Binding Energy and Catalysis: The Implications for Transition-State Analogs and Catalytic Antibodies

Mary M. Mader and Paul A. Bartlett*

Department of Chemistry, Grinnell College, Grinnell, Iowa 50112-0806, and Department of Chemistry, University of California, Berkeley, California 94720-1460

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Contents

I. Introduction

Transition-state analogs, originally synthesized as enzyme inhibitors, now play an important role as haptens for the generation of catalytic antibodies, and indeed have been critical for the development of this technology. Transition-state theory itself, and the application of transition-state analogs as enzyme inhibitors, have both been reviewed, and a critical analysis of their relevance to catalytic antibodies is now appropriate. Important issues to examine are the disparities between the binding affinities typically observed for transition-state analogs and their theoretical values, and the rate accelerations observed for enzymatic processes and what can be expected for catalytic antibodies. It is also useful to review the criteria that have emerged for designating specific enzyme inhibitors as transition-state analogs.

At the heart of these issues are the forces responsible for stabilizing the transition-state form of the protein−substrate complex in preference to the ground-state complex. While the interactions between the protein and its ligand are likely to be similar for enzymes and antibodies, both the mechanism and the goal of the selection processes by which these proteins are tailored differ significantly.

II. Transition-State Theory and Catalysis

Explanations for the extraordinary power of enzymes to accelerate chemical reactions have been sought ever since this behavior was observed. Modern explanations of the catalytic process date from Haldane's classic treatise on enzymatic activity,[1] through comments made by Pauling in the 1940s,[2,3] and have culminated in the currently accepted view that catalysis of a reaction rests on the enzyme's ability to stabilize the transition-state structure of the substrate relative to that of the ground state.[4−7] Catalysis of a transformation often involves alternative reaction pathways from that of the noncatalyzed transformation, usually taking advantage of an enyme's ability to reduce the molecularity of multistep sequences.

Transition-state theory itself can be traced to the 1930s and the work of Eyring.[8] The theory rests on two basic assumptions: (1) that an "activated complex" in a chemical reaction is formed from the reactant(s) as if in equilibrium with them, and (2) that the rate of the chemical reaction is governed by the decomposition of this activated complex to products. In a typical chemical reaction, the potential energy surface consists of two distinct regions, corresponding to reactant(s) and product(s), separated by an energy barrier at a saddle point on the surface. The term "transition state" is applied to the structure represented by this saddle point, although the "activated complex" more realistically corresponds to any of the vibrational states in the vicinity of this saddle point that cross over from reactant to product. The Eyring equation, eq 1, describes the rate constant k of a chemical reaction in terms of a transmission coefficient κ, the frequency of the normal mode oscillation ν, and a pseudoequilibrium constant $K^{\ddagger}$ for formation of the activated complex from reactants. Except for reactions in which tunneling is involved, κ is close to unity. The value of ν depends on the specific reaction; it is the product of translational, rotational, vibrational, and electronic partition functions, and it reflects the rate at which the activated complex itself decomposes. As a pseudoequilibrium constant, $K^{\ddagger}$ indicates that the population of the activated complex, and hence the reaction rate, bears a relationship to temperature and to the activation

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Mary Mader was born in 1963 in Cleveland, OH. She received her B.S. in Chemistry in 1985 from The Ohio State University. After working for a year as a staff editor at Chemical Abstracts Service, she moved to Notre Dame where she worked under the direction of Paul Helquist. Her doctoral work in synthetic methodology focused on the synthesis of streptogramin A antibiotics, and she received her Ph.D. in 1990. An interest in bioorganic chemistry took her to UC Berkeley to work as an NIH Postdoctoral Fellow with Paul Bartlett, where she studied thioacetals as irreversible inhibitors of the cysteine protease, papain. She also began to think really hard about molecular recognition in proteins of all types, hence her participation in this review. In 1993, she joined the faculty at Grinnell College. Her research program is centered around synthetic methods employing organosilanes and rational modification of the antibiotic, vancomycin.

Paul A. Bartlett received his A.B. and A.M. degrees at Harvard University in 1969 and his Ph.D. from Stanford in 1972, working with W. S. Johnson. After a year as an NIH Postdoctoral Fellow at UC San Diego with S. J. Singer, he joined the Chemistry Department at UC Berkeley, where he is currently Professor and Chair. His research interests involve the application of organic synthesis to the understanding of biological problems, and have encompassed stereocontrolled synthesis, the design of enzyme inhibitors and peptidomimetics, and combinatorial chemistry. His recreational interests have moved from skydiving (which he credits with preserving his sanity as a graduate student and junior faculty member) to scuba diving (the other three-dimensional sport) and cycling. It remains to be seen whether these diversions are powerful enough to maintain whatever sanity is left. Notwithstanding his name, he is not related to anyone whom *Chem. Rev.* readers are likely to know.

energy described by the Boltzmann distribution, eq 2.[9]

$$k = \kappa \nu K^{\ddagger} \tag{1}$$

$$K^{\ddagger} = \mathrm{e}^{-E}a^{/RT} \tag{2}$$

Enzymatic processes are multistep transformations involving, at the minimum, binding of substrate to the enzyme, conversion to the product complex, and then dissociation (eq 3). Enzymes that catalyze multisubstrate transformations involve additional association or dissociation steps; moreover, in many cases, several enzyme-bound intermediates are involved, with interconversions that may be partially rate limiting.[10] The transition-state theory as introduced above is applicable to the simplest cases, in which substrate binding is rapid and reversible ($k_{off} \gg k_{cat}$) and where there is a single chemical transformation (k_{cat}) as the rate-limiting step. In these circumstances, the experimentally determined kinetic parameters, the Michaelis constant, K_m, and maximal rate V_{max}/[E], correspond to the reversible dissociation constant K_s (eq 4) and the first-order rate constant k_{cat}, respectively. The Michaelis equation (eq 5) reduces to a second-order expression at substrate concentrations far below K_m (eq 6) and to a first-order expression at saturating substrate (eq 7).

$$\mathrm{E} + \mathrm{S} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{E{\cdot}S} \xrightarrow{k_{cat}} \mathrm{E{\cdot}P} \rightarrow \mathrm{E} + \mathrm{P} \tag{3}$$

$$K_m = \frac{k_{off} + k_{cat}}{k_{on}} \simeq \frac{k_{off}}{k_{on}} = K_s \qquad \text{when } k_{off} \gg k_{cat} \tag{4}$$

$$v = \frac{k_{cat}[\mathrm{E}][\mathrm{S}]}{[\mathrm{S}] + K_m} \tag{5}$$

$$\text{for } [\mathrm{S}] \ll K_m: \qquad v = \frac{k_{cat}}{K_m}[\mathrm{E}][\mathrm{S}] \tag{6}$$

$$\text{for } [\mathrm{S}] \gg K_m: \qquad v = k_{cat}[\mathrm{E}] \tag{7}$$

Interactions between enzyme and substrate may influence K_m and k_{cat} differently. In the simple case where $K_m = K_s$, K_m corresponds to a bimolecular equilibrium constant (expressed as a dissociation constant), hence it reflects the difference in free energy between enzyme plus substrate free in solution and the Michaelis (ground state) complex. k_{cat} is a unimolecular rate constant and reflects the difference in free energy between the ground-state and transition-state forms of the enzyme−substrate complex. There are logical relationships between K_m and substrate concentration [S], and between k_{cat}, enzyme concentration [E], and the noncatalyzed rate constant, k_{un}, that are important for the discussion that follows. Obviously, k_{cat} must be greater than the noncatalyzed rate constant, k_{un}, for the enzyme to be a catalyst under any circumstances. At low substrate concentrations, (k_{cat}/K_m)[E] must be greater than k_{un} if the enzyme is going to accelerate the transformation of substrate into product. More practically from the point of view of synthetic transformations, for high concentrations of substrate ($>K_m$), unless k_{cat}-[E] is greater than k_{un}[S], the conversion is not accelerated by the enzyme. The enzyme must be efficient enough, in terms both of k_{cat} as well as K_m, to make up for the higher reaction order of the catalyzed versus the uncatalyzed process. We will revisit these concepts, with specific examples, in section V.

There are different ways in which the energy that binds a substrate to an enzyme can be used to accelerate the enzyme-catalyzed transformation, de-

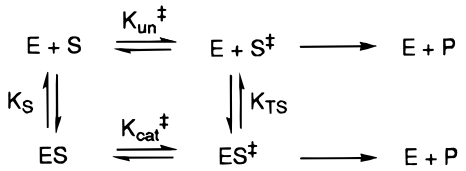


Figure 1. Thermodynamic box illustrating relationship between ground-state and transition-state binding for an enzyme with a single substrate.

pending on the concentration of substrate,[11,12] assuming the association and dissociation steps are not rate-limiting. At low substrate concentration, [S] < K_m, any alteration in enzyme structure that increases the binding interaction between enzyme and substrate will increase the rate of reaction (lowering K_m increases k_{cat}/K_m). However, when [S] > K_m, the enzyme is saturated with substrate and further enhancement in affinity for the ground-state form of the substrate would be without effect. This relationship is readily appreciated by recognizing that the reaction rate at high [S] (eq 7) is independent of K_m. As long as the chemical step (k_{cat}) is rate limiting, any change that leads to an increase in the affinity of the transition-state relative to the ground state will lead to a faster reaction: this difference in binding affinity is directly related to k_{cat}, which appears in both rate expressions eqs 6 and 7. Indeed, preferential binding of the transition-state form of the substrate in comparison with the ground-state form is the central concept in catalysis.

The relationship between transition-state stabilization and enzymatic catalysis is effectively illustrated by the thermodynamic cycle depicted in Figure 1. This formalism originated with Kurz[13] and was later elaborated by Wolfenden[14,15] and Lienhard[16,17] in their reviews of transition-state analogs. The designations E, S, and P refer to enzyme, substrate in the ground-state form, and product, respectively; the transition-state structure is represented by S‡. This thermodynamic box relates the dissociation constant K_S and the hypothetical dissociation constant K_{TS} to the pseudoequilibrium constants $K_{un}^{\ddagger}$ and $K_{cat}^{\ddagger}$ for the noncatalyzed and the enzyme-catalyzed reactions, respectively (eq 8). With the assumption that the values of κ and ν are comparable for the catalyzed and uncatalyzed processes, this equation can be combined with eq 1 to relate the hypothetical dissociation constant for the transition state structure, K_{TS}, to that of the substrate, K_S, by the ratio of the uncatalyzed and catalyzed rate constants, k_{un} and k_{cat}, as reflected in eq 9. The implication of this expression is significant: the transition-state structure is bound to an enzyme with an enhancement in affinity over the ground-state proportional to the increase in the rate constant for the catalyzed over the uncatalyzed reaction.

$$\frac{K_{TS}}{K_{un}^{\ddagger}} = \frac{K_S}{K_{cat}^{\ddagger}} \tag{8}$$

$$K_{TS} = \frac{k_{un}}{k_{cat}} K_S \tag{9}$$

Extrapolation of this analysis to a two-substrate reaction requires introduction of an additional term,

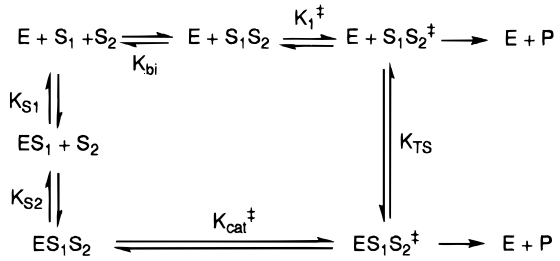


Figure 2. Thermodynamic box illustrating relationship between ground-state, "multisubstrate", and transition-state binding for an enzyme with two substrates.

K_{bi}, the hypothetical dissociation constant of a bimolecular complex of the two substrates (Figure 2, eq 10). This term is required in order to correct for the difference in molecularity between the noncatalyzed and catalyzed reactions. The expressions $1/K_{bi}$ (M^{-1}) and k_1 (s^{-1}) in this case correspond to a dissection of the "observed", bimolecular rate constant for the noncatalyzed process, k_{un} (M^{-1} s^{-1}), into terms that reflect juxtaposition of the two substrate molecules in their ground states and their promotion to the transition state. Enzymatic rate enhancement in this situation results from the entropic advantage of bringing the two molecules together in the active site, in addition to the stabilization of transition state over ground state once they are bound. As eq 11 reveals, this entropic component is also reflected in an additional factor for binding the transition state in preference to the separate substrates; the factor of $1/K_{bi}$ has been estimated by Page and Jencks to be as significant as 10^{-8} M^{-1}.[11] This factor contributes in a similar way to the relationship between the rate constants for the uncatalyzed and catalyzed processes (k_{un} and k_{cat}) and to that between the dissociation constants for the transition state and the individual substrates (K_{TS} and $K_{S1}K_{S2}$, respectively). This analysis (Figure 2) also emphasizes the difference between a transition-state analog and a "multisubstrate" analog. The latter is intended to mimic the combined substrates in their ground state form, and therefore attains a binding advantage over $K_{S1}K_{S2}$ corresponding only to the factor $1/K_{bi}$ (eq 13).

$$\frac{K_{bi}K_{TS}}{K_1^{\ddagger}} = \frac{K_{S1}K_{S2}}{K_{cat}^{\ddagger}} \qquad \frac{K_{bi}K_{TS}}{k_1} = \frac{K_{S1}K_{S2}}{k_{cat}} \tag{10}$$

$$k_{un} = \frac{k_1}{K_{bi}} \tag{11}$$

$$K_{TS} = \frac{k_1}{K_{bi}} \frac{K_{S1}K_{S2}}{k_{cat}} = \frac{k_{un}}{k_{cat}} K_{S1}K_{S2} \tag{12}$$

$$K_{MS} = \frac{1}{K_{bi}} K_{S1}K_{S2} \tag{13}$$

The quantitative relationships embodied in these expressions require that a number of conditions be satisfied. First and foremost is that the noncatalyzed reaction take place by the same mechanism via the same transition state as the enzyme-catalyzed transformation. In reality, this requirement is rarely fulfilled, since most enzymatic mechanisms involve

different catalytic functional groups or follow different paths than their noncatalyzed counterparts in solution. For example, the rate constants for aqueous hydrolysis of a peptide do not reflect a mechanism involving zinc ion catalysis and a carboxylate ion as base, as the solution counterpart to the reactions catalyzed by the zinc peptidases. Second, the chemical step of the enzymatic reaction must be rate limiting, rather than one or more of the association or dissociation steps.[18] For a "sticky" enzyme, whose Michaelis complex is not in equilibrium with the substrates or whose products are slow to dissociate, the rate-limiting step may be a composite of other steps in addition to the chemical step.[19] The practical implication of failure to satisfy these conditions is an underestimation by eqs 9 and 12 of the binding enhancement of the transition state over the ground state. If the "uncatalyzed" process follows a different mechanism than that in the active site, then the rate for the relevant uncatalyzed reaction is slower than that observed in the absence of enzyme and "k_{un}" is overestimated; if the chemical step is faster than the rate of enzymatic turnover, then "k_{cat}" is underestimated.

III. Protein–Ligand Interactions: Forces Available for Binding and Catalysis

A number of factors contribute to the difference in free energy of substrate and enzyme in the unbound (E + S) and bound states (ES), as well as to that between the ground-state and transition-state forms of the enzyme−substrate complex (ES and ES‡, eq 14). The first equilibrium depends on specific interactions between protein and substrate, comprising van der Waals and polar effects in the active site, balanced by the enthalpic and entropic contributions from solvation of both components. The formal equilibrium between ground state E·S and transition state E·S‡ is similarly dependent on ligand−protein interactions, but it is less sensitive to solvation effects since there is little change in solvent-accessible surface at this stage.

$$E_{(solv)} + S_{(solv)} \rightleftharpoons E{\cdot}S \rightleftharpoons E{\cdot}S^{‡} \qquad (14)$$

Estimates of the magnitude of the various forces may be determined from gas-phase measurements with model compounds, but their application to enzyme−substrate equilibria is complicated by the complexity of the molecules involved and the difficulty in compensating for the influence of solvent.[12] Solvation by water gives rise to the hydrophobic effect, for which there is no gas-phase counterpart, and it attenuates drastically the polar interactions which are dominant in the gas phase.[20] While it may not be possible to predict *a priori* the energetic contribution from a specific interaction, these contributions can be determined empirically and with reasonable consistency by comparing systems in which the components differ from each other in a minimal fashion.[21−25]

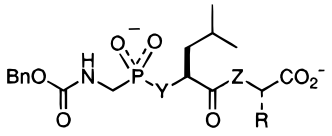
The hydrophobic effect is a stabilization arising from transfer of hydrocarbon surface out of water, typically to the nonpolar interior of a protein or into association with other nonpolar structures. It arises less from attraction of the hydrophobic surfaces for each other and more from the favorable change in free energy as ordered water molecules surrounding the hydrophobic surface are released to bulk solvent.[26,27] As a result, the effect correlates with the area of the hydrophobic surface that is desolvated. A variety of approaches have been taken to determine the magnitude of this effect, and estimates from as little as 25 to as great as 80 cal mol^{-1} Å^{-2} have been proposed.[28,29] Reconciliations between values determined from solvent partitioning and from protein mutagenesis experiments are offered on a regular basis,[30,31] to be corrected in their turn by alternative analyses.[32,33] A value of 25−30 cal mol^{-1} Å^{-2} of desolvated hydrophobic surface appears to be generally accepted.

Although van der Waals interactions contribute significantly to the total energy of a protein−ligand complex, they are often neglected in analyzing contributions to binding equilibria because there is seldom a significant change between the bound and unbound states. van der Waals interaction between elements in the first and second rows of the periodic table is relatively insensitive to the nature of the atoms involved; hence, it does not change significantly on replacing solvent−ligand contacts with solvent−solvent or ligand−protein contacts. Although mismatches between protein and ligand surfaces are possible (for one example, see below), there is usually enough flexibility in the two components that "vacuum" is minimized and van der Waals contact is maintained at a maximum.[34]

Electrostatic and hydrogen-bonding interactions also contribute significantly to the binding affinity between proteins and their ligands, although the effects are greatly moderated by solvation. Indeed, desolvation of a polar moiety on a ligand and the complementary group on a protein may cost as much in terms of enthalpy as is gained by bringing the two groups together.[35] The net balance typically represents a small difference between large numbers.[36,37] The effects are perhaps most significant when there is a mismatch, that is, when an ionic or hydrogen bonding group is desolvated on binding but finds no compensating interaction in the complex. As a result, polar interactions may play less of a role in enhancing association of the correct ligand than they do in creating a penalty for binding the wrong ligand, i.e., in determining ligand specificity.

In spite of the complications from solvation and other effects, there have been a number of attempts to determine the extent to which a hydrogen bond can contribute to binding affinity. These approaches have involved a comparison of the parent system with one in which a hydrogen bonding moiety is replaced with one that cannot. However, because it is impossible to change just one variable in such substitutions, the results observed are complicated by additional factors. The differential binding energies observed in such comparisons vary greatly, ranging from almost zero (phosphonamidate NH vs phosphinate CH_2 in the thermolysin active site,[23] and OH vs H with an uncharged acceptor in tyrosyl-tRNA synthetase[21]), up to 4 to 6 kcal mol^{-1} (O vs NH in thermolysin inhibitors,[23] H vs OH and a charged

donor in tyrosyl-tRNA synthetase,[21] and F vs OH in phosphorylase b substrates[38]). Even for similiar hydrogen-bonding pairs, these values represent small differences between large numbers. For example, the 100-fold gain in affinity ($\Delta G° = -2.7$ kcal/mol^{-1}) on going from the P_1'-P_2' esters **1no** to the amides **1nn** in the thermoysin inhibitors below represents the net of $\Delta G° = +6.8$ kcal/mol^{-1} from the difference in solvation energy (stronger for the amide) and $\Delta G° = -9.5$ kcal/mol^{-1} from the difference in active-site interactions (also stronger for the amide).[23]



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R = H, Me, *i*Bu, Bn

nn: Y = Z = NH

on: Y = O, Z = NH

cn: Y = CH_2, Z = NH

no: Y = CH_2, Z = O

As pointed out above, these assessments of active-site interactions and solvation effects are primarily derived from intermolecular binding studies. For a number of reasons, changes in active-site interactions on going from ground to transition state have seldom been addressed directly. First, more than one substrate is required for this comparison if the contributions from individual functional groups are to be deciphered; there are few systems aside from the peptidases for which information on both K_s (more frequently, K_m) and K_m/k_{cat} is available for a *series* of related substrates. Second, those changes in the substrate molecule that are most directly responsible for the difference in ground-state vs transition-state interactions are usually so significant for the course of the reaction that any alteration in the atoms involved prevents turnover entirely. Effects that are strongly dependent on distance, such as polar and van der Waals interactions, and especially covalent bonding, are crucial for distinguishing ground state from transition state, even though their role in bringing the substrate into the active site is attenuated by solvation. In contrast, the hydrophobic effect, important as it is for overall binding affinity, plays a small role in the catalytic step.

IV. Transition-State Analog Inhibitors: Qualitative and Quantitative Analysis

How much is transition-state stabilization actually worth? And do transition-state analog inhibitors do a very good job in capitalizing on this stabilization? Values of k_{cat}, k_{un}, and K_m for a number of systems are listed in Table 1, along with the dissociation constants calculated for the transition states (K_{TS}) and observed for representative transition state analogs (K_i). While calculated values of K_{TS} range from 10^{-10} to 10^{-24}, for none of the transition-state analog inhibitors is this affinity attained.

There is, of course, a very good reason why the theoretical enhancement in binding affinity is not

Table 1. Catalyzed and Noncatalyzed Rate Constants for Representative Enzyme-Catalyzed Reactions, and Comparison to Transition-State Analog Inhibition Constants[a]

enzyme	k_{un} (s^{-1})	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat}/k_{un}	K_{TS} (M)	inhibitor (ref)	K_i (M)
orotate decarboxylase	2.8×10^{-16}	39	7.0×10^{-7}	5.6×10^{7}	1.4×10^{17}	5.0×10^{-24}	barbituric acid ribotide (39)	$<1 \times 10^{-11}$
adenosine deaminase	1.8×10^{-10}	370	2.6×10^{-5}	1.4×10^{7}	2.1×10^{12}	1.3×10^{-17}	2′-deoxycoformycin (40)	2.5×10^{-12}
acetylcholinesterase[b]	1.1×10^{-8}	1.5×10^{4}	9.4×10^{-5}	1.6×10^{8}	1.4×10^{12}	7.0×10^{-17}	methyl (trimethylammonio)ethyl borinate (41)	3.0×10^{-8}
cytidine deaminase	3.2×10^{-10}	299	1.0×10^{-4}	2.9×10^{6}	1.2×10^{12}	1.1×10^{-16}	phosphapyrimidine riboside (42)	9.0×10^{-10}
carboxypeptidase A[c]	3.0×10^{-9}	52	6.1×10^{-6}	8.5×10^{6}	1.9×10^{11}	3.5×10^{-16}	CbzPheGly{PO_2^-−O}Phe (43)	8.1×10^{-11}
pepsin[d]	3.0×10^{-9}	280	4.0×10^{-5}	7.0×10^{6}	9.3×10^{10}	4.3×10^{-16}	CbzAlaAlaPhe{PO_2^-−O}Phe PP ester (44)	3.4×10^{-10}
triosephosphate isomerase	4.3×10^{-6}	4300	1.8×10^{-5}	2.4×10^{8}	1.0×10^{9}	1.8×10^{-14}	phosphoglycollohydroxamate(45)	4.0×10^{-6}
chorismate mutase	2.6×10^{-5}	50	4.5×10^{-5}	1.1×10^{6}	1.9×10^{6}	2.4×10^{-11}	oxabicyclo[3.3.1]nonene dicarboxylic acid **9** (46)	6.0×10^{-8}

[a] Unless otherwise indicated, enzyme kinetic data taken from ref 47. [b] Substrate data from refs 48 and 49. [c] Substrate Cbz-Phe-Gly-Phe (ref 50). [d] Substrate Cbz-Ala-Ala-Phe-Phe 3-(4-pyridyl)propyl (PP) ester (ref 51).

manifested: no stable molecule can mimic in all details the geometric and electronic characteristics of an unstable transition-state structure, especially with respect to regions where bond cleavage and formation are taking place. A stable mimic of a transition state can only be achieved by replacement of one or more of the atoms with a different element. Although covalent bond lengths and bond angles differ from element to element, there is a limited selection and it does not coincide very well with those anticipated for transition-state structures. However, this is not to say that the transition-state analog concept is without value. Applied appropriately, it provides the conceptual basis for an effective strategy for inhibitor design, and insight into structural and mechanistic details of catalysis.

The majority of TS analog designs have taken advantage of geometric differences between ground and transition states. Many transformations involving carbon entail an interchange between the planar sp^2- and tetrahedral sp^3-hybridizations; hence, stable replacements for enolic or cationic (sp^2) carbons and for tetrahedral adducts (sp^3) have been exploited extensively. In some instances, the electronic characteristics of charged intermediates are important, so stable surrogates for anionic or cationic species have also been sought.

In spite of the plethora of "transition-state analogs" that have been designated as such in the past three decades, as indicated above none has approached the theoretical enhancement in binding affinity that this resemblance should bring. It is usually on a conceptual basis that an inhibitor is classified as a transition-state analog, for example as a reflection of the design process or rationalization of its high affinity, rather than through verification of its actual mode of binding.[52] However, a number of approaches have been described as the basis for a more rigorous characterization of transition state analogy. The observation of binding affinity that is greater than that of the ground state (typically as gauged by the substrate K_m) is not sufficient proof, for many potent inhibitors are known that bind in a very different manner than the transition state. Classic examples are methotrexate, an inhibitor of dihydrofolate reductase with $K_i = 0.15$ pM,[53] and the sulfonyl urea herbicides, some of which bind to acetolactate synthase (ALS) at the picomolar level as well. In spite of the resemblance of methotrexate to dihydrofolate itself, the pterin rings in these two ligands bind in opposite orientations in the active site;[54] and, not only do the sulfonylureas bear no obvious resemblance to the substrate of ALS, it turns out they bind at a vestigial cofactor binding site that happens to be near the active site.[55]

Several high-affinity, transition-state analog inhibitors have been reported to exhibit slow-binding kinetic behavior, which is to say that the rate of formation of the enzyme−inhibitor complex is slower than permitted by diffusion.[56,57] This behavior has been proposed as a criterion for transition-state analogy.[58] But how slow is slow? The generally accepted rate of diffusion-limited encounter between enzymes and typical substrates is on the order of 10^8 M^{-1} s^{-1}. In some instances, slow-binding inhibitors have been observed to bind to their enzymes in two kinetically distinguishable steps, with rapid, reversible formation of a "loose" complex preceding isomerization to a "tightened" complex. It is usually assumed that this isomerization process represents a conformational change on the part of the protein. The initial, loose complex cannot be observed unless the on-rate is determined at concentrations of inhibitor that saturate it, but it is often presumed to exist in every case. That the isomerization process is slower for an analog of the transition state than it is for the substrate is attributed to a mismatch of their structures in the initial encounter complex: the inhibitor cannot trigger the protein conformational change as well as the substrate can.

From another perspective, it is not surprising that inhibitors bind more slowly than substrates, irrespective of their similarity to the transition states. In evolving to facilitate turnover of its substrate to product, an enzyme may acquire the ability not only to accelerate the chemical steps of its reaction, but to speed the entire process, including the substrate association and product dissociation steps.[59] It is likely that different geometric or electronic characteristics would affect the rate at which a molecule loses its conformational freedom, or the path by which it slides into the site, or the ease with which the water molecules that must be displaced are able to dissociate. The correlation that is observed between slow-binding behavior and transition-state similarity could be accidental: it may simply be the case that slow binding is observed more frequently for these analogs because it is more apparent when working at low inhibitor concentrations, as is necessarily the case when determining K_i for a high-affinity ligand.[57,60] Perhaps it would be more appropriate to use "fast-binding" behavior as a criterion for "substrate analogy", rather than the reverse.

More rigorous criteria for transition-state analogy have been proposed. Wolfenden has pointed out that alterations in structure or environment should produce parallel effects on enzymatic rate enhancement and the affinity of a transition-state analog relative to substrate.[48] This relationship is a consequence of the thermodynamic cycle of Figure 1. For an inhibitor, which necessarily is an imperfect mimic of the transition state, and thus for which K_i is clearly different from K_{TS}, the relationship of eq 9 many be translated into eq 15 (equating K_S with K_m), which reflects the proportionality (d), as opposed to equality, between K_i and $K_m(k_{un}/k_{cat})$.

$$K_i = dK_{TS} = dK_m\frac{k_{un}}{k_{cat}} \tag{15}$$

$$\log K_i = \log \frac{K_m}{k_{cat}} + \log dk_{un} \tag{16}$$

Although some studies of the influence of pH on K_i and K_m/k_{cat} have been reported,[61,62] the greatest success in characterizing transition-state analogy has come from analyses of the effects of structural variations, either in the ligands or in the protein. Rewriting eq 15 in logarithmic form and separating the

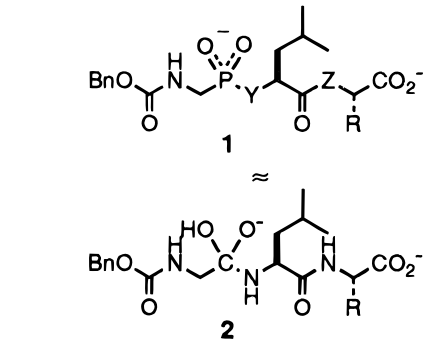
proportionality constant d and the noncatalyzed rate constant k_{un} gives eq 16.

Wolfenden[63] and Thompson[64] were the first to recognize the utility of this expression for evaluating transition-state analogs. If matching structural alterations in a series of inhibitors and substrates are made, such that the alteration does not affect the rate of the noncatalyzed reaction, then a linear relationship with slope of one will be obtained from plotting log K_i against log K_m/k_{cat}, if the inhibitors are transition-state analogs. This relationship holds regardless of the magnitude of the inhibition constants, since the proportionality d from eq 15, along with the unmeasurable k_{un}, become part of the invariant log dk_{un}. For this analysis to be valid, of course, it must also be the case that there is no change in rate-limiting step in the enzyme-catalyzed transformation among the various substrates.

The most extensive applications of this approach have involved the peptidases, because it is for these enzymes that regular variation in substrate structure is most permissible, especially at substrate sites that do not significantly affect the rate of the noncatalyzed reaction. Correlations between inhibitor K_i and substrate K_m/k_{cat} have been demonstrated for the phosphorus-containing peptide inhibitors of thermolysin,[23,65] carboxypeptidase A,[66] and pepsin.[44] The correlations observed for the thermolysin inhibitors already described, the phosphonamidates **1nn**, the phosphinates **1cn**, the phosphonates **1on**, and the esters **1no**, demonstrate many of the points made above. Thermolysin catalyzes the direct addition of a water molecule to the peptide bond, generating a high energy, tetrahedral intermediate **2** (Figure 3). In geometry and in some electronic aspects,[50] the tetrahedral PO_2^- moiety mimics this geminal diol. Each series among these inhibitors shows a slope close to one in the graph of log K_i against log K_m/k_{cat} for the related peptide substrates (Figure 3). Within a given series, the structural variations are at the P_2' residue and should not affect k_{un} significantly, which is an important consideration.

It is important to recognize the meaning of the slope of the line through these data points. It does not represent a measure of how well the inhibitor approximates the transition state; as indicated by eq 16, that metric, d, along with k_{un}, only contributes to the intercept of line. Since the logarithm of an equilibrium constant is directly proportional to the free energy of binding, a slope of 1 in the correlation of eq 16 means that a structural alteration leading to a given incremental change in the binding energy of the transition state produces the same effect in the inhibitors, which is a reasonably intuitive definition of transition-state analogy.

This approach can only gauge the degree to which the *varied* part of the inhibitor structure mimics the corresponding region of the transition state, and it logically depends on the substrates and inhibitors binding in the same fashion across the series. The tightness of the correlation may in fact provide an indication of the degree to which these binding geometries remain constant. The conclusion that can be drawn from each of these correlations is that the P_2' residues interact with the protein active site in



R = H, Me, *i*-Bu, Bn

- **nn**: Y = Z = NH
- **on**: Y = O, Z = NH
- **cn**: Y = CH_2, Z = NH
- **no**: Y = CH_2, Z = O

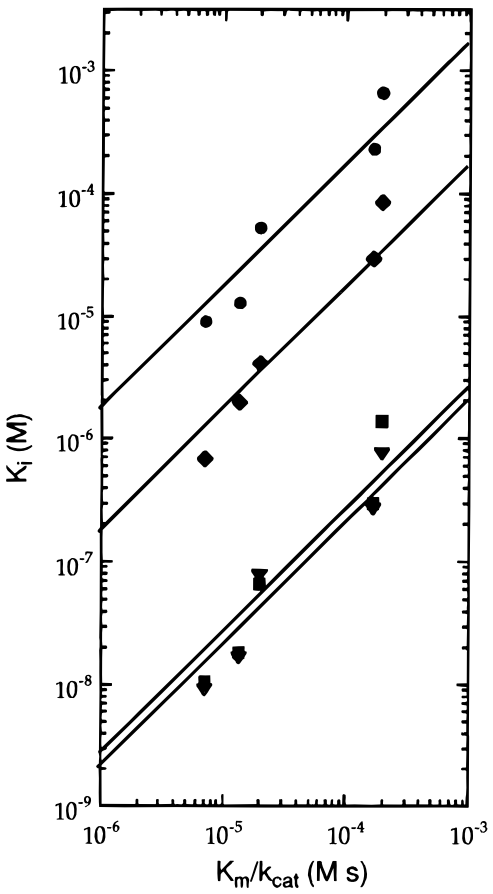


Figure 3. Comparison of K_i values for phosphonate inhibitors of thermolysin with K_m/k_{cat} values for the corresponding substrates.[23,65] The diagonal lines correspond to slopes of 1.

the same manner that they do in the transition state. Except for the geometry of the phosphorus moiety, which presumably allows the P_2' residues to adopt the transition-state orientation, these results do not reveal anything about the transition-state mimicry of any other part of the molecule, since those interactions are a constant feature across each series.

Although the correlations of Figure 3 only probe the geometric similarity of the phosphorus moiety to the tetrahedral carbon, i.e., that characteristic which affects the orientation of the P_2' side chain in

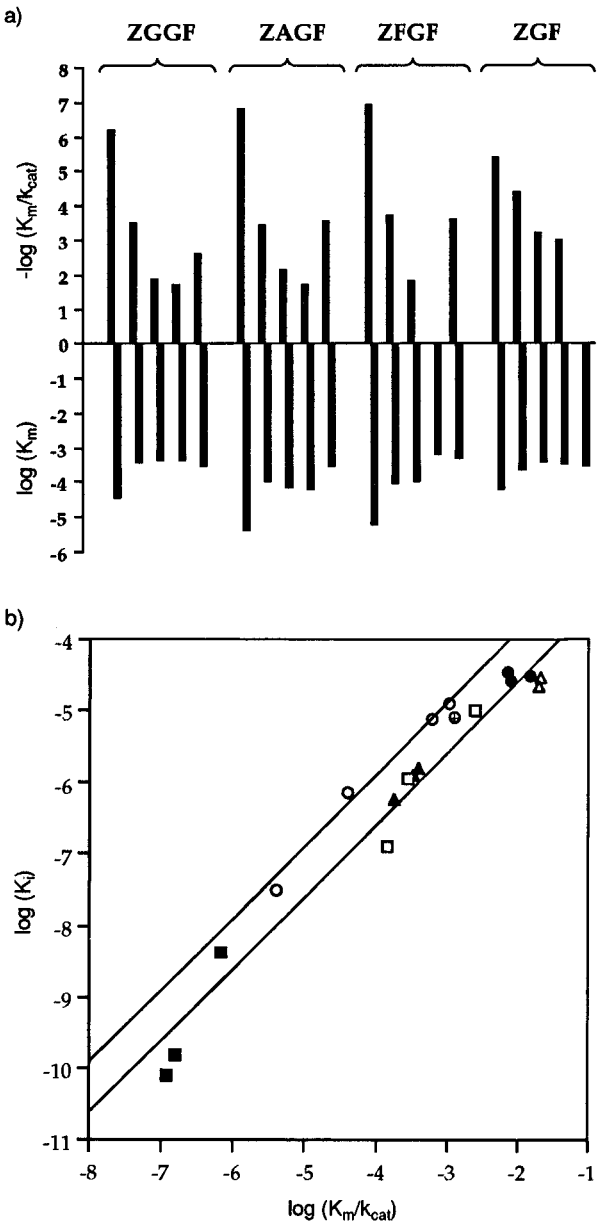
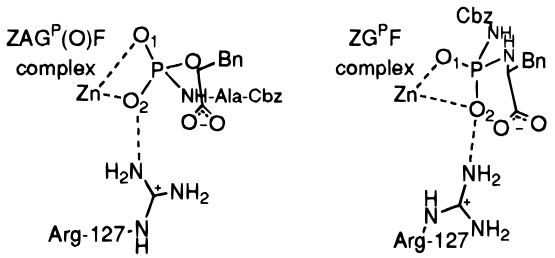


Figure 4. (a) Influence of mutation at Arg^{127} on substrate and transition-state binding to carboxypeptidase A; each group of bars represent, left to right, the wild-type, R127K, R127M, and R127A mutants, and R127A mutant + 0.5 M guanidine hydrochloride, respectively. (b) Correlation of inhibitor K_i and substrate K_m/k_{cat} values for Arg^{127} mutants of carboxypeptidase A. Tripeptide analogs: (■) wild-type enzyme, (▲) R127K, (●) R127M, (△) R127A, (□) R127A in presence of 0.5 M guanidine hydrochloride. Cbz-Gly-Phe analogs: (○) with all enzymes, ⊕ = with R127A mutant in presence of 0.5 M guanidine hydrochloride.

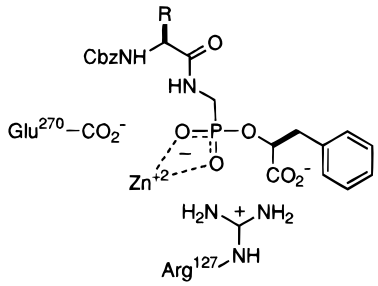
the active site, the electronic similarity was addressed using a series of rat carboxypeptidase A mutants, in which the Arg^{127} residue in the active site was replaced with Lys, Met, or Ala.[50] Mutation of this position has only a modest (~10-fold) impact on binding the ground-state form of the substrate, as revealed by the K_m values (Figure 4), but it has a dramatic effect (up to 10^5-fold) on the enzyme's ability to catalyze hydrolysis, that is, to bind the transition state. The fact that these mutations have the same effect on the K_i values of phosphonate tripeptides **3** indicates that the part of the inhibitor that interacts with the side chain of Arg^{127}, i.e., the phosphorus



Peptidyl phosphonates	Distance (Å) O_1–Zn	O_2–Zn	O_2–N
ZFVP(O)F	2.98	2.19	2.82
ZAAP(O)F	3.07	2.15	2.83
ZAGP(O)F	3.19	1.88	3.02
ZGPF	2.38	3.47	2.64

Figure 5. Differences in complexation geometry of ZXX-{PO_2^-−O}F and ZG{PO_2^-−NH}F to carboxypeptidase A.

oxyanion, does so the same way the carbonyl oxygen does in the transition state, thus demonstrating that the phosphonyl moiety mimics the electronic characteristics of the transition state as well.



3: R = H, Me, Bn

This study also provides insight into the strength and the nature of the active-site interactions that distinguish the transition state from the ground state. The amino acid side chain at position 127 exerts some effect on ground-state binding: mutation of Arg^{127} to lysine increases K_m for ZAGF by 25-fold (2 kcal/mol); further alterations to methionine or alanine do not have any additional effect. The effects on k_{cat} are much more significant: there is a 100-fold (2.8 kcal/mol) decrease in the ability of the Lys^{127} mutant to turn over this substrate in comparison to the wild-type (Arg^{127}) enzyme, and an even greater decrease in going to the methionine and alanine mutants: Arg → Met = 4.7 kcal/mol and Arg → Ala = 5.5 kcal/mol. Similar effects are seen for the ZGGF and ZFGF substrates, but the impact of these mutations on binding and turnover of the dipeptide ZGF is considerably attenuated. X-ray crystallography of the corresponding tetrahedral phosphorus transition-state analogs reveals the basis for this discrepancy by showing that these molecules adopt different orientations in the active site, such that the phosphorus oxyanion is situated differently with respect to the Arg^{127} (or other) side chain. However, this difference is subtle, as shown by the relatively small differences in interatomic distances in the complexes of carboxypeptidase A with ZAG{PO_2^-−O}F and ZG-{PO_2^-−NH}F (Figure 5).[67−69]

Rahil and Pratt have described an interesting thermochemical approach to characterizing the transition-state analogy of inhibitors that become co-

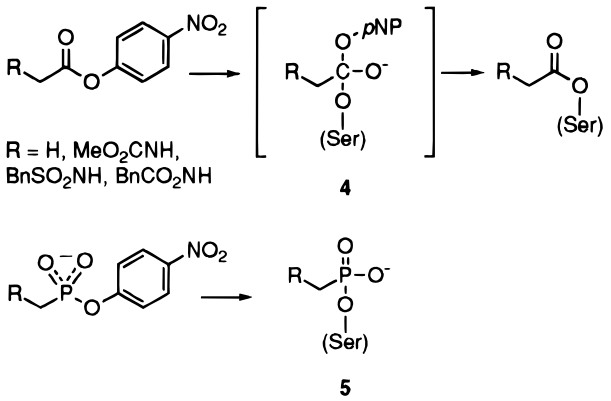
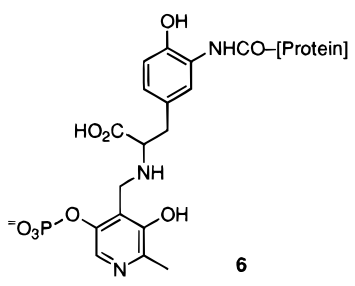


Figure 6. Relationship between the phosphonylated adducts, **5**, of the serine hydrolase PC1 β-lactamase and the tetrahedral intermediate for acylation of the enzyme, **4**.

valently bound to their target enzyme.[70] The *Staphylococcus aureus* PC1 β-lactamase hydrolyzes glycine esters by an acylation−deacylation mechanism, and it is inactivated by electrophilic phosphonate analogs that bind covalently to the active site serine (Figure 6). Rahil and Pratt used thermal denaturation to probe the strength of the *non*covalent interactions between the enzyme and the phosphonate moiety in **5**, and related these values to the noncovalent interactions present in the transition state **4**, which can be derived from the dependence of k_{cat}/K_m on the substrate structure. Phosphonylation of the nucleophilic serine stabilizes the protein against denaturation, and the effect of different inhibitors correlates well enough with the rate of hydrolysis of the substrates to demonstrate that the inhibited enzyme adducts are valid models of the transition state.

V. Transition-State Analogs and Catalytic Antibodies

The suggestion that proteins which bind transition-state analogs strongly should be able to catalyze chemical reactions is a logical consequence of the argument that transition-state analogs should bind tightly as enzyme inhibitors.[71] By generating an antibody to a transition-state analog, a protein may be created that can bind the transition state more tightly than the substrate or products and thus accelerate their interconversion. The first attempt to reduce this idea to practice in 1975 involved the generation of antibodies complementary to the tyrosyl pyridoxamine derivative **6**;[72] however, the polyclonal proteins isolated showed little ability to catalyze formation of the corresponding Schiff base between tyrosine and pyridoxal phosphate. Successful demonstration of this approach had to await the advent of methods for generating monoclonal antibodies as a source of homogeneous proteins.



Enzymes and antibodies are both proteins that develop strong, reversible interactions with small molecules, the former in order to transform the substrate to product and the latter as a result of immune system activation. Antibodies that behave like enzymes can be elicited by immunization with a transition-state analog, which is rendered immunogenic by coupling to a carrier protein that triggers the immune response. As a logical extension of the concepts that led to the development of transition-state analogs as enzyme inhibitors, catalytic antibodies owe their activity to the same principles that govern enzymatic catalysis. Indeed, many of the known motifs for transition-state analog design have been utilized as haptens in the generation of catalytic antibodies. Among the most extensively investigated, for example, are the phosphate and phosphonate analogs that mimic the tetrahedral intermediates in a variety of ester and amide hydrolysis mechanisms. However, the field of catalytic antibodies has extended beyond simple mimicry of known enzymatic processes, with the development of catalysts for transformations that have no biological counterpart.[73] There is practical as well as theoretical interest in such catalysts, not only to facilitate unusual transformations but also to induce substrate selectivites that are different from related enzyme-catalyzed reactions.[74−76]

In spite of the logical basis for their activity, no catalytic antibody has yet been developed that approaches the efficiency of an enzyme, when compared on equal terms.[77,78] A number of strategies have been evaluated in attempts to reach this benchmark, and some of the limitations are beginning to become apparent. Does this situation reflect an inherent limitation in the field, or simply our lack of experience in applying the principles? And, does it matter in any practical sense whether a catalytic antibody is as efficient as an enzyme?

The same panoply of polar and nonpolar interactions are available to bind haptens to antibodies as bind substrates to enzymes, so there should be no fundamental limitation to the ability of catalytic antibodies to accelerate their reactions. Or is there a difference? Rate accelerations of up to 10^7-fold, calculated as k_{cat}/k_{un} have been reported for some hydrolytic antibodies,[79,80] but few approach this number; rate enhancements on the order of $10-10^3$-fold are more typical,[81] less than those of enzymes that catalyze comparable transformations. In this section, we explore some of the possible reasons for this disparity, both from a thermodynamic as well as a structural perspective. Among the points to consider in comparing antibodies to enzymes are the manner in which their rate accelerations are computed, the way in which the proteins interact with their ligands, and the evolutionary pressures that govern their selection processes. How active a catalyst actually must be to be useful in a practical application is a related issue.

A. Kinetic Issues

Most catalytic antibodies, Ab, have been shown to follow traditional Michaelis−Menton kinetics for conversion of substrate, S, to product, P (eqs 17 and 18), at least for the outset of the reaction. The

$$\mathrm{Ab} + \mathrm{S} \underset{K_m}{\rightleftharpoons} \mathrm{Ab{\cdot}S} \xrightarrow{k_{cat}} \mathrm{Ab} + \mathrm{P} \qquad (17)$$

$$V = k_{cat}\frac{[\mathrm{Ab}][\mathrm{S}]}{[\mathrm{S}] + K_m} \qquad (18)$$

assumptions inherent in applying these equations to enzymes are also appropriate for their application to catalytic antibodies, for example that antibody concentration is small in comparison to substrate ([Ab] ≪ [S]). Validation of the antibody mechanism is generally sought by showing that the transition-state analog used to elicit the antibody inhibits catalysis in a competitive fashion, and, moreover, binds with a higher affinity than substrate.[82] Substrate binding to catalytic antibodies is usually assumed to be reversible on the time scale of catalysis, so the Michaelis constant K_m is used for comparison with the hapten inhibition constant K_i.

The kinetic constants of Table 2 are derived from a representative set of antibody-catalyzed reactions (a more extensive compilation is found in Thomas' review[81]). Comparisons with Table 1 provide insight into how substrate binding and catalysis differ for antibodies and enzymes. The Michaelis constant, K_m, in lieu of K_s, is taken as a measure of the affinity of the protein binding site for the substrate, for both enzymes as well as antibodies. There is no general difference between the catalysts with respect to substrate binding; both antibodies and enzymes exhibit K_m values from the micromolar to millimolar ranges.

Comparison of the k_{cat} values is more difficult, however. For enzymatic reactions, the first-order k_{cat} value reflects the difference in energy between the ground-state and transition-state complexes, comprising the changes in protein−ligand interaction energies as well as the higher energy of the partially bonded transition-state structure itself. Many antibody-catalyzed hydrolyses, however, differ from their enzymatic counterparts in that the chemical steps involve attack of hydroxide on the antibody−substrate complex;[89,98] the rates of these reactions are second order, depending both on [Ab·S] and [OH^-]. "k_{cat}" values that are given for these transformation in units of time^{-1} thus represent pseudo-first-order rate constants, which cannot be compared directly to enzymatic k_{cat} values. The pseudo-first-order "k_{cat}" values reported for the second-order antibody-catalyzed processes reflect, in addition to the difference between ground- and transition-state energies, the concentration of hydroxide ion and its association with the substrate−antibody complex.[12] Comparisons made between enzyme- and antibody-catalyzed reactions (k_{cat} vs k_{cat}) or between catalyzed and uncatalyzed processes (k_{cat} vs k_{un}) have to be made on the same basis, as exemplified by the approach of Jacobs et al. in which $V_{Ab} = 1k_{Ab}[OH^-][Ab{\cdot}S]$ is compared to $V_{OH^-} = k_{OH^-}[OH^-][S]$.[99] This complica-

Table 2. Catalyzed and Noncatalyzed Rate Constants for Representative Antibody-Catalyzed Reactions, and Comparison to Hapten Inhibition Constants[a]

antibody (function)	k_{un} (s^{-1})	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat}/k_{un}	K_{TS} (M)	hapten/inhibitor (ref)	K_i (M)
38E1 (phosphatase)	2.5×10^{-9}	0.00002	1.6×10^{-4}	0.13	8.0×10^{3}	1.9×10^{-8}	α-hydroxybenzylphosphonic acid (83)	3.4×10^{-5}
NPN43C9 (anilidase)	6.0×10^{-9}	0.00083	3.7×10^{-4}	2.2	1.5×10^{5}	2.7×10^{-9}	benzylphosphono-*p*-nitroanilidate (84)	8×10^{-10}
13D11 (amide hydrolase)	1.3×10^{-9}	1.7×10^{-7}	4.3×10^{-4}	3.9×10^{-4}	1.3×10^{2}	3.3×10^{-6}	phenylalanine phosphinate (85)	1.4×10^{-5}
14D9 (epoxide cleavage)	3.7×10^{-8}	2.5×10^{-5}	2.5×10^{-5}	1.0	6.7×10^{2}	3.8×10^{-8}	quarternary piperidinium (86)	2×10^{-6}
43D4−3D12 (elimination)	3.5×10^{-8}	0.0031	0.18	1.7×10^{-2}	8.8×10^{4}	2.0×10^{-6}	tertiary benzylamine (87)	2.9×10^{-7}
64D8E10 (biphenyl isomerization)	6.5×10^{-8}	4.3×10^{-5}	4.2×10^{-4}	0.1	6.6×10^{2}	6.4×10^{-7}	dihydrophenanthrene (88)	2.1×10^{-7}
2H12E4 (esterase)[b]	1.2×10^{-6}	0.00031	1.5×10^{-5}	21	2.7×10^{2}	5.6×10^{-8}	tripeptide phosphonate (89)	2.4×10^{-6}
11F1−2E11 (mutase)[c]	4.5×10^{-6}	0.045	2.6×10^{-4}	1.7×10^{2}	1.0×10^{4}	2.6×10^{-8}	oxabicyclic diacid **9** (90)	6×10^{-7}
1F7 (mutase)[d]	6.3×10^{-6}	0.0012	5.1×10^{-5}	24	1.9×10^{2}	2.7×10^{-7}	oxabicyclic diacid **9** (91)	9×10^{-6}
50D8 (esterase)	3.2×10^{-6}	20	1.5×10^{-3}	1.3×10^{4}	6.3×10^{6}	2.5×10^{-10}	benzylphosphonate aryl ester (92)	5×10^{-7}
19F3.1 (thiol S-transferase)	1.2×10^{-6}	0.0022	1.3×10^{-2}	0.17	1.8×10^{3}	7.2×10^{-6}	4-carboxybenzenesulfonamide (93)	4.2×10^{-6}
3B9 (cocaine hydrolase)	3.3×10^{-6}	0.0018	4.9×10^{-4}	3.7	5.4×10^{2}	8.8×10^{-7}	methyl ecgonine phenylphosphonate (94)	$<2 \times 10^{-6}$
34E4 (decarboxylase)	3.1×10^{-5}	0.66	1.2×10^{-4}	5.5×10^{3}	2.1×10^{4}	5.6×10^{-9}	benzimidazole (95)	nr[e]
18C10 (peptide ligase)[f]	1.1×10^{-3} M^{-1} s^{-1}	0.21	4.0×10^{-3} (RCOX) 1.6×10^{-3} (H_2NR')	52 (RCOX) 1.3×10^{2} (H_2NR')	198 M	3.4×10^{-8}	dipeptide phosphonamidate **17** (96)	nr
7D4 (Diels-Alderase)	2.9×10^{-6} M^{-1} s^{-1}	2×10^{-5}	2.7×10^{-3} (diene) 1.0×10^{-2} (d-phile)	7.4×10^{-3} (diene) 2.0×10^{-3} (d-phile)	6.9 M	3.9×10^{-6}	ferrocenyl carboxamide **15** (97)	4.8×10^{-5}

[a] Unless otherwise indicated, antibody kinetic data taken from ref 81. [b] k_{un} determined as $k_{OH^-}[OH^-] = 7 \times 10^{-5}$ min^{-1} at pH 8. [c] Evaluated at 10 °C (ref 90). [d] Evaluated at 25 °C (ref 91). [e] nr = not reported. [f] Substrates: *N*-Acetyl-L-valine p-nitrophenyl ester and L-tryptophan amide.

Table 3. Practical Implications of Catalysis by Enzymes and Catalytic Antibodies[a]

catalyst	k_{cat} (s^{-1})	K_m (μM)	k_{un} (s^{-1})	[Catalyst]$_0$ (μM)[b]	$t_{1/2}$ (h)[c]
carboxypeptidase A	578	89	3.0×10^{-9}	5.3×10^{-9}	0.000 024
adenosine deaminase	370	26	1.8×10^{-10}	4.8×10^{-7}	0.000 037
chorismate mutase	50	45	2.6×10^{-5}	0.053	0.000 28
acetylcholinesterase[d]	15 000	94	1.1×10^{-8}	7.3×10^{-7}	9.2×10^{-7}
7G12 (redox)	6.6	24 000	2.8	10 000[e]	0.07[f]
CPD32A11 (decarboxylase)	4.7	100 000	2.4×10^{-5}	0.51[e]	0.4[f]
6D4 (esterase)	0.027	2	2.8×10^{-5}	1.0	0.51
11F1−2E11 (chorismate mutase)	0.004 5	260	4.5×10^{-7}	5.7	3.1
1F7 (chorismate mutase)	0.001 2	51	4.8×10^{-5}	4.0	12
27H9 (esterase; *m*-NO_2-Bn ester)[g]	0.007 8	1 500	5.6×10^{-9}	1.1×10^{-9} [e]	3.7[f]
AA71.17 (glycosidase)[h]	0.000 28	180	4.4×10^{-6}	16	50
NPN43C9 (anilidase)	0.000 83	370	6.0×10^{-9}	0.007	17
2H12E4 (depsipepidase)	0.000 03	15	1.1×10^{-7}	3.7	460
64D8E10 (biphenyl isomerization)[i]	0.000 043	420	1.5×10^{-8}	0.14[e]	187
14D9 (epoxide cleavage)	0.000 025	25	3.7×10^{-8}	1.5	550
13D11 (amide hydrolase)[j]	1.7×10^{-7}	430	1.3×10^{-9}	7.6	1.4×10^{5}

[a] Unless otherwise indicated, enzyme kinetic data taken from Radzicka & Wolfenden;[47] antibody kinetic data taken from Thomas.[81] [b] Breakeven concentration of catalyst for overall rate to exceed background rate if [S] = 1 mM and K_m < 1 mM; [Catalyst]$_0$ = [S](k_{uncat}/k_{cat}). [c] $t_{1/2}$ (h) for conversion of [S] = 1 mM and [Catalyst] = 10 μM; for K_m < 1 mM, $t_{1/2}$ is calculated from relationship $t_{1/2} = 0.5$ mM/($k_{cat} \times 10\ \mu$M)(1/3600). [d] Substrate data from refs 48 and 49. [e] Since [S] = 1 mM is less than K_m, [Catalyst]$_0$ = $K_m(k_{un}/k_{cat})$. [f] Since [S] = 1 mM is less than K_m, $t_{1/2} = 0.69K_m/(k_{cat}$[Cat]3600). [g] Data from Li et al.[105] [h] Data from Yu et al.[106] [i] Data from Uno et al.[88] [j] Data from Titmas et al.[85]

tion does not apply in the case of the Claisen rearrangements catalyzed by the chorismate mutases,[90,100−102] since these transformations, including the uncatalyzed reaction, are unimolecular. For these catalysts, therefore, k_{cat} values can be compared directly and translated to specific differences between ground and transition state energies.

Comparison of the second-order (or pseudo-second-order) rate constants k_{cat}/K_m for the enzymatic and antibody reactions is complicated by the same issues that intrude on comparisons of k_{cat}, but they do provide a practical assessment of overall catalyst efficacy. As indicated in the introduction, a protein-catalyzed reaction is necessarily a higher-order process than the uncatalyzed transformation; hence, there is a minimum concentration at which the catalyst must be employed if it is to exceed the inherent background rate and affect the overall rate or path of the transformation. This minimum is easily specified for the two limiting cases in which [S] $\gg K_m$ (saturated catalyst) and [S] $\ll K_m$ (catalyst not saturated): eqs 19 and 20.

for [S] $\gg K_m$, catalyzed rate > noncatalyzed rate if

$$k_{cat}[\mathrm{Cat}] > k_{un}[\mathrm{S}] \Rightarrow [\mathrm{Cat}] > [\mathrm{S}]\frac{k_{un}}{k_{cat}} \qquad (19)$$

for [S] $\ll K_m$, catalyzed rate > noncatalyzed rate if

$$\frac{k_{cat}}{K_m}[\mathrm{Cat}][\mathrm{S}] > k_{un}[\mathrm{S}] \Rightarrow [\mathrm{Cat}] > K_m\frac{k_{un}}{k_{cat}} \qquad (20)$$

The biphasic aspect of this lower limit for catalysis is illustrated in Figure 7.

For the sake of comparison of biocatalysts, 1 mM is taken as a practical lower limit on the concentration of substrate in a preparative reaction. Table 3 gives an indication of the concentration of protein above which the catalyzed transformation is faster than the background rate of conversion, according to eqs 19 or 20, whichever is applicable. For further interest, an additional column lists the half-life, $t_{1/2}$, that would be observed for conversion of 1 mM substrate to product at an catalyst concentration of 10 μM. Better than comparisons of k_{cat}/k_{un} or k_{cat}/K_m values, these numbers give an indication of how practical (or impractical) it would be to use the protein as a catalyst to actually carry out a reaction. It should not be overlooked that 10-fold acceleration above the background rate requires a protein concentration 10-fold that of the break-even number, and that a 10 μM concentration of antibody combining sites represents 7.5 mg/mL of protein. These limitations are illustrated by the published examples in which catalytic antibodies have been used preparatively.[103,104]

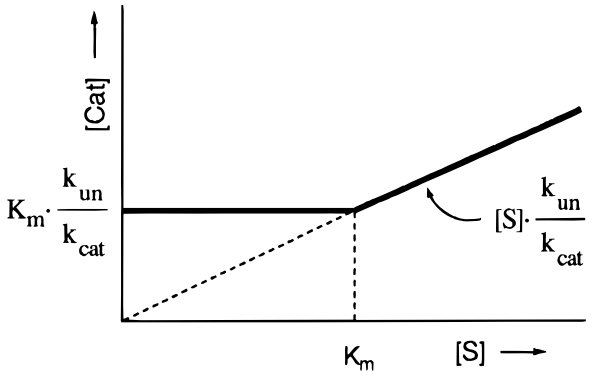


Figure 7. Breakeven concentration of catalyst, [Catalyst]$_0$, at which the catalyzed rate equals the background rate; acceleration of the overall rate occurs when [Catalyst] exceeds this value, in region above the solid line.

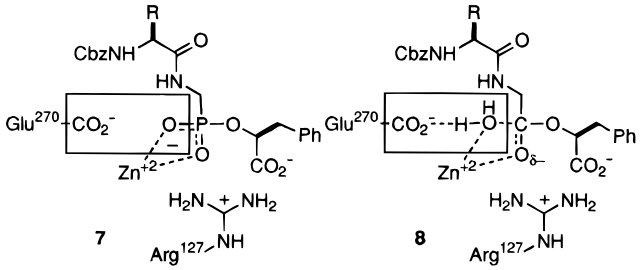
The comparisons of Tables 2 and 3 indicate that catalytic antibodies are limited in their ability to accelerate reactions. Enzymatic k_{cat}/K_m values are typically in the range of 10^5 to 10^8 M^{-1} s^{-1},[47] but for the majority of antibodies, this value ranges from 1 to 10^3 M^{-1} s^{-1}.[81] Are there inherent reasons why antibodies do not do better? Slow release of product from the binding site, k_{off} (as distinct from product inhibi-

tion as an equilibrium effect) has been suggested for a hydrolytic enzyme,[107] but it does not appear to be a general explanation for the slow turnover of Ab·S complexes. Product inhibition may be a more common limitation for ligase-type catalysts, for which the product is likely to bind more tightly than the substrates,[108,109] but it is not responsible when slow turnover is observed at the outset of the reaction. More critical reasons for the poor performance of TS analog-based catalytic antibodies may be (1) our inability to design haptens that can induce the required active-site geometry and functionality; (2) a breakdown in the relationship between substrate binding and catalysis; and (3) fundamental limitations in the biological process that produces the antibodies. We address each of these possibilities in turn.

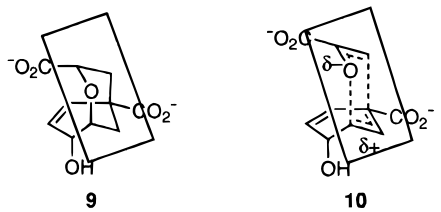
B. Structural Issues

1. Shortcomings in Hapten Design

Our imperfect ability to devise a stable mimic for the fleeting structure of a transition state will always be a limitation. The fractional bond orders, extended bond lengths, expanded valences, distorted bond angles, and high degrees of charge separation in transition state structures cannot be reproduced in any stable organic molecule. Even the "best" transition-state analog enzyme inhibitors fall short of this goal. Phosphonate peptide analogs are potent inhibitors of zinc and aspartic peptidases, and as discussed above, they have been shown to mimic the relevant transition states in some geometric respects,[23,66] and, for one enzyme, in the electronic character of one of the oxyanions.[50] However, there are obvious shortcomings; for example, the unprotonated, anionic nature of the other oxyanion in the phosphonate is the reverse of the proton-donating, partially cationic water molecule in the transition state (**7** vs **8**). The

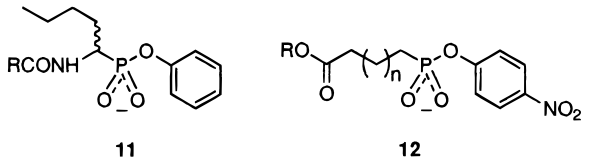


oxabicyclic diacid **9** that inhibits the chorismate mutases[46] is much more compact than the expanded transition state **10** and does not emulate its charge separation.[110] Clearly, we cannot expect even a faithful complement of an imperfect template to compete with an enzyme optimized to bind the true transition state.



Although the general structural features of immunoglobulins are well known, detailed structures are available for relatively few transition state analog−antibody complexes.[101,108,111−114] Selective recognition of antigens is achieved through interactions similar to those involved in enzyme-substrate binding, and include van der Waals interactions, hydrogen bonding, and other electrostatic and hydrophobic effects. However, enzymes are often distinguished from antibodies by covalent bonding to substrates, by the presence of exogenous functional groups such as metals and cofactors, and by the concentration of highly polar side chains near the reacting center of the substrate. Indeed, while an antibody can use an ensemble of weak interactions to bind its antigen with adequate affinity, only enzymes appear to be capable of marshalling the strong forces, with high gradients over short distances, that are necessary to distinguish the subtle differences between ground states and transition states.

The structures of hydrolytic antibodies elicited by three different phosphonate aryl ester haptens are revealing both for their similarities and differences, and in comparison with the enzymes that they imitate. All three antibodies: 17E8, from **11**;[111] CNJ206, from **12** ($n = 1$);[113] and 48G7, from **12** ($n = 2$),[108] bind the aryl ester moieties of their haptens in deep pockets that are lined with aromatic and hydrophobic residues (Figure 8). Moreover, in each



case the anionic phosphonate is found near the entrance of the combining site, interacting with backbone NH groups and different polar side chains. For each antibody, the cluster of polar groups in the vicinity of the phosphonate explains why the anionic, tetrahedral hapten binds with higher affinity than the neutral, planar substrate, and in turn why the transition state is stabilized. One of the key functional groups that may be involved in the catalytic mechanism is His-35 on the heavy chain, one of the residues that is found in all three antibodies. Although its precise role is still not defined, His-35H must be fairly accommodating, since the 17E8-catalyzed reaction proceeds via an acyl−antibody intermediate while the others involve direct attack by water or hydroxide. In antibody 29G11, which was raised from **11** by the same immune response as 17E8, the nucleophilic amino acid (serine-99 in the heavy chain) is replaced with a glycine, yet the antibody retains almost 20% of the activity (measured as k_{cat}/K_m).[112]

One of the obvious differences between the antibody combining sites and the active sites of hydrolytic enzymes is the extent to which the reactive center is exposed to the exterior. While the phosphonate moieties of the bound haptens are near the entrances of the antibody combining sites, in the peptidases the catalytic machinery and the tetrahedral center are buried. By sequestering the reacting groups from the high dielectric environment of the solvent, the enzyme is able to enhance the effect of polar interac-

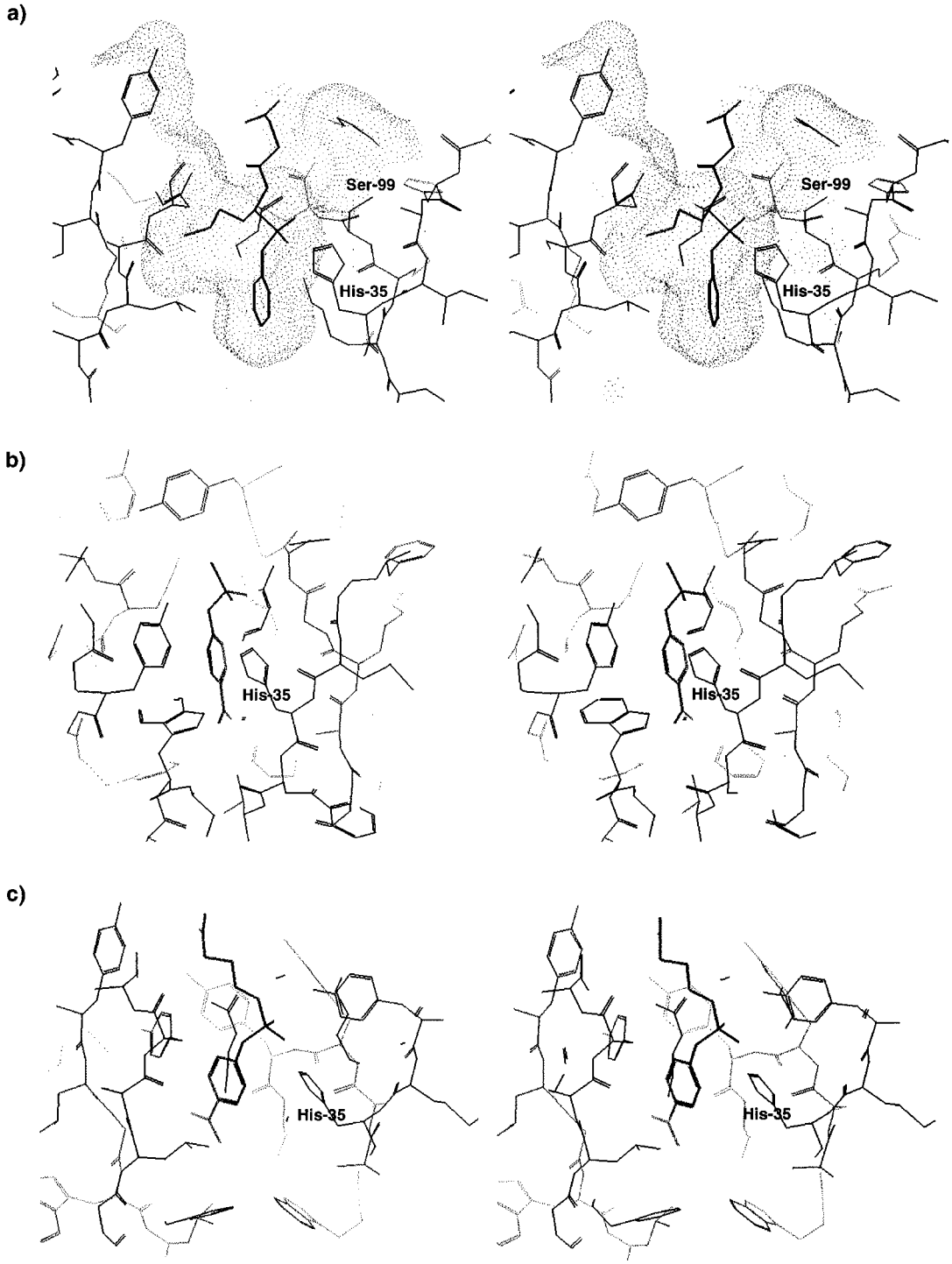
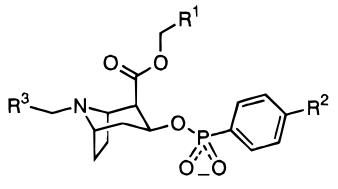


Figure 8. Diagram of active sites of three hydrolytic enzymes complexed to their phosphonate haptens: (a) 17E8 with **11** ($R = {}^{-}O_2CCH_2CH_2$);[111] (b) CNJ206 with **12** ($n = 1$, R = H);[113] and (c) 48G7, with **12** ($n = 2$, R = H).[108]

tions that develop in the transition state.[115] The relatively exposed position of the phosphonate moiety in the antibody−hapten complexes may be a consequence of hapten design: the linear connection of [aryl ester]−[phosphonate moiety]−[linker]−[immunogenic protein] makes the aryl ester the more accessible group for antibody recognition. Perhaps a hapten design in which the aryl ester is between the phosphonate and the immunogenic protein (e.g., [phosponate moiety]−[aryl ester]−[linker]−[immunogenic protein]) would elicit antibodies that bury the phosphonate instead of the aromatic ring. Indeed, the point of attachment of the hapten **13** (R^1, R^2, or R^3) to the immunogenic protein was shown to have a significant effect on the binding and catalytic activities of anti-cocaine catalytic antibodies raised against the conjugates.[116]



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The complexes of the oxabicyclic diacid **9** with two chorismate mutase enzymes[100,117] and one catalytic antibody (1F7)[101] provide one of the most relevant opportunities for direct structural comparisons between enzymes and antibodies. The mutase reaction is unusual in a number of respects; first, as a rearrangement, the uncatalyzed reaction has the same molecularity as that in the active site; second, no covalent linkages appear to form between the enzymes and the substrate in the course of catalysis, and, finally, no cofactors are involved. In all three of the complexes, the inhibitor, and presumably the substrate, are almost completely engulfed by the protein. The negatively charged carboxylates of **9** are major points of recognition in each case, with extensive hydrogen-bonding networks and/or salt bridges to cationic residues in the active sites. The enzymes are also hydrogen bonded to the allylic hydroxyl group of the inhibitor. Because this hydroxyl was the linkage site for tethering the hapten to the immunogenic carrier protein, it is exposed to solvent when **9** is bound to the catalytic antibody. Overall, although each active site interacts with the inhibitor with a different collection of hydrogen bonds and other contacts, the end result is about the same: the dissociation constants of **9** from the three proteins fall within the range 0.6−3 μM.[101,118]

However, the chorismate mutase enzymes are much better catalysts ($k_{cat}/k_{un} \approx 10^6$) than the antibody 1F7 ($k_{cat}/k_{un} = 200$). Can we deduce from their structures why the enzymes complement the actual transition state better than the antibodies do? Both theory and experiment show that the transition state for the Claisen rearrangement is highly dissociative,[118,119] with bond cleavage preceding bond formation and with the build up of considerable positive charge on the allylic moiety and negative charge on the enol group. Wiest and Houk have analyzed in detail the extent to which the neutral, fully bonded framework of hapten **9** falls short of imitating the actual transition state.[110] When viewed from this standpoint, the two enzyme active sites are clearly constructed to complement the expanded, polarized transition state.[102] They make less van der Waals contact with the inhibitor than does the antibody, particularly in the vicinity of the bridging methylene group, consistent with the view that the enolpyruvyl moiety is further from the cyclohexadienyl ring in the transition state. The enzymes position cationic side chains near the ether oxygen (arginine or lysine) and anionic (glutamate) or polarizable (cysteine) groups beneath the allylic region; these arrays complement the charge separation that develops in the transition state. In this respect, the antibody is quite different;[101] there are no residues similarly positioned that can stabilize a polar transition state, and the smaller dimension of the active site could actually impede the expansion of the substrate.

While the crystal structures of these complexes provide a basis for qualitative interpretations, it is more difficult to rationalize the unusual thermodynamic differences between the enzyme- and antibody-catalyzed processes. Whereas the enzymes lower both the entropic as well as enthalpic components to the activation barrier, the rate acceleration of the reaction catalyzed by antibody 1F7 is entirely enthalpic; indeed, the latter transformation is *less* favored entropically than the uncatalyzed rearrangement.[120]

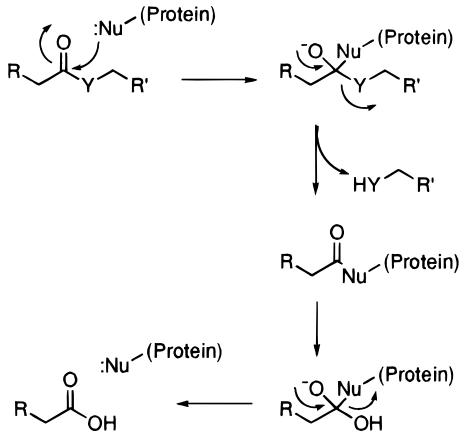
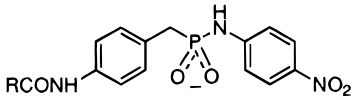


Figure 9. Ping-pong mechanism for acyl transfer.

This example points out the shortcomings of the oxabicycle **9** as a hapten for a catalytic antibody. While it captures some of the geometric characteristics of the transition state, this molecule is unable to induce the polar residues that are key to the enzymatic stabilization of the transition state. It will be interesting to see what the structure of the more active chorismate mutase antibody 11F1-2E11[90] reveals, since this catalyst is much closer to the enzymes in both the degree ($k_{cat}/k_{un} = 10^4$) and the nature ($\Delta H^{\ddagger} = 18.3$ kcal/mol, $\Delta S^{\ddagger} = -1.2$ eu) of the catalysis.[121]

2. Fidelity of the Replica

While the fidelity with which the hapten imitates the transition state is one issue, another is the fidelity with which the antibody combining site complements the hapten. For example, in a number of cases the mechanism of the antibody-catalyzed process is quite different from that for which the transition-state analog was designed. The phosphonamidate **14** and phosphonate **15** give rise to antibodies that are highly active in ester hydrolysis and transesterification, respectively. However, both antibodies have been shown to involve a ping-pong mechanism (Figure 9), with initial acylation of a nucleophilic residue in the combining site followed by deacylation by attack of hydroxide.[80,107] As mentioned above, phosphonate **11** elicits antibodies that are closely related in structure but operate via different mechanisms: 17E8 involves an acyl−antibody intermediate, while 29G11 does not.[111,112,122] In both steps of the acylation−deacylation mecha-



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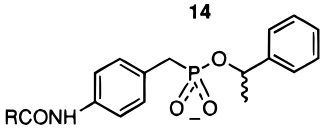

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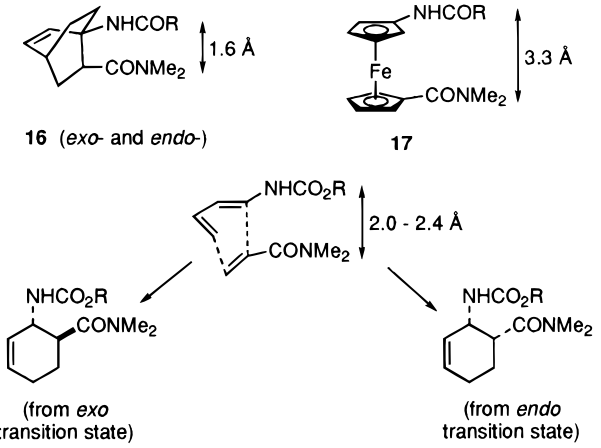
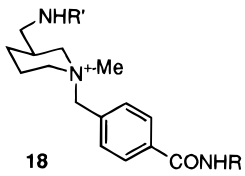
nisms, the tetrahedral intermediates are covalently linked to the protein, a structural feature that is not part of the phosphonate haptens. Such phosphonates only mimic the transition state for direct attack of water on the substrate, and indeed have been shown not to inhibit enzymes, such as the serine proteases, that operate via a ping-pong mechanism.[123] Thus, although the combining site induced by the phosphonate moiety of the hapten is able to stabilize an anionic tetrahedral intermediate, this species differs significantly from that which the phosphonate moiety is supposed to mimic.

Even haptens that are clearly deficient in key respects as transition-state mimics can elicit antibodies with catalytic activity. For example, antibodies that catalyze the Diels−Alder cycloaddition of *N*,*N*-dimethylacrylamide and butadienyl carbamates have been obtained from immunization with the bicyclo[2.2.2]octane and ferrocenyl haptens **16** and **17**, even though the separations they enforce between the polar functional groups are smaller and larger, respectively, than predicted for the transition state.[76,97] Comparable substrate binding affinities (K_m values from 0.7−2.7 mM for diene, 1.7−10 mM for dienophile) and turn-over rates (k_{cat} values from $2-6 \times 10^{-5}$ s^{-1}) were observed for the antibodies they elicited. Although these results conflict with the view that effective hapten structures must be faithful mimics of the transition states their antibodies are intended to promote, the authors do anticipate that more active catalysts may be generated with more accurate analogs.[97]

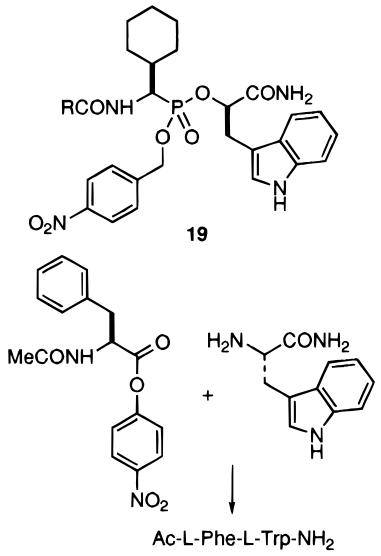


Other examples of the uncoupling of hapten structure and antibody reactivity are provided by antibodies elicited by the piperidinium hapten **18**. Antibody



14D9 catalyzes acetal hydrolysis,[86] enol ether hydrolysis,[124] epoxide hydrolysis,[125] and acetal cyclization,[126] and others raised against **18** have been reported to catalyze epoxide formation,[127] carbon−carbon bond rearrangement,[128] and intermolecular aldol condensation.[129] The common element to these reaction mechanisms is the intermediacy of a cationic or electron-deficient species along the reaction pathway, and it appears to be the positive charge of the piperidinium hapten, rather than structural complementarity to a particular transition state, that is responsible for the wide range of activities displayed by the antibodies that it elicits. An acidic residue in a chiral binding site may be the characteristic of the antibody that is relevant to all of these reactions.

The lack of fidelity between the structure of the hapten and the binding site of a catalytic antibody can be manifested in substrate specificity, in addition to reaction mechanism. For example, antibodies elicited by the phosphonate diester **19** catalyze the coupling of L-Leu, L-Val, and L-Phe as their *N*-acetyl *p*-nitrophenyl esters with L-Trp-NH_2.[96] A positive



feature of these catalytic antibodies is their acceptance of a variety of hydrophobic amino acids as the acylating component, which indicates that the binding affinity is not concentrated in this region. However, it is puzzling that they show a preference for the L enantiomer of tryptophan amide as the amino component, although this moiety had the D configuration in the hapten.

One of the idiosyncracies of the immune system has a significant implication for the development of catalytic antibodies, namely the strong preference for an aromatic ring as part of the hapten structure.[77] Nonaromatic structures, while not devoid of antigenic potential, are less likely to elicit tight-binding antibodies than those incorporating substituted benzene rings.[81] This point has been demonstrated in a dramatic fashion in a comprehensive study by Wallace and Iverson, who found an inverse relationship between the size and hydrophobicity of a series of phosphate haptens and the catalytic activity of antibody hydrolases raised against them.[130] This raises an important question: if the affinity of the antibody for its ligands is focused on the aromatic moieties, how much binding energy is available to discriminate the transition state from the ground state, so that catalysis can be realized? As noted in section III, ground and transition states are more likely to be distinguished by differences in polar and covalent interactions, which are strongly dependent

Table 4. Kinetic Parameters for Esterase 48G7, Its Germline Precursor, and Half-Germline Constructs, for Hydrolysis of *p*-Nitrophenyl Hexanoate[108]

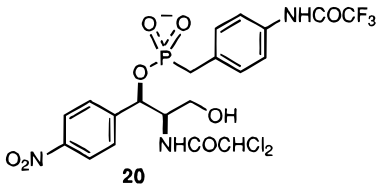
Fab construct (L-H)[a]	k_{cat} (min^{-1})	K_m (μM)	K_{cat}/K_m (M^{-1} min^{-1})	K_d (hapten (μM)
48G7−48G7	5.5	391	14 000	0.004 5
Germ-48G7	<1[b]	−[b]	600	0.33
48G7-Germ	0.83	634	1 300	1.86
Germ-Germ	<1[b]	−[b]	170	135

[a] Designation indicates sequence of light and heavy chains, respectively. [b] Activity too low to determine.

on small changes in geometry, rather than hydrophobic effects.

3. Antibody Diversity

In generating antibodies against an antigen, the immune system makes a multiplicity of proteins that bind the hapten; typically, only a subset of these prove to be catalytic. What is the difference between two antibodies that results in one being catalytic and the other not, when both have high affinity for the transition state analog? How are the antibodies related to each other from the point of view of sequence? Are those that are catalytic similar, or are there multiple solutions to the problem? Fujii et al. have made an extensive investigation in this regard.[131,132] They isolated 11 antibodies elicited against the phosphonate hapten **20**, six of which catalyzed the hydrolysis of ester **21** and five of which did not.



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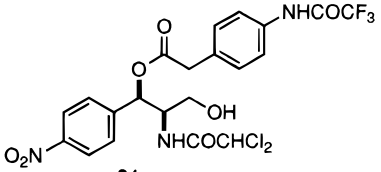

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Five of the catalytic antibodies were closely related in sequence, with 89−95% sequence homologies in the light-chain variable regions and 74−84% homologies in the heavy-chain variable regions. The noncatalytic antibodies show significantly less homology to each other (50−65%) and to the catalytic antibodies. Although three-dimensional structures are not available for these antibodies, chemical modifications of arginine, tyrosine, and histidine residues resulted in comparable reductions in catalytic activity, suggesting that they play similar roles in the active sites.[132] These results suggest that the immune systems may produce a limited number of catalytic sequences, in contrast to a multiplicity that simply bind.

In a study that represents the most revealing analysis of structure and function yet reported for a catalytic antibody, Patten et al. determined the mutational pathway followed by the antibody 48G7 in its transformation from germline sequence to hydrolytic antibody.[108] The catalytic Fab fragment accelerates the alkaline hydrolysis of *p*-nitrophenyl carbonates and esters by 10^4-fold and differs from the germline Fab by nine mutations. The altered amino acid residues are found in both the light and heavy chains, but, interestingly, none contacts the hapten directly in the Fab·hapten complex. The germline Fab, as well as half-germline constructs in combination with the variable regions from either the heavy or the light chains of the catalytic sequence, were evaluated in comparison to the catalytic Fab (Table 4). The activity of 48G7, measured as k_{cat}/K_m, is only 2 orders of magnitude higher than that of the germline Fab, and alterations in both the heavy and light chains contribute to this enhancement. Particularly significant findings from this study were the relationships between hapten binding affinity, association and dissociation rate constants, and catalytic ability. The catalytic Fab binds the hapten 10^4-fold more tightly than the germline Fab; however, the magnitude of the rate enhancement falls short of this value because the hapten is an imperfect transition-state analog. Nevertheless, most of the differences in k_{cat}/K_m for the various Fab's (including other mutants not given in Table 4) result from transition state rather than ground-state binding (i.e, most variation is in k_{cat} rather than K_m). Antibody 48G7 thus behaves fully in accord with predictions from transition-state analog theory, qualitatively as well as quantitatively.

C. Thermodynamic Issues

The thermodynamic box of Figure 1 relates the dissociation constants of ground state ($K_S \approx K_m$) and transition state (K_{TS}, related to K_i for a transition-state analog) and the pseudo-equilibrium constants between ground and transition states, which are in turn related to the rate constants k_{un} and k_{cat}. Are the relationships embodied in this box satisfied for catalytic antibody−hapten−substrate systems? Is there, in fact, a correspondence between enhanced binding of the hapten (= transition state mimic) versus substrate (K_m/K_i) and the rate acceleration observed (k_{cat}/k_{un})? A number of such studies have been reported.[132−135] For the homologous catalytic antibodies elicited by the phosphonate **20** above, a high correlation was found between K_m/K_i and k_{cat}/k_{un}, indicating that the hapten is indeed serving as a transition-state analog and that the interactions which contribute to its enhanced affinity over the substrate are directly manifested in greater binding of the transition state of the substrate over the ground state. This observation not only confirms the expected thermodynamic behavior of the catalysts, but also indicates that the related substrates and inhibitors bind to the antibodies in essentially the same fashion.

For the general class of hydrolytic antibodies elicited by phosphonate haptens such as **13**, there is

a modest correlation between log (K_m/K_i) and log (k_{cat}/k_{un}), although the slope is less than unity.[133] However, the fact that any correlation was found at all suggests that there is some similarity in the binding site interactions across the entire class. It should be noted that even slight changes in ligand orientation in the active site can undermine the expected correlation, as shown for closely related phosphonate inhibitors of carboxypeptidase A.[50] No correlation is observed when log (K_m/K_i) and log (k_{cat}/k_{un}) are compared for different reaction types entirely; this result is not unexpected, however, since the comparison includes antibodies whose mechanisms do not involve the transition states mimicked by their haptens.[135]

In summary, while catalytic antibodies are comparable to enzymes in their ability to bind substrates, to date it has been difficult to elicit antibodies that are as effective at differentiating substrate from hapten (or more relevantly, ground state from transition state).

D. Limitations from the Immune Process Itself

1. Binding Energy Available

A separate issue from the imperfection of a stable hapten as a transition-state mimic is the biological selection process that produces the antibody, which differs from those governing enzyme evolution.[136] There is an obvious difference in time scale. Although the rate at which different enzyme sequences are explored is very much slower than domain interchange in the antibody selection process, the former has operated over millions of years to optimize and modulate catalytic activity, while the latter involves weeks or months at most. Moreover, the criteria according to which the selection processes operate are fundamentally different. For the most part, selection for the enzyme is based on catalytic activity (additional criteria include control of activity by allosteric effectors, etc.), with the result that all steps in the enzymatic cycle are optimized. There appears to be no fundamental limit to the extent to which affinity for a molecule or transition state can be enhanced, if that is required for catalysis. A revealing analysis has been made by Radzicka and Wolfenden, who have determined enzymatic rate enhancements for a number of disparate reactions, many of which are included in Table 2 above.[47] Although the noncatalyzed rate constants range from 10^{-16} to 10^{-2} s^{-1}, the enzymatic k_{cat} values are in the narrower range of 10^2 to 10^4 s^{-1}. The enzymes have evolved to accelerate their reactions to the extent necessary. For the rearrangement of chorismic acid, with a relatively rapid spontaneous rate, the acceleration required is only 10^6-fold; in contrast, orotate decarboxylase must accelerate its rather difficult reaction by 10^{17}-fold to achieve the necessary turnover rate. Since these rate accelerations reflect the differences in binding affinity between ground- and transition-state structures, the hypothetical dissociation constants of the latter can be estimated: they range from 8×10^{-12} M for chorismate mutase (16 μM (K_m) $\div$ 2×10^6-fold acceleration) to 5×10^{-24} M for orotate decarboxylase.

The immune process selects for an antibody simply on the basis of its affinity for the hapten, and there is no mechanism for ensuring that certain parts of the hapten structure will be responsible for the significant interactions in the combining site. If most of the binding energy is derived from an aromatic ring, for example, or from other structural elements that are common to hapten and substrate, the antibody will not be able to stabilize the transition state relative to the ground state. This problem is particularly important when these structures differ only in a small region, as in the hydrolysis of an ester or amide. Too, unless the antibody binding interactions are concentrated on some part of the molecule that changes dramatically during the reaction, product inhibition will be a significant impediment to preparative use of the catalyst.[107,108] This principle served as the basis for design of a catalytic antibody system to hydrolyze prodrugs of chloramphenicol, since intramolecular hydrogen bonding in the product induced a different conformation than in the substrate.[137]

2. Time Scale of Antibody Generation

Finally, there is an upper limit to the affinity that can be developed in an antibody by the immune selection process. Dissociation constants of 10^{-10} or 10^{-12} M are more than adequate for the immune system;[133,138] thus, there is no pressure to enhance affinity beyond this point. The question of time scale becomes important as well: with on-rates for hapten−antibody association on the order of 10^5-10^6 M^{-1} s^{-1},[139] half-lives for dissociation of a picomolar hapten are on the order of weeks to months. No biological selection mechanism can distinguish between ligands that exchange on a time scale longer than that of the biological process itself.

E. Intervention

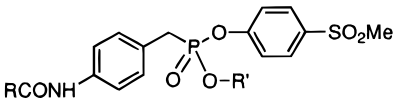
The complementarity to a transition-state analog that can be developed by the immune system does not appear to be sufficient to produce an efficient antibody catalyst except for reactions involving relatively low activation energies. The modest magnitude of the binding interactions induced and their concentration on hydrophobic as opposed to polar regions of the substrate represent significant limitations in the straightforward approach to antibody catalysts. If we as chemists cannot come up with very accurate transition state analogs, and if the immune system cannot develop the kind of transition state affinity that is required, what can we do to make catalytic antibodies more effective? A number of solutions have been proposed to meet this challenge. One has been to devise alternative strategies for immunization and hapten design so as to exaggerate critical features of the transition state structure. The "bait-and-switch" approach to antibody generation,[140,141] and the "reactive immunization" [142] and "heterologous immunization" [143] strategies are examples of such approaches. Another has been to alter the basis for selection so that it is shifted from hapten affinity to actual catalytic activity.[144−146] In contrast to these approaches, in which the inherent diversity of the biological system is maintained, an

alternative strategy has been to employ site-specific mutagenesis and chemical modification to introduce specific functionality as desired into the active site.[147,148]

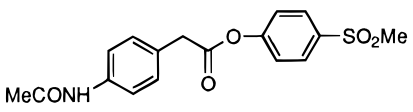
1. Alternative Immunization Strategies

Janda et al. have described what they call a "bait-and-switch" strategy for inducing specific amino acid functionality in the antibody combining site.[140,141] Specifically charged functional groups in the hapten (the "bait") elicit oppositely charged amino acid side chains in the binding site. When an uncharged substrate binds (the "switch"), the lack of charge complementarity results in a strong electrostatic environment that may assist catalysis. This effect is separate from geometric complementarity and, in principle, could enhance the more conventional effects of transition state mimicry. Both cationic and anionic haptens elicited antibodies capable of hydrolyzing an aryl benzoate substrate, while neutral, isosteric analogs did not.

An intriguing alternative to the conventional approach of immunization with stable, unreactive haptens, is to use compounds that can react covalently with amino acid side chains.[142] "Reactive immunization" provides a strategy for inducing this functionality in the antibody combining site and bringing covalent catalysis into the realm of design rather than serendipity. An effective demonstration of this concept has been reported by Janda and Lerner and their co-workers, using the electrophilic phosphonate aryl esters **22** and **23**.[142] Although the intact phosphonate diester **22** is not a transition-state analog, displacement of one of the phenolic esters by an active-site nucleophile affords a mimic of the tetrahedral intermediate in the acylation step of an acylation/deacylation mechanism for ester hydrolysis.[123,149] The antibodies isolated after immunization with a conjugate mixture containing **22** and **23** show an interesting spectrum of activity, comprising not only the ability to hydrolyze the carboxylate ester **24**, but also the diaryl phosphonate **22** (R = Me). Cleav-



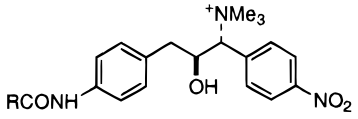
22: R' = *p*-Ph-SO_2Me
23: R' = H



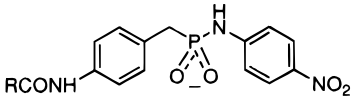
24

age of the phosphonate is accompanied by inactivation of the catalytic antibody; however, in the case of the most active catalyst (49H4), the authors concluded that the inactivated complex does not involve covalent linkage of the phosphonate product to the protein. Nonetheless, hydrolysis of ester **24** catalyzed by 49H4 (initial $k_{cat}/K_m = 1.7 \times 10^3$ M^{-1} s^{-1}) appears to proceed via a covalent intermediate, since an acyl−antibody can be trapped at low pH. A spectrum of kinetic mechanisms for interaction with the phosphonate haptens, as well as the ester substrates, were identified for other antibodies in the series, illustrating the rich chemistry available and the continued role of serendipity in induction of catalytic antibodies.

Heterologous immunization has been proposed as a strategy to cope with the limitations that arise when several electronic or structure features of the transition state cannot be incorporated in a single molecule. According to this strategy, different haptens are used in succession to immunize the host. For example, the quaternary ammonium alcohol **25** and anionic phosphonamidate **26** were employed sequentially in an attempt to generate hydrolytic antibodies containing both acidic and basic residues in the combining site.[143] Antibodies isolated after heterologous immunization (**25** conjugate followed by **26** conjugate) were ~10−fold more active than those from homologous immunization (**25** conjugate only, administered twice). However, since neither the complementary control experiment (**26** conjugate only) nor the reverse heterologous immunization (**26** conjugate followed by **25** conjugate) were reported, the implications of these results and the generality of the strategy are unclear. However, if it proves to be effective, it may offer a way to compensate for the fact that any single hapten structure can only mimic some of the characteristics of a transition state.



25



26

2. Alternative Selection Strategies

In spite of the upper limit on hapten affinity that can be expected from the immune process itself, attempts have been made to improve antibody catalysis by generational mutation, in which the antibodies are allowed to mutate *in vivo* over a period of time.[150] Polyclonal antibodies elicited against phosphonate haptens were isolated from three sheep over a period of 2 years and evaluated for their ability to hydrolyze carbonates. Antibodies isolated from individual animals remained consistent in sequence over this time, and it was shown that activity was not the result of contaminating hydrolases. The kinetic parameters for all of the catalytic antibodies stayed within narrow ranges: 4-fold for K_m, 30-fold for k_{cat}, and, frustratingly, 9-fold for k_{cat}/K_m. The implication that improvements in binding of the ground state and transition state are mutually exclusive is intriguing, however. As discussed above, their differentiation is one of the critical requirements for effective catalysis, although improvements much greater than those observed so far will be necessary. Indeed, Gallacher et al. conclude from this study that "there appear to be substantial

differences in producing enzyme mutants that provide the subtle differences in structure required for catalytically advantageous binding effects in both transition state and ground state complexes".[150]

If the catalytic antibodies isolated so far have arisen as much from serendipity as design, it is possible that selection strategies based solely on affinity for the transition-state analog hapten may work *against* the isolation of the most effective catalysts. It may well be the case that the polyclonal pool contains antibodies with lower affinity for the hapten but higher catalytic efficiency for substrate turnover. Although some work along these lines has been reported for polyclonal antibody catalysis,[150,151] the inhomogeneity of a polyclonal preparation makes rigorous or quantitative analysis difficult.[77] Selection procedures based on catalysis as opposed to simple hapten affinity have been devised,[144,145] of which perhaps the most challenging was to identify an antibody with orotate decarboxylase activity.[146] As noted in Table 1, this transformation takes place extremely slowly without catalysis, and the enzymatic reaction involves one of the greatest rate enhancements so far quantitated for a biological process.[47] After immunization with a hapten designed to elicit orotate decarboxylase activity, a combinatorial library of recombinant Fabs was expressed in an *Escherichia coli* strain auxotrophic for the normal enzyme. Growth and selection were therefore dependent on catalysis rather than hapten binding and afforded activity 10^8-10^{10}-fold higher than the uncatalyzed rate ($k_{cat} \approx 2.7 \times 10^{-4}$ min^{-1} vs $k_{un} = 3 \times 10^{-12}$ to 1.7×10^{-14} min^{-1}).[47,146,152]

3. Site-Directed Mutagenesis and Chemical Modification

With improved techniques for predicting the effects of alterations in protein structure comes the possibility of enhancing catalytic activity by engineering specific modifications to an antibody active site.[153] Early efforts in the Schultz group to enhance the catalytic activity of the dinitrophenyl-binding antibody MOPC, which shows modest hydrolytic activity toward activated esters of *N*-2,4-dinitrophenylglycine, by site-specific introduction of imidazole moieties were quite encouraging in this respect.[147,154] In other systems, seemingly straightforward alterations in active site functionality have not led to the anticipated results. Benkovic and his co-workers have reported an extensive investigation of a hydrolytic antibody to determine the role of active-site residues in substrate binding, catalysis, and product release.[107,148] Systematic mutagenesis of eight residues in the combining site did not yield antibodies with improved k_{cat} or K_m values, and the majority of the five mutants studied had lost all catalytic or binding activity. These sobering results led the authors to conclude that sequential mutations are unlikely to result in significant gains in catalytic antibody efficiency.[107,148] These mixed results can be attributed to our imperfect understanding of protein structure and catalytic function, and they indicate the difficulties that must be surmounted in engineering the subtleties of substrate selectivity and catalytic function.

VI. Conclusion

Antibody catalysis remains an intriguing phenomenon: it is born out of a thermodynamic relationship, yet remains its prisoner. Differential binding affinity gives rise to catalytic behavior, but also sets a limit to its magnitude. There are many ancillary problems that need to be—and are—surmounted in the generation of a catalytic antibody, but there does not seem to be any way around the fact that the immune response alone cannot build enough binding energy into the antibody combining site. Amidst the wide variety of modest catalysts that have been described, a few have been found that demonstrate significant activity; however, it is apparent that these owe their prowess as much to serendipity and selection as design. While the immune system can engender a combining site with affinity for the substrate and a predilection for catalysis, it is now clear this is only the first step in creating a practical catalyst. The strategies proposed for the next step, namely to garner more binding affinity (in particular *differential* binding affinity) in the antibody active site, are considerably more difficult; however, they represent the challenges that must be surmounted if catalytic antibodies are to be successful practically or economically.

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VIII. References

(1) Haldane, J. B. S. *Enzymes*; Longmans, Green: London, 1930.
(2) Pauling, L. *Chem. Eng. News* **1946**, *24*, 1375.
(3) Pauling, L. *Am. Sci.* **1948**, *36*, 51.
(4) *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; pp 1.
(5) Kraut, J. *Science* **1988**, *242*, 533.
(6) Menger, F. M. *Biochemistry* **1992**, *31*, 5368.
(7) Cannon, W. R.; Singleton, S. F.; Benkovic, S. J. *Nature Struct. Biol.* **1996**, *3*, 821.
(8) Eyring, H. *Chem. Rev.* **1935**, *17*, 65.
(9) Levine, I. *Physical Chemistry*; McGraw-Hill: San Francisco, 1983.
(10) Albery, W. J.; Knowles, J. F. *Angew. Chem., Int. Ed. Engl.* **1977**, *16*, 285.
(11) Page, M. I.; Jencks, W. P. *Proc. Nat. Acad. Sci. USA* **1971**, *68*, 1678.
(12) Jencks, W. P. *Adv. Enzymol.* **1975**, *43*, 219.
(13) Kurz, J. L. *J. Am. Chem. Soc.* **1963**, *85*, 987.
(14) Wolfenden, R. *Nature* **1969**, *223*, 704.
(15) Wolfenden, R. *Acc. Chem. Res.* **1972**, *5*, 10.
(16) Lienhard, G. E. *Annu. Rep. Med. Chem.* **1972**, *7*, 249.
(17) Lienhard, G. E. *Science* **1973**, *180*, 149.
(18) Wolfenden, R. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271.
(19) Rocek, J.; Westheimer, F. H.; Eschenmoser, A.; Moldovanyi, L.; Schreiber, J. *Helv. Chim. Acta* **1962**, *45*, 2554.
(20) Creighton, T. E. *Proteins: Structures and Molecular Properties*, 2nd ed.; W. H. Freeman: New York, 1993.
(21) Fersht, A. R.; Shi, J. P.; Knill-Jones, J.; Lowe, D. M.; Wilkinson, A. J.; Blow, D. M.; Brick, P.; Cortes, P.; Waye, M. M. Y.; Winter, G. *Nature (London)* **1985**, *314*, 235.
(22) Lau, F. T.; Fersht, A. R. *Biochemistry* **1989**, *28*, 6841.
(23) Morgan, B. P.; Scholtz, J. M.; Ballinger, M.; Zipkin, I.; Bartlett, P. A. *J. Am. Chem. Soc.* **1991**, *113*, 297.
(24) Vermersch, P. S.; Tesmer, J. J. G.; Quiocho, F. A. *J. Mol. Biol.* **1992**, *226*, 923.

(25) Thorson, J. S.; Chapman, E.; Murphy, E. C.; Schultz, P. G. *J. Am. Chem. Soc.* **1995**, *117*, 1157.
(26) Kyte, J. In *Structure in Protein Chemistry*; Garland Publishing, Inc.: New York, 1995; pp 179.
(27) Tanford, C. *The Hydrophobic Effect*, 2nd ed.; Wiley-Interscience: New York, 1980.
(28) Chothia, C.; Janin, J. *Nature* **1975**, *256*, 705.
(29) Shortle, D.; Stites, W. E.; Meeker, A. K. *Biochemistry* **1990**, *29*, 8033.
(30) Eriksson, A. E.; Baase, W. A.; Zhang, X.-J.; Heinz, D. W.; Blaber, M.; Baldwin, E. P.; Matthews, B. W. *Science* **1992**, *255*, 178.
(31) Sharp, K. A.; Nicholls, A.; Friedman, R.; Honig, B. *Biochemistry* **1991**, *30*, 9686.
(32) Ben-Naim, A. *Curr. Opin. Struct. Biol.* **1994**, *4*, 264.
(33) Holtzer, A. *Biopolymers* **1994**, *34*, 315.
(34) Eriksson, A. E.; Baase, W. A.; Wozniak, J. A.; Matthews, B. W. *Nature* **1992**, *355*, 371.
(35) Kyte, J. In *Structure in Protein Chemistry*; Garland Publishing, Inc.: New York, 1995; pp 159.
(36) Searle, M. S.; Williams, D. H.; Gerhard, U. *J. Am. Chem. Soc.* **1992**, *114*, 10697.
(37) Williams, D. H.; Searle, M. S.; Mackay, J. P.; Gerhard, U.; Maplestone, R. A. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 1172.
(38) Street, I. P.; Rupitz, K.; Withers, S. G. *Biochemistry* **1989**, *28*, 1581.
(39) Levine, H. L.; Brody, R. S.; Westheimer, F. H. *Biochemistry* **1980**, *19*, 4993.
(40) Agarwal, R. P.; Spector, T.; Parks, R. E., Jr. *Biochem. Pharmacol.* **1977**, *26*, 359.
(41) Koehler, K. A.; Hess, G. P. *Biochemistry* **1974**, *13*, 5345.
(42) Ashley, G. W.; Bartlett, P. A. *J. Biol. Chem.* **1984**, *259*, 13621.
(43) Kaplan, A. P.; Bartlett, P. A. *Biochemistry* **1991**, *30*, 8165.
(44) Bartlett, P. A.; Giangiordano, M. A. *J. Org. Chem.* **1996**, *61*, 3433.
(45) Collins, K. D. *J. Biol. Chem.* **1974**, *249*, 136.
(46) Bartlett, P. A.; Nakagawa, Y.; Johnson, C. R.; Reich, S.; Luis, A. *J. Org. Chem.* **1988**, *53*, 3195.
(47) Radzicka, A.; Wolfenden, R. *Science* **1995**, *267*, 90.
(48) Frick, J.; Wolfenden, R. In *Design of Enzyme Inhibitors as Drugs*; Sandler, M., Smith, J. H., Eds.; Oxford University Press: New York, 1989; pp 19.
(49) Wilson, I. B.; Quan, C. *Arch. Biochem. Biophys.* **1958**, *73*, 131.
(50) Phillips, M. A.; Kaplan, A. P.; Rutter, W. J.; Bartlett, P. A. *Biochemistry* **1992**, *31*, 959.
(51) Sachdev, G. P.; Fruton, J. S. *Biochemistry* **1970**, *9*, 4465.
(52) Lolis, E.; Petsko, G. A. *Annu. Rev. Biochem.* **1990**, *59*, 597.
(53) Werkheiser, W. C. *J. Biol. Chem.* **1961**, *236*, 888.
(54) Oefner, C.; D'Arcy, A.; Winkler, F. K. *Eur. J. Biochem.* **1988**, *174*, 377.
(55) Schloss, J. V. *Pestic. Sci.* **1990**, *29*, 283.
(56) Schloss, J. V. *Acc. Chem. Res.* **1988**, *21*, 348.
(57) Morrison, J. F.;. Walsh, C. T. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1987**, *61*, 201.
(58) Kurz, L. C.; Weitkamp, E.; Frieden, C. *Biochemistry* **1987**, *26*, 3027.
(59) Knowles, J. F.; Albery, W. J. *Acc. Chem. Res.* **1977**, *10*, 105.
(60) Bartlett, P. A.; Marlowe, C. K. *Biochemistry* **1987**, *26*, 8553.
(61) Hartman, F. C.; LaMuraglia, G. M.; Tomozawa, Y.; Wolfenden, R. *Biochemistry* **1975**, *14*, 5274.
(62) Dale, M. P.; Ensley, H. E.; Kern, K.; Sastry, K. A. R.; Byers, L. D. *Biochemistry* **1985**, *24*, 3530.
(63) Westerik, J. O.; Wolfenden, R. *J. Biol. Chem.* **1971**, *247*, 8195.
(64) Thompson, R. C. *Biochemistry* **1973**, *12*, 47.
(65) Bartlett, P. A.; Marlowe, C. K. *Biochemistry* **1983**, *22*, 4618.
(66) Hanson, J. E.; Kaplan, A. P.; Bartlett, P. A. *Biochemistry* **1989**, *28*, 6249.
(67) Christianson, D. W.; Lipscomb, W. N. *J. Am. Chem. Soc.* **1986**, *108*, 4998.
(68) Christianson, D. W.; Lipscomb, W. N. *J. Am. Chem. Soc.* **1988**, *110*, 5560.
(69) Kim, H.; Lipscomb, W. N. *Biochemistry* **1991**, *30*, 8171.
(70) Rahil, J.; Pratt, R. F. *Biochemistry* **1994**, *33*, 116.
(71) Jencks, W. P. In *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; pp 288.
(72) Raso, V.; Stollar, B. D. *Biochemistry* **1975**, *14*, 591.
(73) Schultz, P. G.; Lerner, R. A. *Acc. Chem. Res.* **1993**, *26*, 391.
(74) Lerner, R. A.; Janda, K. D. *EXS* **1995**, *73*, 121.
(75) Janda, K. D.; Shevlin, C. G.; Lerner, R. A. *J. Am. Chem. Soc.* **1995**, *117*, 2659.
(76) Gouverneur, V. E.; de Pascual-Teresa, B.; Beno, B.; Janda, K. D.; Lerner, R. A. *Science* **1993**, *262*, 204.
(77) Tawfik, D. S.; Eshhar, Z.; Green, B. S. *Mol. Biotechnol.* **1994**, *1*, 87.
(78) Jacobsen, E. N.; Finney, N. S. *Chem. Biol.* **1994**, *1*, 85.
(79) Jacobsen, J. R.; Prudent, J. R.; Kochersperger, L.; Yonkovich, S.; Schultz, P. G. *Science* **1992**, *256*, 365.
(80) Wirsching, P.; Ashley, J. A.; Benkovic, S. J.; Janda, K. D.; Lerner, R. A. *Science* **1991**, *252*, 680.
(81) Thomas, N. R. *Appl. Biochem. Biotechnol.* **1994**, *47*, 345.
(82) Lerner, R.; Benkovic, S. J.; Schultz, P. G. *Science* **1991**, *252*, 659.
(83) Scanlan, T. S.; Prudent, J. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 9397.
(84) Gibbs, R. A.; Benkovic, P. A.; Janda, K. D.; Lerner, R. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1992**, *114*, 3528.
(85) Titmas, R. C.; Sugasawara, R.; Aman, N.; Darsley, M. J.; Blackburn, G.; Martin, M. T. *Appl. Biochem. Biotechnol.* **1994**, *47*, 277.
(86) Reymond, J.-L.; Janda, K. D.; Lerner, R. A. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1711.
(87) Shokat, K. M.; Leumann, C. J.; Sugasawara, R.; Schultz, P. G. *Nature* **1989**, *338*, 269.
(88) Uno, T.; Ku, J.; Prudent, J. R.; Huang, A.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 3811.
(89) Pollack, S. J.; Hsuin, P.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 5961.
(90) Jackson, D. Y.; Jacobs, J. W.; Sugasawara, R.; Reich, S. H.; Bartlett, P. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 4841.
(91) Hilvert, D.; Nared, K. D. *J. Am. Chem. Soc.* **1988**, *110*, 5593.
(92) Tramontano, A.; Ammann, A. A.; Lerner, R. A. *J. Am. Chem. Soc.* **1988**, *110*, 2282.
(93) Fan, E.; Oei, Y.; Sweet, E.; Uno, T.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 5474.
(94) Landry, D. W.; Zhao, K.; Yang, G. X.-Q.; Glickman, M.; Georgiadis, T. M. *Science* **1993**, *259*, 1899.
(95) Thorn, S. N.; Daniels, R. G.; Auditor, M.-T. M.; Hilvert, D. *Nature* **1995**, *373*, 228.
(96) Hirschmann, R.; Smith, A. B., III; Taylor, C. M.; Benkovic, P. A.; Taylor, S. D.; Yager, K. M.; Sprengeler, P. A.; Benkovic, S. J. *Science* **1994**, *265*, 234.
(97) Yli-Kauhaluoma, J. T.; Ashley, J. A.; Lo, C.-H.; Tucker, L.; Wolfe, M. M.; Janda, K. D. *J. Am. Chem. Soc.* **1995**, *117*, 7041.
(98) Schultz, P. G. *Science* **1988**, *240*, 426.
(99) Jacobs, J.; Schultz, P. G. *J. Am. Chem. Soc.* **1987**, *109*, 2174.
(100) Chook, Y. M.; Ke, H.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 8600.
(101) Haynes, M. R.; Stura, E. A.; Hilvert, D.; Wilson, I. A. *Science* **1994**, *263*, 646.
(102) Lee, A. Y.; Stewart, J. D.; Clardy, J.; Ganem, B. *Chem. Biol.* **1995**, *2*, 195.
(103) Reymond, J.-L.; Reber, J.-L.; Lerner, R. A. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 475.
(104) Sinha, S. C.; Keinan, E. *J. Am. Chem. Soc.* **1995**, *117*, 3653.
(105) Li, T.; Hilton, S.; Janda, K. D. *J. Am. Chem. Soc.* **1995**, *117*, 2123.
(106) Yu, J.; Hsieh, L. C.; Kochersperger, L.; Yonkovich, S.; Stephans, J. C.; Gallop, M. A.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 339.
(107) Stewart, J. D.; Krebs, J. F.; Siudzak, G.; Berdis, A. J.; Smithrud, D. B.; Benkovic, S. J. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 7404.
(108) Patten, P. A.; Gray, N. S.; Yang, P. L.; Wedemeyer, G. J.; Boniface, J. J.; Stevens, R. C.; Schultz, P. G. *Science* **1996**, *271*, 1086.
(109) Stewart, J. D.; Liotta, L. J.; Benkovic, S. J. *Acc. Chem. Res.* **1993**, *26*, 396.
(110) Wiest, O.; Houk, K. N. *J. Org. Chem.* **1994**, *59*, 7582.
(111) Zhou, G. W.; Guo, J.; Huang, W.; Fletterick, R. J.; Scanlan, T. S. *Science* **1994**, *265*, 1059.
(112) Guo, J.; Huang, W.; Zhou, G. W.; Fletterick, R. J.; Scanlan, T. S. *Proc. Natl. Acad. Sci.* **1995**, *92*, 1694.
(113) Charbonnier, J.-B.; Carpenter, E.; Gigant, B.; Golinelli-Pimpaneau, B.; Eshhar, Z.; Green, B. S.; Knossow, M. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 11721.
(114) Hsieh-Wilson, L. C.; Schultz, P. G.; Stevens, R. C. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 5363.
(115) Warshel, A. *Acc. Chem. Res.* **1981**, *14*, 284.
(116) Yang, G.; Chun, J.; Arakawa-Uramoto, H.; Wang, X.; Gawinowicz, M. A.; Zhao, K.; Landry, D. W. *J. Am. Chem. Soc.* **1996**, *118*, 5881.
(117) Lee, A. Y.; Karplus, P. A.; Ganem, B.; Clardy, J. *J. Am. Chem. Soc.* **1995**, *117*, 3627.
(118) Gray, J. V.; Eren, D.; Knowles, J. *Biochemistry* **1990**, *29*, 8872.
(119) Gajewski, J. J.; Jurayj, J.; Kimbrough, D. R.; Gande, M. E.; Ganem, B.; Carpenter, B. K. *J. Am. Chem. Soc.* **1987**, *109*, 1170.
(120) Hilvert, D.; Carpenter, S. H.; Nared, K. D.; Auditor, M.-T. M. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 4953.
(121) Jackson, D. Y.; Liang, M. N.; Bartlett, P. A.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 182.
(122) Guo, J.; Huang, W.; Scanlan, T. S. *J. Am. Chem. Soc.* **1994**, *116*, 6062.
(123) Sampson, N. S.; Bartlett, P. A. *Biochemistry* **1991**, *30*, 2255.
(124) Reymond, J.-L.; Janda, K. D.; Lerner, R. A. *J. Am. Chem. Soc.* **1992**, *114*, 2257.
(125) Sinha, S. C.; Keinan, E.; Reymond, J.-L. *J. Am. Chem. Soc.* **1993**, *115*, 4893.
(126) Shabat, D.; Itzhaky, H.; Reymond, J.-L.; Keinan, E. *Nature* **1995**, *374*, 143.
(127) Koch, A.; Reymond, J.-L.; Lerner, R. A. *J. Am. Chem. Soc.* **1994**, *116*, 803.

(128) Chen, Y.; Reymond, J.-L.; Lerner, R. A. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1607.
(129) Reymond, J.-L.; Chen, Y. *Tetrahedron Lett.* **1995**, *36*, 2575.
(130) Wallace, M. B.; Iverson, B. L. *J. Am. Chem. Soc.* **1996**, *118*, 251.
(131) Miyashita, H.; Hara, T.; Tanimura, R.; Tanaka, F.; Kikuchi, M.; Fujii, I. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 6045.
(132) Fujii, I.; Tanaka, F.; Miyashita, H.; Tanimura, R.; Kinoshita, K. *J. Am. Chem. Soc.* **1995**, *117*, 6199.
(133) Jacobs, J. W. *Bio/technology* **1991**, *9*, 258.
(134) Angeles, T. S.; Smith, R. D.; Darlsey, M. J.; Sugasawara, R.; Sanchez, R. I.; Kenten, J.; Schultz, P. G.; Martin, M. T. *Biochemistry* **1993**, *32*, 12128.
(135) Stewart, J. D.; Benkovic, S. J. *Nature* **1995**, *375*, 388.
(136) Petsko, G. A.; Kenyon, G. L.; Gerlt, J. A.; Ringe, D.; Kozarich, J. W. *Trends Biochem. Sci.* **1993**, *18*, 372.
(137) Miyashita, H.; Karaki, Y.; Kikuchi, M.; Fujii, I. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 5337.
(138) Davies, D. R.; Padlan, E. A.; Sheriff, S. *Annu. Rev. Biochem.* **1990**, *59*, 439.
(139) Foote, J.; Milstein, C. *Nature* **1991**, *352*, 530.
(140) Janda, K. D.; Weinhouse, M. I.; Schloeder, D. M.; Lerner, R. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 1274.
(141) Janda, K. D.; Weinhouse, M. I.; Danon, T.; Pacelli, K. A.; Schloeder, D. M. *J. Am. Chem. Soc.* **1991**, *113*, 5427.
(142) Wirsching, P.; Ashley, J. A.; Lo, C.-H. L.; Janda, K. D.; Lerner, R. A. *Science* **1995**, *270*, 1775.
(143) Suga, H.; Ersoy, O.; Williams, S. F.; Tsumuraya, T.; Margolies, M. N.; Sinskey, A. J.; Masamune, S. *J. Am. Chem. Soc.* **1994**, *116*, 6025.
(144) Tang, Y.; Hicks, J. B.; Hilvert, D. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 8784.
(145) Lesley, S. A.; Patten, P. A.; Schultz, P. G. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 1160.
(146) Smiley, J. A.; Benkovic, S. J. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 8319.
(147) Baldwin, E.; Schultz, P. G. *Science* **1989**, *245*, 1104.
(148) Stewart, J. D.; Roberts, V. A.; Thomas, N. R.; Getzoff, E. D.; Benkovic, S. J. *Biochemistry* **1994**, *33*, 1994.
(149) Oleksyszyn, J.; Powers, J. C. *Biochemistry* **1991**, *30*, 485.
(150) Gallacher, G.; Jackson, C. S.; Searcey, M.; Goel, R.; Mellor, G. W.; Smith, C. Z.; Brockelhurst, K. *Eur. J. Biochem.* **1993**, *214*, 197.
(151) Tubul, A.; Brun, P.; Michel, R.; Gharib, B.; De Reggi, M. *Tetrahedron Lett.* **1994**, *35*, 5865.
(152) Beak, P.; Siegel, B. *J. Am. Chem. Soc.* **1976**, *98*, 3601.
(153) Crowder, M. W.; Stewart, J. D.; Roberts, V. A.; Bender, C. J.; Tevelrakh, E.; Peisach, J.; Getzoff, E. D.; Gaffney, B. J.; Benkovic, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 5627.
(154) Pollack, S.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 1929.

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