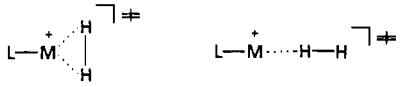
a rather conventional 1,2-elimination mode.

A distinct behavior of Mn^+ and Cr^+ is also observed for the metal ion induced dehydrogenation of 4-octyne. According to extensive labeling experiments,[10c] Mn^+ activates to 83% the C(1)/C(2) positions and to 17% C(2)/C(3), and the hydrogen molecule is reductively eliminated in a formal 1,2-fashion. However, for the Cr^+ complex of 4-octyne, 78% of H_2 loss originates from C(2)/C(3) and only 22% from C(1)/C(2). In spite of these distinct behaviors, the two metal ions have in common the fact that the reductive elimination of hydrogen is rate-determining. The insertion of the (complexed) metal ion into the C–H bond is not associated with a discernible isotope effect. This finding is very reminiscent of the reactions described above for the RCN/Fe^+ system.

With regard to the Cr^+- and Mn^+-induced H_2 loss from 4-octyne, the kinetic isotope effects clearly prove that the reductive elimination proceeds via a transition state that is best described as a "side-on" H_2-coordinated metal ion (**44**, L = C_8H_{12}) and not an "end-on" complex (**45**).[10c,23] This result is in excellent agreement with a theoretical analysis for H_2 *addition* to metal complexes;[24] according to the Saillard–Hoffmann analysis,[24] using extended Hückel molecular orbital theory, the perpendicular (i.e., "end-on") approach of H_2 to, for example, a C_{4v} square pyramidal metal fragment is purely repulsive. In contrast, the parallel approach which eventually generates a "side-on" bond complex is the favored mode of interaction.



44 **45**

Concluding Remarks

This account has attempted to demonstrate that selective activation of remote C–H and C–C bonds of simple, *flexible* organic molecules RX can be easily achieved by "anchoring" a bare transition-metal ion to the functional group. The few systems that surfaced in the last two years already indicate that these reactions seem to be without precedent in oranometallic chemistry performed under more conventional conditions. Moreover, they provide for the first time compelling evidence for Breslow's concept of "remote functionalization" in organometallic systems.

Future work will inter alia focus on questions like the following:

(i) What is the gas-phase chemistry of polyfunctional molecules with "naked" transition-metal ions? Can one expect cooperative effects such that supramolecular structures will be induced by the transition-metal ions?

(ii) With regard to the more traditional chemistry of charged ML_n^+ complexes, it will be interesting to probe whether successive ligation of M^+ will affect the reactions described in this Account. In this context the question of whether stereochemical effects are operative deserves special attention.

(iii) The observation that the reactivity of bare M^+ with gaseous substrates is strongly metal ion dependent suggests the study of heteronuclear complexes $(M'M)^+$. It would be no surprise to observe a chemistry being distinctly different from that of either M^+ and M'^+.[25]

(iv) Lastly, state-selective studies of the electronic states of the metal ions will most certainly add to the understanding of the chemistry of naked M^+ with organic substrates.

No doubt, in spite of the already impressive number of papers published in this rapidly developing area within the last decade, the understanding of the details of the elementary steps is still in its infancy.

The continued and generous financial support of our work by the following institutions is gratefully acknowledged: Stiftung Volkswagenwerk, Fonds der Chemischen Industrie, Deutsche Forschungsgemeinschaft, and the Gesellschaft von Freunden der Technischen Universität Berlin. I am particularly indebted to my students for their dedication and their practical and conceptual contributions to the present work, which we started at TUB around 1986, when Dr. Carlito B. Lebrilla, then a postdoctoral fellow from Berkeley, in a group seminar posed the question, "Can flexible molecules be selectively functionalized by bare transition-metal ions?" The answer is presented in this Account.

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Catalytic Antibodies

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With the advent of hybridoma technology[1] it has become possible to generate homogeneous, high-affinity antibodies that selectively recognize a large number of structurally diverse ligands. Antibodies have been generated against biopolymers such as nucleic acids, peptides, and polysaccharides; natural products such as steroids and prostaglandins; and small synthetic

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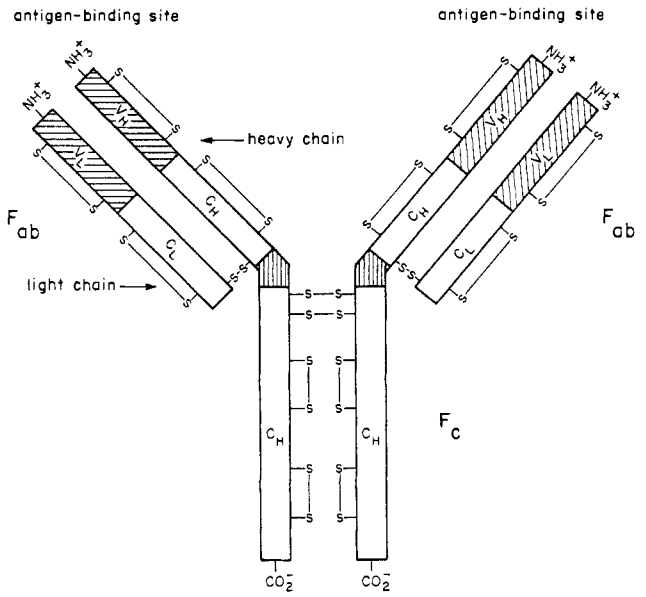

Figure 1. Schematic representation of an IgG molecule (MW ~ 150 000). The two identical light and heavy chains are linked via a series of disulfide bonds. The antibody combining site (F_v) consists of approximately the first 110 amino acids at the amino terminus of the heavy and light chains.

molecules such as phenylphosphonates and benzylamines. Antibodies bind ligands ranging in size from about 6 to 34 Å with association constants in the range of 10^4–10^{14} M^{-1}.[2] The ligand combining site of immunoglobulins (Ig's) consists of six hypervariable regions, regions of extensive amino acid diversity, located in roughly the first 110 amino acids of the light (V_L) and heavy (V_H) polypeptide chains of the Ig[3] (Figure 1). Combinatorial joining of the gene segments encoding the V_L and V_H genes and combinatorial association of different light and heavy chains generate a minimum of 10^8 different antibody molecules; mutations expand this base-line repertoire of receptors still further.[4] This diversity has made antibodies one of the most important classes of receptors in biology and medicine today.

Because antibodies can be generated to virtually any molecule of interest, the development of general strategies for introducing catalytic activity into antibody combining sites should lead to a new class of enzyme-like catalysts *with tailored specificities*. Catalytic antibodies could have considerable value as biochemical or molecular biological tools, as therapeutic agents, or in the synthesis of pharmaceuticals and novel materials. To date, two general approaches have been pursued for the generation of catalytic antibodies. The steric and electronic complementarity of an antibody to its corresponding hapten (the ligand against which the antibody is elicited) has been exploited to generate combining sites that (1) are complementary to a rate-determining transition state, (2) act to overcome the entropy requirements involved in orienting reaction partners, (3) contain an appropriately positioned catalytic amino acid side chain, or (4) contain cofactor binding sites. Alternatively, catalytic groups can be introduced directly into the combining site of an antibody by chemical modification or by site-directed mutagenesis or genetic selection. The ability to generate antibody combining sites with specific catalytic groups and/or microenvironments should, in addition to affording selective catalysts, also help dissect the contribution of various factors involved in enzymatic catalysis, including transition-state stabilization, general acid–base catalysis, nucleophilic catalysis, ground-state strain, and proximity effects.

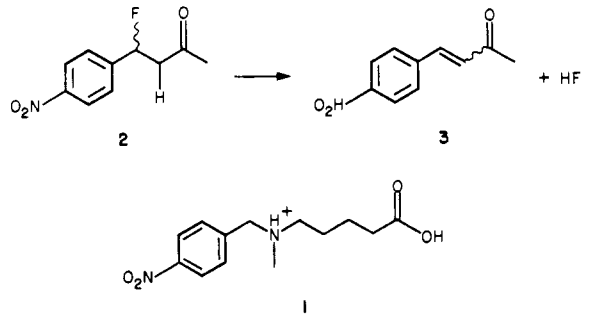

Figure 2. Antibody-catalyzed elimination reaction.

Generation of Antibody Combining Sites That Contain Catalytic Groups

A β-Elimination Reaction. One strategy for inducing catalytic residues, such as basic or acidic amino acid side chains, in the combining site of an antibody involves generating antibodies to a hapten whose structure is complementary to that catalytic group. In fact, an important goal in the design of catalytic antibodies is the development of general rules relating hapten structure to the corresponding catalytic groups or microenvironment in the antibody combining site. One such example involves the use of electrostatic complementarity between a hapten and an antibody to generate a basic amino acid side chain that catalyzes the β-elimination reaction[5] illustrated in Figure 2. This reaction, which involves C–H proton abstraction, is a member of an important class of reactions including elimination and isomerization reactions and aldol and Claisen condensations. Proton abstraction in enzymes that catalyze these reactions is often performed by the carboxylate side chain of glutamic or aspartic acid. These residues typically display higher than normal pK_a values (6.5–8.2) in the hydrophobic active sites of enzymes.[6]

Aspartate and glutamate residues have been generated in the combining sites of antibodies by taking advantage of charge complementarity between haptens and the corresponding antibodies.[2] Consequently, we reasoned that an antibody combining site could be generated that contains a carboxylate within bonding distance of an abstractable substrate proton by using the appropriate positively charged hapten. Importantly, the position of the charged group in the hapten should reflect that of the target C–H group in the substrate. Hapten 1 contains a positively charged ammonium ion replacing the α-CH_2 group of substrate **2**. The fact that the hapten and substrate share a common recognition element, the *p*-nitrophenyl group, should insure rea-

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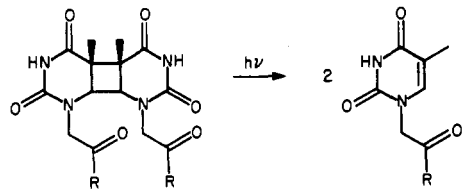

Figure 3. Antibody-catalyzed thymine dimer cleavage.

sonable binding affinity of substrate to the antibodies. 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 since a stabilizing salt bridge interaction is lost.

Of six antibodies generated against the keyhole limpet hemocyanin (KLH) conjugate of **1**, four catalyzed the β-elimination reaction of **2** to **3** (it should be noted that small molecules must be conjugated to a carrier protein in order to elicit an immune response). The IgG 43D4-3D3 obeyed the classical Michaelis–Menten expression 1 (k_{cat} = 0.2 min^{-1}, K_m = 182 μM at 37 °C, pH 6.0), demonstrated substrate specificity, and was competitively inhibited by hapten (K_i = 290 nM). It was

$$\text{Ig} + \text{S} \rightleftarrows \text{Ig·S} \xrightarrow{k_{cat}} \text{Ig} + \text{P} \qquad (1)$$

shown[5] that a Glu or Asp with a pK_a of 6.2 was responsible for catalysis. The increased pK_a of the carboxylate side chain is consistent with loss of a salt bridging interaction when hapten **1** is replaced by substrate in the antibody combining site. The rate enhancement obtained by introduction of the carboxylate residue in the antibody combining site, compared to acetate free in solution, is 8.80 × 10^4 at pH 6.0.[5] This value is similar to the rate acceleration of ~10^4 attributable to the bases Glu 43 in staphylococcal nuclease[7] and Asp 102 in trypsin.[8]

A 2 + 2 Cycloreversion Reaction. A second example in which hapten structure was used to induce a catalytic side chain in an antibody combining site involves the generation of antibodies that catalyze the light-dependent 2 + 2 cycloreversion reaction of thymine dimer **5** (Figure 3).[9] Thymine dimers are the predominant DNA photolesion produced by UV light. Model studies have shown that photosensitizers such as indoles, quinones, or flavins can reversibly transfer an electron to or accept an electron from a thymine dimer, resulting in facile cleavage of the intermediate thymine dimer radical.[10-12] These results suggest that an antibody combining site specific for a thymine dimer and containing an appropriately positioned sensitizer should act as a photorepair enzyme. It seemed reasonable to us that antibodies generated against the polarized π system of a pyrimidine dimer might contain a complementary tryptophan residue in the combining site, much as antibodies generated against positively charged haptens contain complementary aspartate and glutamate residues.[2] In order to test this strategy, antibodies were generated against *cis-syn*-thymine dimer **4** (Figure 3).

Five out of six antibodies generated to the KLH conjugate of carboxymethyl thymine dimer **4** catalyzed the photoreversion of dimer **5** to monomer when irradiated with 300-nm monochromatic light. The k_{cat} of the IgG 29E5 was 1.2 min^{-1} (under conditions of nonsaturating light),[9] close to the k_{cat} of 3.4 min^{-1} for thymine dimer cleavage by the repair enzyme *Escherichia coli* DNA photolyase.[13] The quantum yield of the photocleavage reaction, correcting for the number of tryptophans, was ≥0.75. The antibody did not catalyze the cleavage of the corresponding *N,N'*-dimethyl substrate, consistent with the high specificity of antibodies. The wavelength dependence of the photocleavage quantum yield as well as fluoresence quenching experiments demonstrated the involvement of a tryptophan in catalysis. It remains to be determined whether the reaction proceeds through a dimer radical anion or cation.

In the two examples described above, a high percentage of those antibodies isolated were catalytic. This result may reflect the generality of using electrostatic and π-stacking interactions to elicit complementary combining site residues. One can imagine many other reactions that can be catalyzed by antibodies using this strategy (or this strategy in conjunction with those detailed below) including aldol reactions (a β-aminophosphonate hapten), ester/amide hydrolysis (an *N*-oxide hapten), or oxy-Cope rearrangements (an aminocyclohexane hapten). It should also be noted that monoclonal antibodies have been reported that fortuitously contain nucleophilic residues that react in a stoichiometric fashion with an activated ester analogue of the hapten.[14]

Generation of Antibodies Complementary to Transition-State Analogues

An antibody generated to a haptenic group resembling the transition-state configuration of a given reaction should lower the free energy of activation of the reaction by stabilizing the corresponding transition state relative to reactants or products.[15] In fact, the active sites of many enzymes are complementary in structure and electronic distribution to rate-limiting transition states, as evidenced by studies of enzyme active sites[16] as well as enzyme inhibition by transition-state analogues.[17] Antibodies elicited to transition-state analogues have been demonstrated to catalyze a variety of reactions (vide infra), including carbonate, ester, and

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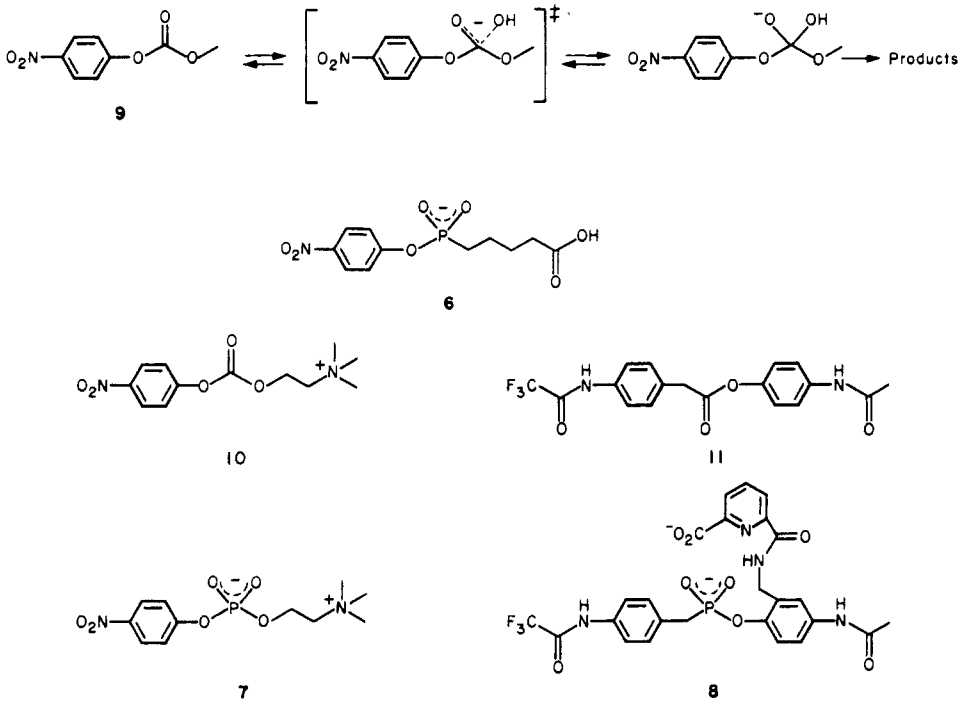

Figure 4. Transition-state analogues and the corresponding substrates.

amide bond hydrolysis, the Claisen rearrangement of chorismic acid to prephenic acid, bimolecular amide bond formation, and a six membered ring lactonization reaction.

Ester, Carbonate, and Amide Hydrolysis. The first examples of catalytic antibodies specific for transition-state (TS) analogues bound tetrahedral, negatively charged phosphate and phosphonate TS analogues for the hydrolysis of carbonates and esters[18,19] (Figure 4).

Monoclonal antibodies specific for tetrahedral transition-state analogues **6**–**8** were found to selectively catalyze the hydrolysis of the corresponding substrates **9**–**11** with kinetics consistent with the Michaelis–Menten rate expression 1.[18,19] The antibody-catalyzed reactions were competitively inhibited by their corresponding transition-state analogues. The inhibition constants (K_i's) are in every case substantially lower than the corresponding K_m's of substrate, suggesting that transition-state stabilization plays a role in catalysis. A comparison of the rates of the antibody-catalyzed reactions ($v_{Ig} = k_{Ig}[Ig{\cdot}S][OH]$) with the rates of hydroxide-dependent hydrolysis ($v_{OH} = k_{OH}[S][OH]$) afforded antibody rate accelerations in the range of 10^3–10^5.[18] These accelerations are in the same range as those attributable, both experimentally and theoretically, to transition-state stabilization in hydrolytic enzymes. For example, a triple mutant of the enzyme subtilisin, in which each amino acid of the catalytic triad of His, Ser, and Asp is replaced by an Ala residue, hydrolyzes a *p*-nitroanilide peptide substrate 3000-fold more rapidly than the corresponding uncatalyzed reaction, which gives an estimate of the extent of catalysis that can be achieved by stabilization of the transition state by binding determinants other than the catalytic triad.[20] In all cases the antibodies also displayed high substrate specificity.

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.[21] 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 Arg52H, which are invariant in all of the phosphorylcholine-binding Ig's sequenced to date,[22] bind the phosphate via hydrogen bonding and electrostatic interactions with the phosphoryl oxygen atoms. The crystal structure shows 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 the hydroxide ion catalyzed hydrolysis of **10**, the phosphorylcholine antibodies should be capable of polarizing the bound carbonate for attack by hydroxide ion in the rate-determining step. In light of the fact that the ground-state structure of **10** differs substantially from the transition-state configuration, the differential binding affinity of the Ig to these two species should be reflected in a lowered free energy of activation for reaction. In fact, the transition-state analogue 4-nitrophenyl phosphorylcholine is bound

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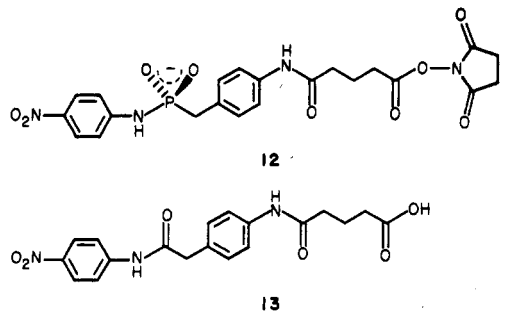

Figure 5. Phosphonamidate and corresponding nitroanilide substrate.

more tightly than the corresponding substrate by both MOPC167 and T15 (K_i = 5 μM, K_m = 208 μM for MOPC167). However, the differential binding of T15 and MOPC167 to carbonate **10** and the transition-state analogue **7** does not fully account for the magnitude of the rate accelerations. Therefore, although the crystallographic and binding data strongly suggest that T15 and MOPC167 selectively stabilize the transition-state configuration and thereby reduce the barrier to reaction, additional factors are contributing to the rate acceleration. Biophysical studies and chemical modification experiments suggest that tyrosine and arginine also play a catalytic role in the antibodies specific for hapten **6**. Again these residues can act to stabilize the tetrahedral transition state. Histidine or tyrosine is thought to be involved in catalysis by antibodies specific for **8**. It is not yet clear what catalytic role these residues play.

It should also be noted that antibodies specific for a phenylphosphonate transition-state analogue were capable of catalyzing the hydrolysis of phenylacetate in reverse micelles.[23] The k_{cat} and K_m values of the antibody solubilized in benzene were 3.74 min^{-1} and 650 μM, respectively [W_0 (molar ratio of water to surfactant) value of 23], compared to the corresponding values of 18.8 min^{-1} and 157 μM in aqueous solution. The optimal value of W_0 is significantly higher than that previously reported for enzymes, consistent with the increased molecular weight of IgG molecules. The ability of antibodies to function in reverse micelles should significantly expand the versatility of antibodies in catalysis and immunoassays.

More recently, antibodies have been generated against the KLH conjugate of arylphosphonamidate **12** (Figure 5). One antibody was found to catalyze the hydrolysis of the nitroanilide amide **13**[24] with a k_{cat} of 0.08 min^{-1} and a K_m of 562 μM. The antibody-catalyzed reaction is characterized by high specificity and a rate enhancement of 2.5 × 10^5 relative to the uncatalyzed reaction. The difference in binding of the phosphonamidate transition-state analogue (K_i) and substrate (K_m) ($\Delta\Delta G^*$ = −2.2 kcal/mol) does not account for the magnitude of the rate acceleration. Therefore other factors such as acid or base catalysis or ground-state destabilization must be involved in catalysis. Generation of an antibody combining site with these additional catalytic interactions may have simply been the result of immunological diversity (one IgG out of 44 was found to be catalytic) or could be a consequence of hapten

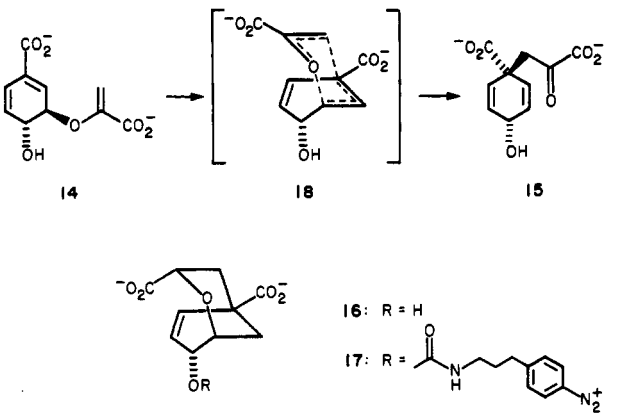

Figure 6. Claisen rearrangement of chorismate to prephenate.

structure. The lone pairs of electrons of both the phosphonamidate P–NH and phosphonate P–O bonds can act as hydrogen-bond acceptors whereas only the phosphonamidate P–NH bond can act as a hydrogen-bond donor. It is not clear, however, how this difference in hydrogen-bonding pattern would affect the nature of the combining site. 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.

A Claisen Rearrangement. It should also be possible to generate antibodies that increase reaction rates by reducing entropic barriers to reactions. The binding energy in this case acts to reduce translational and rotational motions for reaction by properly orienting the reactants in the antibody combining site. This approach should be applicable to intramolecular reactions such as macrocyclic lactonization reactions or intermolecular reactions such as peptide-bond formation or Diels–Alder reactions.

We have used these ideas to guide us in the generation of antibodies that carry out the formal Claisen rearrangement of chorismic acid (**14**) to prephenic acid (**15**) (Figure 6).[25] This thermal 3,3-sigmatropic rearrangement occurs through an asymmetric chair-like transition state (**18**) in which the carbon–oxygen bond is substantially broken while carbon–carbon bond formation has not occurred to any appreciable extent.[26] The activation entropy and enthalpy of reaction have been determined to be −12.85 eu and 20.7 kcal/mol, respectively.[26] The unimolecular rearrangement is catalyzed approximately 10^6-fold by the enzyme chorismate mutase at the branch point in the biosynthesis of aromatic amino acids in bacteria and plants.[27] Although the enzymatic reaction has also been demonstrated to proceed through a chair-like transition state, the mechanism by which chorismate mutase accelerates the rearrangement remains poorly understood.[28]

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One might expect that an antibody combining site, complementary to the conformationally restricted transition-state configuration, but presumably lacking catalytic side chains, could accelerate the Claisen rearrangement of chorismate to prephenate. Monoclonal antibodies were elicited to the endo bicyclic transition-state analogue **16**,[25] the most potent known inhibitor of chorismate mutase with a K_i of 0.15 μM[29] (Figure 6). One of eight antibodies (IgG) generated to the KLH conjugate of **17** was found to catalyze the Claisen rearrangement, with initial rates consistent with the Michaelis–Menten rate expression 1 (k_{cat} = 2.7 min^{-1}, K_m = 260 μM). The rate of the antibody-catalyzed reaction can be directly compared with that of the uncatalyzed thermal rearrangement affording a value of k_{cat}/k_{un} of 1×10^4 at 10 °C, pH 7.0. This factor can be compared with the (3×10^6)-fold acceleration induced by chorismate mutase from *E. coli* under the same conditions. Of the various mechanisms put forth for the enzymatic rearrangement, most can be ruled out for the antibody-catalyzed process. For example, the fact that the (±)-methyl ether of chorismate is converted to the (±) ether of prephenate argues against mechanisms involving loss of the 4-hydroxyl group (such as formation of an oxirinium ion or C-4 cation). Moreover, the observation of a D_2O solvent isotope effect of 1 on the antibody-catalyzed reaction excludes general acid or base catalysis in the rate-limiting step. One is left with the appealing mechanistic alternative that the antibody catalyzes the reaction by providing an environment complementary to the conformationally restricted transition state. Consistent with this notion is the fact that the $\Delta S^{\ddagger}$ for the antibody-catalyzed reaction is −1.2 eu.

A second antibody has been independently generated against an ester linked analogue of **16** which also catalyzed the rearrangement of chorismate to prephenate, but at a 100-fold-lower rate.[30] In this case the ratio of K_i/K_m corresponds roughly with the observed rate enhancement, consistent with preferential binding of the transition-state analogue by the antibody. The stabilization was shown to be largely enthalpic in nature. In addition, catalysis was shown to be highly enantioselective; only the (−) isomer of chorismate was a substrate for the antibody.[30]

Transacylation Reactions. The constraints imposed by the antibody binding pocket should also accelerate an intramolecular cyclization by reducing rotational entropy.[31] Benkovic and co-workers have demonstrated that an antibody elicited to transition-state analogue **19**, which is representative of a six membered ring lactonization reaction, acts as a catalyst for the cyclization of the corresponding substrate **20** (Figure 7).[32] The rate of the antibody-catalyzed reaction relative to the uncatalyzed reaction was 167.

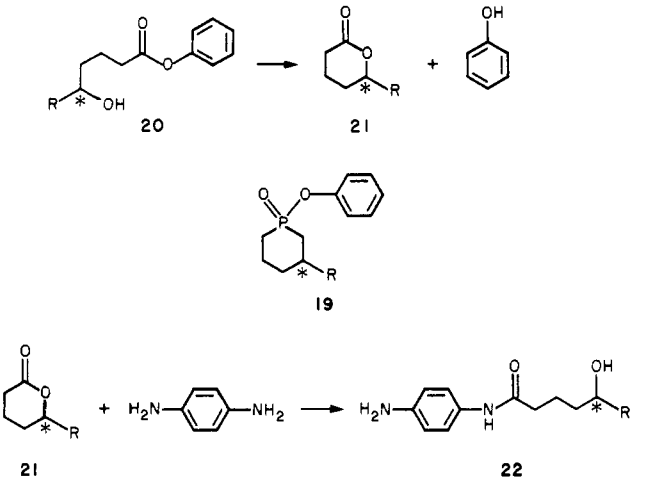

Figure 7. Antibody-catalyzed lactonization reaction.

Again, the antibody-catalyzed reaction was competitively inhibited by the corresponding cyclic phosphonate. The antibody cyclization reaction was stereoselective, permitting isolation of the lactone in an enantiomeric excess of 94%.[32] Antibodies specific for **19** also catalyzed the stereospecific formation of amide **22** from racemic lactone **21** and phenylenediamine.[33] Antibody bound phenylenediamine and lactone **21** with K_m's of 1.2 mM and 4.9 mM, respectively, and the transition-state analogue **19** with a K_i of 75 nM. The acceleration of the antibody-catalyzed reaction relative to the uncatalyzed reaction was 16 M which compares with a K_mK_m/K_i value of 155 M. A second phosphonamidate-specific antibody was also shown to catalyze bimolecular amide bond formation with an effective molarity of 10.5 M.[34] These values are considerably below the value of 10^8 M, which is thought to represent an approximate upper limit for an antibody-catalyzed reaction compared to its bimolecular counterpart.[35] Nonetheless, the demonstration that an antibody can catalyze bimolecular reactions offers the possibility of antibody-catalyzed peptide-bond formation, Diels–Alder reactions, macrolide synthesis, or transglycosylation reactions.

Introduction of Catalytic Groups into Antibody Combining Sites

Semisynthetic Antibodies. One strategy whereby catalytic groups can be introduced into antibody combining sites involves the selective chemical modification of antibodies with natural or synthetic catalysts such as transition-metal complexes, cofactors, bases, or nucleophiles. Kaiser and co-workers demonstrated that enzymes can be selectively modified with cofactors to afford semisynthetic enzymes with new properties.[36] This approach has recently been extended to antibodies, making it possible to combine the exquisite binding specificity of the immune system with the efficient diverse catalysts available from synthetic chemistry.[37,38]

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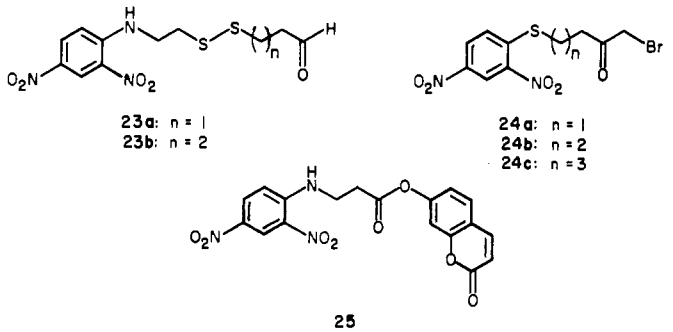

Figure 8. Affinity labels and ester substrate.

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. One such derivatizable group is a free thiol, which, by virtue of its high nucleophilicity and ease of oxidation, can be selectively modified via disulfide exchange or electrophilic reactions. Moreover, introduction of a nucleophilic thiol into an antibody combining site might directly afford a catalytic antibody. We have used cleavable affinity labels to modify selectively the antibody combining site of the IgA MOPC315 with a nucleophilic thiol.[37] Importantly, 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.

MOPC315, which binds substituted 2,4-dinitrophenyl (DNP) ligands with association constants ranging from 5×10^4 to 10^6 M^{-1},[39,40] has been characterized by spectroscopic methods (UV, fluorometry, NMR), chemical modification, and amino acid sequencing of the variable region. Moreover, earlier affinity-labeling studies with reagents of varying structures[41] defined a number of reactive amino acid side chains in the vicinity of the combining site. Cleavable affinity-labeling reagents were synthesized that contain the DNP group linked to electrophilic aldehyde or α-bromo ketone groups via cleavable disulfide or thiophenyl linkages (Figure 8). The geometry of the affinity labels varied with regard to the distance between the DNP group and electrophilic moiety, since the position of a nucleophilic combining site Lys, His, or Tyr side chain was not precisely known. Covalent attachment of the label to the antibody, followed by cleavage of the cross-link and removal of the free ligand, results in site-specific incorporation of a free thiol into the antibody combining site (Figure 9). It was determined that antibody affinity labeled with the aldehyde labeling reagent **23b** in the presence of $NaCNBH_3$ provides, after cleavage of the disulfide linkage, homogeneous antibody containing a free thiol attached to Lys 52H[37] (antibody

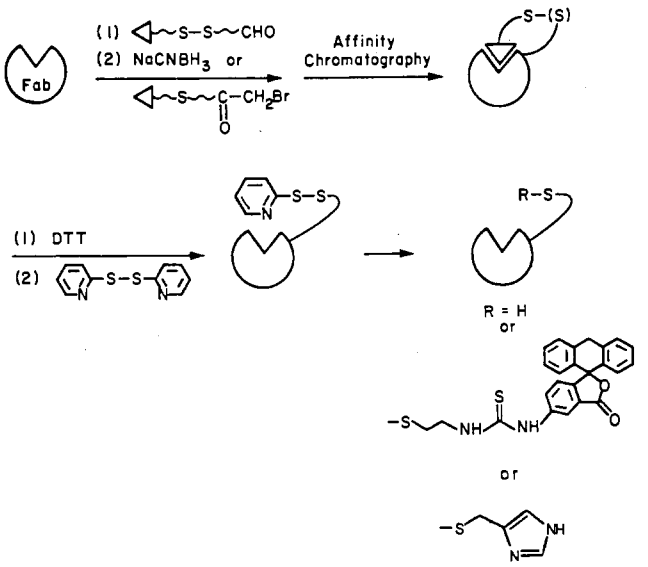

Figure 9. Introduction of thiol "handle" into the antibody combining site of MOPC315 by cleavable affinity label.

derivatized with bromo ketone **24b** was selectively modified at Tyr 34L). The binding affinity of *N*-DNP-glycine to the Lys 52H thiol-modified antibody was similar to that of native MOPC315. The thiol itself is appropriately positioned to act as a nucleophile in the thiolysis of coumarin ester **25**.[37] The semisynthetic antibody demonstrated saturation kinetics and was competitively inhibited by *N*-DNP-glycine with a K_i of 8 μM. The rate acceleration by the thiol-containing antibody relative to that of dithiothreitol was 6×10^4 for ester **25**. This value is quite similar to that found in the antibody-catalyzed β-elimination reaction (Figure 2) and suggests the proximity of a base or nucleophile to a complexed substrate can lower $\Delta G^{\ddagger}$ for reaction by up to 7.0 kcal/mol.

Importantly, the thiol also acted as a handle to introduce other synthetic chemical functionalities into the antibody combining site.[37,38] Imidazole was incorporated in greater than 90% yield by treatment of the thiopyridyl disulfide adduct of the antibody with 4-(mercaptomethyl)imidazole[38] (Figure 9). The resulting semisynthetic antibody catalyzed the hydrolysis of coumarin ester substrate **25** with a rate acceleration of 10^3 above the rate of catalysis by 4-methylimidazole.[37,38] Presumably, imidazole is acting as a general base or nucleophile in the hydrolysis of **25**. The slower rate with this antibody is likely due to the increased flexibility of the imidazole side chain linkage. The semisynthetic antibody showed specificity for the 3-aminopropionate versus the glycinate or 4-aminobutyrate *N*-DNP esters.

The thiol-containing antibody could also be modified with the fluorophore 1-(3-fluoresceinylthioureido)-2-ethanethiol[37] (Figure 9). Addition of the ligand *N*-DNP-glycine to the fluorescein–Fab adduct resulted in a decrease in fluorescence, providing a direct assay of ligand binding. The binding constant for *N*-DNP-glycine obtained from quenching of fluorescein fluorescence was identical within experimental error with that reported in the literature for unmodified MOPC315.[40] Semisynthetic antibodies of this sort may prove useful as sensors and in diagnostics. Derivatization of antibodies with other groups (Zn^{2+}–ligand or

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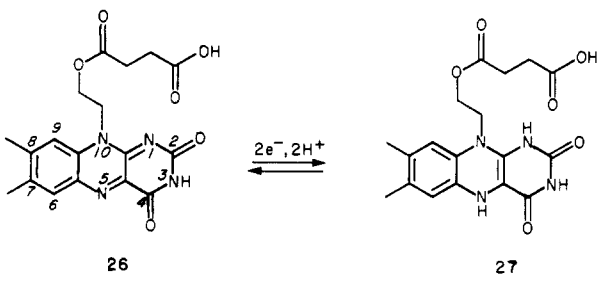

26 27

Figure 10.

Co^{3+}–ligand complexes, flavins, pyridoxamine, etc.) should enable us to generate semisynthetic antibodies with a variety of novel catalytic activities. Therapeutic agents can also be incorporated proximate to the antibody combining site via this strategy.

Site-Directed Mutagenesis. An alternative method for introducing a unique thiol in or proximate to the antibody combining site involves site-specific mutagenesis. In this case, the three-dimensional X-ray structure is required to determine the modification site. This approach is currently being applied to the phosphorylcholine-specific antibody S107 and the dinitrophenyl-specific antibody MOPC315.[42] Moreover, mutagenesis has also been used to introduce a catalytic His side chain into the combining site of MOPC315.[42] Genetic selection or screening might also be used to generate a catalytic residue in the combining site of an antibody already specific for the substrate (or transition-state analogue) of interest.

Cofactor Binding Sites. Another strategy whereby catalytic groups can be introduced into antibody combining sites involves generating, in addition to the substrate binding site, a cofactor (either natural or synthetic) binding site.[43] To this end, antibodies have been generated to oxidized flavin **26** (Figure 10). Because oxidized flavin **26** and the two electron reduced 1,5-dihydroflavin **27** have substantially different electronic and conformational properties,[44] antibodies generated specifically to the oxidized flavin preferentially bind **26** with 4×10^4 higher affinity than 1,5-dihydroflavin **27**. This differential stabilization of the oxidized and two electron reduced flavin by the antibody combining site makes the Ig–flavin complex a substantially stronger reducing agent than free flavin in solution; the reduction potential of flavin **26** bound to antibody is −342 mV, representing a lowering of the reduction potential (E_m(**26**) = −206 mV) by approximately 5 kcal/mol. Because the antibody–flavin complex is a stronger reducing agent than free flavin, the substrate safranine T (E_m = −289 mV) could be rapidly reduced by the antibody–**27** complex, but not by **27** itself. The antibody–flavin complex is able to mediate redox processes not thermodynamically accessible to free flavin. Incorporation of a substrate binding site into these antibodies may lead to catalytic antibodies that can carry out stereocontrolled chemical reductions. It may also be possible to generate antibodies with binding sites for other cofactors such as transition-metal complexes or natural cofactors. Stollar and co-workers have attempted to generate antibodies with pyridoxamine binding sites that catalyze Schiff base formation and transamination.[45] Their lack of success may have been due to the fact that polyclonal antibodies were used.

Conclusion

In the short period of time that has elapsed since the first reports of antibody catalysis in 1986,[18,19] a considerable number of different reactions have been catalyzed by using antibodies. The specificities of catalytic antibodies are high, and rate enhancements approaching those of enzymes have been demonstrated. A number of general strategies have also evolved for generating catalytic antibodies. The development of antibodies with 10^8-fold or higher rate enhancements will require careful mechanistic analysis of those factors affecting antibody catalysis and the generation of antibodies which combine several of these features. This process not only will provide new insight into the nature of molecular recognition and catalysis but also may afford tailor-made catalysts for applications in chemistry, biology, and medicine.

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