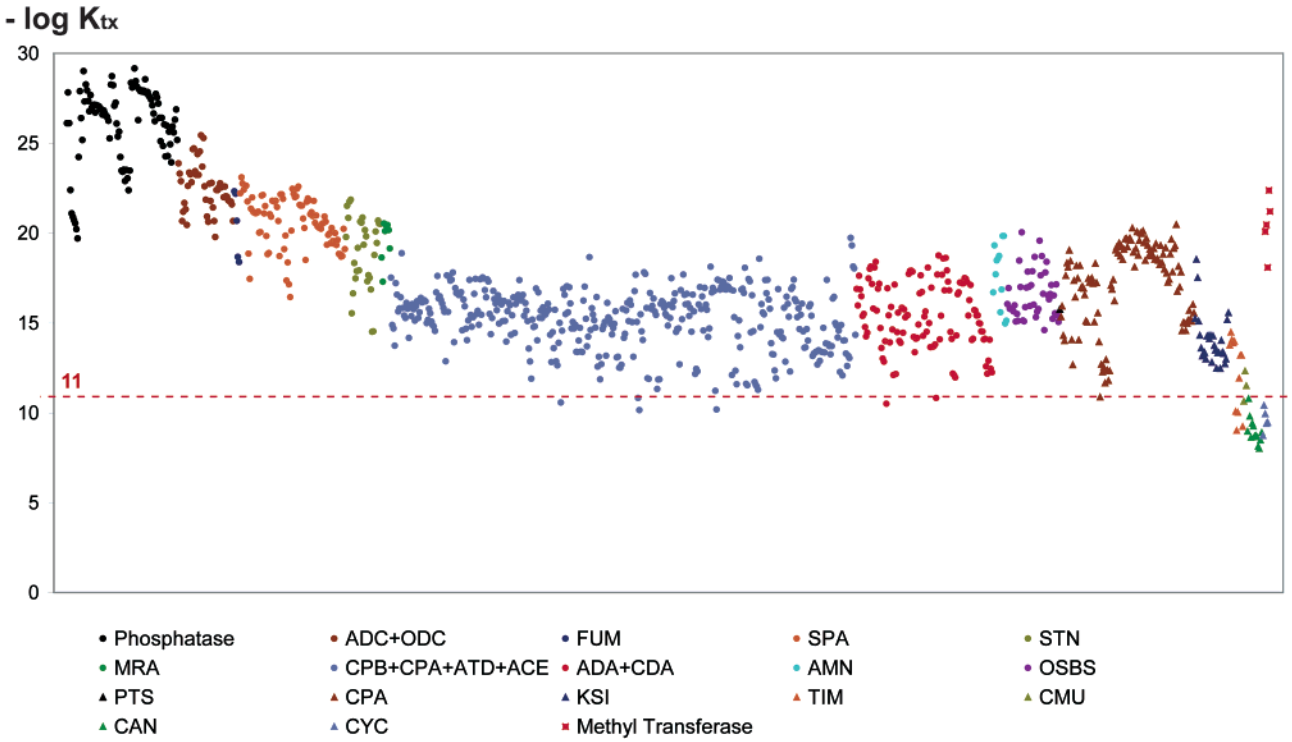

FIGURE 3. Extended proficiency plot for 1017 enzymes. The enzymes are hydrolases, lyases, isomerases, and transferases.

must arise from covalent catalysis, since only 10^{11} M^{-1} proficiency is possible by noncovalent binding.

Chorismate mutase (CMU) and cyclophilin (CYC) are two enzymes that are well established to operate by noncovalent mechanisms involving hydrogen-bonding and electrostatic interactions. These enzymes display only modest proficiencies of $10^{10.6}$ M^{-1} and $10^{8.7}$ M^{-1}, respectively,[34,35] easily explained by noncovalent interactions. Carbonic anhydrase (CAN) operates by a covalent mechanism but has low proficiency because the uncatalyzed reaction in water is quite fast ($t_{1/2}$ = 5 s).[4]

The 24 Wolfenden enzymes can be divided formally into two main groups: (1) the covalent catalysis group exhibiting high proficiencies ($-\log K_{tx} > 11$), including catalysis involving partial covalent bond-breaking or bond-forming processes, the participation of metals, and cofactors; (2) enzymes with relatively low proficiencies ($-\log K_{tx} < 11$), often involving catalysis that may occur through nonbonded interactions, generally hydrogen-bonding, electrostatic, and hydrophobic in nature. Covalent catalysis is not excluded from the low-proficiency class, but it is not necessary to achieve the modest accelerations required to accomplish diffusion-control. In terms of free energies, transition state binding of >15 kcal/mol reflects covalent binding in the transition state, while lower transition state binding can occur by either noncovalent or covalent mechanisms. The 15 kcal/mol dividing line corresponds to the maximum binding generally observed in noncovalent protein−ligand binding.[29,30] While covalent catalysis is known in many cases, we propose that all enzymes with proficiency >10^{11} M^{-1} must involve covalent catalysis.

Estimations of Proficiencies for More than 1000 Enzymes. The mechanistic evidence presented in favor of this hypothesis is based on only 24 enzymes studied by Wolfenden. There are about 100 000 unique genes for all living organisms, among which over 30 000 are involved in metabolic processes. Moreover, there are on the order of 10 000−15 000 enzymes in the human proteome.[36] Relative to these, our hypothesis has been checked with only a tiny number of enzymes. To prove that all proficient enzymes involve covalent catalysis, we need to establish the proficiencies and mechanisms of many more enzymes.

Predictions regarding the magnitude of proficiency ($(k_{cat}/K_M)/k_{uncat}$) vary depending on the uncatalyzed or nonenzymatic reactions chosen for comparison with the enzymatic reactions of interest. We have used the definition by Wolfenden for uncatalyzed reactions as reactions taking place in water at pH 7 without the presence of enzymes or cofactors.[4] A reference state involving catalytic groups, acids, bases, and metal ions in water will give a lower apparent proficiency. The uncatalyzed rate for a specific substrate in water, k_{uncat}, is often difficult to obtain experimentally, since the uncatalyzed reactions are extremely slow. However, rates in water at 25 °C can often be estimated relatively accurately: when enzymes are arranged by the reaction type they catalyze, reactions involving one type of functionality have k_{uncat} values in a reasonably small range. For example, the phosphatases, FBP, PPA, and IMP, catalyze the hydrolysis of different phosphate monoester dianions; Wolfenden has used the same estimated k_{uncat} (2×10^{-20} s^{-1}) for all of these to estimate their proficiencies.[9] Another case is the hydrolysis of peptide bonds. The experimental k_{uncat} values for the peptide hydrolysis by CPA(N), ATD, ACE, and CPB range from 10^{-9} to 10^{-11} s^{-1}.[37] ADA and CDA both catalyze deaminations, and the k_{uncat} values are basically the same, 10^{-10} s^{-1}.[8] Both ADC and ODC catalyze decarboxylations

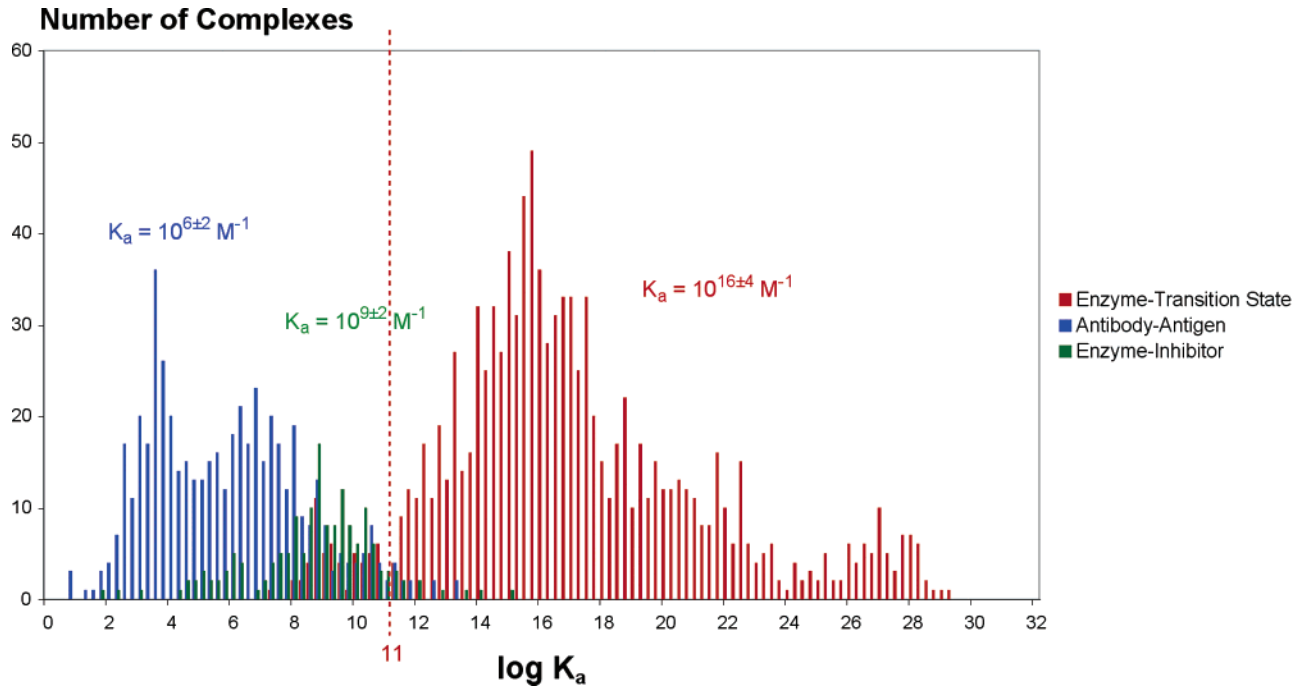

FIGURE 4. Frequency plot of association constant for 507 antibody−antigen complexes, 160 enzyme−inhibitor complexes, and 1017 measurements of enzyme−transition state complex.

to give unstabilized carbanions, and the k_{uncat} ranges merely from 10^{-16} to 10^{-17} s^{-1}.[8] CYC catalyzes the isomerization of Suc-Ala-Ala-*cis*-Pro-Phe-*p*NA to Suc-Ala-Ala-*trans*-Pro-Phe-*p*NA; k_{uncat} of the reaction of the cis substrate in water is 2.8×10^{-2} s^{-1},[8] nearly the same as the cis to trans isomerization rate of *N*-acetyl proline in water, 2.4×10^{-2} s^{-1}.[38]

With this information at hand, we have extended the original Wolfenden treatment from 24 enzymes to 1012 enzymes (information on the generic k_{uncat} is available in Supporting Information) (Figure 3). Many of these are of the same families as those in the original Wolfenden treatment, some are orthologs from different species, and some enzymes are repeated with different substrates. The Brenda protein database provides the source for k_{cat} and K_M values.[39] Mutated enzymes are excluded, so the enzymes in Figure 3 include only wild-type (native) enzymes and some recombinant enzymes with similar activities as the native enzymes.

Only first-order reactions are considered in Table 1. However, the proficiencies of enzymes that catalyze second-order reactions can also be estimated as $(k_{cat}/(K_M K_M'))/k_{uncat}$ where the two Michaelis constants are for the two substrates. For example, the rate of methyl transfer reaction of trimethylsulfonium ion as a donor and dimethylamine as an acceptor in dilute aqueous solution was determined by Callahan and Wolfenden as 1.5×10^{-8} M^{-1} s^{-1}.[40] When this second-order k_{uncat} is compared with the rate constants $(k_{cat}/(K_M K_M')$ of reactions catalyzed by tRNA methyltransferase [E.C.2.1.1.31], uroporphyrinogen methyltransferase [E.C.2.1.1.107], and sterigmatocystin methyltransferase [E.C.2.1.1.110], all of which use *S*-adenosylmethionine (SAM) as a methyl donor, five proficiency values were estimated.[41] Putting all of these data together, the extended proficiency plot for 1017 measurements of enzyme catalysis is obtained.

Based on the survey, in most cases (987 out of 1017, or 97%), enzymes are proficient catalysts with $-\log K_{tx}$ higher than 11. According to our covalent hypothesis, most enzyme mechanisms involve covalent interactions in the transition state. The enzymes in Figure 3 include hydrolases, lyases, isomerases, and transferases. Oxidoreductases and ligases are not covered, but these reactions require additional oxidants−reductants or ATP cofactor and require covalent interactions to occur at all.

Comparison of Covalent and Noncovalent Binding Affinities. A direct comparison of antibody binding with antigens, enzyme binding to strongest-binding reversible inhibitors, and enzyme binding to transition states is made in Figure 4. This is the frequency plot for 507 antibody−antigen complexes (including antibody−small molecule and antibody−biomolecule complexes in Figure 1),[7] 160 enzyme−inhibitor complexes in Figure 1,[29] and the 1017 enzyme−transition state measurements in Figure 3. The naturally evolved antibodies show the lowest average binding constant, $K_a = 10^{6\pm2}$ M^{-1}. It has been observed that during in vivo affinity maturation, the B-cell response exhibits an apparent affinity ceiling, $K_a < 10^{10}$ M^{-1}.[42] These operate by noncovalent binding, and only an antibody artificially engineered to involve covalent binding displays very high ("infinite") affinity in vitro.[43] Enzyme−inhibitor complexes exhibit a higher average binding constant, $K_a = 10^{9\pm2}$ M^{-1}, as a result of better noncovalent binding. Finally, enzyme−transition state complexes achieve exceptional binding, $K_a = 10^{16\pm4}$ M^{-1}, originating from the partial covalent bond formation between enzymes and the

real transition states. The dichotomy between noncovalent binding of antigens by antibodies and inhibitors by enzymes versus covalent binding of transition states by enzymes is apparent in Figure 4. It is also noteworthy that since there is no real limit of how weak covalent forces can be, free energy of binding lower than the 15 kcal/mol threshold may still have a partial covalent character.

Concept of Covalent Catalysis. The covalent catalysis in enzymes that we refer to includes electrophilic catalysis and nucleophilic catalysis, such as Schiff base formation, the participation of cofactors, such as pyridoxal phosphate and thiamine phosphate, or the participation of nucleophilic groups, such as the serine hydroxyl in serine proteases and the cysteine thiol in cysteine proteases. Binding of the substrates and enzyme side chains to metal cations provides direct electrophilic catalysis (involving covalent and ionic bonding) or promotes general acid−base catalysis to activate water molecules. Many metals, especially transition metals such as Fe, Mn, and Cu, are redox active and are required by oxidoreductase as reactants. Proton transfer (general acid−base catalysis) in the transition state is another type of covalent catalysis commonly observed in the enzymes with high proficiencies. Thornton and co-workers analyzed the residues directly involved in catalysis in 178 enzyme active sites.[44] Amino acid side chains that can enter into acid−base, nucleophile, and electrophilic bonding account for the majority of all residues in contact with the substrate in the active site.

Some of the strong binding that is included in our concept is ionic bonding, and the relative importance of covalent and ionic bonding is subject only to theoretical analysis. The degree of covalency involved in general acid−base catalysis is not known accurately, but a covalent bond-order of $^1/_2$ is expected in a proton transfer. The degree of covalency involved in low barrier hydrogen bonding (LBHB), clearly related to the transition state for proton transfer, has been studied. For ionic systems, the bonding above the normal 5 kcal/mol due to electrostatic factors of normal hydrogen bonding is attributed to covalent interactions [45−47]

Conclusions

Proficiency greater than 10^{11} M^{-1} signals covalent interactions between enzyme and transition state; the corresponding transition state is different in the uncatalyzed reaction in aqueous solution and in the enzyme-catalyzed process. Still, a hypothetical equilibrium between the transition state in water and the (altered) transition state in the enzyme can be defined and is $\Delta G_{tx} = RT \ln K_{tx}$.[5] The enzyme assembles catalytic groups in the active site and brings all the noncovalent factors such as electrostatic stabilization, proximity, and environmental factors together to achieve spectacular covalent catalysis not achieved by the same functionality distributed randomly in aqueous solution.

The covalent hypothesis postulated here is supported by available experimental data and has significant implications for the design of drugs and artificial catalysts, such as catalytic antibodies and engineered proteins. The types of noncovalent interactions or dynamic factors involved in enzyme catalysis are of major significance only for that small class of only modestly proficient enzymes with $K_{tx}^{-1} < 10^{11}$ M^{-1} that operate by noncovalent transition state-binding mechanisms.[48] The proficient enzymes with $K_{tx}^{-1} > 10^{11}$ M^{-1} are true chemical catalysts, entering into the reaction to alter the mechanism, in a way that could not have been anticipated by Pauling.

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Supporting Information Available: The reactions catalyzed by the 24 Wolfenden enzymes, literature cited for Table 1 in the text, uncatalyzed reaction rates reported by Wolfenden, intrinsic k_{uncat} abstracted from the 24 Wolfenden enzymes, and PDB codes for the X-ray crystal structure of enzyme−substrate complexes and calculated ΔSASA. This material is available free of charge via the Internet at http://pubs.acs.org.

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