Catalytic Antibodies as Designer Proteases and Esterases

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1. Introduction

Most chemical transformations in the biological world are catalyzed by enzymes. Enzymes participate in biological systems by means of their catalytic activity along with their selectivity and specificity. Creating designer protein catalysts that exhibit such selectivity and specificity facilitates an understanding of how enzymes work and evolved. In addition, such designer proteins can catalyze chemical transformations on demand even when no natural enzyme does so. Preparation of catalytic antibodies is one strategy for accessing such designer protein catalysts.

Fujie Tanaka was born in Hiroshima, Japan. She received her Ph.D. from Kyoto University in 1992 under the direction of Kaoru Fuji. After postdoctoral studies with Chi-Huey Wong in the Frontier Research Program, Riken, with Ikuo Fujii at the Protein Engineering Research Institute (Biomolecular Engineering Research Institute), and Carlos F. Barbas III at The Scripps Research Institute, she was appointed Assistant Professor of the Department of Molecular Biology, The Scripps Research Institute, in 2000. Her research interests include molecular recognition and catalysis, including the development of antibody catalysts, the development of catalytic and functional small peptides, and the development of synthetic methodology.

The concept of catalytic antibody was described by Jencks: "If complementarity between the active site and the transition state contributes significantly to enzymatic catalysis, it should be possible to synthesize an enzyme by constructing such an active site. One way to do this is to prepare an antibody to a haptenic group which resembles the transition state of a given reaction. The combining site of such antibodies should be complementary to the transition state and should cause an acceleration by forcing bound substrates to resemble the transition state." [1] According to this concept, the first monoclonal catalytic antibodies were reported by the two groups.[2,3] The Lerner group generated catalytic antibodies by immunization with a phosphonate transition-state analogue designed for ester hydrolysis, and Schultz's group demonstrated that the antibody that binds to a phosphate diester transition-state analogue is a catalyst. Since then, antibody catalysts have been prepared for many types of reactions by using anti-

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body diversity and specificity. Catalytic antibodies have been obtained not only with transition-state analogues but also with other designed compounds.

In this review I describe monoclonal catalytic antibodies that catalyze the hydrolysis of ester, amide, carbonate, and carbamate. The focus is on aspects of hapten design, selection strategy, and their consequences. The purpose is to provide an understanding of how designer protein catalysts are created using the immune system. Information about naturally occurring catalytic antibodies, anti-idiotypic catalytic antibodies, and polyclonal catalytic antibodies is not included in this review.

2. Hydrolytic Antibodies Generated against Transition-State Analogues

2.1. Transition-State Theory and Hydrolytic Antibodies

Ester and amide hydrolysis is known to involve the formation of a high-energy intermediate that subsequently decomposes to become the corresponding acid and alcohol or amine depending on the pK_a of the leaving group. This high-energy tetrahedral intermediate and its transition state can be mimicked by using phosphonates and phosphonamidates (Scheme 1). (In the case of unactivated amides, i.e., amides of a poor leaving group, decomposition of the tetrahedral intermediate is another high-energy step for hydrolysis.[4]). Antibodies specific for the transition-state analogue might be more specific for the transition state of the reaction than the ground-state substrate. According to transition-state theory,[5] under ideal conditions, the rate enhancement can be expected from the transition-state stabilization from a thermodynamic cycle.[6,7] The constant K_S is defined as the dissociation constant for the antibody−substrate complex, K_{TS} is the dissociation constant for the antibody−transition-state complex, k_{cat} is the rate constant for the reaction of the antibody−substrate complex, and k_{uncat} is the rate constant for the reaction without antibody. The system depicted in Scheme 1b represents the equation

$$k_{cat}K_{TS} = k_{uncat}K_S$$

$$k_{cat}/k_{uncat} = K_S/K_{TS}$$

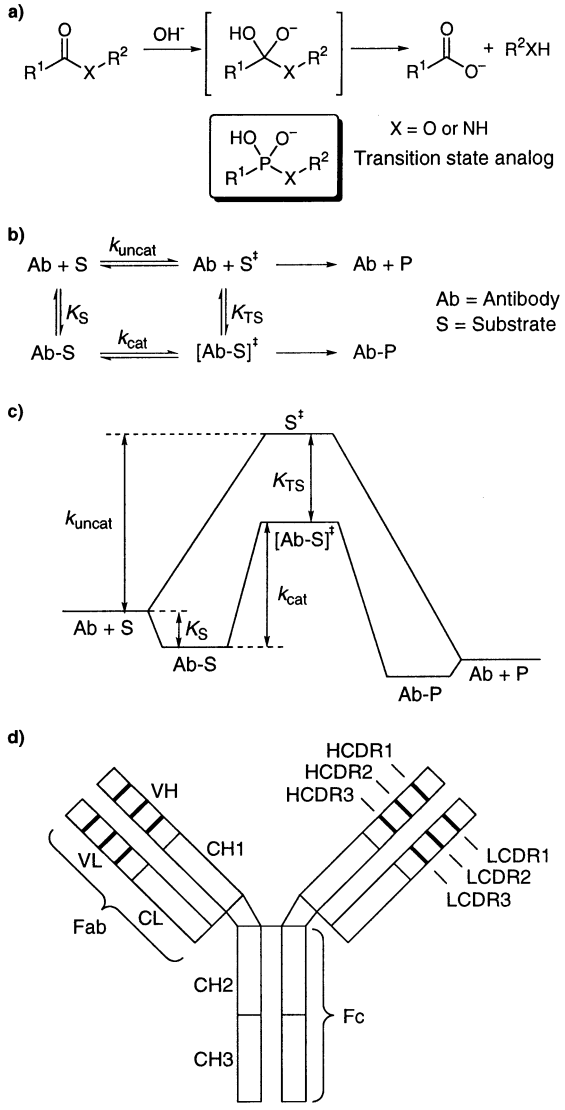
If a hapten is a true analogue of the actual transition state of a reaction, the rate enhancement of an antibody-catalyzed reaction can be predicted from the ratio of the affinity for the substrate to the affinity for the transition-state analogue (K_{TSA}). Assuming $K_m \approx K_S$ and $K_i \approx K_{TSA}$ provides the following relationship:

$$k_{cat}/k_{uncat} = K_S/K_{TS} = K_S/K_{TSA} \approx K_m/K_i$$

This relationship of K_i values of a series of related transition-state analogue inhibitors with the kinetic parameters of the parallel series of related substrates has also been observed in enzymes.[8]

A summary of ester and amide hydrolytic antibodies whose structures were determined by X-ray

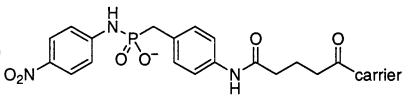
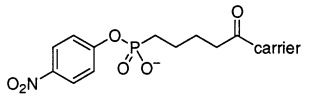
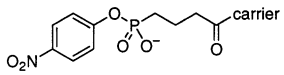
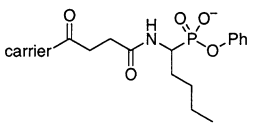
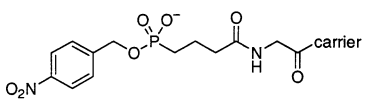
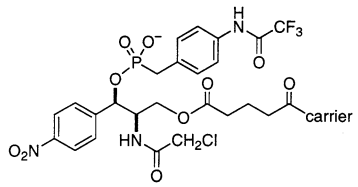
Scheme 1[a]



[a] (a) The mechanism of ester and amide hydrolysis involves a tetrahedral intermediate and the corresponding phosphonate (X = O) or phosphonamidate (X = NH) transition-state analogue. (b) A thermodynamic cycle interrelating substrate and transition-state binding for an antibody. (c) An energy profile for the antibody-catalyzed reaction. (d) Schematic representation of an antibody (IgG) molecule. One antibody molecule has two identical active sites. A light chain is built up from one variable domain (VL) and one constant domain (CL), and a heavy chain from one variable domain (VH), followed by constant domains (CH1, CH2, and CH3). Complementarity-determining regions, CDR1−CDR3, vary among different immunoglobulins and determine the specificity of the antigen−antibody interactions.

structural analysis in addition to characterization of the catalyzed reactions appears in Table 1. About half of these antibodies follow the relationship $k_{cat}/k_{uncat} = K_S/K_{TSA}$, but the remaining antibodies do not fit the equation, although stabilization of the transition state formed by nucleophilic attack of a hydroxide or an amino acid residue on the substrate carbonyl is the important mechanism of catalysis for all antibodies listed in Table 1. The antibody−phosphonate hapten complex structures show hydrogen bonds between antibody amino acid residues and phosphonate oxygen atoms. The similarities between these active site structures, particularly their hydrogen bonds, have been discussed in other reviews.[9] In the

Table 1. Kinetic Parameters of Hydrolytic Antibodies and Their Amino Acid Residues Forming Hydrogen Bonds with Phosphonate Oxygens in Antibody−Hapten Complexes

Antibody	Hapten[a]	Type of substrate	pH	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/k_{uncat}	K_S/K_{TSA} or K_m/K_i	H-bond residues[b]	Mechanism[c]	Ref.
43C9		Amide	9.0	0.08	562	2.5×10^5	225[d] 7.0×10^{5} [e]	Arg L96, His H35	T + N (His L91)	19-22
48G7		Ester	8.2	5.5	391	1.6×10^4	3.9×10^4	Arg L96, Tyr H33, His H35	T + [N or G]	23-26
CNJ206		Ester	8.0	0.39	110	1.6×10^3	208	His H35, (Asp H96), (Tyr H97)	T + [N (Tyr) or G]	27-30
17E8		Ester	8.7	101	259	1.3×10^4	5.2×10^2	Arg L96, Lys H93, (Tyr H96)	T + N or G	31-33
29G11		Ester	9.5	59	639	2.2×10^3	2.4×10^4	Arg L96, Lys H93, (Tyr H96)	T + N or G	33
D2.3		Ester	8.3	3.1	280	1.1×10^5	1.1×10^5	Asn L34, Trp H95, Tyr H100d, H_2O	T	10-13
D2.4		Ester	8.3	1.0	260	3.6×10^4	3.3×10^4	Asn L34, Trp H95, Tyr H100e, H_2O	T	10-13
D2.5		Ester	9.0	0.39	180	1.9×10^3	1.3×10^3	Trp H95, Tyr H100d, H_2O-Ser L34	T	10-13
6D9		Ester	8.0	0.145	50	9.0×10^2	9.0×10^2	His L27d	T	14-18
7C8		Ester	8.0	0.115	3.8	7.1×10^2	12	Tyr H95	T + N or G	14-17, 34

[a] Hapten−carrier protein conjugate used for immunization to produce catalytic antibody. [b] Antibody residues donating hydrogen bonds to oxygen atoms of phosphonate or phosphonamide in the antibody−hapten cocrystal structure (except 43C9). The residues are numbered according to Kabat et al. (Kabat, E. A.; Wu, T. T.; Perry, H. M.; Gottes-man, K. S.; Foeller, C. *Sequences of Proteins of Immunological Interest*, 5th ed.; U.S. Public Health Service, National Institutes of Health: Bethesda, MD, 1991). Residues in parentheses from a hydrogen bond from the backbone amide NH. Water molecules within hydrogen-bonding distance are also indicated. H_2O−Ser L34 (D2.5) indicates that a water molecule forms a hydrogen bond with phosphonate oxygen, and Ser L34 forms a hydrogen bond with the water. [c] Mechanism of the antibody-catalyzed hydrolysis. T = stabilization for the transition state, formed by a hydroxy anion or nucleophilic residue's attack, through hydrogen bonds between the antibody and the oxyanion. N = nucleophilic catalysis. Residues that form acyl intermediates are indicated in parentheses. G = general-base catalysis. Possible mechanisms are indicated in brackets. [d] $K_i = 2.5$ μM, determined by inhibition assay.[19] [e] $K_i = 0.8$ nM, determined by quenching of intrinsic antibody fluorescence.[19]

catalyzed reactions, these residues would be expected to form hydrogen bonds to the oxyanion of the transition state produced by hydroxyl anion or the nucleophilic residue's attack on the substrates, lowering the activation energy. Antibodies D2.3,[10−13] D2.4,[10−12] D2.5,[10−12] and 6D9[14−18] catalyze the hydrolytic reaction by transition-state stabilization. In addition to transition-state stabilization, other catalytic mechanisms, such as nucleophilic and/or general-base catalysis, have been observed or suggested for antibodies 43C9,[19−22] 48G7,[23−26] CNJ206,[27−30] 17E8,[31−33] 29G11,[33] and 7C8.[14−17,34] Although the haptens employed to generate these antibodies do not have a structural entity that induces amino acid residues capable of contributing to nucleophilic and general-base catalysis, antibody diversity has the potential to provide catalytic antibodies possessing these properties in addition to the transition-state stabilization. Tyrosine[35] and histidine[21,22] have been identified as the nucleophilic residues involved in nucleophilic catalysis when antibodies were generated by immunization, whereas nucleophilic serine and cysteine residues are found in many naturally evolved proteases.[36] This difference may originate from (1) the difference in the selection strategies, i.e., binding selection for catalytic antibodies and functional selection (rate acceleration for a certain substrate) for natural enzymes, (2) limitation of V gene availability, i.e., limitation of catalytic residue availability in the immune responses (see section 2.3), and (3) conformational and dimensional restrictions in antibody CDRs to form proper arrangement of catalytic residues. Functional selection, expanding the antibody repertoire, and using combinatorial antibody libraries in vitro to search for catalytic antibodies are discussed below.

Most ester hydrolytic antibodies generated against phosphonate transition-state analogues catalyze their reactions with rate acceleration values (k_{cat}/k_{uncat}) from 10^2 to 10^5, and the best value of k_{cat}/k_{uncat} performed by an ester hydrolytic antibody is 6.25×10^6.[37] One or two hydrogen bonds between antibody residues and the hapten phosphonate oxyanion are exhibited in the complex structures. The typical binding energy for one hydrogen bond between charged and uncharged groups in enzyme−substrate

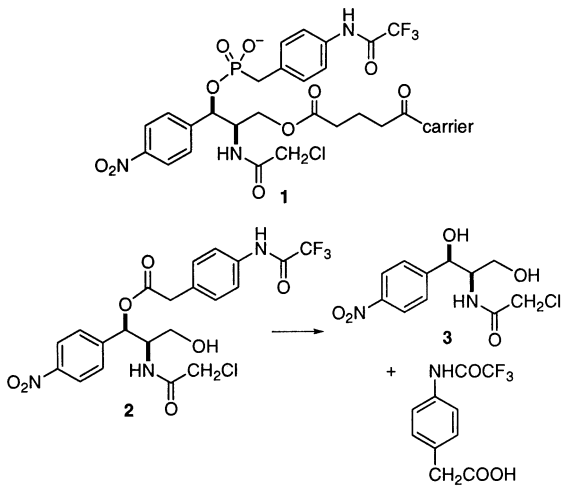
(or inhibitor) complexes is ~4 kcal/mol, and that between two uncharged groups is 0.5−1.5 kcal/mol.[38] Therefore, the maximum value of k_{cat}/k_{uncat} that can be expected in antibody-catalyzed reactions is $\sim 10^5$ when two hydrogen bonds (4 kcal/mol plus 1.5 kcal/mol) contribute to the rate acceleration by transition-state stabilization. The rate accelerations (10^2−10^5) achieved by antibodies that were generated with phosphonate transition-state analogues reflect the type of selection strategy, binding selection against the transition-state analogues. This range of rate accelerations is excellent for designer catalysts, although it may seem to be moderate compared to that of natural protease and esterase enzymes. Generation of catalytic antibodies is the most powerful and successful procedure available for creating protein catalysts that possess substrate specificity at will. Many antibody catalysts can process substrates that natural enzymes cannot. No method other than generation of catalytic antibodies can provide such a variety of designer protein catalysts from a single scaffold.

2.2. Comparison of a Set of Antibodies Generated against a Single Hapten

When mice are immunized with a phosphonate transition-state analogue−carrier protein conjugate to generate catalytic antibodies, a few and occasionally several of the dozens of antibodies that bind the hapten are catalytic. These catalytic antibodies are very similar or are divisible into a few groups, each of which is similar in catalytic mechanism, biochemical properties, and structures, but varied in the degree of rate enhancement of the catalytic activity.

Six catalytic antibodies elicited with transition-state analogue phosphonate **1**[14] were dissected on the basis of transition-state analysis.[15] These antibodies were generated for catalyzing the hydrolysis of prodrug ester **2** to yield the drug chloramphenicol (**3**) (Scheme 2). The plot of $\log(K_S/K_{TSA})$ versus $\log(k_{cat}/k_{uncat})$ for four of these catalytic antibodies−6D9 (Table 1), 4B5, 8D11, and 9C10−had a linear relationship (slope 0.99), but the values for the remaining two antibodies−7C8 (Table 1) and 3G6−deviated from this line (Figure 1). The magnitude of the slope

Scheme 2



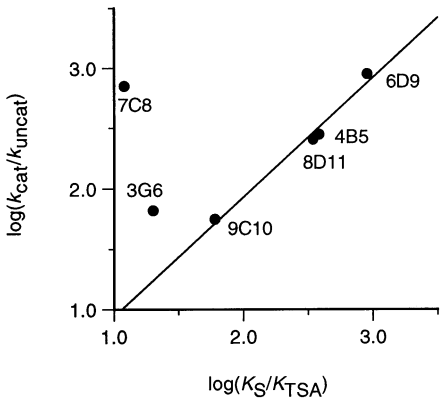
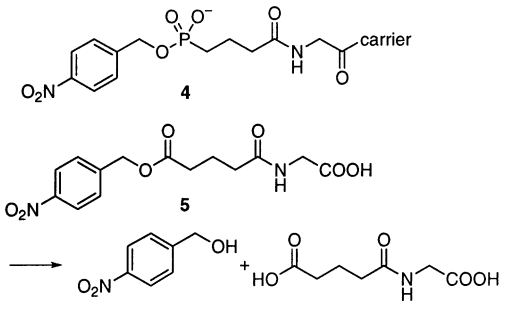


Figure 1. Plot of $\log(K_S/K_{TSA})$ versus $\log(k_{cat}/k_{uncat})$ for catalytic antibodies generated against transition-state analogue **1**.

for the linear relationship (0.99) suggests that the entire differential binding energy of the transition state versus the ground state was available for the rate enhancement and that the transition-state analogue matched the transition-state structure of the uncatalyzed reaction almost exactly. The four antibodies bearing a linear relationship shared highly homologous amino acid sequences, with the essential catalytic residue His at light chain 27d (L27d), and had similar substrate specificity.[15−18] Antibody 3G6 had amino acid sequences similar to those of the four related antibodies, with the exception of a Tyr residue at L27d. The five antibodies 6D9, 4B5, 8D11, 9C10, and 3G6 were derived from the same germline gene in both light and heavy chains.[16] Antibody 7C8 had sequences and substrate specificity different from those of the other five antibodies.[15,16] This difference in substrate specificity was attributed to a differing hapten recognition mode observed in the crystal structures of 7C8 and 6D9.[18,34] The majority of these catalytic antibodies generated against a single transition-state analogue were highly homologous in biochemical and structural properties, and they catalyzed the reaction with the same mechanism, as expected from the designed transition-state analogue. However, noncatalytic (binding) antibodies generated from the same hapten **1** from the same hybridoma fusion as the catalytic antibodies also bound to the transition-state analogue with high affinity.[14] Subsequent analyses of noncatalytic binding antibodies showed that higher binding affinities to the transition-state analogue did not correlate with their catalytic activities, but differential binding between the transition-state analogue and the substrate is important for catalysis, as explained by transition-state theory. That is, many antibody V genes can provide diverse modes and types of interactions with a transition-state analogue, but for catalysis, a certain orientation of phosphonate transition-state analogue and formation of hydrogen bonds with the phosphonate oxyanion and oxygen are necessary. Such interactions can be provided by only a few V gene combinations.

Antibodies D2.3,[39] D2.4, and D2.4 generated with phosphonate **4** catalyzed the hydrolysis of **5** (Scheme 3, Table 1).[10−13] These antibodies had rate accelerations that correlated to their affinity for the transi-

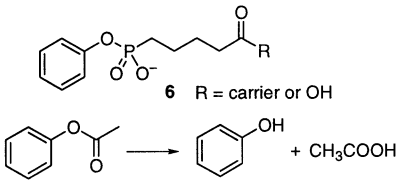
Scheme 3



tion-state analogue relative to the substrate ($K_S/K_{TSA} = k_{cat}/k_{uncat}$) (Table 1).[10] The mechanism of the reactions catalyzed by these antibodies is delineated by the transition-state stabilization and by X-ray structural studies.[12] The sequences of these three antibodies were derived from the same germline gene and share a high degree of identity,[11] and structural convergence in the active sites was observed in the crystal structures.[12]

In the case of antibodies elicited from phosphonate **6**, five antibodies including 20G9[40] (Scheme 4) have

Scheme 4



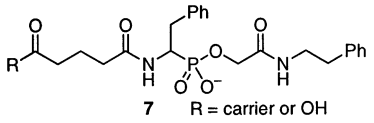
Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
6	20G9	18.9 min^{-1} [14700] at pH 8.5	167 μM [39 nM for **6** (R = OH)]

been analyzed.[41] All these antibodies displayed a burst phase followed by a steady-state phase, and their pH profiles were similar in the catalytic reactions. In accord, their amino acid sequences were highly homologous. A plot of log K_i versus log(K_m/k_{cat}) gave a linear relationship (slope 0.5). A possible reason for a slope deviating from unity is that the hapten does not perfectly mimic the transition state. The linearity of this plot indicates the importance of hapten affinity to the transition-state analogue for transition-state stabilization, and the slope suggests the critical importance of designing haptens that closely resemble the true transition state. These catalytic antibodies catalyzed the reaction with a mechanism involving both an acyl−tyrosyl intermediate and transition-state stabilization.[35]

A series of six catalytic antibodies, including CNJ206 (Table 1), generated with a single hapten were also studied for biochemical properties and primary structures.[27] These catalytic antibodies were divided into three groups by sequence analyses. Group I antibodies (CJN157, CJN2, CJN19) had similar light and heavy chain variable regions (VL and VH, respectively). The other three antibodies had similar VL regions, and of these, two (group II, CNJ206 and CNJ174) had similar VH regions, the remaining one (CNJ123) forming group III. Group I catalyzed the hydrolysis of the ester and the corresponding carbonate, but groups II and III catalyzed only the hydrolysis of the ester. Group I exhibited tighter binding to the hapten than groups II and III. All antibodies were inactivated by tyrosine modification, but tryptophan or arginine modification produced different results for each group.

Immunization of autoimmune mice expanded the structural repertoire of catalytic antibodies. The occurrence of catalytic antibodies in autoimmune mouse strains, such as MRL/*lpr* and SJL, was dramatically higher than in immunologically normal mouse strains, such as BALB/c and wild-type MRL/++, when *p*-nitrobenzyl phosphonate **4** was injected to elicit hydrolytic antibodies.[42] The results were similar when phosphonate **7** (Chart 1) was the

Chart 1



hapten used to elicit hydrolytic antibodies.[43,44] Amino acid sequences in the variable region of catalytic antibodies generated in autoimmune-prone mouse strains differed from those of catalytic antibodies made by normal mice. Since the antibody repertoire in autoimmune mouse strains is virtually unrestricted, the opportunity may be increased to provide a certain interaction with a transition-state analogue for catalysis. However, these catalytic antibodies also had homologous primary amino acid squences within a series of catalytic antibodies generated by immunization with a single phosphonate transition-state analogue.[44] In MRL/*lpr* mice, catalytic subsets that existed in the initial repertoire were effectively captured by the phosphonate hapten.

2.3. Affinity Maturation to a Transition-State Analogue in Vivo

Antibody 48G7 (Table 1) catalyzes the hydrolysis of both *p*-nitrophenyl acetate and the corresponding carbonate.[23] X-ray structures of the mature 48G7 and its germline antibody have been solved with and without the hapten.[24−26] Nine amino acid residues are different in the variable region between 48G7 and the germline antibody. These nine somatic mutations, which were introduced during maturation, improved affinity for the hapten by 3000-fold (K_d = 16 nM for 48G7 and 50 μM for the germline antibody).[26] Although germline antibody 48G7 has lower affinity to the hapten than affinity-matured 48G7, the germline antibody is still catalytic ($k_{cat}/K_m = 1.4 \times 10^4$ min^{-1} M^{-1} for matured 48G7 and 1.7×10^2 min^{-1} M^{-1} for the germline antibody).[26]

In the structures of matured 48G7, comparison of the unliganded 48G7 with the 48G7−phosphonate hapten complex revealed that very few structural changes occurred upon binding of hapten.[25] On the other hand, in the case of germline antibody 48G7, binding of the hapten led to significant structural changes.[24] Such structural changes in the germline−

hapten complex become preorganized in the combining site of the mature antibody. Somatic mutations improved complementarity to the hapten in a preorganized fashion, and as a result, the hapten bound to the mature antibody 48G7 by a lock-and-key mechanism.[24]

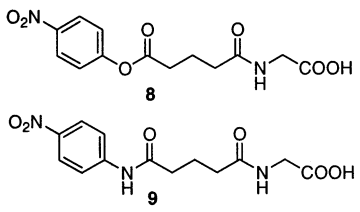
Although affinity-matured 48G7 and the germline 48G7 bound different conformations of the hapten in their complex structures, the positions of the phosphonate group were essentially the same in the two structures.[45] None of the nine residues in which somatic mutations have been fixed directly contacted the hapten. Arg L96 and His H35 formed an oxyanion hole for the phosphonate group in both complexes. Tyr H33 formed a hydrogen bond to the phosphonate oxygen in the affinity-matured antibody complex but not in the germline antibody complex. Tyr H33 packed against the methylene linker in the germline complex. The affinity maturations appear to play a conformational role, either in reorganizing the active site geometry or in limiting side chain and backbone flexibility of the germline antibody. Individual somatic mutations were either positive or neutral in their effects on affinity for the hapten and contributed to affinity in a nonadditive fashion. Significant cooperativity was found between proximal as well as nonproximal mutations.[45]

The residues that donated hydrogen bonds to the oxygen atoms of the hapten phosphonate, His H35 (CNJ206),[27] Asn L34 (D2.3 and D2.4),[11] and His L27d (6D9)[16] (Table 1), were also found in their germline antibodies, in addition to Tyr H33 and His H35 (48G7).[45] Maturation of each germline antibody provided a set of antibodies that were markedly similar in amino acid sequences and biological properties.

2.4. Conformational Change of the Active Site upon Binding and Catalysis

Kinetic studies indicated that antibodies D2.3, D2.4, and D2.5 bound phosphonate hapten **4** (Scheme 3, Table 1) with an induced-fit mechanism,[46] although comparison of the unliganded structures of these antibodies with their hapten complexes indicates that their conformation does not change upon binding to the hapten.[12,13] In the catalytic assay of antibody D2.3, a gradual increase of the initial rate of hydrolysis was observed for ester **8** (Chart 2), reaching a

Chart 2



steady-state constant rate after several minutes. The lag was resolved after multiple turnovers. The kinetic behavior of antibody D2.3 indicated a slow, rate-determining, substrate-induced conformational change leading from a less active (or nonactive) complex to a more active complex. The active conformer had increases of both affinity of binding to the substrate and catalytic activity. When the substrate was readded to an ongoing reaction before the completion of the first reaction, the active conformation still prevailed, and the second reaction proceeded with no lag. Preincubation with substrate **5** and with the amides **9** (nonhydrolyzable substrate analogues) also provided the same effect. Although phosphonate **4** (R = OH) inhibited catalysis, the binding of **4** (R = OH) to antibodies D2.3, D2.4, and D2.5, tested by fluorescence quenching, exhibited biphasic kinetics that implied an induced-fit mechanism. Since the incubation of D2.3 in crystallization buffer altered the preequilibrium to favor the active form, crystallization might provide the active conformer both in the presence and in the absence of the hapten.

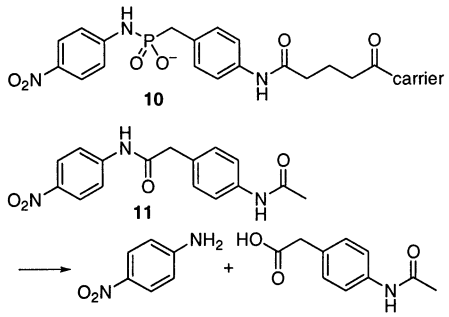
In the case of antibody CNJ206, comparison between the CNJ206−hapten complex[28] and unliganded CNJ206[30] indicates the conformational changes occurring during hapten binding.

2.5. Amide Hydrolyis

2.5.1. Aryl Amide Hydrolysis

Immunization with phosphonamidate **10** provided catalytic antibody 43C9 (Table 1), which catalyzes amide hydrolysis of **11**[19] (Scheme 5) as well as

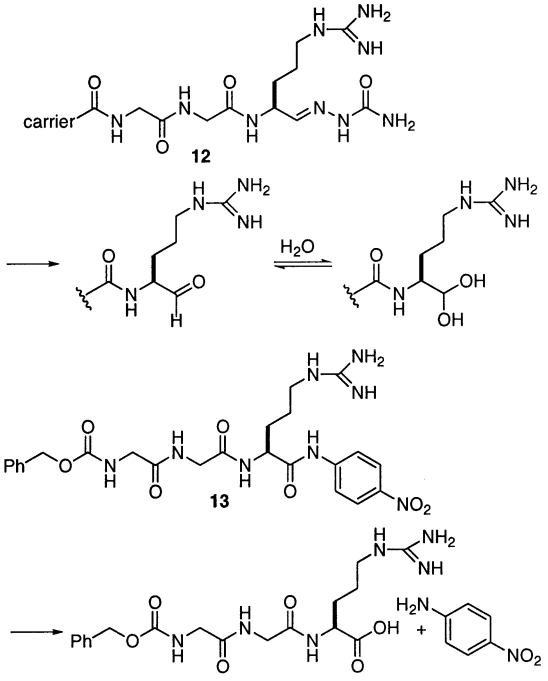
Scheme 5



esters.[20,21] A covalent acyl−antibody intermediate formed during this catalysis. Key steps of the amide hydrolytic process include transition-state stabilization through a hydrogen bond network and nucleophilic attack by an imidazole of His L91 to form the first tetrahedral intermediate.[22] Tyr L36 and Tyr H95 may assist nucleophilic attack of His L91 by a proton transfer from the imidazole. Arg L96 and His H35 stabilize the negative charge of the transition state produced by nucleophilic attack of the imidazole and of a hydroxyl anion. Nucleophilic attack by imidazole may account for the amide hydrolysis by 43C9. Although antibodies 48G7 and D2.3 provided a similar or higher value of k_{cat}/k_{uncat} compared to 43C9 in the ester hydrolysis, these antibodies did not catalyze the hydrolysis of *p*-nitroanilide.[23,46] Therefore, the catalytic machinery of 43C9 is distinctive.

Tripeptide aldehyde was used for the generation of antibodies that catalyze the amide hydrolysis of the corresponding tripeptide *p*-nitroanilide (Scheme 6).[47] Peptide aldehydes have been studied as inhibitors of serine and cysteine proteases.[8b,48] The aldehydes exist in water predominately as hydrates, and these *gem*-diols resemble the tetrahedral intermediates formed during the attack by water on an amide.

Scheme 6



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m
12	2C9	0.23 min^{-1} [1400] at pH 7.4	550 μM

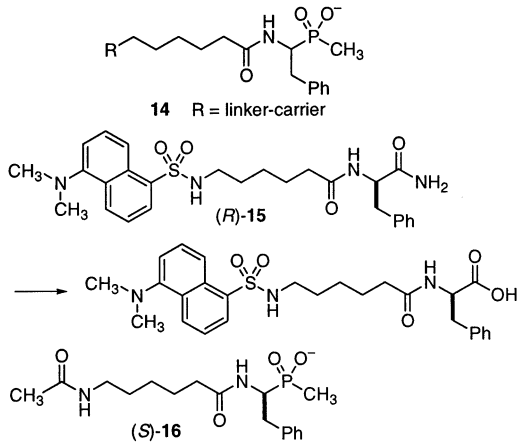
Semicarbazone derivative **12** was used for immunization to generate the corresponding aldehyde and the hydrated form as a transition-state analogue in vivo. Antibody 2C9, which was obtained by this concept, catalyzed hydrolysis of the aryl amide bond of Cbz-Gly-Gly-Arg-*p*-nitroanilide (**13**) with a k_{cat}/k_{uncat} of $\sim 10^3$ but did not catalyze the hydrolysis of acetyl-Leu-Leu-Arg-*p*-nitroanilide.

2.5.2. Unsubstituted Amide Hydrolysis

Phosphinate **14** was used for the generation of antibodies that catalyze the hydrolysis of an unsubstituted amide (Scheme 7).[49] Antibody 13D11, generated with a racemic mixture of **14**, catalyzed the hydrolysis of the primary amide of (*R*)-**15** with a k_{cat}/k_{uncat} of 132, and the catalyzed reaction was inhibited with phosphinate (*S*)-**16** possessing the same stereochemistry as the substrate. This antibody did not catalyze the corresponding methyl ester or (*S*)-**15**.

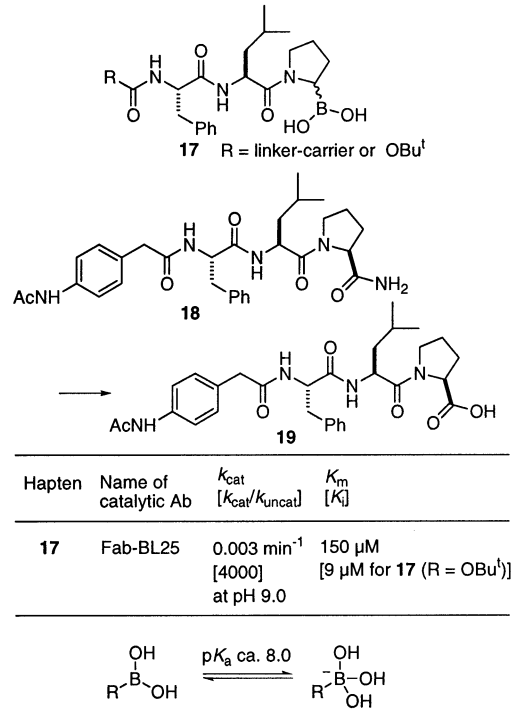
Boronic acid was used as the hapten for the generation of primary amide bond hydrolytic antibodies (Scheme 8).[50] Boronic acid inhibitors of serine proteases are known to bind by coordination with the active-site serine or histidine to form a high-affinity tetrahedral anionic mimic of the transition state for peptide bond cleavage.[51] Antibody selection of such a Lewis base may lead to recruitment of nucleophilic catalysis and/or transition-state stabilization by recognition of the tetrahedral adduct formed after coordination. A hapten **17**−carrier protein conjugate was used for immunization, and a combinatorial Fab library was prepared using an immunized mouse spleen. The library was selected against **17** by phage

Scheme 7



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
14	13D11	9.9 x 10^{-6} min^{-1} [132] at pH 9.0	432 μM [14 μM for (*S*)-**16**]

Scheme 8



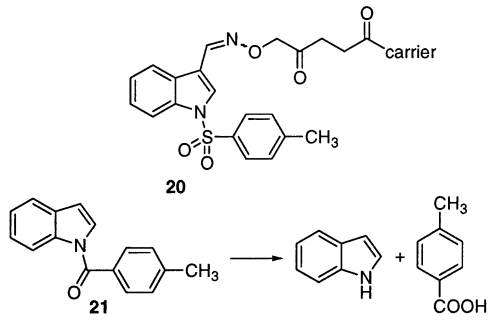
Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
17	Fab-BL25	0.003 min^{-1} [4000] at pH 9.0	150 μM [9 μM for **17** (R = OBu^t)]

display. Fab BL25 catalyzed the hydrolysis of **18** and gave **19** with a k_{cat}/k_{uncat} of 4000. The pH profile of competitive ELISA binding studies showed a marked increase in BL25 affinity for the inhibitor **17** (R = OBu^t) with an elevation in pH. The 50% displacement point occurred at 8.0, which is the approximated pK_a for the hydration of a trigonal α-amino boronic acid to its tetrahedral form. These data are strong evidence that BL25 binds the tetrahedral form, suggesting that the catalytic power of BL25 is linked to transition-state stabilization of a process involving the catalytic addition of water to the primary amide rather than by elicitation of a complementary Lewis base in the antibody binding site.

2.5.3. Heterocyclic Amide Hydrolysis

Sulfonamide **20** was used for the generation of antibodies that catalyze the hydrolysis of acylindole **21** (Scheme 9).[52]

Scheme 9

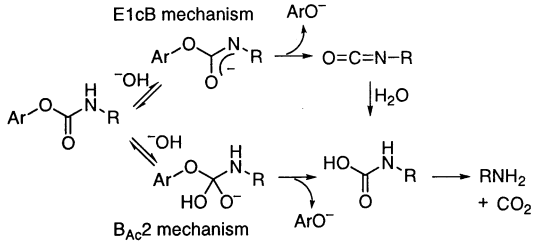


Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m
20	312D6	4.5×10^{-4} min^{-1} [750] at pH 8.0	35.7 μM

2.6. Carbamate Hydrolysis

Aryl carbamate hydrolysis is known to occur dominantly via an E1cB (elimination−addition) mechanism, whereas the $B_{Ac}2$ (addition−elimination) pathway involving a tetrahedral intermediate operates effectively only for carbamates that lack an ionizable N−H group (Scheme 10).[53] The rate of alkaline

Scheme 10[a]

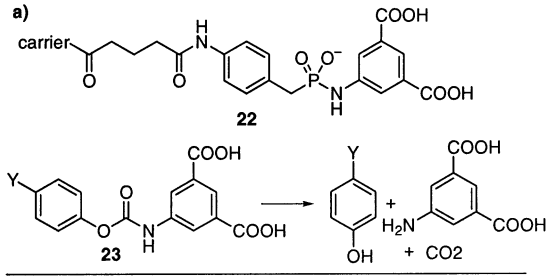


[a] Mechanism for alkaline hydrolysis of carbamate.

hydrolysis via the $B_{Ac}2$ process is up to 10^8 times slower (depending on substutution in the aryl ring) than that of the E1cB process. Immunization with phosphonamidate **22** generated antibody DF8-D5, which catalyzes the hydrolysis of N−H carbamates **23** (Y = NO_2, Br, F, OCH_3) with a k_{cat}/k_{uncat} range of 3.0×10^3 to 1.2×10^6 (Scheme 11a).[53] The significantly smaller Hammett ρ value obtained for the antibody-catalyzed reactions compared to that obtained for the uncatalyzed reactions suggested that the antibody-catalyzed reactions proceeded via the disfavored $B_{Ac}2$ mechanism rather than the E1cB process found for the uncatalyzed reactions.

Antibody ST51 was generated against **24**, a transition-state analogue of the $B_{Ac}2$ process for the hydrolysis of *N*-methylcarbamates (Scheme 11b).[54] Although *N*-methylcarbamate **25** has a *p*-nitrophenyl leaving group, it is very resistant to hydrolysis, exhibiting a half-life of approximately 5.7 years at pH 9.0. Antibody ST51 catalyzed the hydrolysis of **25** with a k_{cat}/k_{uncat} of 6500.

Scheme 11



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m
22	DF8-D5	0.17 min^{-1} for **23** (Y = F) [4000] at pH 6.5	41 μM
24	ST51	0.0015 min^{-1} [6500] at pH 9.0	260 μM
26	33B4F11	1.5 min^{-1} [260] at pH 7.0	5.5 μM

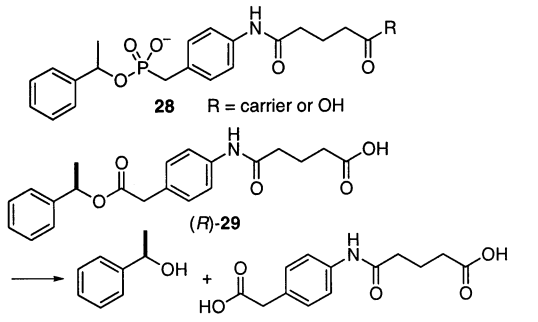
Phosphonate **26** provided antibody 33B4F11, which catalyzed the hydrolysis of carbamate **27**, whose rate acceleration (k_{cat}/k_{uncat} = 260) was moderate (Scheme 11c).[55] This antibody also catalyzed the hydrolysis of *p*-nitrophenyl acetate.

2.7. Selectivity and Scope of Substrate Specificity

2.7.1. Enantioselective and Stereoselective Hydrolysis

Immunization with racemic phosphonates resulted in the generation of antibodies that catalyze stereoselective hydrolysis of either the (*R*)- or (*S*)-ester substrate. When racemic phosphonate **28** was used for the immunization, 11 antibodies that catalyzed the hydrolysis of (±)-**29** were identified.[56] Nine of them, including 2H6, catalyzed the hydrolysis of (*R*)-**29**, and the other two, including 21H3, catalyzed the hydrolysis of (*S*)-**29** (Scheme 12). Since less than 2%

Scheme 12



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
28	2H6	4.6 min^{-1} for (*R*)-**29** [8.3 x 10^4] at pH 9.0	4.0 mM [2.0 μM for **28** (R = OH)]
28	21H3	0.09 min^{-1} for (*S*)-**29** [1619] at pH 9.0	394 μM [0.19 μM for **28** (R = OH)]

of the opposite stereoisomer underwent hydrolysis in the antibody-catalyzed reactions, presumably antibodies that bound either (*R*)- or (*S*)-**28** were induced separately, when racemic hapten was used for the immunization. This is a convenient feature for the preparation of antibody catalysts, because the chiral synthesis or separation of enantiomers of the transition-state analogue is not necessary.

When a diastereomer mixture of **30** was used for immunization, the antibodies induced, including antibody 2H12E4, catalyzed the stereoselective ester hydrolysis of **31** containing (*R*)-phenylalanine (Scheme 13).[57] The preference for **31** was >200/1 relative to **32** containing (*S*)-phenylalanine.

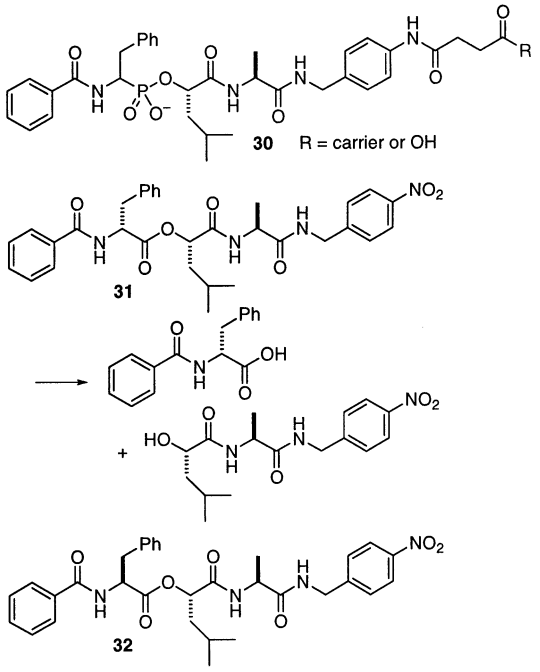
Antibodies 17E8 and 29G11 (Table 1) were generated against **33** and catalyzed the enantioselective hydrolysis of (*S*)-**34** (Scheme 14).[31−33] Antibody 17E8 used hydrophobic binding interactions of the alkyl side chain remote from the reaction center to controll enantioselectivity and catalytic activity.[58] Removing methylene groups from the side chain of substrate **34** decreased k_{cat} values and increased K_m values. For example, the catalyzed reactions of norvaline, α-aminobutyric acid, and alanine esters gave the lowered transition-state stabilization up to $\Delta\Delta G = 2.5$ kcal/mol compared to that of substrate **34**.

Antibody-catalyzed enanioselective hydrolysis of *meso*-ester substrate[59] and antibody-catalyzed enol ester hydrolysis following enantioselective protonation[60] have been reported.

2.7.2. Relaxing Substrate Specificity

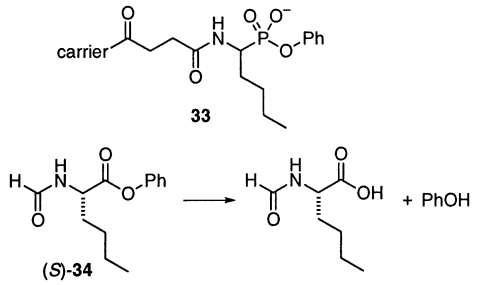
Although high enantioselectivities have been accomplished, the substrates available for antibody-catalyzed reactions are typically limited within a narrow range due to the inherent binding specificity of antibodies. For a catalytic antibody to be useful, depending on the purpose, it must be able to accept a broad range of substrates on demand, yet retain high selectivity. A strategy for relaxing substrate specificity has been reported in antibody-catalyzed enantioselective hydrolysis of *N*-Cbz-amino acid esters (Scheme 15).[61] For this strategy, a transition-state analogue was designed on the basis of the size

Scheme 13



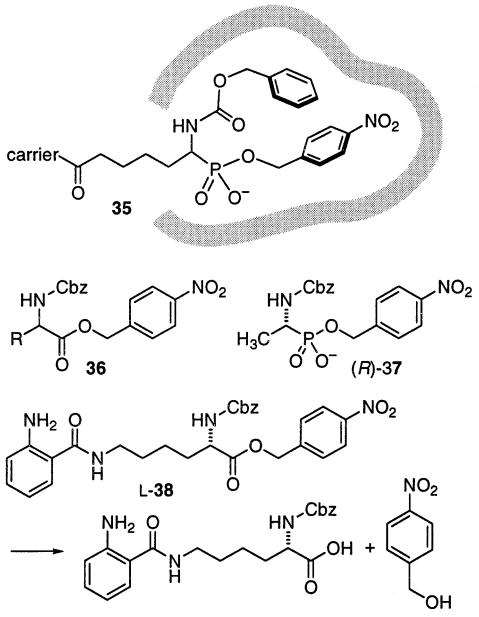
Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
30	2H12E4	0.0187 min^{-1} for **31** at pH 8.0 (k_{32}/k_{31} <0.005)	14.8 μM [2.4 μM for **30** (R = OH)]

Scheme 14



of the antigen-combining sites of antibodies observed in X-ray structures of antibody−small hapten complexes.[62] Relatively small haptens, such as fluorescein and progesterone, are buried deep in the antigen-combining sites in the complex structures. To induce high enantioselectivity in antibody-catalyzed reactions, an immunizing haptenic epitope including a chiral center should be within this size, by design, so that the haptenic epitope occupies the antigen-combining site. In addition, since the linker to the hapten is outside the antigen-combining site, the attachment site of the linker to the hapten should be positioned to allow broad substrate specificity. Accordingly, phosphonate transition-state analogue **35** was designed with these requisites and used to generate catalytic antibodies that enantioselectively hydrolyze amino acid ester derivatives **36** possessing various α-substituents. The surface area (solvent-accessible area) of the packed conformation of transition-state analogue **37** fell into the range of the size of antigen-combining sites described above. Ten antibodies including 7G12 catalyzed the hydrolysis

Scheme 15



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
35	7G12	0.07 min^{-1} for L-**38** [3700] at pH 8.0	13 μM [19 nM for (*R*)-**37**]
		0.028 min^{-1} for L-**36** (R = CH_3)	13 μM
		0.037 min^{-1} for L-**36** (R = $(CH_3)_2CHCH_2$)	23 μM
		0.028 min^{-1} for L-**36** (R = $CH_3(CH_2)_3$)	36 μM
35	3G2	0.033 min^{-1} for D-**38** [1700] at pH 8.0	5.4 μM [47 nM for (*S*)-**37**]

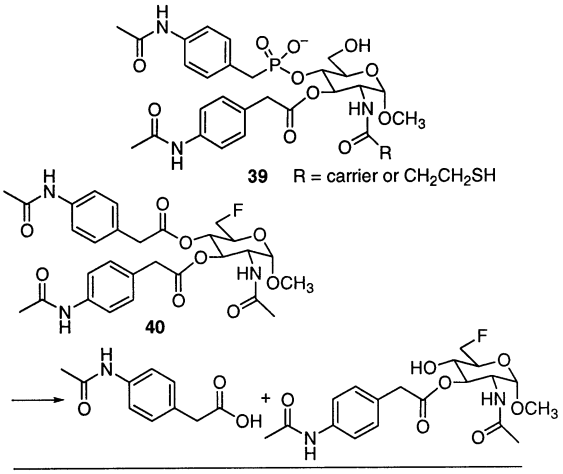
of L-**38**, and four antibodies including 3G2 catalyzed the hydrolysis of D-**38**. Hydrolysis of less than 3% of the opposite enantiomer was detected. The 7G12- and 3G2-catalyzed reactions were inhibited by addition of the corresponding enantiomer of **37**, and both antibodies preferentially bound to the corresponding enantiomer of **37**. Antibodies 7G12 and 3G2 were able to accept a broad range of substrates **36** possessing various α-substituents, for example, R = CH_3, $(CH_3)_2CHCH_2$, $CH_3(CH_2)_3$, $CH_3SCH_2CH_2$, $PhCH_2$, $(CH_3)_2CH$, and Ph, with high enantioselectivity. In addition, the K_m values of the preferred enantiomers of these substrates were in a narrow range, indicating that alteration of the α-substituents of the substrates had little effect on the binding affinity to the antibodies.

Although antibody 17E8 (Table 1) catalyzed the enantioselective hydrolysis of *N*-formyl amino acid phenyl esters possessing a set of α-side-chain substituents as well as **34** (Scheme 14), the side-chain structures of the substrates of 17E8 were restricted within a certain range.[58] Since antibody 17E8 was generated with **33**, this antibody has the hydrophobic pocket to accept the *n*-butyl side chain of substrate **34**. Therefore, the side-chain structures of the substrates must be accepted in this pocket for hydrolysis by 17E8. Using haptens that fit into the antigen-combining site while leaving the linker moiety outside is an effective approach for the generation of catalytic antibodies with broad substrate applicability while retaining high stereoselectivity.

2.7.3. Deprotection Reaction

Regioselectively protected and/or deprotected carbohydrates are essential in oligosaccharide synthesis. Antibody-catalyzed regioselective hydrolysis of carbohydrate esters has been reported.[63] Antibody 17E11 generated against **39** catalyzed the hydrolysis of ester **40** at C4 with high regioselectivity, affording 4-OH and 3-OH in a ratio of 20:1 at 26% conversion (Scheme 16). In the spontaneous reaction, the rate

Scheme 16



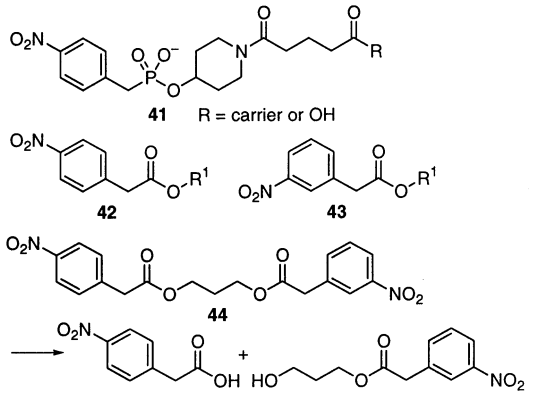
Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
39	17E11	0.182 min^{-1} [2730] at pH 8.2	6.6 μM [26 nM for **39** (R = CH_2CH_2SH)]

of acyl migration was much faster than that of deacylation, giving an equilibrium mixture of 4:1 of 4-OH and 3-OH. In addition to high regioselectivity, the antibody demonstrated an ability to discern the stereochemistry at C-4 by hydrolyzing a glucopyranoside, **40**, but not a corresponding galactopyranoside bearing an axial ester group at C-4.

Antibody-catalyzed reactions were applied to the selective removal of a class of similar protecting groups.[64] Antibody 27H9 was generated by immunization with hapten **41**, and catalyzes the selective cleavage of a *p*-nitrophenylacetyl group of ester **42** (Scheme 17). Esters of *m*-nitrophenylacetate **43** were not substrates for this antibody. In the case of diester **44**, only the *p*-nitrophenylacetyl group was removed by this antibody. Antibody 27H9 accepted a wide variety of primary alcohols protected as *p*-nitrophenylacetates, indicating the potential of this antibody as a deprotecting catalyst in organic synthesis.

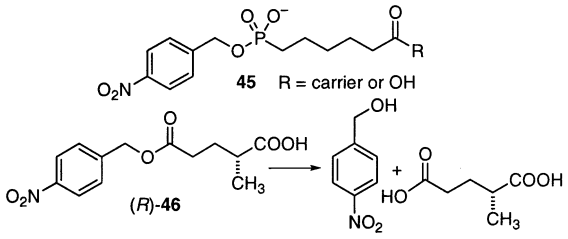
Antibodies D2.3, D2.4, and D2.5 generated with **4** (Scheme 3, Table 1) were also available for the selective hydrolysis of *p*-nitrobenzyl ester as described above. These antibodies did not catalyze the hydrolysis of *m*-nitrobenzyl ester.[10] The same haptenic epitope as in **4** has been used to generate antibodies for deprotecting reactions (Scheme 18).

Scheme 17



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
41	27H9	0.062 min^{-1} for **42** (R^1 = allyl) [2900] at pH 9.0 0.47 min^{-1} for **42** (R^1 = *m*-nitrobenzyl) [1.4 x 10^6] at pH 9.0	1.5 mM [12 μM for **41** (R = OH)]

Scheme 18



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
45	7B9	0.0454 min^{-1} for (*R*)-**46** [1820] at pH 8.0 0.0201 min^{-1} for (*S*)-**46** [804] at pH 8.0	1.5 mM [31.1 nM for **45** (R = OH)]

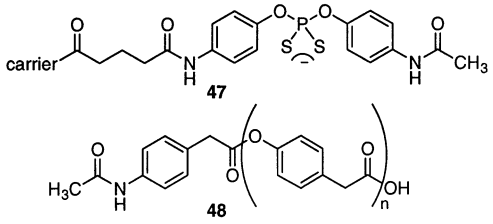
Antibody 7B9 generated with **45** catalyzed the hydrolysis of *p*-nitrobenzyl ester derivatives,[65] and this antibody accepted a set of esters including both enantiomers of ester **46**. Antibody 4A1 generated with *p*-nitrophenylphosphonate catalyzed the hydrolysis of a set of *p*-nitrophenyl carbonates with a k_{cat}/k_{uncat} of up to 6.4×10^4.[66]

2.7.4. Other Substrates

Antibodies capable of catalyzing hydrolysis of oligomeric esters and polyesters have potential for the degradation of synthetic polymers. Antibody OB2-48F8 generated with **47** catalyzed degradation of oligomeric ester substrate **48** (Chart 3; $n = 4$, 5, and 6) as well as a monoester.[67]

Antibody-catalyzed ester hydrolysis of a glycerol derivative,[68] phosphatidylcholine derivative,[69] and tyrosine benzoate[70] has been reported. These antibodies were generated with the corresponding phosphonate transition-state analogues.

Chart 3

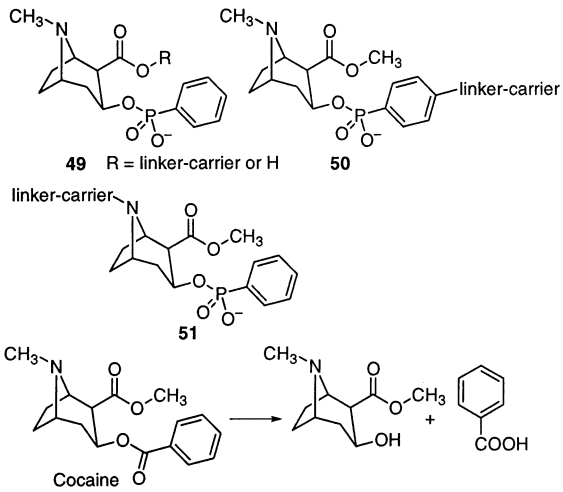


2.8. Drug Degradation and Prodrug Activation

2.8.1. Drug Degradation

One of the most important applications for catalytic antibodies is their use for human therapies, for example, degradation of poisonous or narcotic drugs. Antibodies that catalyze cocaine hydrolysis have been reported.[71−74] As discussed above, immunization with a single hapten conjugate typically provides catalytic antibodies homologous in both biochemical properties and amino acid sequences. To increase the diversity of an immune response to a transition-state analogue for cocaine hydrolysis, tether sites for the attachment of carrier protein were examined at different points on the molecule (Scheme 19).[72,74] The three conju-

Scheme 19



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
49	15A10	2.3 min^{-1} [2.3 x 10^4] at pH 7.8	220 μM [9 nM for **49** (R = H)]
49	3B9	0.11 min^{-1} [1100] at pH 7.8	490 μM [10 nM for **49** (R = H)]

gates **49**, **50**, and **51**, which include the same transition-state analogue for cocaine hydrolysis but different linker positions, provided catalytic antibodies with differential capacities for binding to the three transition-state analogue conjugates,[72] the difference in the binding properties corresponding to dissimilar amino acid sequences. Although the linker attachment position on the transition-state analogue increased the diversity of catalytic antibodies produced, seven of the nine antibodies showed a linear relationship in the plot of $\log(K_S/K_{TSA})$ versus $\log(k_{cat}/k_{uncat})$. The best of these catalytic antibodies was 15A10,

obtained from immunization with **49**, and this antibody catalyzed cocaine hydrolysis with a k_{cat}/k_{uncat} of 2.3×10^4. Antibody 15A10 prevented cocaine's reinforcing and toxic effects in rats.[73]

2.8.2. Prodrug Activation

Another important application of catalytic antibodies in human therapy is prodrug activation. Site-specific delivery of a prodrug at the tumor cells and activation of the prodrug into an active drug enable the selective destruction of those tumor cells. This type of site-specific targeting is known as antibody-directed enzyme prodrug therapy (ADEPT).[75,76] The antibody−enzyme conjugate is delivered to the target cells that express tumor antigens on the surface, and the enzyme part of the conjugate catalyzes the prodrug activation reaction at the tumor site. This system enhances the efficiency of anticancer drugs and reduces the peripheral cytotoxicity because of the prodrug's low toxicity. Most ADEPT systems incorporate a bacterial enzyme, and the problems of this system are (1) identification of an enzyme not already present in humans and (2) the immunogenicity of such a bacterial enzyme. These problems can be circumvented by use of a humanized catalytic antibody, which catalyzes the activation reaction of the prodrug selectively, in place of the foreign enzyme. Although humanization of catalytic antibodies is another issue, antibodies have been generated for prodrug activation reactions.

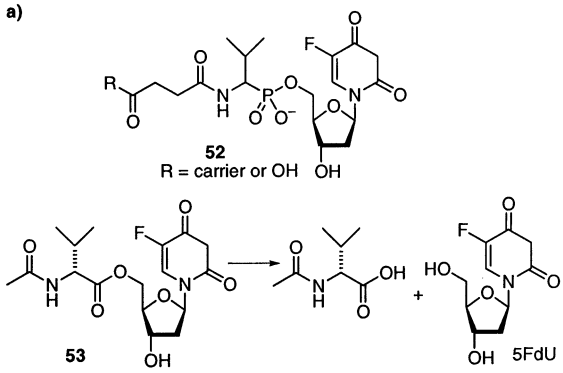
Transition-state analogues including the structural entities of certain drugs were used to elicite catalytic antibodies for prodrug activation (Scheme 20). Antibody produced in response to **52** catalyzed the activation of a prodrug ester, **53**, to generate 5-fluorodeoxyuridine (5-FdU) (Scheme 20a).[77] Antibody EA11-D7 generated with **22** catalyzed the carbamate hydrolysis of prodrug **54** to produce cytotoxic agent **55** (Scheme 20b).[76] Furthermore, studies performed in vitro with antibody EA11-D7 and prodrug **54** reduced the viability of cultured human colonic carcinoma cells relative to controls.[76]

Another approach for prodrug activation by a catalytic antibody is catalyzing the decomposition of a substrate linker that masks a drug (Scheme 21). Antibody recognizes the linker and catalyzes its degradation to form the drug. Since the drug's structure is not incorporated into the hapten design, a variety of drugs can be used in this approach, as demonstrated in aldolase antibody-mediated prodrug activation.[78,79]

N-Methylcarbamate-hydrolyzing antibody ST51[54] generated with **24** (Scheme 11b) was also applied to prodrug activation.[80] Since hydrolysis of *N*-methylcarbamate is very slow under physiological conditions and unwanted background generation of the drug from a prodrug can be minimized, antibodies that catalyze hydrolysis of this functional group have promise for prodrug activation. When the drug-conjugated linker **56** (as in the model drug H_2N-Drug = L-tryptophan) was treated with this antibody, free drug was generated by hydrolysis and subsequent spontaneous elimination (Scheme 21a).[80] The rate enhancement (k_{cat}/k_{uncat}) was about 5000 at pH 9.0,

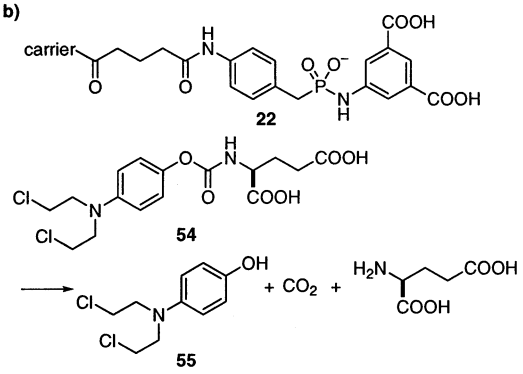
Scheme 20

a)



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
52	49.AG.659.12	0.03 min^{-1} [968] at pH 8.0	218 μM [270 nM for **52** (R = OH)]

b)



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m
22	EA11-D7	1.88 min^{-1} at pH 7.4	201 μM

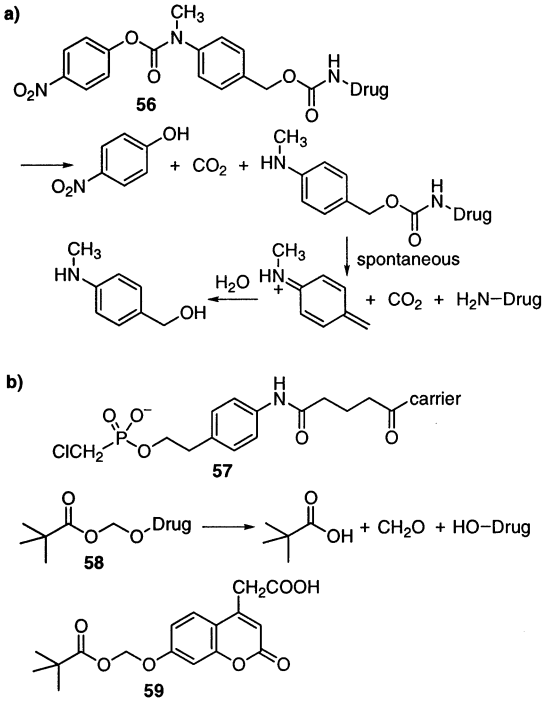
although the rate of the catalyzed reaction was too slow at physiological pH for ADEPT.

When an ester hydrolytic reaction is used for prodrug activation, high background may be a problem. Less reactive esters, for example, sterically hindered esters, are suitable for this prodrug activation strategy. Since the pivaloyloxymethyl group can be used to protect a variety of functional groups, such as phenols and amines that are found in many drugs, antibodies that catalyze the hydrolysis of pivaloyl esters were generated with **57** for the activation of prodrug **58** to form the drug HO-Drug (Scheme 21b).[81] These antibodies catalyzed the hydrolysis of **59**, a prodrug based on the **58** model (HO-Drug = phenol derivatives).

2.9. Efficient Screening of Catalysts

Typical screenings based on the affinity of hybridoma supernatants toward the transition-state analogue provide both catalytic and noncatalytic binding antibodies. A repertoire is selected to include not only antibodies that recognize the transition-state structural element in the transition-state analogue, but also antibodies with affinities for other parts of the transition-state analogue. Selection with a truncated transition-state analogue representing only the es-

Scheme 21

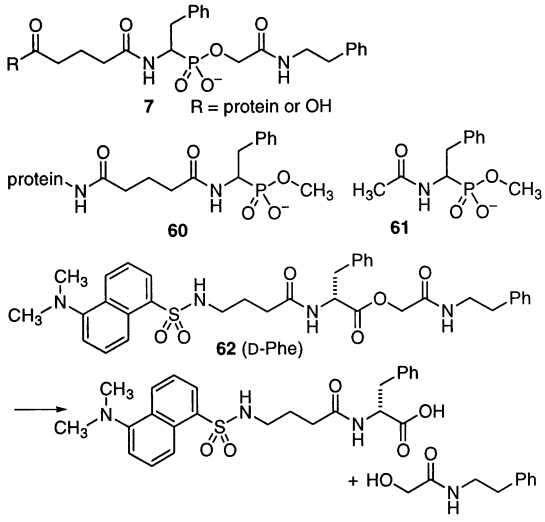


Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m
57	8A9	0.17 min^{-1} for **59** [2450]	270 μM

sential structural element maximized relative contributions of the transition structure and enabled selection of potential catalytic antibodies more efficiently than a full-length transition-state analogue employed for the immunization.[82,83] In the selection of catalytic antibodies induced with **7**,[84] a three-step screening process was devised for selection with a truncated transition-state analogue (Scheme 22). Step 1 was an enzyme-linked immunosorbent assay (ELISA) for **7**, step 2 was an ELISA for **60**, and step 3 was competitive inhibition by **61**. Ten of 12 monoclonal antibodies selected by the three-step screening were catalytic for the hydrolysis of **62** (D-Phe), and 5 of 14 selected by step 1−step 2 were catalytic, but 0 of 6 screened only by step 1 were catalytic. The selection with a truncated transition-state analogue enriched for catalytic antibodies in the screening. These selected antibodies catalyzed the stereoselective hydrolysis of **62** (D-Phe), but did not catalyze the hydrolysis of its isomer possessing L-Phe instead of D-Phe.

A convenient catalytic assay for screening is the catELISA (which detects the reaction product by ELISA).[39] However, this procedure needs specific antibodies that bind to a product of antibody-catalyzed reactions. Genetic screening of recombinant catalytic antibodies in *E. coli*[23] and screening using a chromogenic substrate[85] have been described as efficient approaches for accessing catalytic antibodies. Catalysis-oriented screening with reactive haptens and evolution of catalytic antibodies in vitro are described in sections 3.3, 3.4, and 4.

Scheme 22



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
7	MS6-164	0.43 min^{-1} for **62** (D-Phe) [4300] at pH 7.0	2.8 μM [0.61 nM for **7** (R = OH)]

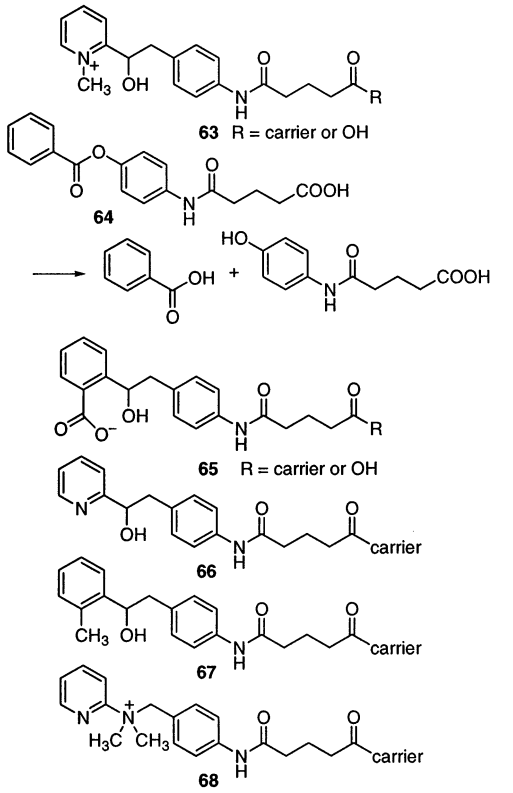
3. Alternative Hapten Design and Selection Strategies for Hydrolytic Antibodies

3.1. Bait and Switch, and Charged Compounds

Haptens carrying positive or negative charges were used for the induction of complementary charged amino acid residues in active sites.[86,87] The positively and negatively charged amino acid residues in an active site contributed to catalysis of the hydrolytic reactions as general-acid/base or nucleophilic catalysts. This strategy has been named "bait and switch", because the hapten serves as "bait" for attracting catalytic functions during the induction of the antibody, which is then "switched" for the substrate. Immunization with *N*-methylpyridinium hapten **63** provided antibodies that catalyzed ester hydrolysis of **64** (Scheme 23). Antibody 30C6 generated with **63** catalyzed the hydrolysis with a rate acceleration of 10^6. Negatively charged hapten **65** provided additional antibodies that catalyzed the same reaction. On the other hand, neutral haptens **66** and **67** did not yield any catalysts. Haptens **63**, **65**, and **66** possessed a hydroxy group having a tetrahedral geometry that could serve as an adequate representation of the developing transition state. Hapten **68** failed to provide any catalysts for the hydrolysis of **64**.

1,2-Amino alcohol haptens **69** and **70** and zwitterionic hapten **71** were used for the immunizations that induced antibodies for catalyzing the hydrolysis of ester **72** (Scheme 24).[88] (A possibility was discussed that the corresponding aziridine was actually used instead of amino alcohol **69**.[89]). These haptens have the following features: The protonated primary amine in **69** and the quaternized ammonium salt in **70** act as positive charges to generate a basic residue in the antibody combining site and form an empty cavity for the incoming water molecule. The second-

Scheme 23



Hapten	Name of catalytic Ab	k_{cat}	K_m [K_i]
63	30C6	0.005 min^{-1} at pH 7.2	1.12 mM [83 μM for **63** (R = OH)]
65	27A6	0.01 min^{-1} at pH 8.5	243 μM [6 μM for **65** (R = OH)]

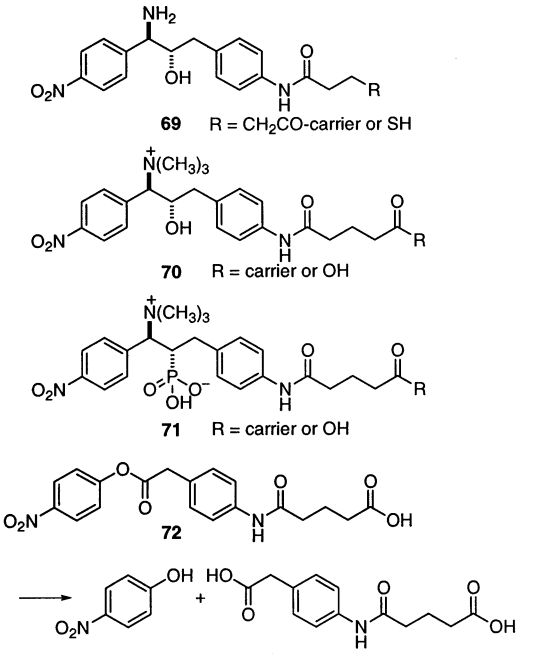
ary hydroxy group in **69** and **70** represents the tetrahedral geometry required to stabilize the transition state. Hapten **71** carries an additional negatively charged phosphate group attached to the hydroxy group to induce an acidic residue that further stabilizes the oxyanion transition state. Antibodies elicited from these haptens catalyzed the hydrolysis of **72** with rate accelerations (k_{cat}/k_{uncat}) of up to 3300.

Zwitterionic hapten **73** was used to induce catalytic antibodies for the hydrolysis of ester **74** (Scheme 25).[90] The phosphonate is expected to induce an acidic residue in the catalytic site that would stabilize the oxyanion of the transition state, whereas the protonated amine should induce a basic residue that deprotonates a water molecule to transform it into hydroxide for the nucleophilic attack. Antibody H8-1-2D5 catalyzed the ester hydrolysis of **74** with a rate acceleration of 10^5.

Hapten **75** carrying two negatively charged phosphonate groups was used for the induction of antibodies destined to catalyze the hydrolysis of **76** (Scheme 26).[91] This hapten was designed by combining the concepts of "bait and switch" and "transition-state mimic", since the one phosphonate is a transition-state analogue and the other induces a positively charged amino acid residue.

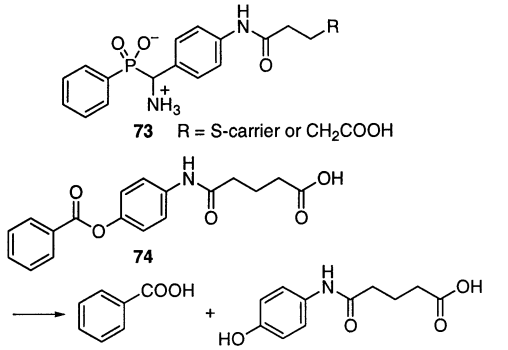
Cyclopropenone hapten **77** was also used for the induction of hydrolytic antibodies (Scheme 27).[92] The

Scheme 24



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
69	H6-39	0.72 min^{-1} [2400] at pH 7.8	880 μM [490 μM for **69** (R = SH)]
70	H5-38	1.0 min^{-1} [3300] at pH 7.8	870 μM [5 μM for **70** (R = OH)]
71	H7-59	0.49 min^{-1} [1600] at pH 7.8	1100 μM [230 μM for **71** (R = OH)]

Scheme 25



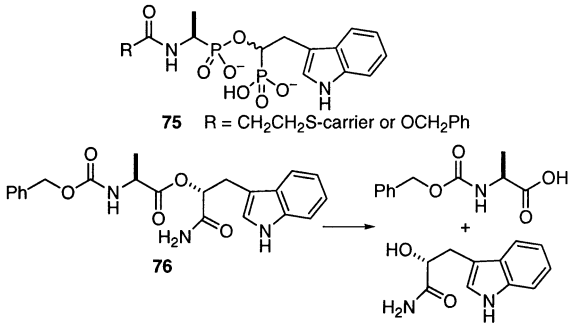
Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
73	H8-1-2D5	3.5 min^{-1} [1.3 x 10^5] at pH 7.8	488 μM [1.6 nM for **73** (R = CH_2COOH)]

carbonyl bond of **77** is highly polarized, and the zwitterionic structure is contributed during the induction process. Although this hapten does not have tetrahedral geometry, antibody 12G2 generated with **77** catalyzed the hydrolysis of **78** with a k_{cat}/k_{uncat} of ~1000.

3.2. Hetrologous Immunization

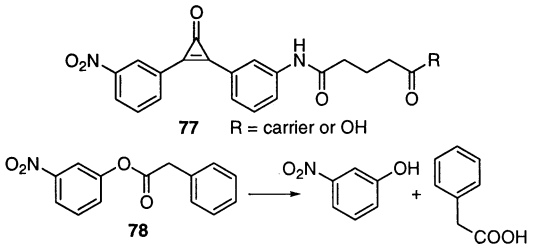
Efficient esterolytic catalysis could be achieved with the aid of both acidic and basic amino acid

Scheme 26



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
75	12F12	0.36 min^{-1} [5000] at pH 7.5	3.3 mM [4.4 μM for **75** (R = OCH_2Ph]

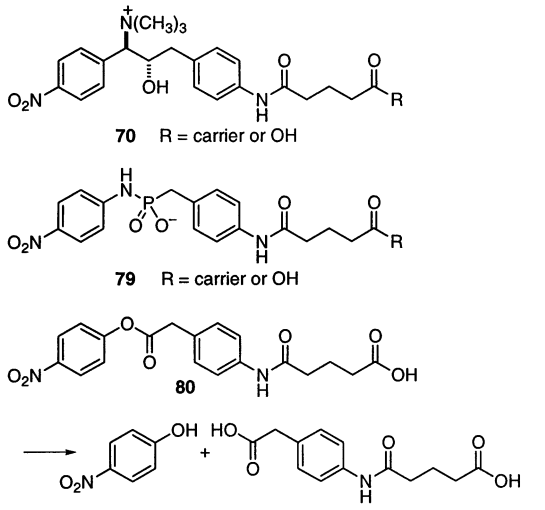
Scheme 27



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m
77	12G2	0.035 min^{-1} [970] at pH 6.5	500 μM

residues appropriately disposed in the combining site of an antibody. These residues would be induced by a zwitterionic hapten. However, this type of hapten synthesis is not a simple task. One strategy, known as heterologous immunization, has been reported to serve as an alternative to the use of a zwitterionic hapten for the generation of catalytic antibodies.[93,94] For heterologous immunization, an animal is injected successively with two different but structurally related haptens. The host cross-reactively responds to the secondary antigen and produces antibodies that have affinity for the primary antigen, the secondary antigen, or both antigens. The positively charged quaternary ammonium alcohol **70** and the negatively charged phosphonamidate **79** were used for immunization to induce antibodies with the capacity to bind both functionalities as a result of introducing more than one essential amino acid residue into an antigen-combining site. Two injections of **70** followed by **79** provided antibodies that catalyzed the hydrolysis of ester **80** with significantly higher catalytic efficiency than did antibodies formed after homologous immunization (immunization with one hapten) with either **70** or **79** (Scheme 28). In this heterologous immunization, antibodies showed higher catalytic activity, i.e., with a k_{cat}/k_{unct} of >10000, and antibody H5H2-42 produced by heterologous immunization catalyzed the reaction with a k_{cat}/k_{uncat} of 68000. The plots of pH versus log k_{cat} of H5H2-42-catalyzed reactions appeared to have a bell-shaped profile, indicating that the antibody could have two ionizable

Scheme 28



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
70/70/79 (heterologous immunization)	H5H2-42	12.5 min^{-1} [68000] at pH 7.0	240 μM [300 μM for **70** (R = OH)] [22 μM for **79** (R = OH)]
27 (homologous immunization)	H5-25	0.79 min^{-1} [2700] at pH 7.0	970 μM [5 μM for **70** (R = OH)] [>1.0 mM for **79** (R = OH)]
79 (homologous immunization)	H2-23	3.2 min^{-1} [11000] at pH 7.0	160 μM [>1.0 mM for **70** (R = OH)] [4.6 μM for **79** (R = OH)]

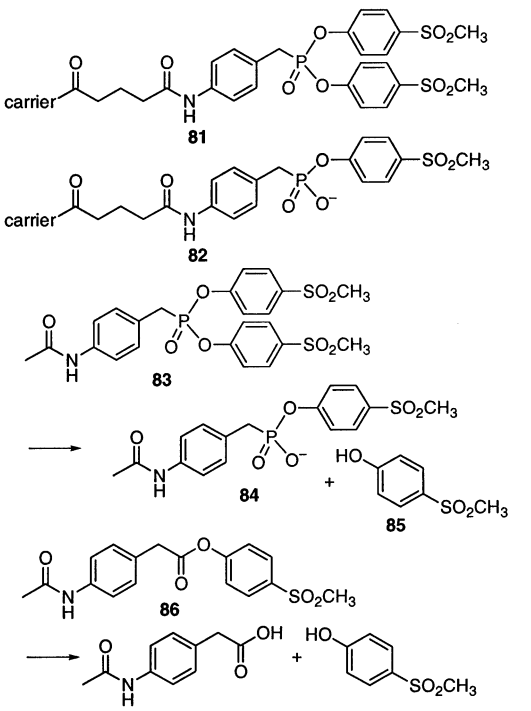
residues with pK_a values of 5.6 and 8.7 upon forming the complex with the substrate. These results indicate that heterologous immunization is a potential means of introducing more than one functional residue into the antigen-combining site.

3.3. Reactive Immunization

Natural selection of enzyme catalysis occurs as a consequence of improved function or fitness during evolution. By contrast, selection of antibody catalysts is typically based on noncovalent binding to transition-state analogues of the reactions or charged compounds designed from the reaction coordinates. If the selection criteria are switched from simple binding to function in the immune system, efficient catalytic antibodies should be generated. Adapting this type of selection has afforded a new strategy, reactive immunization. Reactive immunization provides a means to select antibody catalysts in vivo on the basis of their ability to carry out a chemical reaction.[95−97] A designed reactive antigen is used for immunization, after which a chemical reaction(s) such as the formation of a covalent bond occurs in the binding pocket of the antibody during its induction. The chemical reactivity and mechanism integrated into the antibody by the covalent trap with the reactive immunogen are used for the catalytic reaction of the corresponding substrates.

A labile phosphonate diester hapten, **81**, was used for reactive immunization to induce ester hydrolytic antibodies (Scheme 29).[95] The phosphonate diester could be either hydrolyzed at physiological pH or

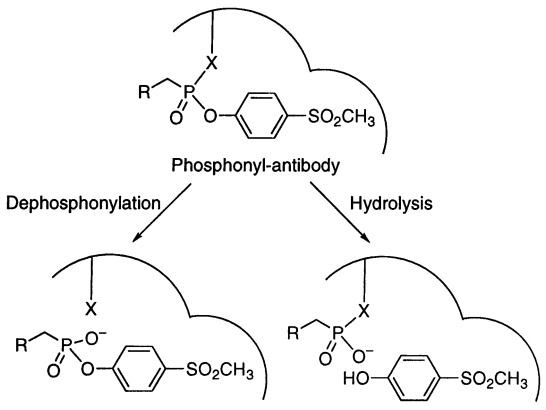
Scheme 29



Hapten	Name of catalytic Ab	k_{obs} for **83**	k_{cat} for **86** [K_i]
81 and **82**	SPO49H4	6.4 min^{-1} (Formation of **84**) 6.4 min^{-1} (Formation of **85**)	31 min^{-1} at pH 8.0 k_{cat}/k_{uncat} 6700 [3.0 μM for **87**] [0.11 μM for **88**]

trapped by a nucleophile at the B-cell level of the immune response (Scheme 30). The reactive nucleophile induced with **81** in the antibody binding pocket should act as a nucleophile for ester hydrolysis, and

Scheme 30[a]



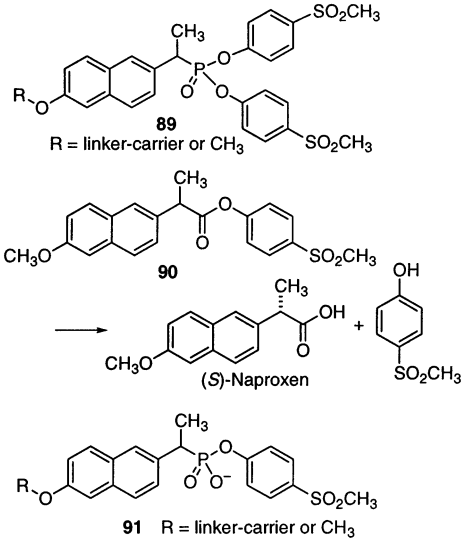
[a] Chemical opportunities that can take place in an antigen-combining site when it binds to the phosphonate diester. X = the nucleophile in the antibody.

the partly hydrolyzed phosphonate monoester **82** should contribute as a transition-state analogue during the reactive immunization.

Protein conjugate containing equal amounts of diester **81** and monoester **82** was used for immunization because of the high reactivity of **81**. Eleven of the 19 resulting antibodies catalyzed the hydrolysis of phosphonate diester **83** in a one-turnover inactivation of the antibody. When an excess of phosphonate diester **83** was rapidly mixed with antibody SPO49H4, approximately 1 equiv of each product, phosphonate **84** and alcohol **85**, was produced. The rates of formation of **84** and **85** were the same, an indication that there was no significant accumulation of an intermediate and that phosphonylation was largely rate determining. This antibody catalyzed the hydrolysis of activated ester **86**. The reaction was inhibited by the addition of phosphodiester **87** and phosphonate **88**. Turnover of these antibodies was limited only by the rate of product release. There was no significant pre-steady-state accumulation of an intermediate, and an acyl antibody was trapped at low pH in a stoichiometric amount. These results suggested that both covalent catalysis and transition-state stabilization were operative. The acylation with **86** substantiated the presence of an antibody nucleophile. Antibody SPO50C1 obtained from the same immunization yielded esters from **86** with a wide variety of alcohols by way of a covalent acyl intermediate.[98]

A reactive immunization strategy employing phosphonate diester was applied for the generation of catalytic antibodies for a kinetic resolution of a racemic mixture of esters of naproxen (Scheme 31).[99] Antibody 15G12 generated with **89** catalyzed the

Scheme 31



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
89	15G12	28 min^{-1} for (*S*)-**90** [6.6 x 10^5] 0.23 min^{-1} for (*R*)-**90** at pH 8.0	300 μM [950 nM for **89** (R = CH_3)] [150 μM for **91** (R = CH_3)]
91	6G6	81 min^{-1} for (*S*)-**90** [1.9 x 10^6] at pH 8.0	890 μM [4 nM for **91** (R = CH_3)]

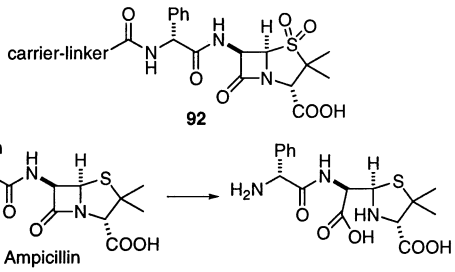
hydrolysis of (*S*)-**90** and gave (*S*)-naproxen. Antibodies obtained by reactive immunization and by transition-state analogue immunization have been compared on the basis of transition-state analysis.[100] A set of antibodies elicited with transition-state analogue hapten **91** showed a linear relationship between $\log(k_{cat}/k_{uncat})$ and $\log(K_m/K_i)$, yet antibodies elicited with reactive immunization had no such linear relationship. Each strategy offered antibodies exhibiting different catalytic behaviors.

Although reactive immunization provided a relatively higher range of rate enhancement ($k_{cat}/k_{uncat} > 10^5$), such enhancement has also been achieved by antibodies obtained from transition-state analogue immunization.

3.4. Mechanism-Based Inhibitor

Since mechanism-based inhibitors react to form covalent adducts with the enzymes that process them along a defined reaction pathway, such inhibitors should facilitate the direct selection of catalytic antibodies that utilize particular features of a designed mechanism. A mechanism-based inhibitor of β-lactamase, **92**, was used to obtain β-lactam hydrolytic antibodies (Scheme 32).[101] The inhibitor conjugate was used to immunize mice, and a single-chain antibody (scFv) library was prepared by using the spleen of an immunized mouse. Covalently binding antibodies against **92** were selected with a phage display system. An acidic washing condition was used during binding selection to remove noncovalently bound phage. ScFv FT6 obtained from this selection catalyzed ampicillin hydrolysis.

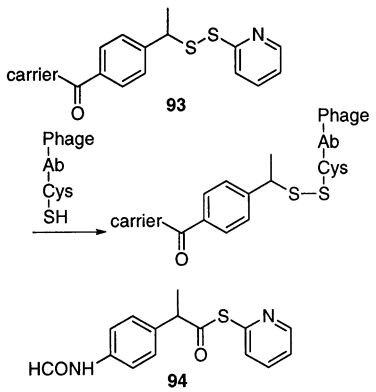
Scheme 32



Hapten	Name of catalytic Ab	k_{cat} $[k_{cat}/k_{uncat}]$	K_m
92	FT6 (scFv)	0.029 min^{-1} [5200] at pH 7.4	560 μM

To select antibodies possessing a reactive cysteine residue within the active site, semisynthetic Fab libraries were panned with pyridyl disulfide **93**, which undergoes disulfide interchange (Scheme 33).[102] The selected Fab 32-7 had a sulfur nucleophile and formed a covalent intermediate with **94**.

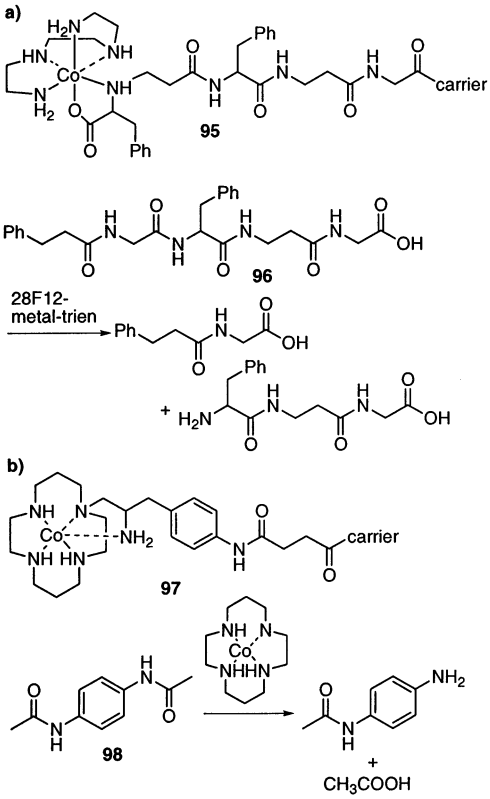
Scheme 33



3.5. Cofactor-Mediated Reactions

Antibodies that catalyze site-specific hydrolysis of a Gly−Phe peptide bond with metal cofactors have been elicited using a Co−triethylenetetramine−peptide hapten, **95** (Scheme 34a).[103] Antibody 28F11 formed in this way catalyzed the hydrolysis of **96** at pH 6.5 in the presence of metal−triethylenetetramine (trien). Active cofactor metals were Zn(II), Ga(III), In(III), Fe(III), Cu(II), NiII), Lu(II), Mn(II), and Mg(II). This metal-mediated concept was used for the generation of an arylamide hydrolytic antibody. Antibody generated with **97** catalyzed Co(II)-mediated hydrolysis of **98** (Scheme 34b).[104]

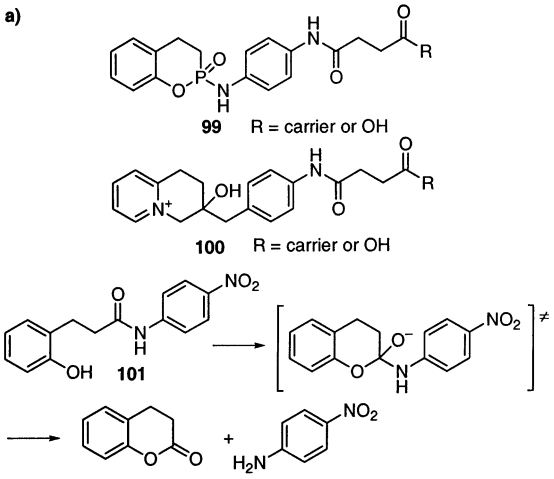
Scheme 34



Phenol was used as the auxiliary nucleophile for the antibody-catalyzed reaction.[105] Haptens **99** and **100** were originally designed for the N−O acyl transfer reaction of **101** (Scheme 35a).[106] Heterologous immunization with the two haptens provided catalytic antibody 14-10. This antibody also catalyzed the phenol-assisted cleavage of *p*-nitroanilide **102** (Scheme 35b), but in the absence of phenol, this antibody did not catalyze the cleavage of **102**. Since the rate of hydrolysis of ester **103** in the buffer was 3 orders of magnitude faster than that of amide **102**, this antibody achieved hydrolysis of the amide bond in **102**.

A thiol nucleophile was site specifically introduced by the chemical modification of a binding antibody

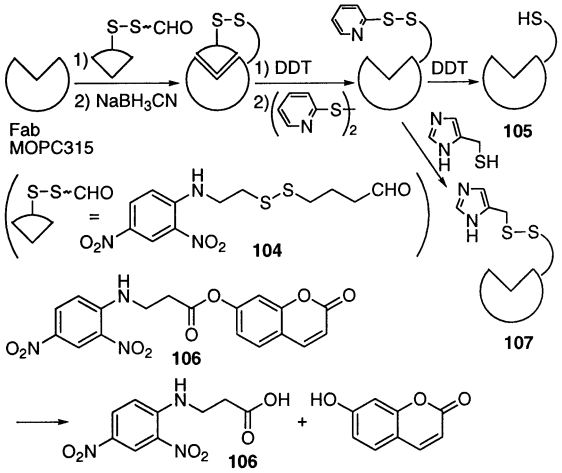
Scheme 35



Hapten	Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_i]
99/99/100 (heterologous immunization)	14-10	0.0093 min^{-1} [20000] at pH 6.8	1.4 mM [51 μM for **99** (R = OH)] [790 μM for **100** (R = OH)]

Hapten	Name of catalytic Ab	k_{cat}	K_m
99/99/100 (heterologous immunization)	14-10	1.3 x 10^{-4} min^{-1} at pH 8.0	136 μM for phenol 370 μM for **102**

Scheme 36



Catalyst	k_{cat}	K_m
105	0.87 min^{-1} at pH 7.0	1.2 μM
107	0.052 min^{-1} at pH 7.0	2.2 μM

(Scheme 36).[107] Antibody MOPC315 bound 2,4-dinitrophenyl ligands with association constants ranging from 5×10^4 to 1×10^6 M^{-1}. This antibody Fab was modified with **104**, the 2,4-dinitrophenyl ligand part was removed, and a free thiol group was generated with DTT. Greater than 95% of label **104** was incorporated at Lys H52. The thiol-containing Fab **105** catalyzed the cleavage of ester **106**, and imidazole-containing Fab **107** also catalyzed the cleavage of ester **106**.[108]

4. Evolution of Catalytic Antibodies in Vitro

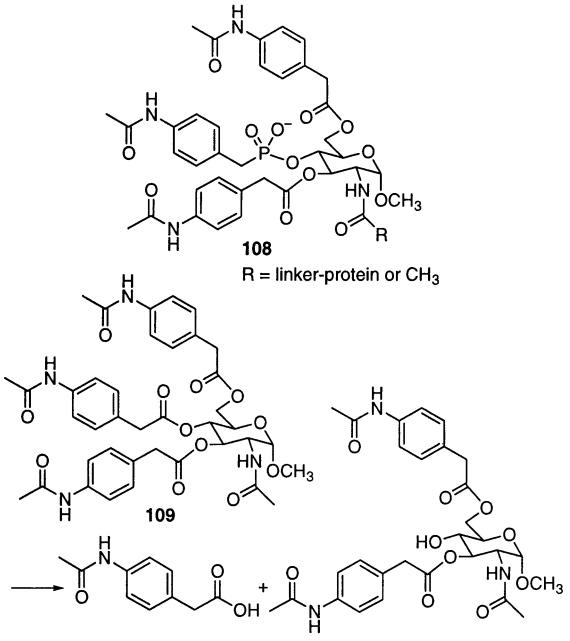
Phage-displayed antibody libraries have been explored as an efficient strategy for the identification of monoclonal antibodies that bind to target molecules.[109] Since antibody phage libraries can be made from both immune and nonimmune sources, selection with designed haptens using antibody phage libraries in vitro allows access to catalytic antibodies that are not limited by animal sources or immune responses. For example, antibody libraries can be made by combining information gained from the study of existing catalytic antibodies with synthetic combinatorial libraries or a naive V gene repertoire. In addition, a selection system in vitro enables one to use multiple haptens without reimmunizing animals. Antibodies possessing altered substrate specificity and enhanced catalytic activity have been selected by phage display in vitro.

Murine-derived catalytic antibody 17E8[31−33] (Scheme 14, Table 1) was humanized by grafting murine CDR sequences onto a human VL κ subgroup I and VH subgroup III variable framework.[110] The hapten binding affinity and catalytic activity of humanized Fab 17E8 (hu17E8) were similar to those of the parent murine (chimeric) antibody Fab17E8, which contains a murine variable region and a human constant region. To improve affinity to the phosphonate transition-state analogue, combinatorial libraries of hu17E8 were prepared and panned by phage display.[110] The libraries were prepared by randomly mutating antibody residues (five and six residues) in either direct contact with or close proximity to the hapten in the 17E8−hapten crystal structure. The selection provided variants with up to 8-fold improvement in binding affinity for the phosphonate hapten. However, this improved binding to the phosphonate did not correlate with higher catalytic activity. By contrast, a weaker binding mutant exhibited improved catalytic activity. The libraries of light chain CDR2 and heavy chain CDR3 of 17E8 were also used for the phage selection against the phosphonate transition-state analogue, but variants with improved catalytic activity were not obtained.[111] These variants did not give any relationship between log(K_m/K_i) and log(k_{cat}/k_{uncat}). This observation is consistent with the fact that antibody 17E8 has a lower affinity for the phosphonate hapten (K_d = 500 nM) and a lower value of K_m/K_i (520) than the less catalytically active antibody 29G11 (K_d = 27 nM, K_m/K_i = 24000) obtained from the same immunization.[33] On the other hand, mutants that were prepared on the basis of the gained information afforded about 3-fold improvement in the turnover (k_{cat}). Since stabilization of the transition state produced by hydroxide attack is not the only factor responsible for the catalytic mechanism of antibody 17E8, improved binding to the transition-state analogue did not enhance catalytic activity. Other mechanisms of the hydrolysis by 17E8, for example, a general-base-assisted mecha-

nism, may be unrelated to the improvement of binding affinity to the phosphonate hapten.

Upon affinity maturation in vitro to phosphonate transition-state analogue **108**, antibody 17E11 became an improved catalyst for a substrate different from its original substrate (Scheme 37).[112] Antibody

Scheme 37

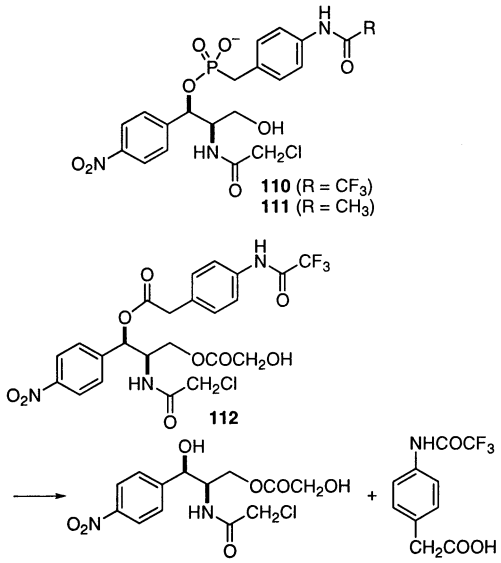


Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [K_m/K_{TSA}]
Wild type17E11	0.018 min^{-1} [184] at pH 8.0	15 μM [100]
mutant 115	0.22 min^{-1} [2248] at pH 8.0	130 μM [1440]

17E11 originated from immunization with **39** and catalyzed the stereoselective hydrolysis of 6-floride sugar ester **40** at the C-4 position with a k_{cat}/k_{uncat} of 2730 (Scheme 16).[63] When the fully protected glucosamine **109** was used as a substrate, 17E11 had a reduced activity ($k_{cat}/k_{uncat} = 184$). A molecular model of 17E11 docked with **39** indicated that the heavy chain CDR3 (HCDR3) loop interacted in the neighborhood of C-6 and that reduced activity for substrate **109** resulted from steric hindrance between the HCDR3 loop and the bulky ester group at C-6 in **109**. Accordingly, a library of HCDR3 with six randomized residues was prepared, and affinity binding selection to **108** using the phage display system was performed to obtain catalysts that have improved catalytic activity for **109**. Mutant 115 obtained from this selection provided a higher activity ($k_{cat}/k_{uncat} = 2248$) than the wild-type 17E11 in the hydrolysis of **109**. The kinetic values obtained for both the wild type and mutant 115 appeared to be in close agreement with $k_{cat}/k_{uncat} = K_m/K_{TSA}$, suggesting that they use the same reaction mechanism, transition-state stabilization, to accelerate the reaction.

Antibody 6D9 (Scheme 2, Table 1) generated with **110** also provided better catalysts after affinity maturation in vitro (Scheme 38).[113] According to

Scheme 38



Name of catalytic Ab	k_{cat} [k_{cat}/k_{uncat}]	K_m [$K_m/(K_i$ for **110**)]
6D9	0.129 min^{-1} [935] at pH 8.0	61.4 μM [1104]
8Hf	2.264 min^{-1} [1.6 x 10^4] at pH 8.0	141.5 μM [2.0 x 10^4]
8Hg	2.593 min^{-1} [1.9 x 10^4] at pH 8.0	283.5 μM [2.8 x 10^4]

transition-state theory, rate acceleration correlates with differential affinity for the transition state relative to the ground state. Therefore, to evolve antibodies toward those with high catalytic activity, the differential affinity must be maximized. To optimize the differential affinity, transition-state analogue **111** was employed, whose functionality differed at an important epitope for the binding to 6D9. Because the trifluoroacetyl group of **110** was buried deep in the antigen-combining site of 6D9 in the X-ray structure, the binding affinity of 6D9 for the acetyl derivative **111** was weakened. Panning of an antibody phage library derived from 6D9 against **111** would rescue variants that bound predominantly to the tetrahedral phosphonate group. The resulting variants would have higher binding affinities for the transition state relative to the ground state for the hydrolysis of **112**. A library was used possessing six randomized residues around the essential catalytic residue His L27d, which forms a hydrogen bond to the transition-state structure produced by a hydroxyl anion attack. The variants obtained, including 8Hf and 8Hg, catalyzed the hydrolysis of **112** with 20-fold higher activity than 6D9. Compared with 6D9, the variants showed increased K_m values for substrate **112**, although affinities of the variants for **110** remained similar to that of 6D9, resulting in higher values for K_m/K_i. This improvement in catalytic activity was attributed to the optimized differential affinity of the antibodies for the transition state relative to the substrate. The rate enhancement (k_{cat}/k_{uncat}) of the variants was almost identical to the ratio of the affinity for substrate **112** to the affinity for phosphonate **110** (K_m/K_i), suggesting that the vari-

ants retained the same catalytic mechanism for stabilizing the transition state produced by a hydroxyl anion attack.

An antibody V gene shuffling strategy for the evolution of aldolase antibodies in vitro has been reported.[114] This strategy may also be useful for developing antibodies that catalyze ester hydrolysis and related reactions. Although the immune repertoire provides a highly diverse array of V genes from which to select catalysts, immune responses to haptens are often highly restricted to a few favored V genes. This fact acts to limit opportunities to probe the structural repertoire available to antibodies more completely. To search for novel aldolase antibodies, antibody libraries were prepared by recombining the catalytic residues of parental antibodies with a naive V gene repertoire. Binding selection against an altered diketone hapten along with the original hapten provided aldolase antibody Fab 28 possessing altered substrate specificity and turnover while retaining the catalytic residues and mechanism of the parental catalytic antibodies. Since the naive V gene library was generated using human bone marrow cDNA, Fab 28 is a humanized aldolase antibody with respect to its primary sequence. Antibody libraries of this type may provide new evolutionary opportunities for catalysis not accessible through relatively small, five or six amino acid residue changes. In addition, this strategy is also useful in providing humanized antibodies for the catalytic antibody-mediated prodrug activation described above. Since several humanized antibodies (binding antibodies) are now approved for antibody-based therapeutics,[115] application of humanized catalytic antibodies may be the next step.

Evolution strategies that have been employed in the improvement of enzyme activity in vitro[116] may also be useful for improving antibody catalysts. Since naturally occurring antibodies that catalyze peptide bonds have been reported,[117] it might be possible to obtain highly active hydrolytic antibodies by using a designer system, i.e., functional or catalytic selection in vitro from antibody libraries.

5. Conclusion

Catalytic antibodies that catalyze hydrolysis of ester, amide, carbonate, and carbamate have been surveyed. Preparation of hydrolytic antibodies is successful in terms of the selectivity of the reactions, for example, substrate selectivity and enantioselectivity. Such selectivities of antibody-catalyzed reactions cited in this review are attributed to the design of the haptens used for immunization and selection. In addition, mechanism and rate acceleration of the catalyzed reactions are also explained by the hapten design in many cases. Functional or catalytic selection from antibody libraries prepared in vitro by combination with a naive V gene repertoire or with synthetic combinatorial antibody libraries may provide access to antibody catalysts that perform desired reactions with higher rate accelerations. Endeavors to create and evolve antibody catalysts should supply information for obtaining useful designer protein catalysts.

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