At the Crossroads of Chemistry and Immunology: Catalytic Antibodies

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Immunochemistry has historically focused on the nature of antigenicity and antibody-antigen recognition. However, in the last 5 years, the field of immunochemistry has taken a new direction. With the aid of mechanistic and synthetic chemistry, the vast network of molecules and cells of the immune system has been tapped to produce antibodies with a new function—catalytic antibodies. Because antibodies can be generated that selectively bind almost any molecule of interest, this new technology offers the potential to tailor-make highly selective catalysts for applications in biology, chemistry, and medicine. In addition, catalytic antibodies provide fundamental insight into important aspects of biological catalysis, including the importance of transition-state stabilization, proximity effects, general acid and base catalysts, electrophilic and nucleophilic catalysis, and strain.

CHEMISTS HAVE BECOME INCREASINGLY SOPHISTICATED IN their understanding of reaction mechanisms and their ability to synthesize complex organic molecules. For example, the total synthesis of small proteins and genes is now accessible through solid-phase methodology (*1, 2*). New powerful asymmetric synthetic methods have been developed (*3*) and complex natural products such as palytoxin (*4*) (a molecule with 68 stereogenic centers) have been synthesized. In addition, unnatural molecules with novel properties such as desoxyglucose DNA (*5*), cavity-containing molecules such as carcerands and cryptands (*6*), and synthetic DNA cleaving agents (7) have been designed and synthesized. Crossed molecular beam techniques (*8*) and femtosecond laser spectroscopy (*9*) have provided new insights into the dynamics of elementary chemical processes, and theoretical calculations can accurately predict the electronic structure and reactivity of simple molecules (*10*).

Yet given these spectacular advances, chemistry cannot yet begin to match nature's ability to generate the complex molecular structures that carry out the remarkable processes of life, including signal transduction, molecular recognition, catalysis, motility, and gene regulation. One example is the immune system, which is capable of synthesizing large folded polypeptides (immunoglobulins or antibodies) that bind virtually any natural or synthetic molecule with high affinity and exquisite selectivity (*11*). Antibodies protect organisms through their ability to discriminate nonself molecules (foreign invaders such as pathogenic bacteria, viruses, or parasites) from self. Selective recognition is achieved through a large number of weak bonding interactions involving hydrogen bonds, van der Waals, and electrostatic interactions, and solvent effects that chemists do not yet fully understand and are still far from being able to mimic.

Nature solves the complex chemical problems associated with the generation of these sophisticated receptors through its ability to synthesize and screen tremendous numbers of molecules for one with a desired property (in contrast to chemists who typically synthesize one molecule at a time). Biologists have long been fascinated by this process and have begun to unravel the basic mechanism by which the immune system functions (*12*). B-lymphocytes, the cells of the immune system that produce antibody molecules, make use of genetic recombination to generate a pool of antibody molecules, each possessing a unique combining site amino acid sequence. The genes encoding each antibody are spliced together from a battery of gene segments, which enables an organism to mount a primary immune response of some 10^8 different antibody molecules. A complex screening system coupled with further cellular events such as somatic mutation and affinity maturation provide up to another million-fold structural variants, which allows fine tuning of antibody-ligand specificity and affinity.

The ability of the highly evolved machinery of biology to produce structurally and functionally complex molecules like antibodies offers tremendous opportunities for chemists in the coming decade. Chemistry provides the framework for understanding the molecular basis of biomolecular structure and function. In addition, by using the tools and principles of chemistry in combination with the highly evolved synthetic and selection processes of nature, new classes of molecules with novel functions are likely to emerge. We describe one example of the productive interplay of chemistry and biology—the generation of catalytic antibodies (*13*).

Background

Chemistry has played an important role in the development of our current understanding of antibody structure and function. Much of our insight into the nature of antibody specificity came from studies of polyclonal antibodies by Landsteiner and his co-workers (*14*) more than 50 years ago and was extended by work of Pauling, Pressman, Grossberg, and others (*15*). More recently, x-ray crystallographic studies (*16*) have provided structures of antibody molecules and have revealed the nature of antigen-antibody recognition. Antibodies are large proteins (~150,000 daltons in the case of an

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immunoglobulin G) that consist of four polypeptide chains: two identical heavy chains and two identical light chains. The combining site consists of roughly the first 110 amino acids of the heavy and light chains and is termed the variable region. The basic fold of the variable region is that of an eight-stranded β-barrel onto which six loops of extended chain have been grafted; three each from the light- and heavy-chain variable domains (*16*). These loop segments, which are often called hypervariable, display a high degree of sequence variability and provide the basis for the diversity of the antibody molecule.

Antibodies bind molecules with association constants that range from 10^4 to 10^{14} M^{-1} (*11*). Small molecules are typically bound in a cleft, but for large molecules the binding site can be an extended surface that can cover 600 to 800 $Å^2$ (*16*). The specificity of antibodies for their ligands can exceed that of enzymes for substrates. In fact, Pauling (*17*) first pointed out more than 40 years ago that the fundamental difference between enzymes and antibodies is that the former selectively bind transition states and the latter bind ground states. Of course, the other major distinction is that antibody specificity evolves on a time scale of weeks, whereas enzyme specificity evolves over millions of years.

An important recent advance in immunology occurred when Kohler and Milstein (*18*) demonstrated that it was possible to generate in vitro monoclonal antibodies, antibody molecules consisting of a single distinct molecular structure. The generation of large amounts of homogeneous antibodies of a desired specificity reproducibly has dramatically expanded the role of antibodies in biology and medicine.

Catalytic Antibodies

The chemical potential of the immune system was underscored in 1986, when the research groups of Schultz (*19*) and Lerner (*20*) showed that antibodies raised to tetrahedral, negatively charged phosphate and phosphonate transition state analog could selectively catalyze the hydrolysis of carbonates and esters, respectively (*21, 22*). Since that time, antibodies have been generated that catalyze a wide array of chemical reactions ranging from pericyclic reactions to peptide bond cleavage (Tables 1 and 2). The specificity of these antibody-catalyzed reactions rivals or exceeds that of enzymatic reactions. In some cases rates approaching those of enzymes have been achieved, but typically the antibody-catalyzed reactions proceed with rates 10^3- to 10^6-fold faster than the uncatalyzed reaction. A number of general strategies have been developed for generating catalytic antibodies, including (i) the use of antibodies to stabilize negatively and positively charged transition states, (ii) the use of antibodies as entropic traps, and (iii) the generation of antibodies with catalytic groups and cofactors in their combining sites.

Antibodies and Transition States

The use of antibodies as a starting point to tailor-make highly selective catalysts has the obvious advantage that the challenging problem of engineering substrate specificity is solved by nature. Consequently, once an approach has been developed for generating antibodies that catalyze a specific class of reaction, such as stereospecific ester hydrolysis, substrate specificity can be "tuned" by appropriate modifications to hapten structure (screening may even provide antibodies with broad substrate specificities). An additional advantage of catalytic antibodies is that the structural framework of all antibodies is conserved, simplifying purification, structural studies, biochemical engineering, and bacterial expression of each individual catalyst.

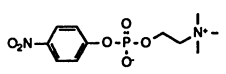
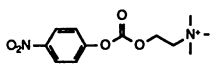
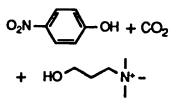
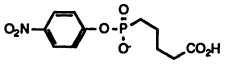
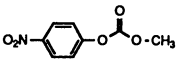
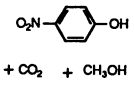
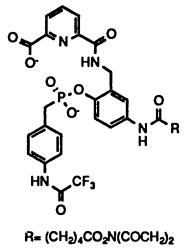
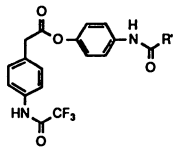
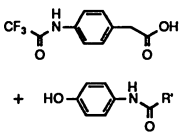
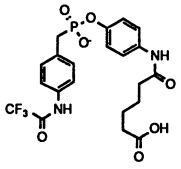
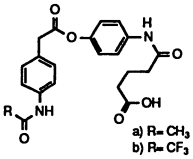
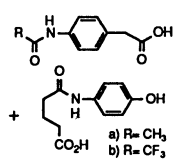
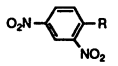
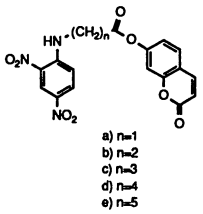
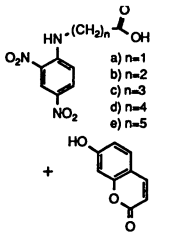
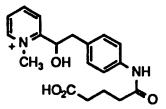
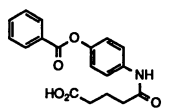
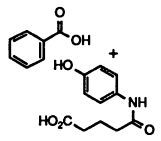
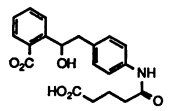
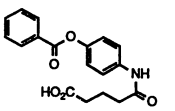
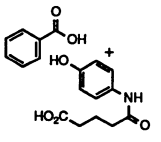
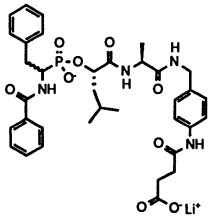
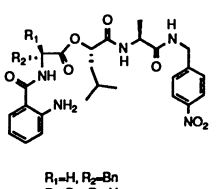
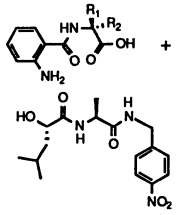
The challenge posed by antibody catalysis is that of chemical reactivity—how does one exploit the binding affinity and specificity of the antibody molecule (weak noncovalent bonds) to make and break covalent chemical bonds? The first solution to this problem was proposed by Jencks (*23*), who suggested that antibodies could be used to selectively stabilize the rate-determining transition state on a reaction pathway. This proposal reflects the fact that many enzymes provide an active site that is sterically and electronically complementary to the rate-determining transition state (*24*).

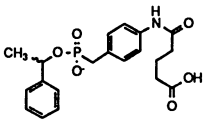
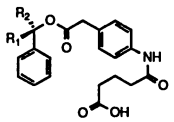
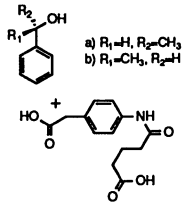
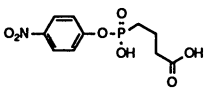
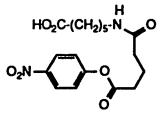
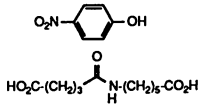
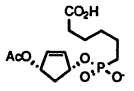
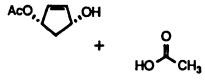
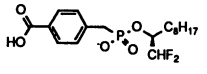
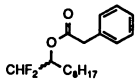
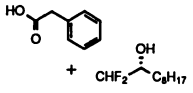
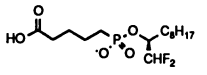
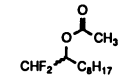
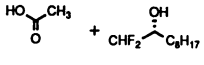
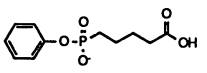
The first experimental demonstration of this notion involved acyl transfer reactions, specifically simple hydrolytic reactions. It seemed logical to begin with acyl transfer reactions for two reasons. First, these reactions have been an object of interest to physical organic chemists for decades and are among the most thoroughly understood organic reactions (*23*). Second, the steric and electronic nature of the transition state for these reactions (tetrahedral and negatively charged) is sufficiently different from substrate (planar and neutral) that it should be possible to generate antibodies that selectively stabilize the high-energy transition state. The features of these species are mimicked by relatively stable phosphates, phosphonates, and phosphonamidates, which were already known to be potent inhibitors of some acyl-transfer enzymes and thus were presumed to be good transition state analogs (*25*). The use of these "mechanism-based" haptens was expected to induce antibody-combining sites that are sterically and electronically complementary to the transition state configuration. Antibodies specific for these transition state analogs acted as catalysts with rate acceleration on the order of 10^3 to 10^4 over the uncatalyzed reactions (*19, 20*). The antibodies followed classical Michaelis-Menten kinetics, displayed substrate specificity, and bound the transition state analog considerably more tightly than substrate, consistent with the notion of transition state stabilization.

It is important to note that these experiments, and virtually all of the experiments detailed in Tables 1 and 2, were carried out with monoclonal antibodies. The use of a single homogeneous catalytic species (versus a polyclonal sera) greatly simplifies kinetic, mechanistic, and structural characterization of catalytic antibodies and allows reproducible generation of large quantities of these proteins for characterization. The ability to prepare highly purified antibodies or Fab fragments by a series of well-known procedures is also critical to the characterization of antibody catalysts in cases where enzymes are known to catalyze a similar reaction. For example, in attempting to generate antibodies with ribonuclease activity, one should (after purifying antibodies to homogeneity and constant specific activity) generate and characterize the activity of Fab fragments, demonstrate hapten inhibition and hapten-related specificity, and characterize the activity of antibodies to an unrelated hapten.

Since these early experiments, more than 20 acyl transfer reactions (*26–51*) have been catalyzed with rate accelerations approaching 10^8 M over the uncatalyzed reactions (Tables 1 and 2). Important experiments that point to commercial applications of antibodies include the demonstration that antibodies can carry out the stereospecific hydrolysis of unactivated esters with an enantiomeric excess of greater than 100 to 1 (*36*). These results are significant because at present there exists no general chemical method for generating stereospecific esterolytic catalysts. Specificities for both the alcohol and acid components of the esters were demonstrated as well as for *R* and *S* configurations (reactions 8, 9, 11, and 12 in Table 1) (*36, 37, 39, 40*). Such antibodies might find applications in the chiral resolution of synthetic intermediates containing acid or alcohol functionality. In fact, the specificity of esterolytic antibodies has already been exploited in the production of a biosensor (*52*).

Table 1. Reactions catalyzed by antibodies; adapted in part from (*40*).

Antigen	Substrates	Products
	Carbonate hydrolysis	
	 Reaction 1 (*19*)*	
	 Reaction 2 (*26,27*)	
	Ester hydrolysis	
 R= $(CH_2)_4CO_2N(COCH_2)_2$	 a) R'= CH_3 b) R'= $(CH_2)_2CO_2H$ Reaction 3 (*20,28*)	 a) R'= CH_3 b) R'= $(CH_2)_2CO_2H$
	 a) R= CH_3 b) R= CF_3 Reaction 4 (*29*)	 a) R= CH_3 b) R= CF_3
	 a) n=1 b) n=2 c) n=3 d) n=4 e) n=5 Reaction 5 (*30–33*)†	 a) n=1 b) n=2 c) n=3 d) n=4 e) n=5
	 Reaction 6 (*34*)	
	 Reaction 7 (*35*)	
	 R_1=H, R_2=Bn R_1=Bn, R_2=H Reaction 8 (*36*)‡	

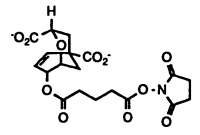
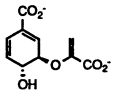
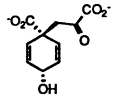
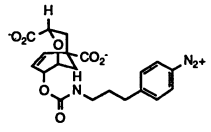
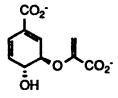
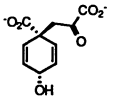
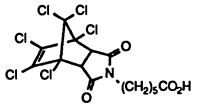
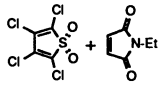
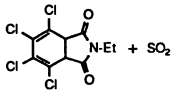
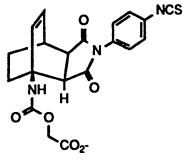
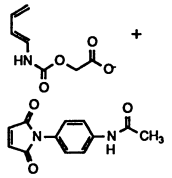
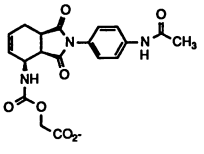
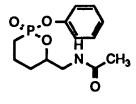
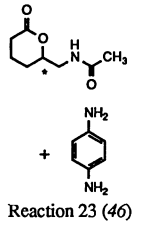
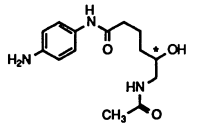
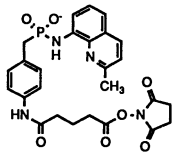
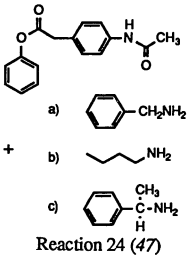
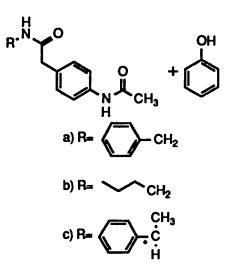
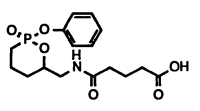
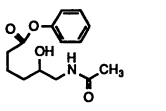
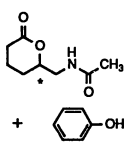
Antigen	Substrates	Products
	Ester hydrolysis	
	 a) R_1=H, R_2=CH_3 b) R_1=CH_3, R_2=H Reaction 9 (*37, 38*)	 a) R_1=H, R_2=CH_3 b) R_1=CH_3, R_2=H
	 Reaction 10 (*50*)	
	 Reaction 11 (*39*)§	
	 Reaction 12 (*40*)	
	 Reaction 13 (*40*)	
	Reaction 14 (*41,42*)	
	Reaction 15 (*45*)	
	Amide hydrolysis	
	Reaction 16 (*43*)	
	a) n=2 b) n=3 Gly Phe βAla Gly-CO_2H Reaction 17 (*44*)	a) n=2 b) n=3 +H_3N-Gly-Phe-βAla Gly-CO_2H
Unknown	Reaction 18 (*86*)	

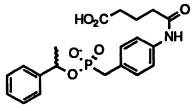
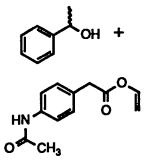
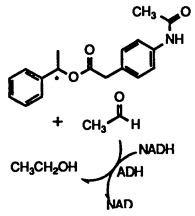
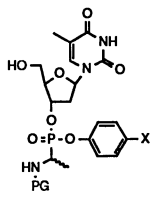
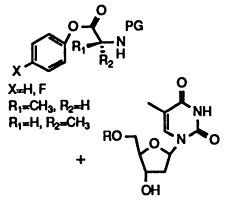
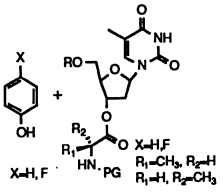
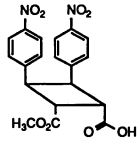
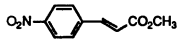
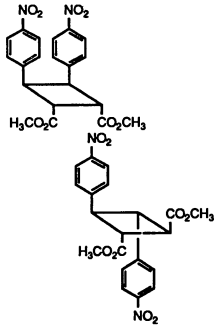
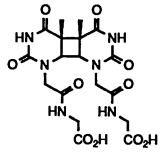
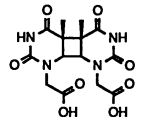
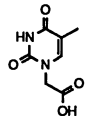
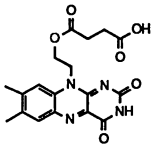
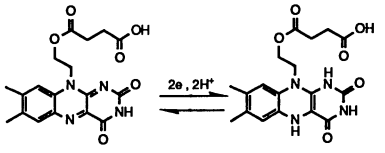
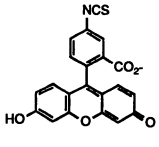
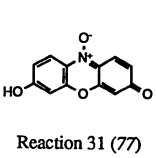
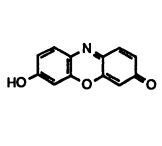
*Used preexisting murine melanoma protein MOPC167. †The antibody used was prepared by chemical and genetic modification of MOPC315. ‡*m*-NO_2 compound is a competitive inhibitor Bn, benzyl. §AcO, acetate.

Related experiments that might bear on commercial applications of catalytic antibodies were the demonstrations that (i) antibodies can function as catalysts in organic solvents by solubilization in reverse micelles (*41*) and (ii) immobilized antibodies retain activity and specificity (with enhanced stability) in organic solvents (*38*).

One indication that antibodies are complementary to rate-determining transition states comes from a comparison of dissociation

Table 2. Reactions catalyzed by antibodies; adapted in part from (*40*).

Antigen	Substrates	Products
	Claisen rearrangement	
		
	Reaction 19 (*65,66*)	
		
	Reaction 20 (*67*)	
	Diels-Alder	
		
	Reaction 21 (*54*)*	
		
	Reaction 22 (*68*)	
	Amide bond formation	
		
	Reaction 23 (*46*)	
		
	Reaction 24 (*47*)	
	Lactonization	
		
	Reaction 25 (*48*)	

Antigen	Substrates	Products
	Transesterification	
		
	Reaction 26 (*49*) †	
		
	Reaction 27 (*57*)	
	Photo-induced dimerization (2+2)	
		
	Reaction 28 (*85*)	
	Photo-induced cleavage (retro 2+2)	
		
	Reaction 29 (*84*)	
	Redox	
		
	Reaction 30 (*76*)	
		
	Reaction 31 (*77*)	

*Secondary products. Et, ethyl. †NADH, reduced form of nicotinamide adenine dinucleotide; ADH, alcohol dehydrogenase.

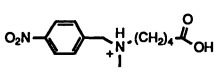
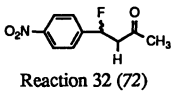
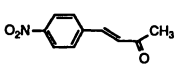
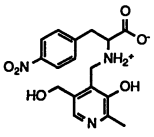
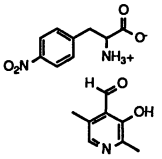
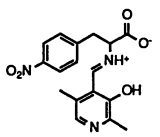
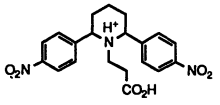
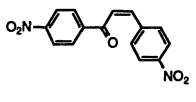
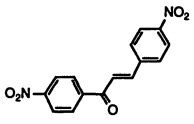
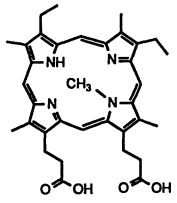
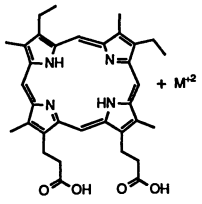
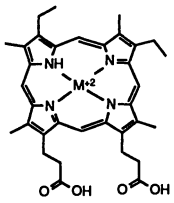
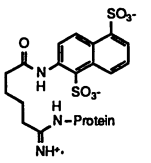
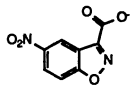
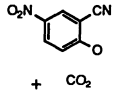
constants for substrates and transition state analogs with the rate of the antibody-catalyzed reaction over the uncatalyzed reaction (*53*):

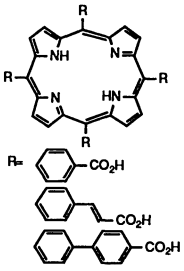
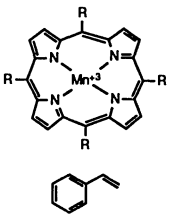
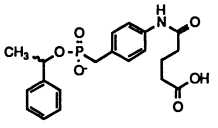
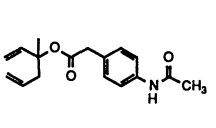
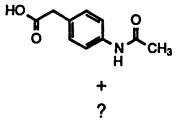
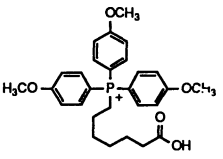
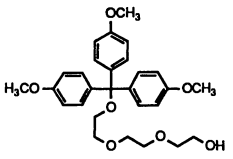
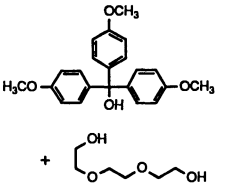
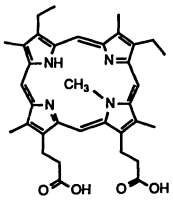
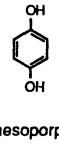
$$\begin{array}{ccc} \text{Ab} + \text{S} & \overset{K_N^{\ddagger}}{\rightleftharpoons} & [\text{S}]^{\ddagger} + \text{Ab} \rightarrow \text{P} + \text{Ab} \\ \Big\downarrow\uparrow K_S & & \Big\downarrow\uparrow K_T \\ \text{Ab•S} & \overset{K_{Ab}^{\ddagger}}{\rightleftharpoons} & [\text{Ab•S}]^{\ddagger} \rightarrow \text{P•Ab} \rightarrow \text{P} + \text{Ab} \end{array}$$

where $\frac{K_{Ab}^{\ddagger}}{K_N^{\ddagger}} = \frac{K_S}{K_T} = \frac{k_{Ab}}{k_N}$.

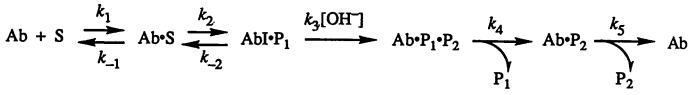
where K_S and K_T correspond to the dissociation constants for substrate and transition state, respectively, and k_{Ab} and k_N are the corresponding rate constants. A number of antibodies specific for phosphonate transition-state analogs show close agreement between the calculated $K_{Ab}^{\ddagger}/K_n^{\ddagger}$ and experimentally derived K_S/K_T ratios. These reactions include a unimolecular lactonization reaction (*48*), bimolecular amide bond formation (*46*), and the hydrolysis of an aryl ester (*41, 42*) [other antibody-catalyzed reactions, including Diels-Alder (*54*) and porphyrin metallation (*55*) reactions, show similar behavior].

In some cases, factors in addition to those attributable to hapten design contribute to catalysis. This observation reflects an important principle involved in the generation of catalytic antibodies: the diversity of the immune response may provide catalytic functions not anticipated a priori. The diversity of the immune response can be viewed as the counterpart to the refinements achieved in enzyme evolution through the processes of mutation and selection. One important example is an antibody that catalyzes the hydrolysis of an arylamide bond (*43*). In this case, antibodies generated to a phosphonamidate (reaction 16 in Table 1) catalyzed the hydrolysis of the corresponding amide substrate with a rate acceleration of 2.5×10^5 relative to background hydrolysis (the analogous ester is also cleaved). However, this rate enhancement results only in part from differential stabilization of the transition state by the antibody. A minimal kinetic sequence for the antibody-catalyzed hydrolysis has been proposed based on a combination of pre–steady-state and steady-state kinetic methods, $H_2{}^{18}O$ tracer measurements, and the determination of D_2O solvent isotope effects (*56*). One mechanism which is consistent with the experimental data is a multistep kinetic pathway that

Antigen	Substrates	Products
	β-Elimination	
		
	Reaction 32 (*72*)	
	Imine formation	
		
	Reaction 33 (*78*)	
	Cis-trans isomerization	
		
	Reaction 34 (*74*)	
	Metal chelation	
		
	Reaction 35 (*55*)	
	Decarboxylation	
		
	Reaction 36 (*87*)	

Antigen	Substrates	Products
	Epoxidation	
		
	Reaction 37 (*80*)	
	Ester hydrolysis	
		
	Reaction 38 (*88*)	
	Trityl hydrolysis	
		
	Reaction 39 (*61*)	
	Oxidation	
		
	H_2O_2, mesoporphyrin•Fe(III)	
	Reaction 40 (*79*)	

processes substrate through a putative acyl antibody intermediate:



where k_i are the rate constants, I represents the acyl portion of the substrate, P_1 refers to its parent acid, and P_2 to the leaving group.

There are some particularly instructive comparisons between the proposed mechanism and that of a serine esterase such as chymotrypsin (*57, 58*). Both make use of acyl intermediates to process their substrates, but the deacylation step for chymotrypsin uses an internal general acid-base mechanism, whereas the antibody requires hydroxide ion. For chymotrypsin, both products are lost rapidly from the enzyme in the steps in which they are formed; for the catalytic antibody, both cleavage products are carried through the entire pathway and dissociate at the end of the turnover cycle. Nevertheless, this antibody is a potent catalyst: for the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-Phe-*p*-nitrophenyl ester, the catalytic rate constant k_{cat} is 77 s^{-1}, while for *N*-acetyl-L-Tyr-*p*-nitroanilide k_{cat} is 0.051 s^{-1} (25°C, pH 7); for antibody-catalyzed ester hydrolysis, k_{cat} is 40 s^{-1}, whereas for amide hydrolysis k_{cat} is 0.002 s^{-1}. Further experiments with this antibody may provide us the opportunity to study the evolution of a primitive enzyme, as well as provide important insight into the generation of antibodies that catalyze the related, but more energetically demanding, hydrolysis of peptide bonds.

Mechanistic experiments have also provided insight into the catalytic mechanism of the phosphorylcholine (PC)-binding antibodies, MOPC167, T15, and S107, which catalyze the hydrolysis of the corresponding choline carbonates (reaction 1 in Table 1) (*19, 59*). These antibodies belong to a class of highly homologous, well-characterized antibodies. The three-dimensional structure of McPC603, a representative member of this class, has been solved by x-ray crystallography (*60*). Site-directed mutagenesis experiments in combination with chemical studies and crystallographic data, demonstrated that Arg^{52} residue of the heavy chain plays an important catalytic role by electrostatically stabilizing the negatively charged transition state, as is the case with many enzymes.

Antibodies with esterase (*45*) and tritylase (*61*) activity have been generated by using neutral (hydroxymethylene) and positively charged transition-state analogs (phosphonium salts), respectively. Negatively and positively charged transition state analogs are also being tested for their ability to produce antibodies with phosphodiesterase and glycosidase activity.

A recent advance in antibody catalysis involved the use of a hapten resembling a distorted conformation of substrate to catalyze the metallation of porphyrin (*55*). Ferrochelatase, the terminal enzyme in the heme biosynthetic pathway, catalyzes the biological insertion of iron(II) into protoporphyrin (*62*). A potent inhibitor of this enzyme is the bent porphyrin, *N*-methylprotoporphyrin. The distorted structure of the methylated porphyrin macrocycle results from steric crowding due to the internal methyl substituent and is thought to resemble the transition state of porphyrin metallation catalyzed by ferrochelatase. This distortion of the macrocyclic ring system forces the chelating nitrogen lone pairs into a position that is more accessible for binding to the incoming metal ion. Antibodies generated to *N*-methylmesoporphyrin IX (reaction 35 in Table 2) catalyzed the metallation of the planar substrate, providing good evidence for the role of distortion in the transition state of this reaction.

The catalytic properties for this antibody are similar to those of the enzyme ferrochelatase. The reported values of the Michaelis constant K_m of ferrochelatase for metal ion are similar to those of the antibody, and both enzyme and antibody catalysis are subject to product inhibition. Both enzyme and antibody can insert a variety of divalent transition metals into mesoporphyrin with similar turnover numbers (k_{cat}). A calculated value of k_{cat} for ferrochelatase with Zn(II) and mesoporphyrin is 800 $hour^{-1}$; the experimentally determined value of k_{cat} for the antibody is 80 $hour^{-1}$; representing the closest correlation of turnover number achieved to date for a catalytic antibody and the analogous enzyme. This work not only suggests that antibody binding energy can be used to distort substrate structure (for example, twist an amide bond), but also illustrates the utility of antibodies for testing fundamental notions of enzymic catalysis.

Antibodies and Entropy

There has been much discussion and debate over the relation between proximity effects and rate accelerations in biological catalysis (*63*). Jencks and Page have argued that entropic effects can account for effective molarities of up to 10^8 M in enzyme-catalyzed reactions (*64*). If this is in fact the case, antibodies should be capable of efficiently catalyzing reactions with unfavorable entropies of activation ($\Delta S^{\ddagger}$) by acting as "entropy traps": the binding energy of the antibody being used to freeze out the rotational and translational degrees of freedom necessary to form the activated complex.

These notions have recently been tested in the design of antibodies that catalyze unimolecular reactions [both a lactonization reaction (*48*) and Claisen rearrangement (*65–67*)] as well as bimolecular reactions [including Diels-Alder (*54, 68*) and transacylation reactions (*47–49, 51*)]. An antibody induced to a bicyclic hapten that resembles the conformationally restricted chairlike transition state for the Claisen rearrangement of chorismic acid to prephenic acid accelerated this pericyclic rearrangement 10^4-fold over the uncatalyzed rate (reaction 20 in Table 2) (*67*). For comparison, the well-studied enzyme chorismate mutase from *Escherichia coli* accelerates the reaction approximately 3×10^6–fold over the background reaction. For this antibody, mechanisms involving a cationic substituent effect or general acid catalysis were ruled out. As expected, the entropy of activation of the antibody-catalyzed reaction was close to zero, compared with a $\Delta S^{\ddagger}$ of −13 entropy units (eu) for the uncatalyzed reaction (*13*). Another antibody catalyzed the reaction at a lower rate, but was regioselective, catalyzing only the conversion of the (−)-isomer of chorismate (*65, 66*). Interestingly, this antibody functioned primarily by lowering the enthalpy of activation ($\Delta H^{\ddagger}$) for reaction. Again, these experiments demonstrate an important aspect of antibody catalysis, namely, that experiments with similar haptens, through the rich diversity of the immune response, may produce more than one antibody that can catalyze a particular reaction (and, at the same time, provide additional insight into catalytic mechanism). Thus there may be many mechanistic alternatives available to antibodies and enzymes for catalyzing a specific reaction. Because the conversion of chorismate to prephenate is an essential step in the biological synthesis of aromatic amino acids in bacteria, this system offers the possibility of applying random mutagenesis and selection to the evolution of antibodies with improved catalytic efficiency (perhaps rivaling or exceeding that of the enzyme).

Another example of the use of antibodies to act as entropic traps involves an antibody-catalyzed Diels-Alder reaction. This reaction, which has long been one of the most powerful transformations in synthetic organic chemistry, is a reaction between a diene and an alkene giving rise to a cyclohexene product. Given the importance of this reaction in organic synthesis, it is surprising that there is not yet a documented example of an enzyme-catalyzed Diels-Alder reaction. This observation prompted the question of whether a Diels-Alderase antibody could be developed.

The transition state for this reaction involves a highly ordered

cyclic array of interacting orbitals in which carbon-carbon bonds are broken and formed in a single concerted mechanistic event. As a result, an unfavorable entropy of activation on the order of −30 to −40 eu is generally observed. The design of haptens that would lead to catalytic antibodies for this bimolecular reaction must not only make use of proximity effects but must also provide a mechanism for eliminating product inhibition. Hilvert and co-workers were successful in designing a system which satisfies both criteria (*54*). The particular Diels-Alder reaction chosen was that of tetrachlorothiophene dioxide with *N*-ethylmaleimide (reaction 21 in Table 2). Antibodies raised to a stable tricyclic hapten catalyze the formation of the initial Diels-Alder adduct that spontaneously extrudes SO_2 to give the secondary dihydrophthalimide product which is bound weakly by the antibody. Significant rate accelerations were achieved with multiple turnovers.

Another, perhaps more general, strategy for Diels-Alder catalysis involved the synthesis of a hapten that contained an ethano bridge that locks the cyclohexane ring into a conformation resembling the Diels-Alder transition state (reaction 22 in Table 2) (*68*). Because the cyclohexene product does not contain this hydrophobic bridge and has a conformation different than that of hapten, it should not be bound tightly by antibody (thereby avoiding product inhibition). Antibodies elicited to the conformationally locked cyclohexane ring system should also bind the acyclic diene in the reactive cis-syn conformation. An antibody generated to this hapten catalyzed the formation of the Diels-Alder adduct with apparent second-order rate constants (k_{cat}/K_m) of 900 M^{-1} s^{-1} for the diene and 583 M^{-1} s^{-1} for the dienophile (the rate of the uncatalyzed reaction in acetonitrile is 0.001 M^{-1} s^{-1}). The dissociation constant for product was 10 μM (*68*). These reactions not only demonstrate that antibodies can be generated that catalyze a reaction for which no known enzymes exist but also illustrate how hapten design can be used to solve the problem of product inhibition.

One of the most exciting recent results in the catalytic antibody field was the discovery of a remarkably efficient catalytic antibody for the transesterification reaction in water between *sec*-phenethyl alcohol and an enolic ester to form the corresponding chiral ester (reaction 26 in Table 2) (*49*). The reaction was found to be highly efficient with an effective molarity of approximately 10^6 to 10^8 molar, verifying the predictions of previous workers (*63, 64*). That this acceleration is largely entropic in nature is reflected by a reduction of $\Delta S^{\ddagger}$ of 35 eu. The steady-state kinetics showed classic "ping-pong" behavior in double-reciprocal plots, indicating that the reaction proceeds through two half-reactions in which the initial step consists of formation of a covalent enzyme complex. Even more remarkable is the finding that the formation of the acyl-intermediate depends on an induced fit phenomenon (*69*) in that a close analog of *sec*-phenethyl alcohol, such as the corresponding chloride or bromide, increases the rate of the antibody acylation half-reaction. Perhaps the most interesting feature of this antibody is the finding that it uses catalytic mechanisms previously thought to be the purview of highly evolved enzymes. Such antibodies should be useful for important transacylation reactions such as formation of chiral esters or condensation of peptide fragments. Other reactions amenable to antibody catalysis by proximity might include macrocyclization reactions, transamidation, transphosphorylation, and transglycosylation reactions, and aldol condensations.

Antibodies and General Acid-Base Catalysis

Enzymes achieve their remarkable rate accelerations by the concerted use of a number of catalytic mechanisms, including covalent catalysis, general acid-base catalysis, entropic effects, and strain. For example, the enzyme triose-phosphate isomerase contains an active site carboxylate that is involved in proton transfer, as well as a histidine that is thought to play a role in stabilizing the negatively charged transition state (*70*). These and perhaps other factors allow the enzyme to function at a diffusion-controlled rate. Strategies have already been described for generating antibodies that stabilize negatively charged transition states. The next challenge was to ask whether suitably designed haptens might induce antibody combining sites with specific functional groups (such as a base or acid) positioned for effective catalysis.

An examination of binding studies (*11*) and crystallographic data (*16*) on antibodies convincingly shows that structural features of the hapten induce complementary structural features in the antibody combining site: charged groups are stabilized by oppositely charged entities and hydrophobic groups are surrounded by an apolar environment [a simplistic view of antibodies is as a heterogeneous microsolvent (*71*)]. That the complementarity of hapten and antibody can be used to induce an active site base was first demonstrated in an antibody-catalyzed β-fluoride elimination reaction (reaction 32 in Table 2) (*72*). A hapten was synthesized that resembled the β-fluoroketone substrate, with the exception that an ammonium group replaced the abstractable α-proton of the substrate. The positively charged alkyl ammonium ion was expected to induce a complementary negatively charged carboxylate residue in the antibody combining site, positioned to function as a general base for β-elimination of hydrogen fluoride. From a panel of six monoclonal antibodies, four were able to catalyze the elimination reaction; one catalyzed the reaction with a rate acceleration of approximately 10^5 over that of the corresponding background reaction. Chemical modification and affinity labeling experiments confirmed the presence of an active site carboxylate (*73*). Isotope effects demonstrated that the rate-determining step involves deprotonation of the Cα proton (*73*). In addition, the reaction displays a pH profile indicative of an ionizable active site residue with a pK_a of 6.2 (K_a is the acidity constant), suggesting that hapten design can also be used to modulate the pK_a's of active site bases and acids.

More recently this approach has been applied to a cis-trans isomerization reaction of a disubstituted-α,β-unsaturated ketone (reaction 34 in Table 2) (*74*) (isomerization reactions play an important role in chemistry and biology; examples include the industrial synthesis of vitamin D and the role of retinal in vision). One mechanistic model for an antibody-catalyzed isomerization reaction involves 1,4-nucleophilic addition of an active site group to the enone, followed by rotation around the resulting α-β single bond and subsequent collapse of the intermediate to afford the isomerized product. Consequently, not only must the antibody contain an active site nucleophile, it must also accommodate the orthogonal transition state for bond rotation. It was expected that antibodies raised to the appropriate trans-disubstituted piperidinium system would meet both criteria. The positively charged ammonium ion should generate an active site carboxylate that could act as a nucleophile or general base. In addition, computer modeling suggested that the trans configuration of the aryl groups in the hapten mimics the geometry of a transition state in which the α,β-bond is rotated 90°. Consistent with this observation, antibodies specific for the trans hapten significantly accelerated the isomerization reaction, whereas those specific for the cis hapten had no catalytic activity. Evidence for a catalytic residue and corresponding covalent antibody-substrate adduct followed from chemical modification experiments including epoxide affinity labeling. This example suggests that haptens can be designed that accommodate more than one mechanistic criteria.

Catalytic groups have also been introduced into antibody combining sites to generate acyl transfer catalysts. In one case, the hapten

contained an *N*-methyl-pyridinium group in order to induce a negatively charged carboxylate that might act as a general base or nucleophile (reaction 6 in Table 1) (*34*). In addition, a secondary hydroxyl group was present in the hapten in an effort to mimic the tetrahedral transition state for the acyl transfer reaction. Antibodies generated against the hapten catalyzed the hydrolysis of the corresponding acyl ester with a millionfold rate enhancement over the uncatalyzed reaction. Again, a high percentage of antibodies generated were catalytic. Importantly, antibodies generated against an analog of the hapten in which the pyridine group was not alkylated (and therefore neutral at physiological pH) had no catalytic activity. These successes suggest that hapten-antibody complementarity should prove to be a general strategy for generating catalytic antibodies for a variety of reactions, including condensation, isomerization, and hydrolytic reactions. A next important step in antibody catalysis would be induction of two catalytic groups in the combining site (two acids, an acid and a base, or two bases) in an effort to achieve a multiplicative rate effect.

Another important approach for generating antibodies that contain active site catalytic groups makes use of the molecular biological method of site-directed mutagenesis. This technique has been used to introduce catalytic histidines into the active sites of dinitrophenyl (*32*) and phosphorylcholine-specific antibodies (*59*). The resulting antibodies have significant estereolytic activity, up to 10^5 times that of the corresponding imidazole-catalyzed reaction. This strategy for enhancing the catalytic efficiency of antibodies generated by hapten-based methods is similar to the "second-site revertant" experiments of mechanistic enzymology. Recent experiments in the development of bacterial expression systems for antibodies (*75*) should facilitate the use of random mutagenesis coupled with screens and selection experiments to improve the catalytic efficiency of antibodies. The use of cofactor selection schemes and of fluorogenic (fluorescence energy transfer) and chromogenic screens to enhance the activity of esterolytic antibodies are being explored.

Antibodies and Cofactors

The range of reactions that can be catalyzed by enzymes composed of only the 20 natural amino acids might be limited but for the fact that nature makes use of a powerful set of nonpeptidyl catalytic auxiliaries termed cofactors. These include metal ions, hemes, thiamine, flavins, and pyridoxal. Strategies that allow incorporation of cofactors into antibody combining sites should, by analogy, expand the scope of antibody catalysis. The diversity of the immune response should allow one to use not only the natural cofactors, but also a host of unnatural cofactors not accessible to enzymes.

There are in principle three ways in which this could be accomplished. First, the antibody could be elicited to a multisubstrate analog in which binding sites for the cofactor and substrate are generated in a single immunization. The antigen must be carefully designed to insure correct approximation of the functional portion of the cofactor and the substrate. This principle was used to achieve sequence-specific cleavage of a peptide bond with Zn(II) as a cofactor (reaction 17 in Table 1) (*44*). The substitution-inert cobalt(III) trien was linked to a peptide substrate for immunization so that an antibody binding pocket that would accommodate substrate, trien, and Zn(II) was induced. The open coordination site on the Zn(II) ion presumably delivered hydroxide anion to the carbonyl carbon of the scissle amide bond of bound substrate. This strategy has also been applied to a number of redox-active cofactors, including flavin (*76*) and resazurin (*77*), as well as a pyridoxamine-dependent reaction (*78*). Recently an antibody has been characterized that binds Fe(III)-mesoporphyrin IX and catalyzes the H_2O_2-dependent oxidation of a number of substrates (reaction 40 in Table 2) (*79*). The antibody-porphyrin complex is capable of at least 200 to 500 catalytic turnovers. The N-alkyl functionality of the porphyrin-derived hapten should serve as a handle to introduce selective substrate binding sites into the antibody, possibly leading to antibodies with hydroxylase or epoxidase (*80*) activity.

A second possibility for introducing cofactors is by semisynthesis (*81*), in which cofactors are covalently linked to the antibody molecule in close proximity to the substrate binding site. A general two-step strategy has been developed in which cleavable affinity-labeling reagents are used to selectively introduce a reactive functionality into the binding site that can act as a handle for the subsequent introduction of a variety of catalytic groups and cofactors (*30–33, 82*). Semisynthetic hydrolytic antibodies that contain active site thiols (*30*) and imidazoles (*31*) have been generated through this strategy, and efforts are currently under way to introduce natural and unnatural cofactors (reaction 5 in Table 1). Importantly, this strategy does not require knowledge of the three-dimensional structure of the antibody of interest.

A third approach for bringing cofactors to bear on abzymic transformations is to take advantage of the fact that antibodies have two chains, and to use one of the chains to bring the cofactor into the binding pocket. Both combinatorial methods for preparing and screening large numbers of antibodies as well as transgenic mouse experiments might facilitate this process, since only a limited number of cofactor-substrate binding geometries would be catalytically productive. An important step in this direction was taken recently when it was shown that a cofactor (metal-ion) binding site could be introduced into the light chain of an antifluorescein antibody combining site (*83*). Metal ions were chosen because of the key role they play as cofactors in many biological transformations. The zinc binding domain of carbonic anhydrase was used as a structural guide to design a series of mutants of a single chain fluorescein-specific antibody that incorporated from one to three histidine mutations in CDR1 and CDR3 of the light chain. The mutant constructs were expressed in *E. coli*, and the recombinant antibodies were analyzed for metal-dependent fluorescence quenching. The mutant containing the three histidine substitutions was found to bind a variety of metal ions with micromolar dissociation constants.

A final example of the use of cofactors in antibody catalysis was the demonstration that light could be used by an antibody to break two carbon-carbon bonds of a cis-syn thymine dimer (reaction 29 in Table 2) (*84*). Upon irradiation at 300 nm, the antibody-catalyzed photorepair reaction proceeded at a rate (k_{cat} of 1.2 min^{-1}) comparable to that of the reaction catalyzed by the well-studied *E. coli* enzyme, DNA photolyase (k_{cat} of 3.4 min^{-1}). This work suggests that excited-state chemistry should find considerable application in the catalytic antibody field.

Prospectus

Although there was speculation about antibody catalysis (*23,89*), it was not until 1986 that catalytic antibodies were discovered. Since that time the number and diversity of antibody-catalyzed reactions has been growing rapidly as new strategies are being developed for their generation. Catalytic antibodies are characterized by high substrate specificity, share many mechanistic features with enzymes, including catalysis by proximity, transition-state stabilization, and covalent catalysis, and in some cases their rates are comparable to enzymes. The next few years should likely see an emphasis on increasing the catalytic efficiency of antibodies by generating active

sites in which catalytic groups, cofactors, and entropic effects work in concert. This may well be achieved by augmenting mechanism-based hapten design with genetic screens and selections. Recent advances in bacterial expression systems for antibodies, as well as the combinatorial λ and phage libraries, should greatly facilitate these efforts. At the same time, the ability to engineer catalyst specificity should be increasingly applied to the development of novel catalysts for biological, chemical, and medical applications. Finally, this work underscores the new opportunities that result when chemistry and biology are used synergistically to tap the wealth of molecules available from nature.

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