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CATALYTIC ANTIBODIES

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INTRODUCTION

The synthesis of catalysts with tailored specificities and catalytic properties is a challenging problem to chemists and biologists alike. A key element in the design of enzyme-like catalysts is the rational generation of receptors capable of discriminating ligands with high selectivity. Most efforts in this regard have focused on either the synthesis and functionalization of relatively small cavity–containing hosts or on the alteration of the natural specificity of enzymes by chemical modification, oligonucleotide-directed mutagenesis, or genetic selections or screens. Although these approaches hold great promise, only a limited number of successes have been realized to date.

Hybridoma technology (1) permits the exploitation of the tremendous diversity (10^8–10^{10} different specificities) of the humoral immune system to generate monoclonal antibodies specific for virtually any biopolymer, natural product or synthetic molecule of interest. Consequently, the development of strategies for introducing catalytic activity into antibody combining sites should lead to a new class of enzyme-like catalysts with tailored specificities. Recently, principles of enzymic catalysis have been applied to the generation of antibodies that catalyze a wide variety of chemical reactions including ester and amide bond hydrolysis, concerted Claisen rearrangements, elimination reactions, photochemical thymine dimer cleavage, redox reactions, lactonization reactions, and bimolecular amide bond formation. In every example the high specificity of antigen recognition is reflected in high substrate selectivity.

The development of catalytic antibodies of defined specificity promises to be of considerable value to biology, chemistry, and medicine. Catalytic

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antibodies may find use as therapeutic agents to selectively hydrolyze protein or carbohydrate coats of viruses, cancer cells, or other physiological targets. It may also be possible to selectively cleave or ligate complex biomolecules such as polynucleotides, carbohydrates, and proteins, thereby facilitating structure/function studies or allowing the synthesis of new biomolecules with novel properties. The ready availability of large quantities of monoclonal antibodies may allow for their use as synthetic tools for the production of pharmaceuticals or new materials. The ability to generate antibody combining sites with specific catalytic groups and/or microenvironments should also serve to test fundamental notions of enzymatic catalysis.

Thus far, two major approaches have been applied to the design of antibody catalysts. The most widely used method exploits the electronic and steric complementarity of an antibody to its corresponding hapten. This approach allows for (*a*) generation of precisely positioned catalytic amino acid side chains in the combining site, (*b*) stabilization of high energy transition states in reactions, (*c*) reduction in the entropy of reactions by orienting reaction partners in reactive conformations, (*d*) and the incorporation of cofactor binding sites into antibody combining sites. The second general approach to the generation of catalytic antibodies involves direct introduction of catalytic groups into the antibody combining site by either selective chemical modification, site-directed mutagenesis, or genetic selections or screens. The development of a general set of "rules" for generating catalytic antibodies is essential in order to fully exploit the diversity of the humoral immune system. For example, once a general approach to the hydrolysis of a glycosidic linkage by an antibody has been developed, a large number of sequence-specific glycosidases should become available simply by altering the hapten structure accordingly. Therein lies the enormous advantage of using antibodies as catalysts for chemical transformations.

ANTIBODY GENERATION

The protocols used to generate catalytic monoclonal antibodies are generally no different than those used for the production of monoclonal antibodies for other uses (1, 5). The haptens described below have been conjugated to the carrier protein keyhole limpet hemocyanin (KLH) for immunization and to bovine serum albumin (BSA) for use in ELISA assays to identify hapten specific antibodies (6). The coupling strategies used have been designed to be compatible with hapten structure and in vivo stability. Couplings generally involve amide bond formation between carboxyl

groups on haptens and ε-amino groups of surface lysine residues on carrier proteins (7, 8). Some haptens have been coupled via diazo linkages to surface tyrosine residues, disulfide exchange reactions, and reductive amination (7). Typically the length of the spacer arm between the hapten and carrier protein is greater than 6 Å, so as to preclude any steric interference from the carrier (8). Epitope densities between 4 and 30 have been successfully used. Several strains of mice have been used including Balb/c, Swiss Webster, B10Q, and AJ1. In the examples below traditional PEG fusions of spleenocytes with the SP2/0 myeloma cell line have been used (9, 10). Antibodies were screened by ELISA for cross-reactivity with BSA-hapten conjugate, for inhibition of binding to the BSA-hapten conjugate by free hapten, and for lack of cross-reactivity with KLH. Those with maximum binding affinity for the hapten carrier conjugate were characterized. Recently, a fluorescent energy transfer (FET) assay has been developed to assay hydrolytic catalytic activity (see below) which should make possible direct screening of cell supernatants for catalytic activity (11). In our experience only IgG and IgA antibodies have proven to function as catalysts, even though a large number of IgM antibodies have been tested. Either Protein-A affinity- or ion-exchange chromatography have been used to purify antibodies to homogeneity (12).

Characterization of the kinetic parameters, specificity, mechanism and structural properties of catalytic antibodies is complicated and difficult to interpret unless a monoclonal antibody is used. In addition, reproducibility becomes an important concern when a polyclonal antibody preparation is generated. It should also be noted that over 15 years ago Raso & Stollar attempted to use pyridoxamine binding antibodies to catalyze Schiff base formation and transamination reactions (4). Their lack of success may have been due to the use of polyclonal rather than monoclonal antibodies.

The purity of monoclonal antibodies is tremendously important, especially when an enzyme is known to catalyze the reaction of interest. For example, if the turnover number, k_{cat}, for a catalytic (IgG) antibody is 1 min^{-1} and a natural enzyme has a k_{cat} of 5×10^4 min^{-1}, contamination of the antibody with 1×10^{-3} % (on a mol/mol basis) of the natural enzyme would lead one to believe that the antibody is catalytic when in fact the contaminating enzyme is responsible for the rate-enhancement observed. Kinetic analysis, specificity, or inhibition data cannot always distinguish between catalysis by an antibody and catalysis by an enzyme impurity. Rigorous checks of specific activity must be performed after each purification step, along with a comparison between different methods of purification and comparison of whole antibody versus isolated Fab. For example, we have found it very difficult even after considerable purification

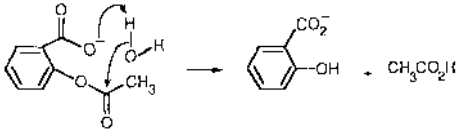
(including affinity chromatography) to remove glycosidase, adenosine deaminase, and ribonuclease impurities from what appears by gel electrophoresis to be homogeneous antibody.

DESIGN, GENERATION, AND EXAMPLES

The strategies used to generate catalytic antibodies are based largely on principles of enzymatic catalysis including transition state stabilization (13), general acid-base catalysis, nucleophilic and electrophilic catalysis, strain and proximity effects (14, 15). Enzymes typically employ several such catalytic mechanisms simultaneously, to achieve large rate enhancements. The introduction of catalytic function into the binding sites of antibodies via any one of these mechanisms allows us to dissect its contribution to catalysis. To date, antibody catalyzed rate enhancements over a background range from 10^2 to 10^6. The generation of antibodies with rate accelerations on the order of 10^8 or greater will likely involve the simultaneous application of two or more strategies for introducing catalytic activity into antibodies. Clearly then, strategies that yield a high percentage of catalytic antibodies relative to the total number of hapten-specific antibodies isolated are the most desirable and generalizable. The development of such strategies will require a thorough characterization of the structure and mechanism of the catalytic antibodies being generated.

Eliciting Catalytic Groups

The introduction of a general acid or base into an antibody combining site should be an effective method of catalyzing a variety of reactions including condensation, isomerization, elimination, and hydrolytic reactions (16–18). The high, effective concentration of the catalytic group in the antibody combining site as well as favorable orbital alignment should lead to considerable lowering of the entropy ($\Delta S^{\neq}$) and enthalpy ($\Delta H^{\neq}$) of activation for reaction (14). For example, the rate of hydrolysis of aspirin (Eq. 1) is 100-fold greater than the uncatalyzed rate of hydrolysis (19).



1.

This rate acceleration is achieved by intramolecular general base catalysis by the ortho carboxylate group in aspirin. Two key features of this form of catalysis are the base strength (pK_a) and the position of the base relative to the group being hydrolyzed. If a catalytic group (A) is free in solution

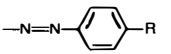
as opposed to attached covalently to the group being hydrolyzed (B), then the rate equation is shown in Equation 2, where the rate constant, k_1, has units $M^{-1}s^{-1}$. If (A) is attached covalently to (B) the rate equation becomes Equation 3, where the dimensions of the rate constant are s^{-1}. The ratio of the rate constants k_2/k_1 for the unimolecular (Eq. 3) and bimolecular reactions (Eq. 2) gives the value (13 M) for the effective molarity of the catalytic carboxylate.

$$v_1 = k_1[A][B] \quad 2.$$

$$v_2 = k_2[A\,B] \quad 3.$$

That is, a 13 M solution of external carboxylate anion would be necessary to give the same first order rate of hydrolysis of aspirin. Model systems with effective molarities greater than 10^7 M have been realized and theoretical arguments suggest accelerations up to 10^8 M can be achieved by approximation of reactive groups in enzyme active sites (20).

Experiments by Pressman & Siegel in 1953 suggest a strategy whereby the electrostatic complementarity between haptens and antibodies can be used to introduce a catalytic carboxylate in an antibody combining site (21). Negatively charged, aspartate or glutamate residues were found in the combining sites of antibodies raised toward *p*-azobenzenetrimethylammonium cation, *1*[1] (22). Conversely, positively charged arginine and lysine residues were identified in the combining sites of antibodies elicited against negatively charged *p*-azobenzoate, *2* (23).



1: R = $-\overset{+}{N}(Me)_3$

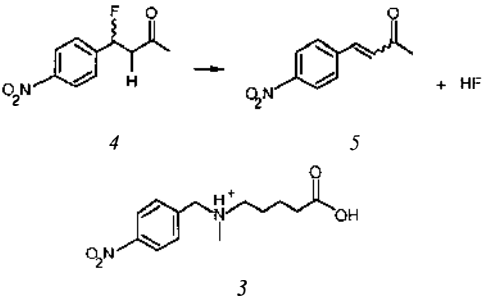
2: R = $-CO_2^-$

Besides electrostatic forces, hydrophobic and hydrogen bonding interactions can be used to induce certain amino acids in antibody combining sites. A tryptophan in MOPC315 has been shown to π-stack with the aryl ring of 2,4-dinitrophenyl containing ligands (24). The predictable nature of such complementary interactions allows for the rational generation of catalytic antibody combining sites.

β-ELIMINATION Isomerizations, eliminations, and many condensation reactions involve proton abstraction from carbon centers (16–18). Such reactions are of great importance in many biological transformations. In general, enzymes that catalyze such reactions use carboxylate groups and imidazoles as the catalytic bases to deprotonate the substrate (25). In

[1] Numbers in italic refer to corresponding molecular structures in figures.

order to determine whether antibodies could be generated that carry out reactions involving abstraction of a proton, hapten *3* was used as an immunogen to generate an antibody that catalyzed the elimination of HF from β-fluoroketone, *4* (26).



The position of the ammonium group corresponds to the position of the abstractable proton in substrate *4* and should elicit a complementarity catalytic carboxylate within bonding distance. The *p*-nitrophenyl ring was included to serve as a common recognition element between hapten and substrate. Moreover, replacement of hapten by substrate in the antibody combining site should lead to an increase in the pK_a of the catalytic carboxylate group (making it a better base) since a stabilizing salt bridge interaction is lost.

Of six antibodies tested which bound *3*, four accelerated the conversion of *4* to *5*, and were inhibitable by hapten. Competitive hapten inhibition demonstrated that catalysis occurs in the binding site of the antibody. One of the four antibodies, 43D4-3D12, was further characterized. The kinetics of the 43D4-3D12 catalyzed reaction obeyed the Michaelis-Menten rate expression (Eq. 4) indicating reversible substrate binding followed by an essentially irreversible unimolecular catalytic step.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad 4.$$

$$v = \frac{k_{cat}[E_T][S]}{K_M + [S]}, \qquad K_M = \frac{k_{cat} + k_{-1}}{k_1}$$

K_M, the Michaelis constant, is equal to the concentration of substrate (S) that produces one half the maximal ($k_{cat}[E_T]$) catalyzed rate, E_T is the total enzyme concentration, and k_{cat} is the unimolecular rate constant for the catalytic step. The k_{cat} and K_M for substrate *4* were 0.2 sec^{-1} and 182 μM, respectively. In general the K_M values of antibody catalysts are in the same range as those of enzymes. The rate acceleration by antibody compared to the background rate with acetate ion was 8.8×10^4, reflecting the contribution of proximity of substrate and a catalytic group in a protein binding

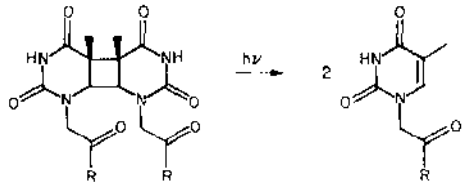
site to rate enhancement. This value is similar to the rate acceleration of $\approx 10^4$ attributable to the bases Glu 43 in staphylococcal nuclease (27) and Asp 102 in trypsin (28). The antibody discriminated between the *p*-nitro substrate *4* and the *m*-nitro analog by a factor of ten in overall rate.

Chemical modification studies demonstrated that a carboxylate group was indeed responsible for catalysis. In the absence of inhibitor selective chemical modification with the carboxylate specific reagent, diazoacetamide, inactivated the antibody almost completely. When inhibitor was present, activity was retained due to protection of the carboxylate group responsible for catalysis. The pH dependence of catalysis indicated an essential residue with a pK_a of 6.3, active in the deprotonated form. Carboxylate groups on surfaces of proteins have pK_a's between 2.0 and 5.5 (29). However, in the hydrophobic cavities of enzymes they become stronger bases with pK_a's in the range of 6.5 to 8.2 (29).

The high success rate of this charge-charge complementary design in generating antibodies with catalytic activity [greater than 65% (4/6)], suggests that this approach should be broadly applicable to other reactions in which negatively or positively charged amino acid side chains serve as catalysts. An important challenge will be the introduction of uncharged amino acids capable of nucleophilic catalysis into antibody combining sites. Perhaps haptens with reactive labeling groups such as α-halo ketones, epoxides, and maleimides can be exploited for this purpose. (A covalent bond between immunogen and antibody would be the basis for antibody selection rather than purely noncovalent interactions.)

THYMINE-DIMER CLEAVAGE Thymine-dimers are the primary photolesion product of damage to DNA by UV light. The repair enzyme, DNA photolyase, catalyzes the light-dependent cycloreversion reaction to thymine, but its mechanism is not fully understood (30). From model systems it is known that compounds such as indoles, quinones, and flavins can inefficiently photosensitize the reaction by reversibly accepting or donating an electron from or to the dimer to generate a radical cation or anion, respectively, both of which might be expected to undergo facile ring opening (31–35).

Antibodies were elicited toward the planar thymine dimer derivative *6* (36).



6 : R = $NHCH_2COOH$

7 : R = OH

The extended π-system of *6* should elicit a complementary π stacking aromatic amino acid (such as tryptophan) in the antibody combining site, as was the case with MOPC315. An appropriately positioned tryptophan should efficiently sensitize the cycloreversion reaction of *7* to thymine. In fact, five of six antibodies generated to hapten *6* showed light dependent acceleration of the conversion of *7* to thymine. One of these five, 15F1-B1, was further characterized. The substrate analog, which is methylated at the two N-3 positions, is not turned over even at relatively high concentrations consistent with the high specificity of antibodies. Antibodies not specific for *6* but which are known to contain binding site tryptophan residues do not catalyze the reaction. At nonsaturating light intensity the k_{cat} and K_M values were measured to be 1.2 min^{-1}, and 6.5 μM, respectively. This k_{cat} value resembles the value for *Escherichia coli* DNA photolyase, 3.4 min^{-1} (37).

One important measure of the efficiency of a catalyst that requires light is the quantum yield of reaction (Φ_R), the ratio of the number of molecules undergoing reaction to the number of photons absorbed (a Φ_R of 1 indicates that every photon absorbed results in conversion of one substrate molecule to product). The corrected quantum yield of the 15F1-B1 catalyzed reaction is ≥ 0.75 (unpublished results, A. G. Cochran & P. G. Schultz), close to the value of 1 estimated for DNA photolyase. The value for the background reaction from model studies is 0.05 (38). The increased quantum efficiency of this antibody compared to the background reaction suggests that the antibody efficiently partitions the intermediate thymine dimer radical to the desired products. The mechanism of this partitioning is not yet known. The wavelength dependence of catalysis and fluorescence quenching of tryptophan by thymine is strong evidence that a tryptophan residue in the combining site is responsible for the observed catalysis. The high success rate in using π-π complementarity to generate catalytic antibodies suggests that this approach also should prove quite generalizable. The ability to generate combining sites with defined functional groups may lead to the generation of discrete microenvironments within antibody combining sites. These microenvironments could demonstrate unusual solvation, dielectric, or hydrophobic properties for studies of molecular recognition and other structure-function relationships.

Transition State Stabilization

The first successful approach used in the design of catalytic antibodies was that of transition state stabilization (the transition state is defined as the highest energy species in the reaction pathway—Figure 1). Whereas intermediates and ground state species lie in free energy wells and correspond to structures in which bonds are fully formed or fully broken, the

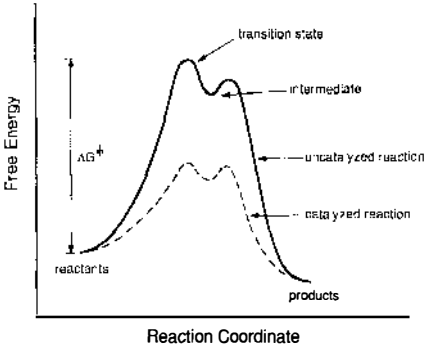
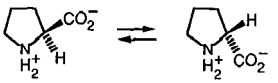


Figure 1 Energy profile of a chemical reaction; uncatalyzed reaction (——), catalyzed reaction (- - - -). The transition state configuration is the highest point on the diagram and intermediates, reactants and products occupy wells.

transition state configuration corresponds to species in which bonds are partially formed and partially broken and has a lifetime on the order of a bond vibration (10^{-13} sec). Many enzymes have evolved to provide an active site environment that is sterically and electronically complementary to the rate determining transition state (39). The active site stabilizes the transition state *relative* to reactants or products, thereby lowering the free energy of activation for reaction and increasing reaction rate. [The free energy of activation ($\Delta G^{\neq}$) is the energy difference between the reactants and the transition state.] For example, racemization of proline by the enzyme proline racemase proceeds through a planar trigonal α carbon transition state (Eq. 5).



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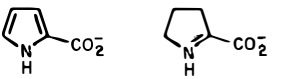
The two planar transition state analogs, *8* and *9*, are bound 160 times more tightly by the enzyme than proline, reflecting the complementarity of the enzyme to the transition state configuration (40).



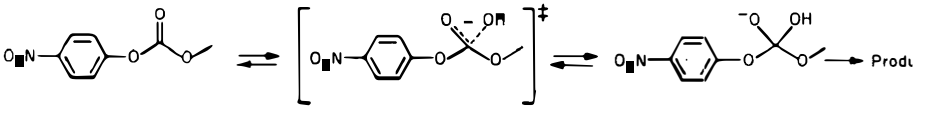
8 *9*

The more closely a given planar analog of proline resembles the transition state structure, the more tightly it should be bound by the enzyme.

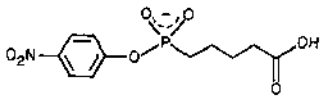
Design of an antibody that takes advantage of transition state

stabilization to accelerate a reaction requires an immunogen that closely resembles the transition state for the reaction. Since transition states are only transient species, stable analogs of the proposed transition state must be used as immunogens. These analogs are in many instances derived from known classes of enzyme inhibitors. Because antibodies are elicited toward transition state analogs, they should have lowered affinity for the reaction products which can then rapidly diffuse from the combining site.

ESTER, CARBONATE, AND AMIDE BOND HYDROLYSES The first antibodies characterized as having catalytic properties accelerated the hydrolysis rate of esters and carbonates *14–16* (41–47). The rate limiting step in these reactions is formation of the negatively charged tetrahedral transition-state structure *10*.



14 *10*



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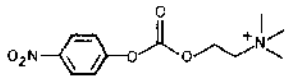

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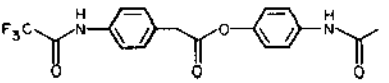

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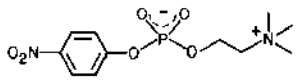

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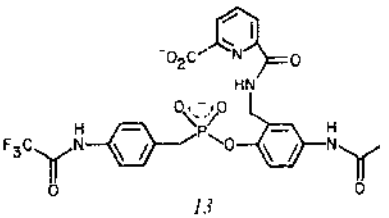


13

A stable analog of such a structure is formed by substitution of a tetrahedral phosphorous center for the tetrahedral carbon, affording structure *11*. Because a typical P-O single bond is 10–15% longer than a C-O single bond, these analogs are quite similar to the negatively charged tetrahedral transition state which has longer, partially formed bonds (48). Such compounds are potent transition-state analog inhibitors of proteolytic enzymes (12, 48). Antibodies specific for *11–13* accelerate the hydrolysis of sub-

strates *14–16*, respectively. The antibodies also demonstrated saturation kinetics, and the kinetic constants for these reactions are tabulated in Table 1. In each case, both competitive inhibition by the respective hapten and high substrate specificity were observed. That these antibodies function by selectively stabilizing the transition state structure, is evidenced by the substantially higher affinities for the haptens (transition state analogs) versus the substrates. However, the difference in affinity of the antibody for the hapten (K_I) and the substrate (K_M) fails to fully account for the large rate accelerations observed (10^3–10^5) over the hydroxide dependent background rates (13).

The three-dimensional structure of the antibody McPC603, which is highly homologous to the catalytic phosphorylcholine-binding antibodies, T15 and MOPC167, has been solved. The crystal structure makes possible direct identification of the combining site residues responsible for catalysis (Figure 2; 49–52). The hapten is bound in the cavity of McPC603, with the choline group deep in the interior and the phosphate toward the exterior, in contact with aqueous solvent. The heavy-chain residues Tyr 33H and Arg 52H, which are invariant in all of the phosphorylcholine-binding antibodies sequenced to date (53), bind the phosphate via hydrogen bonding and electrostatic interactions with the phosphoryl oxygen atoms. The X-ray structure demonstrates that the combining site of McPC603 is both sterically and electronically complementary to the tetrahedral, negatively charged phosphate moiety of phosphorylcholine. Inasmuch as this tetrahedral phosphate mimics the transition state for hydroxide ion-catalyzed hydrolysis of *15*, the phosphorylcholine antibodies should be capable of polarizing the bound carbonate for the attack of hydroxide ion in the rate-determining step. Because the ground state structure of *15* differs substantially from the transition state configuration, the differential binding affinity of the antibody to these two species is reflected in a lowered free energy of activation for reaction. Biophysical studies and chemical modification experiments suggest that tyrosine and arginine also play a catalytic role in the antibodies specific for hapten *11* (41, 43). Again,

Table 1 Kinetic constants for catalytic antibodies catalyzing ester and carbonate hydrolysis reactions

Hapten	Substrate	k_{cat} (min^{-1})	K_M (μM)	K_I (μM)	k_{cat}/K_m	Reference
11	*14*	7.3	430	0.3	4200	43
12	*15*	0.4	208	5.0	770	41
13	*16*	1.6	1.9	0.16	960	43

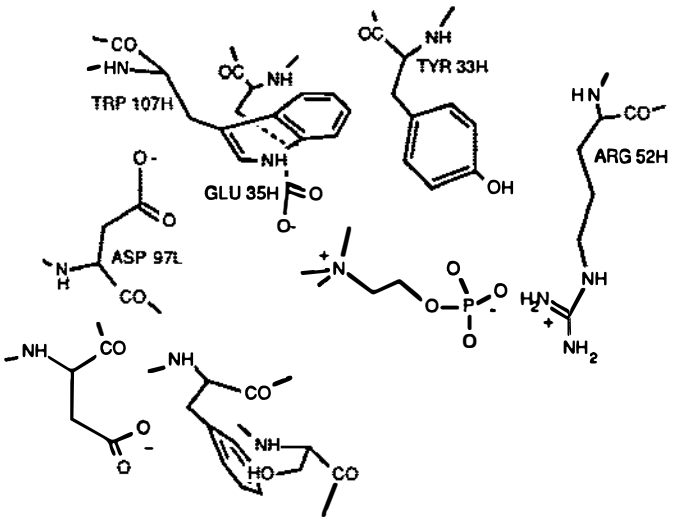
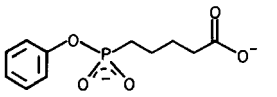


Figure 2 Schematic representation of binding interactions between phosphorylcholine and amino acid side chains in the combining site of McPC603.

these residues can stabilize the tetrahedral transition state. Histidine or tyrosine is thought to be involved in catalysis by antibodies specific for *13* (54), but it is not yet clear what catalytic role these residues play.

Antibodies have been shown to catalyze the hydrolysis of water insoluble substrates. Antibodies specific for phenyl phosphonate *17* were shown to accelerate the hydrolysis of *18* in reverse miscelles (55).



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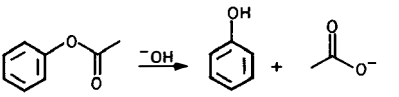

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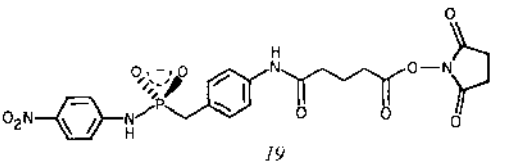
Reverse miscelles are formed when water/surfactant mixtures are dissolved in water immiscible solvents. The k_{cat} and K_M values of the antibody solubilized in isooctane in reverse miscelles were 3.89 min^{-1} and 569 μM, respectively. The optimal ratio of water to detergent (W_o) for antibody catalysis is significantly larger than the values found for enzymes, consistent with the increased molecular weight of IgG molecules. The expan-

sion of antibody catalysis to water insoluble substrates in reverse miscelles should greatly enlarge the scope of antibody catalysis.

An antibody, NPN43C9, raised against aryl phosphonamidate *19* has been demonstrated to catalyze the hydrolysis of the activated amide bond in *20* (56).



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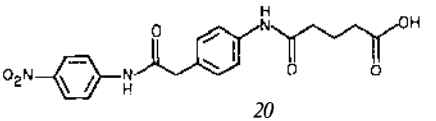


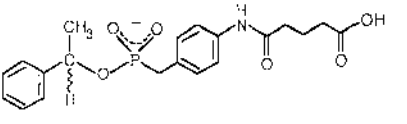
20

Hapten *19* is again an analog of the tetrahedral transition state in the hydrolysis of *20*. A rate acceleration of 2.5×10^5 by NPN43C9 over the background rate 2.5×10^5 was reported. This rate acceleration is inconsistent with a mechanism that solely involves transition state stabilization, since the difference in affinity for hapten *19* and substrates *20* is small, $\Delta\Delta G^{\neq} = -2.2$ kcal mol^{-1}. This difference in binding energy only accounts for a 100-fold rate acceleration. Other factors such as acid/base catalysis must be responsible for the observed rate acceleration. In this context it was found that 150 mM NaCl completely inhibited antibody catalysis. The structural feature of this hapten responsible for induction of such acid/base catalysis is not apparent because the phosphonamidate NH group is not charged in the immunogen. In fact the low success rate, 2% (1/44), suggests that immunological diversity may be responsible for the catalysis observed. Mechanistic analysis of this antibody catalyzed–reaction should provide important insight into the generation of antibodies that catalyze the related, but considerably more energetically demanding, hydrolysis of peptide bonds.

The strategy of transition state stabilization should be applicable to the hydrolysis of other biopolymers such as carbohydrates and polynucleotides. Lysozyme (57) and RNAse S (58) are classic examples of enzymes that function by transition state stabilization. The well-characterized transition state analog inhibitors of these enzymes, nojirimycin for lysozyme and uridine vanadate complex for RNAse S, if used as haptens may yield antibodies capable of catalyzing these important transformations.

STEREOSPECIFIC HYDROLYSES OF UNACTIVATED ESTERS Antibodies elicited toward phosphonates *21* and *22* were shown to catalyze the hydrolysis of

23 (59) and *24* (60), respectively, in a stereospecific manner. In both cases antibodies were elicited against a 50/50 mixture of both stereoisomers. Of 18 antibodies specific for *21*, 9 catalyzed the hydrolysis of (R)-*23* and 2 catalyzed the hydrolysis of (S)-*23*. Two antibodies, one of each specificity, were further characterized. The rate acceleration for the hydrolysis of (R)-*23* by antibody 2H6 was 80,000, whereas the (S)-*23* specific antibody 21H3 only showed a modest 1600-fold rate acceleration over background. The R/S selectivity is greater than 98%.



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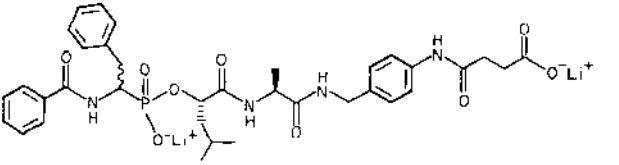

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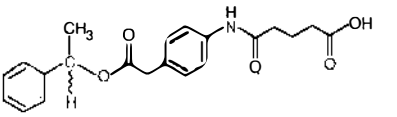
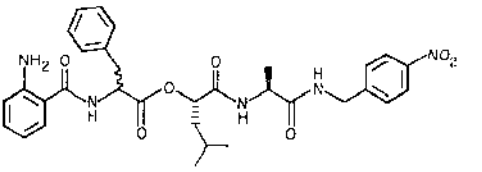


23

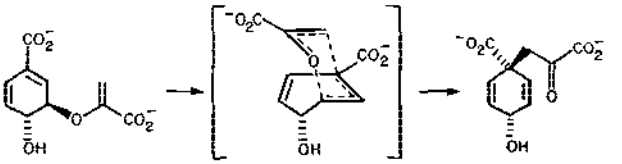


24

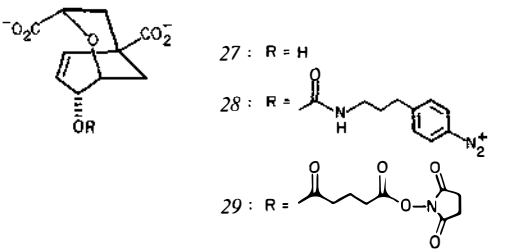
Of 25 antibodies isolated that were specific for the tripeptide phosphonate *22*, 18 accelerated the hydrolysis of the corresponding ester substrates (60). It is interesting that an exclusive preference for antibodies of one specificity was observed. All 18 antibodies were selective for the D-phenylalanyl isomer of *24*. The selectivity for D over L phenylalanine at this position was greater than 99.5% for three of the five antibodies characterized. The modest rate accelerations of 50–300 fold may be a result of the size of the immunogen. The tetrahedral phosphonate contributes proportionally less to the overall binding energy of hapten to antibody

than it does in smaller haptens. Note that the hapten also contains analogs of fluorogenic and quenching groups at the amino and carboxy termini of substrate *24*, respectively. These groups allow hydrolysis of the substrate to be monitored by observing the fluorescence increase which occurs when the fluorescent 2-aminobenzoyl group is separated from the quenching 4-nitrobenzylamide group in the reaction (11). This sensitive assay may allow direct screening of ELISA plates for catalytic activity, facilitating the production of catalytic antibodies. These two examples suggest that excellent stereoselectivities can be achieved by antibodies with large or small substrates and chiral centers in the alcohol portion or the acyl group portion of the substrate. This strategy could be readily applied to the chiral resolution of racemic alcohols and esters in the production of pharmaceuticals.

CLAISEN REARRANGEMENT The Claisen rearrangement of chorismate, *25*, to prephenate, *26*, involves the concerted cleavage of a carbon-oxygen bond and formation of a carbon-carbon bond.



25 *26*



27 : R = H

28 : R =

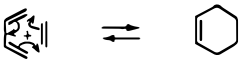
29 : R =

The enzyme that catalyzes this reaction, chorismate mutase, is at the branchpoint of aromatic amino acid biosynthesis in bacteria and plants. Bicyclic diacid *27* is the most potent known transition state analog inhibitor of the enzyme (61). Two independent groups used *28* (62) and *29* (63, 64) as haptens for generating antibody catalysts for the Claisen rearrangement of *25* to *26*. In one case, one (11F1) out of eight antibodies specific for *28* was shown to catalyze the reaction with a k_{cat} of 2.7 min^{-1} and a K_M of 260 μM at 10°C, pH 7.0. This corresponds to a rate enhancement of 10^4 over the uncatalyzed rate. This factor compares with the 3×10^6-

fold rate enhancement achieved by *E. coli* chorismate mutase assayed under the same conditions. Notably this is the first example of a rate enhancement with respect to a unimolecular background reaction. In all the other examples the background reaction is bimolecular. With *29* as the immunogen, 1 (1F7) out of 15 antibodies specific for *29* was catalytic, with a k_{cat} equal to 0.025 min^{-1} and a K_M of 22 μM at 13°C, pH 7.5. Antibody 1F7 accelerates the reaction 190-fold over the uncatalyzed rate. This antibody was shown to be highly stereoselective with a 90 : 1 preference for the (−) over the (+) isomer of chorismate.

Several mechanisms proposed for chorismate mutase can be ruled out in the case of antibody 11F1. No D_2O solvent isotope effect ($k_H/k_D = 1$) was observed for the reaction ruling out general acid or general base catalysis in the rate-limiting step. The fact that (±)-methyl ether of chorismic acid is converted to the prephenate methyl ether by the antibody rules out loss of the hydroxyl group to form a C-4 cation or oxirinium ion. Furthermore, the dimethyl ester of chorismic acid is not a substrate for this antibody, suggesting that the carboxylate groups are important recognition elements. To test whether the antibody is catalyzing the reaction by acting as an entropic trap, the entropy and enthalpy of activation were measured for both the antibody catalyzed process and the background reaction (D. Y. Jackson, P. G. Schultz, unpublished results). The rearrangement catalyzed by antibody 11F1 has $\Delta S^{\neq}$ and $\Delta H^{\neq}$ values of −1.2 eu and 18.3 kcal/mol, respectively. The background reaction has activation parameters of −12.9 eu and 20.5 kcal/mol for $\Delta S^{\neq}$ and $\Delta H^{\neq}$, respectively, under the same conditions. The fact that the $\Delta S^{\neq}$ is almost zero for the antibody catalyzed process suggests that locking the substrate in a conformationally restricted conformation is the mechanism by which the antibody catalyzes the reaction. By contrast, similar studies with 1F7 suggest a purely enthalpic stabilization by the antibody (63). The enormous amount of antibody diversity apparently can lead to catalysts that operate in fundamentally different ways even when elicited against virtually identical immunogens.

This approach can be applied to other important carbon-carbon bond formation reactions such as Diels-Alder (Eq. 6) and Cope rearrangements (Eq. 7) for which no enzymes are known.



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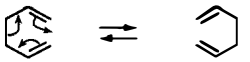
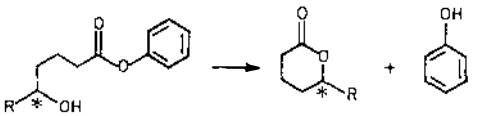


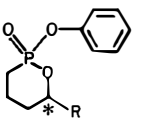
7.

ACYL-GROUP TRANSFER REACTIONS The constraints imposed by the antibody binding pocket should also accelerate acyl or phosphoryl group

transfer reactions by reducing rotational and translational entropy of the reactants (20). The catalysis of an intramolecular acyl group transfer (lactonization) reaction was the first example of the use of this strategy to design an antibody catalyst. Antibodies were elicited against the cyclic phosphonate ester *30* (65).



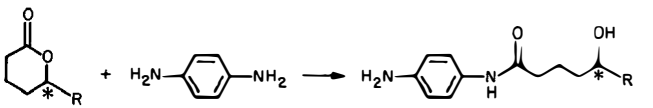
31 *32*



30

This hapten is a transition-state analog for the six-membered ring cyclization reaction of *31* to *32*. The kinetic parameters were determined to be $k_{cat} = 0.5\ min^{-1}$ and $K_M = 76\ \mu M$; the rate acceleration afforded by this antibody is 167-fold over the background rate. The antibody catalyzed reaction was stereoselective, affording a 94% excess of one product isomer when provided with a 50/50 mixture of isomeric starting materials. This reaction is a member of an important class of reactions that includes formation of 12–16 membered ring macrolides as well as cyclic peptides. These reactions are a considerable challenge in the syntheses of antibiotics such as erythromycin and immunosuppressants such as FK506. Using the strategy just described, antibodies could be used to reduce the entropic requirements for bringing the reactive end groups of these linear precursors into the orientation required for ring closure.

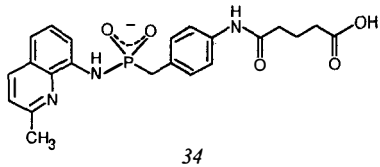
A significant development in antibody catalysis has been the use of antibody binding energy to overcome the entropic barriers of bimolecular reactions. The antibody that was shown to catalyze the conversion of *31* to *32* also catalyzes bimolecular amide bond formation reaction to give *33* (66).



32 *33*

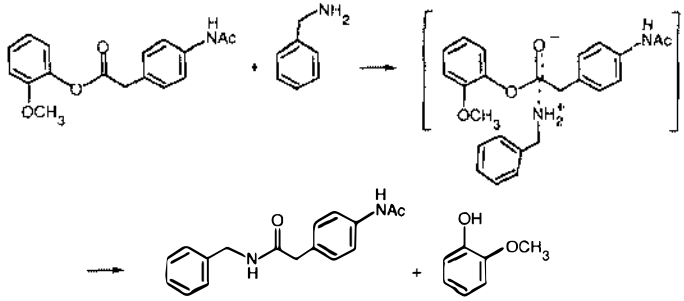
The antibody binds phenylenediamine and lactone *32* with K_M's of 1.2 mM and 4.9 mM, respectively. The inhibitor *30* was shown to be competitive for

both reactions with a K_I of 75 nM for the bimolecular condensation reaction and 250 nM for the cyclization reaction. The effective molarity for the bimolecular reaction was measured to be 16 M which is considerably below the theoretical limit of 10^8 M. This upper bound is based on the calculated value of approximately 45 eu for the translational and rotational entropy of activation of a bimolecular reaction (20).



34

Another antibody generated against phosphonamidate *34* (67) was shown to catalyze a bimolecular amide formation reaction (Eq. 8).



8.

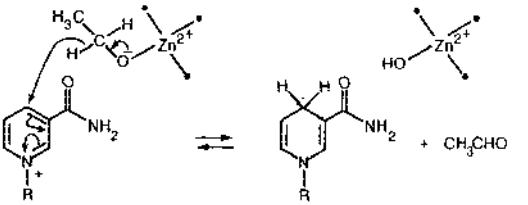
This antibody was shown to provide an effective molarity of 10.5 M. Here the hapten did not include the ester leaving group, an important element of the proposed transition state for the reaction. This design feature may explain why less than 2% (1/55) antibodies specific for *34* were able to catalyze the reaction of interest.

The condensation of large peptide fragments synthesized on a peptide synthesizer is slow in solution and subject to many side reactions. Thus, the ligation of large (> 50 aa) peptide fragments represents a significant obstacle to the de novo synthesis of large proteins. Antibodies could be designed using the strategy described above to bring the appropriate carboxyl and amino termini together (protecting groups would not be necessary) in the combining site and catalyze peptide bond formation. Another important class of bimolecular reactions that could be catalyzed by this strategy is the aldol condensation. An example of this class of reaction is the formation of fructose 1,6-diphosphate from dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate which is catalyzed by aconitase.

Recently antibodies elicited aginst a reduced Schiff's base formed between *p*-nitro phenylalanine and pyridoxal have been shown to catalyze a transamination reaction. In the presence of imidazole buffer all seven antibodies isolated catalyze the transamination between pyridoxamine and the corresponding α-ketoacid (A. G. Cochran, P. G. Schultz, unpublished results).

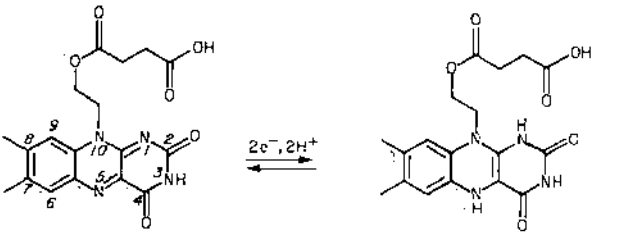
Cofactor Chemistry

Many enzymes utilize nonamino acid cofactors to catalyze reactions. Important members of this class of enzymes include cytochrome P_{450} (Fe-heme), α-ketoacid dehydrogenases (thiamine pyrophosphate), D-amino acid oxidase (flavin), and alanine racemase (pyridoxal phosphate). For example, the enzyme alcohol dehydrogenase uses an electrophilic Zn^{2+} atom to polarize the alcohol oxygen in order to facilitate hydride transfer from the alcohol to a second cofactor, NAD^+, that acts as an electron sink (Eq. 9) (68).



9.

In order to expand the scope of antibody catalysis to redox reactions and to energetically demanding hydrolytic reactions, strategies that allow incorporation of cofactors into antibody combining sites will have to be used. To this end antibodies have been elicited against flavin cofactor *35* (69).

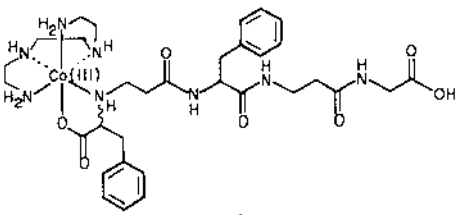


35 *36*

The three rings are coplanar in the oxidized flavin, *35*. The reduced dihydro flavin, *36*, has a substantially different electron distribution about the rings and is also conformationally distinct from the oxidized *35*. The reduced form is butterfly shaped due to a pucker about the two central nitrogen

atoms in the central ring. Antibodies elicited toward oxidized *35* bind this form with an affinity 4×10^4 higher than *36*. This differential binding energy manifests itself in the reduction potential (E_m) of the bound flavin: the reduction potential of flavin in the absence of antibody is -206 mV; in the antibody bound state, the flavin E_m is -343 mV. Hence, bound flavin is a substantially stronger reducing agent than free flavin. Because the antibody-flavin complex is a stronger reductant than free flavin, the substrate Safranine T ($E_m = -289$ mV) could be rapidly reduced by the antibody flavin complex. This process does not occur with free flavin. The antibody-flavin complex is therefore able to mediate redox reactions not thermodynamically accessible to free flavin. Here, antibody binding energy is used to destabilize the reduced form of a flavin, creating a more powerful donor of electrons to substrates. By incorporating substrate binding sites adjacent to flavins, stereocontrolled chemical reductions could be carried out.

An exciting recent result in the area of catalytic antibodies has been the generation of antibodies to a peptide-cofactor complex, *37*, that are capable of catalyzing the hydrolysis of a Gly-Phe peptide bond (70).



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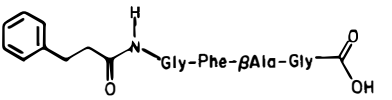


38

Although a structurally inert Co(III) trien complex was used as the cofactor in the immunogen, the 14 antibodies elicited could accommodate any of 13 different trien metal complexes. In the presence of a variety of metal trien complexes two of the antibodies catalyzed the cleavage of the Gly-Phe peptide bond in *38*. A turnover number of 6×10^{-4} sec^{-1} was measured. Somewhat surprisingly, the Gly-Phe bond hydrolyzed is next to the bond one might have expected to have been cleaved, based on model chemistry by Buckingham and others (71). Nonetheless, the extension of antibody catalysis to biopolymers such as peptides is of great value. The general rules of using cofactors in such systems must be further developed.

Another example of antibody catalyzed peptide hydrolysis has recently been reported (72). Two out of six immune human IgG autoantibody preparations cleaved [Tyr^{10}-^{125}I] vasoactive intestinal peptide (VIP) between residues Gln^{16} and Met^{17}. The k_{cat} and K_M values reported were 15.6 min^{-1} and 37.9 nM, respectively. Of considerable concern is the fact that the *background* rate for the hydrolysis of the iodinated substrate used in the study is remarkably fast considering a measurable amount of conversion could be observed in as little as 3 hours. One possible explanation of the fast hydrolytic rates is that radioactive decay of ^{125}I is actually responsible for the fast background rate. The normal half-life ($\tau_{1/2}$) of peptide bonds is 7 years (73). Clearly, it is essential that monoclonal antibodies with this activity be generated and carefully characterized.

Genetic and Chemical Modification of Existing Antibodies

A second major approach toward the design of catalytic antibodies involves introduction of catalytic activity into existing antibodies of the desired specificity either by selective chemical methods or by site-directed mutagenesis. Both approaches also allow for the incremental increase in rate-enhancements achieved by antibodies generated via other strategies.

SITE-DIRECTED MUTAGENESIS To date, efforts to modify enzyme specificity rationally via the use of site-directed mutagenesis have been successful in only a few cases. Modification of steric and hydrophobic interactions in the binding site of the protease subtilisin increased specificity toward smaller hydrophobic substrates, compared to the wild-type enzyme (74). The charge specificity of aspartate amino transferase was inverted in such a way that a lysine/arginine transaminase was produced by a single arginine to aspartate mutation at position 292 of the enzyme (75). In spite of these examples, the large number of such studies that yield functionally incompetent enzymes even when X-ray crystal structures are available attests to the difficulty of this approach for the design of selective catalysts. The application of this method to antibody catalysts is complicated by the small number of X-ray crystal structures of antibodies. A recent paper has reported attempts to introduce catalytic activity into an antilysozyme antibody, Gloop2 (76). Mutations were made, using aspartyl peptidases as a model, in order to introduce peptidase activity into the combining site. Unfortunately the mutations introduced were either silent or eliminated lysozyme binding significantly.

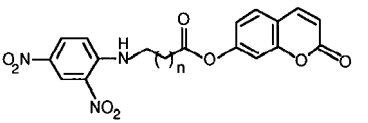
Site-directed mutagenesis has been successfully applied to the antibody MOPC315 (77). MOPC315 binds substituted 2,4-dinitrophenyl (DNP) ligands with association constants ranging from 10^3 to 10^7 M^{-1} (24, 78). The combining site has been characterized by spectroscopic methods (UV,

fluorimetry, NMR), chemical modification, and amino acid sequencing of the variable region. Moreover, earlier affinity-labeling studies with reagents of varying structures defined a number of reactive amino acid side chains in the vicinity of the combining site (79).

In the absence of an X-ray crystal structure of MOPC315, chemical modification experiments were used to guide the mutagenesis. Although there are 14 potentially reactive side chains in the hypervariable region (2 histidines, 2 lysines, 3 arginines, and 7 tyrosines), DNP-containing affinity labels alkylate primarily 2 residues, tyrosine 34L and lysine 52H (37). Tyrosine 34L was chosen as the initial target for mutagenesis since it appeared that histidine at residue 34 would be situated to catalyze the hydrolysis of esters. The role of tyrosine 34L in DNP binding has not been clearly established; affinity labeling of the tyrosine prevents binding of ligands (79), perhaps by sterically blocking the entrance to the site. Nitration of the tyrosine ring has no effect (80).

In order to generate mutants of MOPC315, a recombinant Fv fragment was generated. MOPC315 IgA can be proteolyzed with pepsin to yield functional Fab or Fv fragments (81). The Fv fragment (26 kD) contains all the sequences necessary for folding of the binding domain and recognition of the DNP hapten. Reduced and separated V_H and V_L can be efficiently formed by oxidation of the reduced forms prior to reconstitution (81). This property has been exploited to produce a hybrid Fv, in which recombinant V_L produced in *E. coli* is reconstituted with V_H derived from MOPC315 IgA. Functional Fv and Fab fragments of other antibodies have been previously expressed in *E. coli* (82, 83). A gene encoding the N-terminal 115 amino acids of MOPC315 light chain was chemically synthesized and expressed in *E. coli* as a fusion with the lambda cII gene (84). The resulting hybrid protein was cleaved at one site with Factor Xa; the liberated V_L peptide was reconstituted with V_H; and the resulting Fv was purified to homogeneity by gel filtration and affinity chromatography.

Wild type Fv [Fv(315)] and a phenylalanine mutant [Fv($Y34F_L$)] bound DNP lysine with similar affinities at pH 6.8 ($K_D = 250$ nM), while the histidine mutant [Fv($Y34H_L$)] bound DNP lysine with six-fold lower affinity ($K_D = 1400$ nM). The histidine mutant Fv accelerated hydrolysis of *39*, 9×10^4-fold over the background reaction with 4-methyl imidazole at pH 6.8.



39 : n = 1

40 : n = 3

The initial rate of ester cleavage is 50 times faster than that of wild type Fv or the Fv($Y34F_L$) mutant. In addition, the hydrolyses of aminopropanoic and aminohexanoic homologs were not significantly accelerated, consistent with the postulated location of His 34 side chain in the active site. Site-directed mutagenesis should prove a powerful tool for either augmenting the rate enhancements of catalytic antibodies generated via other strategies, or for the stepwise evolution of catalytic activity in antibodies to produce efficient selective catalysts.

SEMISYNTHETIC ANTIBODIES Selective chemical modification of antibodies should make it possible to extend the catalytic groups available to carry out a given reaction beyond the common natural 20 amino acids. For example, it may be possible to introduce transition metal catalysts, cofactors, strong nucleophiles, or electrophiles into antibody combining sites to generate novel catalytic antibodies. Kaiser and associates introduced a flavin coenzyme into the active site of the enzyme papain via covalent attachment to catalytically active cysteine 25 (see Ref. 85). This flavin imparted NADH dehydrogenase activity into papain. Site-directed mutagenesis has been used to introduce a unique surface cysteine into the relatively nonspecific phosphodiesterase staphylococcal nuclease (86). A thiol derivatized DNA oligomer was attached via a disulfide bond to this cysteine to afford a hybrid enzyme that in sequence selectively hydrolyzes DNA or RNA substrates at defined recognition sequences.

The key to the generation of semisynthetic catalytic antibodies is the development of mild methods for selectively introducing derivatizable groups of unique reactivity into or near the combining site. These groups can then be modified in a second step to incorporate the chemical functionality (cofactor, metal-ligand complex, fluorophore, etc) of interest. As noted above, one such derivatizable group is a free thiol, which, by virtue of its high nucleophilicity and ease of oxidation, can be selectively modified via sulfide exchange or electrophilic reactions. Moreover, introduction of a nucleophilic thiol into an antibody combining site might directly afford a catalytic antibody. Cleavable affinity labels have been used to selectively modify the antibody combining site of the IgA MOPC315 with a nucleophilic thiol (87). It is important that this method does not require knowledge of the three-dimensional structure of the antibody and should therefore be applicable to a large number of proteins of interest.

The generalized scheme for generation of a semisynthetic antibody (Figure 3) begins with the generation of an antibody specific for a given hapten. This hapten could be a transition state analog or simply a substrate for the reaction of interest. An affinity labeling reagent is synthesized with a cleavable linkage between the hapten and the affinity labeling group (e.g.

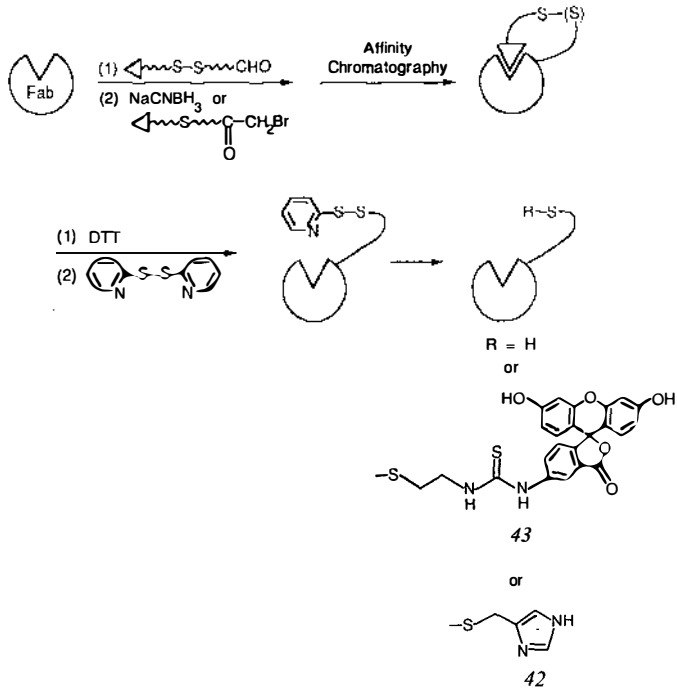
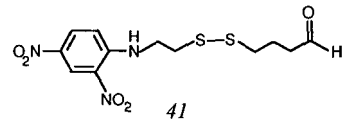


Figure 3 Outline for the generation of a semisynthetic catalytic antibody using cleavable affinity labeling reagents.

aldehyde, α-bromoketone). It is also important that the hapten or substrate analog provides the specificity necessary to direct the labeling group to a residue (e.g. tyrosine, lysine, serine) in or near the combining site. Once the labeling reagent has reacted with the antibody the linkage to the hapten is cleaved under mild conditions; leaving behind a thiol "handle" adjacent to the combining site which can be subsequently derivatized with a wide variety of synthetic cofactors.

Cleavable affinity labeling reagents have been used to introduce nucleophilic thiols (87, 88), imidazole groups (89), and fluorescent reporter molecules into MOPC315 (87). Treatment of the Fab fragment of MOPC315 with *41* in the presence of the reducing agent $NaCNBH_3$ resulted in specific labeling (>90%) of lysine 52H.



Treatment with dithiothreitol cleaved the disulphide bond of *41* (without affecting the internal disulfides of the Fab) leaving a free thiol. This synthetically introduced thiol was positioned so that it could act as a nucleophile to accelerate the thiolysis of coumarin ester *40*. The k_{cat} and K_M for this reaction were determined to be 0.87 min^{-1} and 1.2 μM, respectively. A competitive inhibitor for the antibody is N-DNP glycine with a K_i of 8 μM. Compared to the reaction in the presence of 0.1 μM dithiothreitol, the rate acceleration provided by the thiolated antibody is 6×10^4. This value is quite similar to that found in the antibody-catalyzed β-elimination reaction, and the value suggests that proximity of a base or nucleophile to a complexed substrate can lower $\Delta G^{\neq}$ for reaction up to 7.0 kcal/mole.

An imidazole group, *42* (Figure 3), was covalently attached to the thiolated Fab via a disulfide exchange reaction (89). This imidazole group facilitated the catalytic hydrolysis of the coumarin ester, *9*. Whether the mechanism is direct nucleophilic attack or general-base catalysis has not been determined. The rate acceleration compared to 4-methyl imidazole as a catalyst is 1.1×10^3. Perhaps a shorter more rigid tether between the imidazole and the protein would lead to a greater rate acceleration. It is interesting to compare catalysis by $F_v(Y34H_L)$ and by the MOPC315 that has been chemically derivatized with imidazole at lysine 52H by the flexible eight bond tether. $F_v(Y34H_L)$ hydrolyzes *39* ($k_{cat} = 0.001$ min^{-1}, pH 7.0) approximately 16 times more rapidly than imidazole derivatized Fab. This difference is likely due to the decreased number of degrees of freedom of the histidine 34 side chain.

By incorporating a derivatizable group adjacent to the combining site, it is possible to attach reporter molecules to the antibody. Such reporter groups provide homogeneous, fast, and sensitive assays for ligand binding. The thiol containing fluorescein group *43* (Figure 3) was attached to the derivatized Fab fragment of MOPC315 (87). When N-DNP glycine was added to this derivatized antibody, a decrease in the fluorescence of fluorescein was observed due to quenching by the DNP bound in the nearby binding site. This provided a direct assay of ligand binding. Semisynthetic antibodies of this sort may prove useful as sensors and in diagnostics. It should be noted that all binding constants measured with Lys 52H derivatized MOPC315 were identical to those measured with underivatized Fab using the same ligands. This is in contrast to derivatives at tyrosine 34L where ligand binding was significantly decreased by introduction of a thiol containing side chain (87). Cleavable affinity labels provide a fast and selective method for the introduction of a wide variety of molecules (including catalysts, reporter molecules, and therapeutic agents) adjacent to the antibody combining site.

Derivatization of antibodies with other groups such as copper phen-

anthroline, porphyrin cofactors, or flavins should provide novel redox catalysts. Electrophilic metal centers such as Zn^{2+} could also be incorporated via this strategy to polarize carbonyl groups of amide or phosphate groups thereby facilitating hydrolysis.

CONCLUSION

In the short period of time which has elapsed since the first reports of antibody catalysis in 1986 (41, 42), a considerable number of reactions have been catalyzed using antibodies. The substrate specificities of antibody catalyzed reactions are high, and rate accelerations up to 10^6 have been achieved (54). A number of general strategies have also evolved for generating catalytic antibodies including transition state stabilization, catalysis by approximation, introduction of catalytic groups via hapten antibody complementarity and chemical modification, generation of cofactor binding sites and site-directed mutagenesis. Characterization of catalytic antibodies is providing insight into fundamental notions of enzymatic catalysis.

A number of strategies have yet to be exploited for the generation of catalytic antibodies. Genetic selections or screens may allow the generation of catalytic antibodies from substrate specific antibodies. However, this approach will require more efficient yeast and bacterial expression systems for antibodies. Antibodies could also be used to destabilize ground state species by introducing strain. For example, antibodies may be able to twist the RC(O)-NHR′ bond of an amide and consequently reduce the barrier for hydrolysis. Antiidiotypic antibodies generated against enzyme active sites may also prove to have catalytic activity. Clearly, the most promising method for the generation of new catalytic antibodies with rate enhancements comparable to enzymes is the combination of two or more highly successful strategies. New efficient approaches will become increasingly important as different strategies are combined to make efficient tailorable catalysts for application in biology, chemistry, and medicine.

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