3. Wash the wells four times with a 0.85% sodium chloride solution.
4. Incubate with 100 μl urease substrate solution for 5 min at room temperature. Read absorbance at 590 nm.

Results of such an experiment are shown in Fig. 4. This assay detected 25 mIU/ml hCG using bsMAb-containing ascitic fluid or protein A-purified bsMAb. This level of detection is equivalent to the traditional MAb–urease assay, even though monospecific parental antibodies are still present. Using HPLC-purified bsMAb (which lacks the parental anti-hCG antibody), the level of detection is slightly enhanced to 15 mIU/ml.

[17] Catalytic Antibodies

By KEVAN M. SHOKAT and PETER G. SCHULTZ

Introduction

The specificity and affinity of antibody molecules have made them important tools in biology and medicine and as diagnostics. Our efforts have focused on exploiting the vast repertoire of unique antibody specificites for the immune system to generate novel catalysts. To date antibodies have been demonstrated to catalyze a wide variety of reactions, which have recently been reviewed (Table I).[1-4] Several general strategies for the production of antibody catalysts have emerged since the first reports of catalytic antibodies in 1986, and these are reviewed here.

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 (1) generation of precisely positioned catalytic amino acid side chains in the combining site, (2) stabilization of high-energy transition states in reactions (3) reduction in the entropies of reactions by orienting reaction partners in reactive conformations, and (4) 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

[1] P. G. Schultz, *Angew. Chem., Int. Ed. Engl.* **28**, 1283 (1989).
[2] P. G. Schultz, *Acct. Chem. Res.* **22** 287 (1989).
[3] K. M. Shokat and P. G. Schultz, *Annu. Rev. Immunol.* **8**, 355 (1990).
[4] P. G. Schultz, R. A. Lerner, and S. J. Benkovic, *Chem. Eng. News* **68**, 26 (1990).

TABLE I
ANTIBODY-CATALYZED REACTIONS

Reaction	Ref.[a]
Ester, carbonate hydrolysis	*1–6*
Stereospecific lactonization	*7*
Amide bond formation	*7, 8*
Redox	*9*
Thymine dimer cleavage	*10*
Chorismate mutase	*11–13*
Stereospecific ester hydrolysis	*14, 15*
β-Elimination	*16*
Diels–Alder	*17, 18*
Porphyrin metallation	*19*
Porphyrin-mediated oxidations	*20*
Activated amide bond hydrolysis	*21*
Peptide bond hydrolysis	*22, 23*

[a] Key to references: (*1*) K. D. Janda, M. Weinhouse, D. M. Schloeder, R. A. Lerner, and S. J. Benkovic, *J. Am. Chem. Soc.* **112,** 1274 (1990); (*2*) A. Tramontano and D. Schloeder, this series, Vol. 178, p. 535; (*3*) K. M. Shokat, M. K. Ko, T. S. Scanlon, L. Kochersperger, S. Yonkovich, S. Thairivongs, and P. J. Schultz, *Angew. Chem. Int., Ed. Engl.* **29(11),** 1296 (1990); (*4*) C. N. Durfor, R. J. Bolin, R. J. Sugasawara, R. J. Massey, J. W. Jacobs, and P. G. Schultz, *J. Am. Chem. Soc.* **110,** 8713 (1988); (*5*) E. Baldwin and P. G. Schultz, *Science* **245,** 1104 (1989); (*6*) D. Y. Jackson, J. R. Prudent, E. P. Baldwin, and P. G. Schultz, *Proc. Natl. Acad. Sci. U.S.A.* in press (1990); (*7*) S. J. Benkovic, A. D. Napper, and K. A. Lerner, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 5355 (1988); (*8*) K. D. Janda, R. A. Lerner, and A. Tramontano, *J. Am. Chem. Soc.* **110,** 4835 (1988); (*9*) K. M. Shokat, C. J. Leumann, R. Sugasawara, and P. G. Schultz, *Angew. Chem. Int. Ed. Engl.* **27,** 1172 (1988); (*10*) A. G. Cochran, K. Sugasawara, and P. G. Schultz, *J. Am. Chem. Soc.* **110,** 7888 (1988); (*11*) D. Y. Jackson *et al., J. Am. Chem. Soc.* **110,** 4841 (1988); (*12*) D. Hilvert, S. H. Carpenter, K. D. Nared, M.-T. M. Auditor, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 4953 (1988); (*13*) D. Hilvert and K. D. Nared, *J. Am. Chem. Soc.* **110,** 5593 (1988); (*14*) K. D. Janda, S. J. Benkovic, and R. A. Lerner, *Science* **244,** 437 (1989); (*15*) S. J. Pollack, P. Hsuin, P. G. Schultz, *J. Am. Chem. Soc.* **111,** 5961 (1989); (*16*) K. M. Shokat, C. J. Leumann, R. Sugasawara, and P. G. Schultz, *Nature (London)* **338,** 269 (1989); (*17*) D. Hilvert, K. W. Hill, K. D. Nared, and M.-T. M. Auditor, *J. Am. Chem. Soc.* **111,** 9261 (1990); (*18*) A. Braisted and P. G. Schultz, *J. Am. Chem. Soc.* **112,** 7430 (1990); (*19*) A. G. Cochran and P. G. Schultz, *Science* **249,** 781; (*20*) A. Cochran and P. G. Schultz, *J. Am. Chem. Soc.* **112,** 9414 (1990); (*21*) K. D. Janda, D. Schloeder, S. J. Benkovic, and R. A. Lerner, *Science* **241,** 1188 (1988); (*22*) B. L. Iverson and R. A. Lerner, *Science* **243,** 1184 (1989); (*23*) P. Sudhir, S. Paul, D. J. Volle, C. M. Beach, D. R. Johnson, M. J. Powell, and R. J. Massey, *Science* **244,** 1158 (1989).

generating catalytic antibodies is essential in order to exploit fully the diversity of the humoral immune system. For example, once a general approach to the hydrolysis of peptides by an antibody has been developed, a large number of sequence-specific peptides should become available simply by altering hapten structure accordingly. Therein lies the enormous advantage of using antibodies as catalysts for chemical transformations.

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

Strategies

The strategies used to generate catalytic antibodies are based largely on principles of enzymatic catalysis including transition state stabilization, general acid–base catalysis, nucleophilic and electrophilic catalysis, strain, and proximity effects. To date, antibody-catalyzed rate enhancements over background rates range from 10^2 to 10^6. The generation of antibodies with rate accelerations of 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 which 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. Each strategy is discussed in the context of specific reactions which have been catalyzed to date.

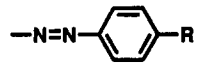
Eliciting Catalytic Groups

The introduction of a general acid or base into an antibody combining site should be an effective method of catalyzing a variety of chemical reactions using antibodies. 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 ($\Delta S^{\ddagger}$) and enthalpy ($\Delta H^{\ddagger}$) of activation for reaction.[5] Model systems with effective molarities greater than 10^7 M have been realized, and theoretical arguments suggest

[5] W. P. Jencks, "Catalysis in Chemistry and Enzymology." McGraw-Hill, New York, 1969.

accelerations up to 10^8 *M* can be achieved by approximation of reactive groups in enzyme active sites.[6]

Experiments by Pressman and Siegel in 1953 suggested a strategy whereby the electrostatic complementarity between haptens and antibodies can be used to introduce a catalytic carboxylate in an antibody combining site.[7] Negatively charged aspartate or glutamate residues were found in the combining sites of antibodies raised toward *p*-azobenzenetrimethylammonium cation. **(1).**[8] Conversely, positively charged arginine and lysine residues were identified in the combining sites of antibodies elicited against negatively charged *p*-azobenzoate **(2).**[9] Besides electrostatic forces, hydro-

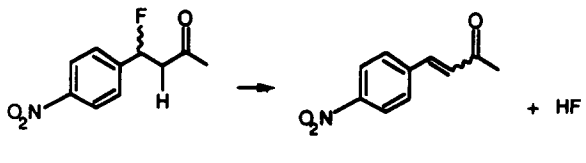


1: R= $-\overset{+}{N}(Me)_3$
2: R= $-CO_2^-$

phobic and hydrogen bonding interactions can be used to induce certain amino acids in antibody combining sites. A tryptophan in MOPC 315 has been shown to π stack with the aryl ring of 2,4-dinitrophenyl-containing ligands.[10]

β-Elimination. Isomerizations, eliminations, and many condensation reactions involve proton abstraction from carbon centers.[11-13] Such reactions are of great importance in many biological transformations. In general, enzymes which catalyze such reactions use carboxylate groups and imidazoles as the catalytic bases to deprotonate the substrate.[14] In order to generate an antibody capable of catalyzing a reaction of this type an Asp, Glu, or His residue needed to be positioned in the correct orientation to the bound substrate. Hapten **3** was used as an immunogen in order to generate an antibody that catalyzed the elimination of HF from β-fluoroketone **(4).**[15] The position of the ammonium group corresponds to the position of

[6] M. J. Page and W. P. Jencks, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1678 (1971).
[7] D. Pressman and J. Siegel, *J. Am. Chem. Soc.* **75**, 686 (1953).
[8] A. L. Grossberg and D. Pressman, *J. Am. Chem. Soc.* **82**, 5478 (1960).
[9] M. H. Freeman, A. L. Grossberg, and D. Pressman, *Biochemistry* **7**, 1941 (1968).
[10] S. K. Dower and R. A. Dwek, *Biochemistry* **18**, 36687 (1979).
[11] K. J. Shray, E. L. O'Connell, and I. A. Rose, *J. Biol. Chem.* **248**, 2214 (1973).
[12] D. W. Banner, A. C. Bloomer, G. A. Petsko, D. C. Phillips, C. I. Pogson, I. A. Wilson, P. H. Corran, A. J. Furth, J. D. Milman, R. E. Offord, J. D. Priddle, and S. G. Waley, *Nature (London)* **255**, 609 (1975).
[13] H. M. Miziorko and M. D. Lane, *J. Biol. Chem.* **252**, 1414 (1977).
[14] S. M. Parsons and M. A. Rafferty, *Biochemistry* **11**, 1623 (1972).
[15] K. M. Shokat, C. J. Leumann, R. Sugasawara, and P. G. Schultz, *Nature (London)* **338**, 269 (1989).



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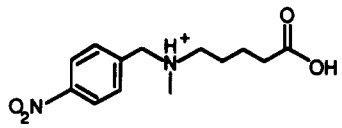

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the abstractable proton in substrate **4** and should elicit a complementary catalytic carboxylate within bonding distance. The *p*-nitrophenyl ring was included to serve as a common recognition element between hapten and substrate. Moreover, replacement 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.

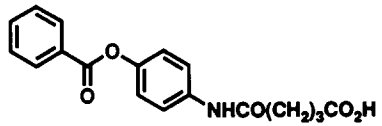
Of six antibodies tested which bound **3**, four accelerated the conversion of **4** to **5**, and were able to be inhibited by hapten. Hapten inhibition demonstrates that catalysis occurs in the binding site of the antibody. One of the four antibodies, 43D4-3D12, was further characterized. The kinetics of the 43D4-3D12-catalyzed reaction obeyed the Michaelis–Menten rate expression. 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 $((k_{cat}/K_m)/k_{uncat})$ 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 10^4 attributable to the bases Glu-43 in staphylococcal nuclease[16] and Asp-102 in trypsin.[17] The antibody discriminated between the *p*-nitro substrate **4** and the *m*-nitro analog by a factor of 10 in overall rate (k_{cat}/K_m).

Chemical modification studies demonstrated that a carboxylate group was indeed responsible for catalysis. Selective chemical modification with the carboxylate-specific reagent diazoacetamide showed almost complete inactivation in the absence of inhibitor. When inhibitor was present activity was retained owing to protection of the carboxylate group responsible

[16] E. H. Serpersu, D. Shortle, and A. S. Mildvan, *Biochemistry* **26**, 1289 (1987).
[17] C. S. Craik, S. Roczniak, C. Largman, and W. J. Rutter, *Science* **237**, 909 (1987).

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 values between 2.0 and 5.5.[18] However, in the hydrophobic cavities of enzymes they become stronger bases with p*K*a values in the range of 6.5 to 8.2.[18] Further mechanistic studies have shown there to be a kinetic isotope effect ($k_{cat,H}/k_{cat,D}$) for the antibody of 2.4.[19] This value can be compared to the kinetic isotope effect for the background reaction catalyzed by acetate ion, $k_H/k_D = 3.7$. The isotope effect argues against a mechanism involving rate-limiting S_N2 displacement of fluoride followed by elimination.

Charge–charge complementarity has also been applied by Janda *et al.* to design an esterolytic catalytic antibody.[20] These workers targeted the hydrolysis of the mildly activated substituted phenyl ester **6**. Hapten **7** catalyzed the desired reaction. Interestingly, the pH dependence of the catalyzed reaction indicates the catalysis is due to the presence of the basic form of a residue with $pK_a = 6.23$. This is identical to the pK_a dependence found in the β-elimination catalytic antibody.



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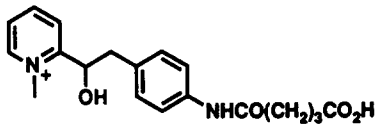

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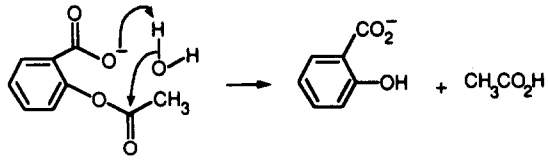


(1)

dependence found in the β-elimination catalytic antibody.

The high success rate of the charge–charge complementary design in generating antibodies with catalytic activity [65% (4/6) and 30% (7/23)] suggests that this approach should be broadly applicable to other reactions

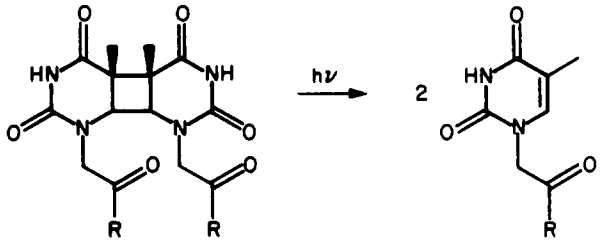
[18] A. Fersht, "Enzyme Structure and Mechanism." Freeman, New York, 1985.

[19] K. M. Shokat and P. G. Schultz, unpublished results (1989).

[20] K. D. Janda, M. Weinhouse, D. M. Schloeder, R. A. Lerner, and S. J. Benkovic, *J. Am. Chem. Soc.* **112**, 1274 (1990).

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.[21] 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.[22-26] In order to generate a tryptophan residue in close proximity to the bound thymine dimer substrate antibodies were elicited toward the planar thymine dimer derivative **8**.[27] The extended π system of **8** should elicit a complementary π stacking aromatic amino acid (such as tryptophan) in the antibody combining site as was the case with MOPC 315. An appropriately positioned tryptophan should efficiently sensitize the cycloreversion reaction of **9** to thymine. In fact, five of six antibodies generated to hapten **8** showed light-dependent acceleration of the conversion of **8** to



8: R = $NHCH_2COOH$
9: R = OH

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

[21] B. M. Sutherland, *Enzymes* **14**, 909 (1981).
[22] C. Helene and M. Charlier, *Photochem. Photobiol.* **25**, 429 (1977).
[23] J. R. Van Camp, T. Young, R. F. Hartman, and S. D. Rose, *Photochem. Photobiol.* **45**, 365 (1987).
[24] H. D. Roth and A. A. Lamola, *J. Am. Chem. Soc.* **94**, 1013 (1972).
[25] S. E. Rokita and C. T. Walsh, *J. Am. Chem. Soc.* **106**, 4589 (1984).

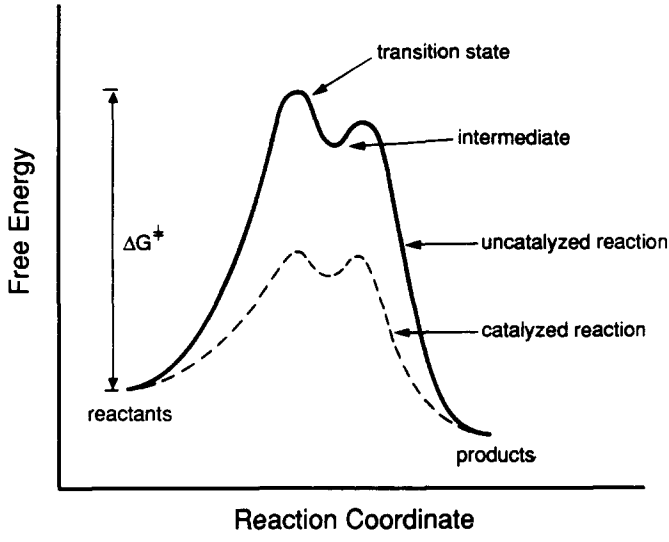


FIG. 1. Transition state stabilization.

over even at relatively high concentrations, consistent with the high specificity of antibodies. Antibodies not specific for **8** 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 is quite close to the value for *Escherichia coli* DNA photolyase, 3.4 min^{-1}.[28]

The high success rate in using $\pi-\pi$ 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 microenvionments could demonstrate unusual solvation, dielectric, or hydrophobic properties for studies of molecular recognition and other structure–function relationships.

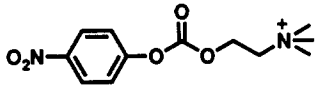
Transition State Stabilization

The first successful approach used in the design of catalytic antibodies was that of transition state stabilization (Fig. 1), a proposal first made by Linus Pauling in 1948 to explain enzymatic catalysis.[29] Design of an antibody which takes advantage of transition state stabilization to acceler-

[26] M. S. Jorns, E. T. Baldwin, G. B. Sancar, and A. Sancar, *J. Biol. Chem.* **262**, 486 (1987).
[27] A. G. Cochran, R. Sugasawara, and P. G. Schultz, *J. Am. Chem. Soc.* **110**, 7888 (1988).
[28] M. S. Jorns, G. B. Sancar, and A. Sancar, *Biochemistry* **24**, 1856 (1985).
[29] L. Pauling, *Am. Sci.* **36**, 51 (1948).

ate a reaction requires an immunogen which 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 and allow them to diffuse rapidly from the combining site.

Ester, Carbonate, and Amide Bond Hydrolysis.. The first antibodies characterized as having catalytic properties accelerated the hydrolysis rate of esters and carbonates **10–12.** These examples have been extensively



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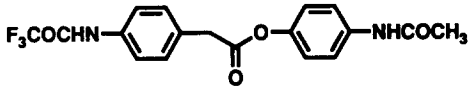

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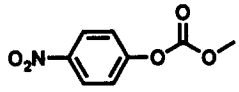

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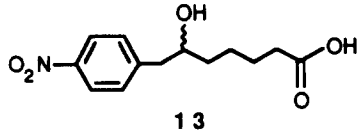
reviewed in a previous volume of this series.[30] In each of these cases negatively charged phosphonates or phosphates served as analogs of the tetrahedral negatively charged transition states. In addition to this well-known class of charged peptidase inhibitors, neutral inhibitors containing an hydroxyl group as an analog of the tetrahedral transition state are also potent protease inhibitors.[31] For example, pepstatin has a K_I of 50 n*M* for pepsin even though it lacks the negative charge which distinguishes the transition state from the products and substrate.[32]



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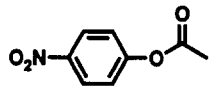
Antibodies elicited against alcohol **13** have been shown to catalyze the hydrolysis of **12** and **14**.[33] One out of four antibodies specific for **13** were catalytic with kinetic constants, $k_{cat,14}$ 0.72 min−1; $K_{m,14}$ 3.65 m*M*;K_I 140

[30] A. Tramoantano and D. Schloeder, this series, Vol. 178, p. 535.
[31] W. M. Kati, D. T. Pals, and S. Thaisrivongs, *Biochemistry* **26**, 7621 (1987).
[32] M. W. Holladay, F. G. Salituro, and D. H. Rich, *J. Med. Chem.* **30**, 374 (1987).
[33] K. M. Shokat, M. K. Ko, T. S. Scanlon, L. Kochersperger, S. Yonkovich, S. Thairivongs, and P. G. Schultz, *Angew. Chem., Int. Ed. Engl.* **29(11)**, 1296 (1990).



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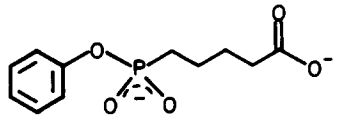
μM. The ratio K_m/K_I of 26 is several orders of magnitude lower than the rate acceleration achieved by the antibody, k_{cat}/k_{uncat} 2250. The disparity between the differential stabilization of the transition state relative to the substrate compared to the rate acceleration suggests that another factor such as acid–base catalysis is contributing to the catalysis observed. Chemical modification and affinity labeling experiments may suggest candidate residues in the combining site responsible for the additional catalytic activity.

Antibodies have been shown to catalyze the hydrolysis of water-insoluble substrates.[34] Antibodies specific for phenyl phosphonate **15** were shown to accelerate the hydrolysis of **16** in reverse miscelles. Reverse miscelles are



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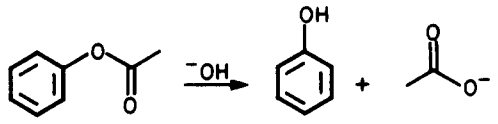

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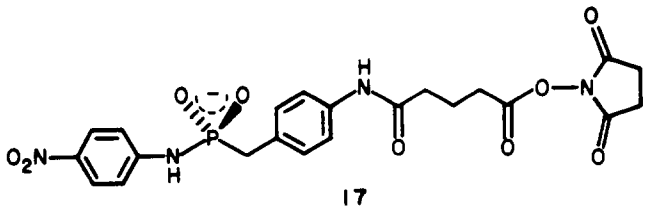
formed when water–sufactant mixtures are dissolved in water-immiscible solvents. The k_{cat} and K_m values of the antibody solubilized in isooctane in reverse miscelles were 3.89 min^{-1} and 569 μM, respectively. The optimal ratio of water to detergent (W_o) for antibody catalysis is significantly larger than values found for enzymes, consistent with the increased molecular weight of immunoglobulin G (IgG) molecules. The expansion of antibody catalysis to water-insoluble substrates in reverse miscelles or organic solvents should greatly expand the scope of antibody catalysis.

An antibody, NPN43C9, raised against aryl phosphonamidate **17** has been demonstrated to catalyze the hydrolysis of the activated amide bond in substrate **18**.[35] Hapten **17** is again an analog of the tetrahedral transition

[34] C. N. Durfor, R. J. Bolin, R. J. Sugasawara, R. J. Massey, J. W. Jacobs, and P. G. Schultz, *J. Am. Chem. Soc.* **110**, 8713 (1988).

[35] K. D. Janda, D. Schloeder, S. J. Benkovic, and R. A. Lerner, *Science* **241**, 1188 (1988).



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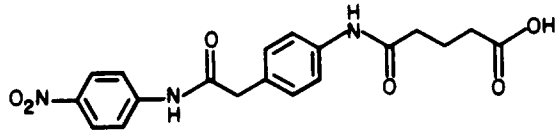


18

state in the hydrolysis of **18**. A rate acceleration by NPN43C9 over the background rate of 2.5×10^5 was reported. This rate acceleration is inconsistent with a mechanism which solely involves transition state stabilization since the difference in affinity for hapten **17** and substrate **18** is only $\Delta\Delta G^{\ddagger}$ − 2.2 kcal/mol/. The 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 m*M* NaCl completely inhibited antibody catalysis. The structural feature of this hapten responsible for induction of such acid–base catalysis is not apparent since 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.

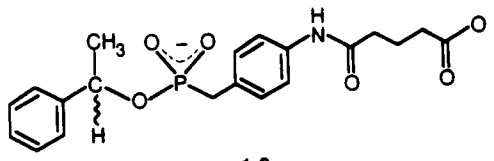
The strategy of transition state stabilization should be applicable to the hydrolysis of other biopolymers such as carbohydrates and polynucleotides. Lysozyme[36] and RNase S[37] are classic examples of enzymes which function by transition state stabilization. The well-characterized transition state analog inhibitors of these enzymes, nojirimycin for lysozyme and uridine vandate complex for RNase S, if used as haptens may yield antibodies capable of catalyzing these important transformations.

Stereospecific Hydrolysis of Unactivated Esters. Antibodies elicited toward phosphonates **19**[38] and **20**[39] were shown to catalyze the hydrolysis

[36] T. I. Secemski and G. E. Liengard, *J. Biol. Chem.* **249**, 2932 (1974).

[37] F. M. Richards and H. W. Wycoff, "Bovine Pancreatic Ribonuclease." Academic Press, New York and London, 1970.

[38] K. D. Janda, S. J. Benkovic, and R. A. Lerner, *Science* **244**, 437 (1989).



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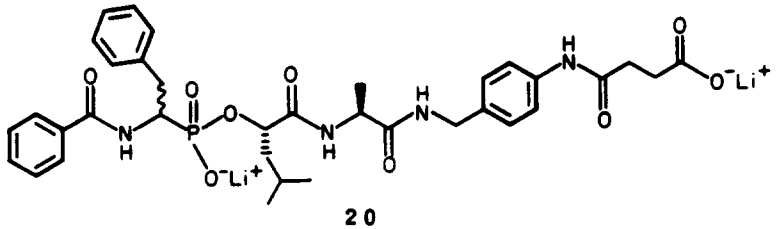

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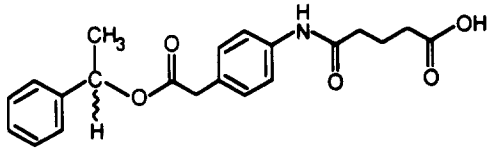

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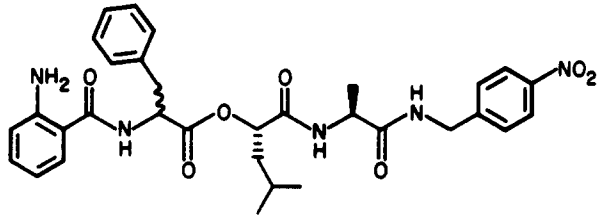


22

of **21** and **22**, respectively, in a stereospecific manner. In both cases antibodies were elicited against a 50/50 mixture of both stereoisomers. Of 18 antibodies specific for **19**, nine catalyzed the hydrolysis of (*R*)-**21** and two catalyzed the hydrolysis of (*S*)-**21**. Two antibodies, one of each specificity, were further characterized. The rate acceleration for the hydrolysis of (*R*)-**21** by antibody 2H6 was 80,000, whereas the (*S*)-**21** specific antibody 21H3 showed only a modest 1600-fold rate acceleration over background. The *R*/*S* selectivity is greater than 98%.

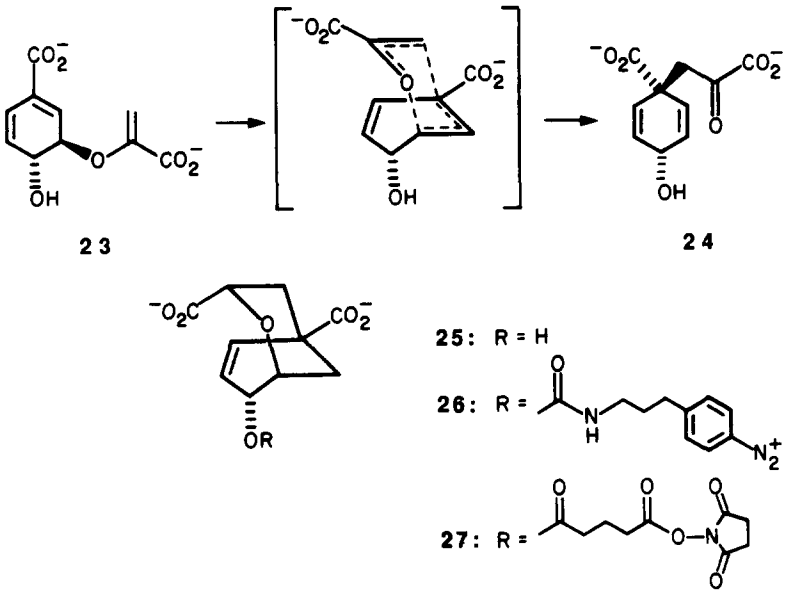
Of 25 antibodies isolated that were specific for the tripeptide phosphonate **20**, 18 were shown to accelerate the hydrolysis of the correspond-

[39] S. J. Pollack, P. Hsuin, and P. G. Schultz, *J. Am. Chem. Soc.* **111**, 5961 (1989).

ing ester substrates. Interestingly, an exclusive preference for antibodies of one specificity was observed. All 18 antibodies were selective for the D-phenlyalanine isomer of **22**. 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 to 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 in smaller haptens. Note that the hapten also contains analogs of fluorogenic and quenching groups at the amino and carboxy termini of substrate **22**, respectively. These groups allow hydrolysis of the substrate to be monitored by observing the increase in fluorescence which occurs when the fluorescent 2-aminobenzoyl group is separated from the quenching 4-nitrobenzylamide group in the reaction.[40] This sensitive assay may allow direct screening of enzyme-linked immunosorbent assay (ELISA) plates for catalytic activity or the screening of λ libraries of antibody F_{ab} fragments,[41] 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 Rearangement. The Claisen rearrangement of chorismate, **23**, to prephenate, **24**, formally involves the concerted cleavage of a carbon–oxygen bond and formation of a carbon–carbon bond. The enzyme which catalyzes this reaction, chrismate mutase, is at the branchpoint of aromatic amino acid biosynthesis in bacteria and plants. Bicyclic diacid **25** is the most potent transition state analog inhibitor of the enzyme known.[42] Two independent groups used **26**[43] and **27**[44,45] as haptens for generating antibody catalysts for the Claisen rearrangement of **23** to **24**. In one case one (11F1) out of eight antibodies specific for **26** was shown to catalyze the reaction with a k_{cat} of 2.7 min^{-1} and a K_m of 260 μM at 10°, pH7.0. This corresponds to a rate enhancement of 10^4 over the uncatalyzed rate and a decrease in $\Delta S^{\ddagger}$ from -12.9 e.u. for the uncatalyzed reaction to -1.2 e.u. for the 11F1 catalyzed reaction.[46] This factor compares with the 3×10^6-

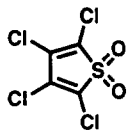
[40] N. Nishino and J. C. Powers, *J. Biol. Chem.* **255**, 3482 (1980).
[41] W. D. Huse *et al.*, *Science* **246**, 1275 (1989).
[42] P. A. Bartlett and C. R. Johnson, *J. Am. Chem. Soc.* **107**, 7792 (1965).
[43] D. Y. Jackson *et al.*, *J. Am. Chem. Soc.* **110**, 4841 (1988).
[44] D. Hilvert, S. H. Carpenter, K. D. Nared, and M.-T. M. Auditor, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4953 (1988).
[45] D. Hilvert and K. D. Nared, *J. Am. Chem. Soc.* **110**, 5593 (1988).
[46] D. Y. Jackson and P. G. Schultz, unpublished results (1989).



fold rate enhancement achieved by *E. coli* chorismate mutase assayed under the same conditions. Notice that 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 **27** as the immunogen, one (1F7) of 15 antibodies specific for **27** was catalytic, with a k_{cat} equal to 0.025 min^{-1} and a K_m of 22 μM at 13°, pH 7.5. Antibody 1F7 accelerates the reaction 190-fold over the uncatalyzed rate. Most of the rate acceleration is attributable to a decrease in $\Delta H^{\ddagger}$. Antibody 1F7 was also shown to be highly stereoselective, having a 90:1 preference for the (−) over the (+) isomer of chorismate. Complementation experiments underway in yeast and *E. coli* will provide formats to increase catalytic activity via genetic selection techniques.

Diels–Alder Reactions. Two groups have described the generation of antibodies capable of catalyzing Diels–Alder reactions. This reaction proceeds through a concerted transition state involving simultaneous formation and breakage of carbon–carbon bonds. It is one of the most important reactions in synthetic organic chemistry because it is a highly stereospecific route to cyclohexene derivatives. Hilvert and co-workers chose to catalyze the Diels–Alder reaction between tetrachlorothiophene 1,1-dioxide (**28**) and *N*-ethylmaleimide (**29**).[47] The hapten chosen was the bicyclic adduct

[47] D. Hilvert, K. W. Hill, K. D. Nared, and M.-T. M. Auditor, *J. Am. Chem. Soc.* **111**, 9261 (1990).



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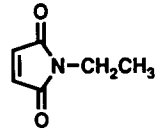

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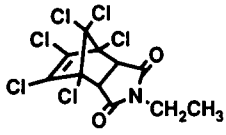

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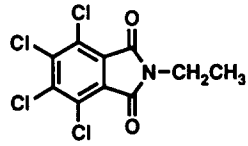

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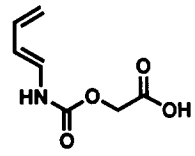
30. Tetrachlorothiophene 1,1-dioxide was used as the diene since extrusion of sulfur dioxide from the Diels–Alder adduct minimizes product inhibition. One of five antibodies specific for hapten **30** was shown to ctalyze the desired Diels–Alder reaction. Because of low solubility of the substrates the true k_{cat} value was not measurable.

An example of a more generalizable approach to catalyzing Diels–Alder reactions is the conversion of **32** and **33** to **34**.[48] This reaction does not depend on extrusion of sulfur dioxide. Hapten **35** contains an ethano



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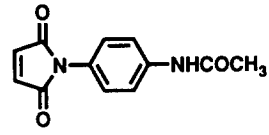

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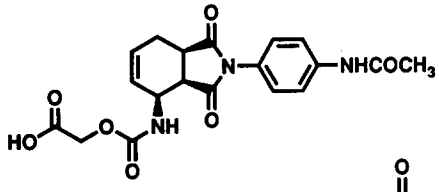

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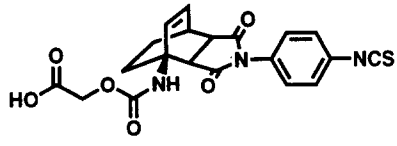


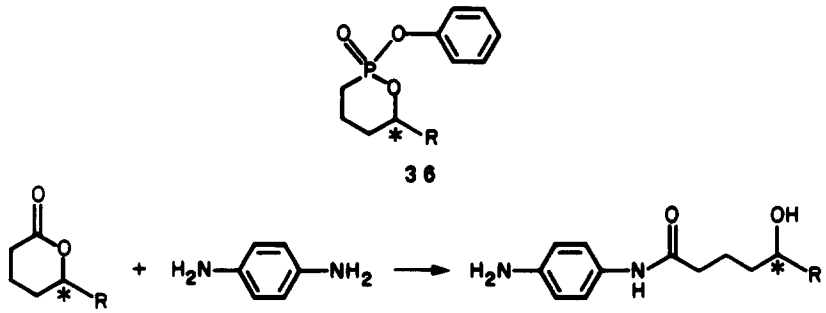
35

[48] A. Braisted and P. G. Schultz, *J. Am. Chem. Soc.* **112**, 7430 (1990).

bridge locking the cyclohexene ring into the proposed pericyclic transition state conformation. Furthermore, the high-energy boatlike conformation of the hapten should be distinct from the lowest energy conformer of the Diels–Alder product **34**, and hence product inhibition should be minimized. One of ten antibodies specific for **35** showed a large rate enhancement over the background rate: $k_{uncat,pH7.5}$ 1.9 M sec^{-1}, $k_{uncat,acetonitrile}$ 0.002 $M^{-1}sec^{-1}$. The catalytic rate constrants were measured to be k_{cat} 0.67 sec^{-1}, $K_{M,32}$ 1130 μM, and $K_{m,33}$ 740 μM. The K_D values for the hapten and product were determined to be 126 nM and 10 μM, respectively. The 100-fold difference in binding energy between the hapten and the substrates does lead to product inhibition after 5% reaction completion.

Combining strategies used to catalyze complex organic reactions such as Diels–Alder reactions with the ability to catalyze reactions in reverse miscelles provides intriguing possibilities for control of stereoselectivity.

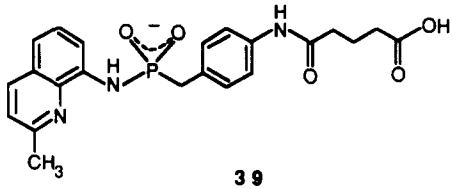
Acyl-Group Transfer Reactions.. The first examples of antibody-catalyzed bimolecular reactions involved acyl-transfer reactions. Antibodies specific for bisubstrate analog **36** were shown to catalyze the bimolecular amide bond formation reactions of **37** to **38** involving phenylenediamine.[49]



The antibody binds phenylenediamine and lactone **37** with K_m values of 1.2 and 4.9 mM, respectively. The inhibitor (**36**) was shown to be competitive for both reactions having 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 e.u. for the translational and rotational entropy of activation of a bimolecular reaction.[6]

[49] S. J. Benkovic, A. D. Napper, and R. A. Lerner, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5355 (1988).

Another antibody generated against phosphonamidate **39** was shown to catalyze a bimolecular amide formation reaction [Eq.(2)][50].



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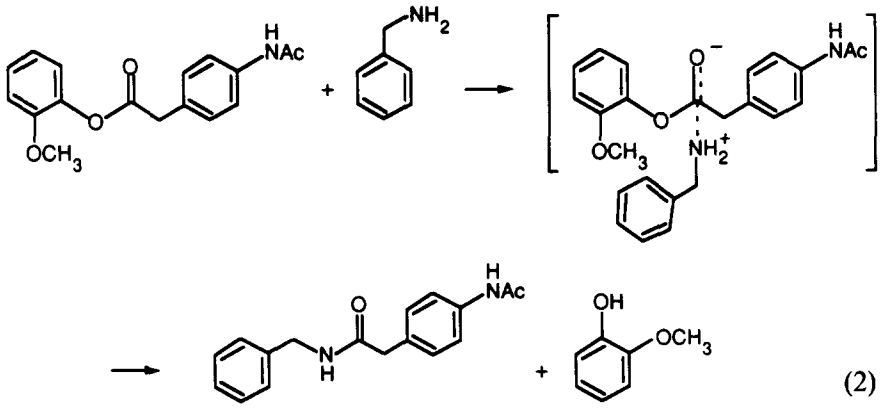


(2)

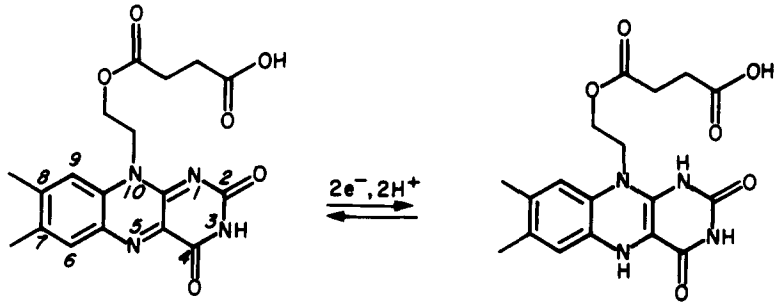
The antibody was shown to provide an effective molarity of 10.5 *M*. Here the hapten did not include the ester leaving group, an important element of the proposed transition state for the reaction. This design feature may explain why less than 2% (1/55) of antibodies specific for **39** were able to 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 peptide fragments (> 50 amino acids) 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 aldol condensation. An example of this class of reaction is the formation of fructose 1,6-diphosphate from dihydroxyacetone phosphate and glyceraldehyde 3-phosphate catalyzed by aconitase.

[50] K. D. Janda, R. A. Lerner, and A. Tramontano, *J. Am. Chem. Soc.* **110**, 4835 (1988).

Cofactor Chemistry

Many enzymes utilize non-amino acid cofactors to catalyze reactions. Important members of this class of enzymes include cytochrome P_{450}(Fe-heme), α-keto-acid dehydrogenases (thiamin pyrophosphate), D-amino-acid oxidase (flavin), and alanine racemase (pyridoxal phosphate). In order to expand the scope of antibody catalysis to redox reactions and 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 **40**.[51] The three rings



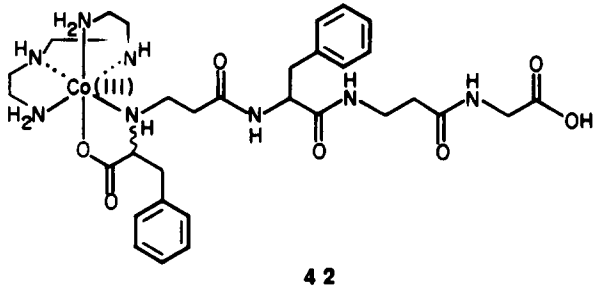
40 **41**

are coplanar in the oxidized flavin, **40**. The reduced dihydroflavin, **41**, has a substantially different electron distribution about the rings and is also conformationally distinct from the oxidized compound (**35**). The reduced form is butterfly shaped owing to a pucker about the two central nitrogen atoms in the central ring. Antibodies elicited toward oxidized **40** bind this form with a 4×10^4 higher affinity than **41**. 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, and 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

[51] K. M. Shokat, C. J. Leumann, R. Sugasawara, and P. G. Schultz, *Agnew. Chem. Int. Ed. Engl.* **27**, 1172 (1988).

adjacent to flavins, stereocontrolled chemical reductions could be carried out.

Antibodies have been generated against a peptide–cofactor complex (**42**) that are capable of catalyzing the hydrolysis of a Gly-Phe peptide



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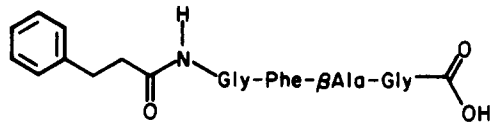

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bond.[52] Although a structurally inert Co(III) trien [*N*,*N*′-bis(2-aminoethyl)-1,2-ethanediamine] complex was used as the cofactor in the immunogen, the 14 antibodies elicited could accommodate any of 13 different trien metal complexes. In the presence of a variety of metal trien complexes two of the antibodies were shown to catalyze the cleavage of the Gly-Phe peptide bond in **43**. A turnover number of 6×10^{-4} sec^{-1} was measured. Somewhat surprisingly, the Gly-Phe bond hydrolyzed is one removed from the bond one might have expected to have been cleaved based on model chemistry by Buckingham and others.[53] 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.

A second example of antibody-catalyzed peptide hydrolysis has been reported. Two of six immune human IgG autoantibody preparations were shown to cleave ^{125}I labeled [Tyr^{10}] vasoactive intestinal peptide (VIP) between residues $Gln\text{-}^{16}$ and $Met\text{-}^{17}$.[54] The k_{cat} and K_m values reported were 15.6 min−1 and 37.9 n*M* respectively. Surprisingly the *background* rate for

[52] B. L. Iverson and R. A. Lerner, *Science* **243**, 1184 (1989).

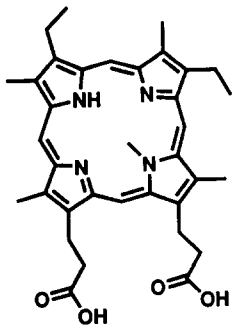
[53] D. A. Buckingham, D. M. Foster, and A. M. Sargeson, *J. Am. Chem. Soc.* **92**, 6151 (1970).

[54] P. Sudhir, S. Paul, D. J. Volle, C. M. Beach, D. R. Johnson, M. J. Powell, and R. J. Massey, *Science* **244**, 1158 (1989).

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 hrs. Radiolysis may contribute to the fast background rate and to the "antibody-catalyzed" reaction. The normal half-life ($t_{1/2}$) of peptide bonds is 7 years.[55] Clearly, it is essential that monoclonal antibodies with this activity be generated and carefully characterized.

One of the most interesting recent results in the field of catalytic antibodies has been catalysis of a porphyrin metallation reaction.[56] This system expands the scope of antibody-catalyzed reactions to ligand substitution reactions and is the first report of antibody binding of natural porphyrins. Two of three antibodies specific for the strained *N*-methymesoporphyrin (**44**) catalyze the metallation of mesoporphyrin IX (**45**). One of the antibodies catalyzes metallation with zinc(II) and copper(II). The kinetics of the zinc(II) metallation k_{cat} 80 hr^{-1}, compare favorably with the same reaction catalyzed by the enzyme ferrochelatase, k_{cat} 800 hr^{-1}. The antibody shows high substrate specificity in that protoporphyrin IX (**46**) and deuteroporphyrin IX (**47**) are not accepted as substrates in the metallation reactions.

Furthermore, this antibody also binds two biologically relevant metalloporphyrins, iron(III) mesoporphyrin and iron (III) protoporphyrin. The complex of antibody–iron(III) mesoporphyrin was recently shown to catalyze the reduction of hydrogen peroxide by several typical chromogenic peroxidase substrates: pyrogallol, hydroquinone, *o*-dianisidine, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid).[57] Addition of a stoichiometric amount of hapten **44** completely inhibited antibody catalysis of the



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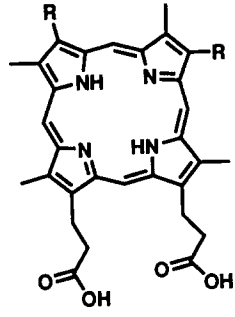


45: R = ethyl
46: R = vinyl
47: R = H

[55] D. Kahne and W. C. Still, *J. Am. Chem. Soc.* **110**, 7529 (1988).
[56] A. G. Cochran, and P. G. Schultz, *Science* **249**, 781 (1990).
[57] A. G. Cochran and P. G. Schultz, *J. Am. Chem. Soc.* **112**, 9414 (1990).

peroxidation reactions. Kinetic analysis of the oxidation of *o*-dianisidine catalyzed by the antibody afforded a $k_{cat}/k_m(H_2O_2)$ ratio of 274 $M^{-1}sec^{-1}$. For comparison, peroxidases are among the most efficient enzymes known, with typical $k_{cat}/K_m(H_2O_2)$ values of 10^7 $M^{-1}sec^{-1}$. The identity of axial ligands to the hemin iron in these complexes will be investigated in order to determine whether any are contributed by the antibody itself. By incorporating both a porphyrin and an oxidation substrate in an antibody combining site perhaps stereo and regioselective oxidations can be catalyzed.

Genetic and Chemical Modification of Existing Antibodies

A second major approach toward the design of catalytic antibodies involves introduction of catalytic activity into extant 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. Site-directed mutagenesis has been successfully applied to the antibody MOPC 315.[58] MOPC 315 binds substituted 2,4-dinitrophenyl (DNP) ligands with association constants ranging from 10^3 to 10^7 M^{-1}.[59] 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.[60]

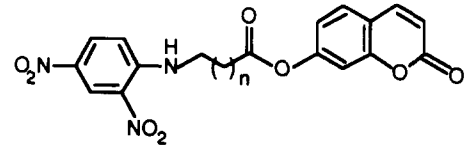
In the absence of an X-ray crystal structure of MOPC 315, chemical modification experiments were used to guide the mutagenesis. In spite of the fact that there are 14 potentially reactive side chains in the hypervariable region (2 histidines, 2 lysines, 3 arginines, and 7 tyrosines), DNP-containing affinity labels alkylate primarily two residues, tryrosine-34L and lysine-52H.[60] 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,[60] perhaps by sterically blocking the entrance to the site. Nitration of the tyrosine ring has no effect.[61]

In order to generate mutants of MOPC 315, a recombinant Fv fragment was generated. MOPC 315 IgA can be proteolyzed with pepsin to

[58] E. Baldwin and P. G. Schultz, *Science* **245**, 1104 (1989).
[59] D. Haseldorn, S. Friedman, D. Givol, and I. Pecht, *Biochemistry* **13**, 2210 (1974).
[60] J. Haimovich, H. W. Eisen, E. Hurwitz, and E. Givol, *Biochemistry* **11**, 2389 (1972).
[61] R. J. Leatherbarrow, W. R. C. Jackson and R. A. Dwek, *Biochemistry* **21**, 5124 (1982).

yield functional Fab or Fv fragments.[62] The Fv fragment (26 kDa) 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.[62] 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 MOPC 315 IgA. Functional Fv and Fab fragments of other antibodies have been previously expressed in *E. coli*.[63] A gene encoding the N-terminal 115 amino acids of MOPC 315 light chain was chemically synthesized and expressed in *E.coli* as a fusion with the λ *c*II gene.[64] 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-*L*-lysine with similar affinities at pH 6.8 (K_D 250 n*M*), whereas the histidine mutant [Fv(Y34H$_L$)] bound DNP-lysine with 6-fold lower affinity (K_D 1400 n*M*). The histidine mutant Fv acelerated hydrolysis of **48** 6×10^5-fold over the background reaction with 4-methylimida-



48: n = 1

zole at pH 6.8. 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 the His-34 side chain in the active site. Site-directed mutagenesis should prove to be a powerful tool either for augmenting the rate enhancements of catatytic antibodies generated via other strategies or for the stepwise evolution of catalytic activity in antibodies to produce efficient selective catalysts.

Site-directed mutagenesis has also been used to probe the catalytic mechanism of phosphorylcholine (PC) binding antibody S107.[65] S107 is one member of a well-characterized class of PC binding antibodies. Two antibodies in this class, T15 and MOPC 167, have been shown to catalyze

[62] J. Hochman, D. Inbar, and D. Givol, *Biochemistry* **12**, 1130 (1973).
[63] A. Skerra and A. Pluckthun, *Science* **240**, 1038 (1988).
[64] K. Nagai and H. C. Thogersen, *Nature (London)* **309**, 810 (1984).
[65] D. Y. Jackson, J. R. Prudent, E. P. Baldwin, and P. G. Schultz, *Proc. Natl. Acad. Sci. U.S.A.* in press (1990).

the hydrolysis of PC *p*-nitrophenyl carbonate (**10**). Additionally, an X-ray crystal structure has been solved for the highly homologous PC binding antibody McPC 603.[66] Based on the three-dimensional structure and earlier kinetic and chemical studies, it is thought that T15 and MOPC 167 stabilize the tetrahedral negatively charged transition state for carbonate hydrolysis.[67] Two residues, Tyr-33 and Arg-52, are thought to play critical roles in the catalyzed reaction. Mutagenesis was performed in a pUC-fl plasmid containing the V_H binding domain of S107. After *in vitro* mutagenesis the V_H sequence was inserted back into a modified SV2 expression vector, (pSV2-S107). The vector allows coexpression of the S107 κ light chain and heavy chain variable region with a γ_{2b} constant region from a BALB/c liver cell. The wild-type and mutant genes were transfected into a myeloma cell line which produces no endogenous antibody and is gpt negative. Expression of up to 5 μg of antibody per milliliter of cell culture supernatant was obtained.

Mutations of Arg-52 to lysine, glutamine, and cysteine were made. Both the glutamine and cysteine mutants showed a large decrease in catalytic efficiency ($k_{cat,Glu}$ 0.04 min^{-1},$k_{cat,Cys}$ 0.02 min^{-1}), presumably because they lack a positive charge to stabilize the negatively charged transition state for hydrolysis of carbonate **10**. The lysine mutant displays catalytic activity similar to wild-type, consistent with molecular modeling which shows the ϵ-nitrogen of lysine to be in close proximity (3.4Å) to the phosphoryl oxygen of PC. These mutations emphasize the importance of electrostatic interactions in transition state stabilization. In light of this result it is interesting that uncharged transition state anaolgs successfully elicit catalytic antibodies for hydrolysis reactions. In contrast, mutations at Tyr-33 had little effect on catalytic activity with the exception of a Tyr-His mutant which showed a 6,700-fold rate enhancement of carbonate hydrolysis over the rate for 4-methylimidazole. No pre-steady-state burst of nitrophenolate was observed in the hydrolysis of **10** by the His mutant. This study successfully demonstrates that a general base catalysis can be combined with electrostatic catalysis in an additive fashion to improve the efficiency of an antibody by suing site-directed mutagenesis.

An important breakthrough in antibody technology is the recent development of a method to express the murine immunological repertoire in *E. coli*.[41] The method uses the polymerase chain reaction to make separate libraries of the gene segments encoding the antigen binding portion of the heavy-and light-chain proteins. The libraries are then recombined at ran-

[66] D. M. Segal, E. A. Padlan, G. H. Cohen, S. Rudikoff, D. Potter, and D. R. Davies, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4298 (1974).

[67] S. J. Pollack, J. W. Jacobs, and P. G. Schultz, *Science* **234**, 1570 (1986).

dom in a bacteriophage λ vector system. Infection of *E. coli* with the phage produces up to 10^{12} heavy–light chain combinations. Currently, several million clones can be screened in 2 days for antigen binding, compared with 6 months or longer needed to screen fewer than 1000 clones produced by conventional hybridoma technology. With this method, it may soon be possible to screen large numbers of antibody clones directly for catalytic activity. Such genetic screens can be combined with directed mutagenesis and chemical approaches to develop highly efficient catalytic antibodies.

Semisynthetic Antibodies. A comprehensive review of efforts in generating semisynthetic antibodies has recently appeared in this series.[68]

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.[69,69a,70] 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.[71] 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.[72] Some haptens have been coupled via diazo linkages to surface tyrosine residues, via disulfide exchange reactions, and via reductive amination.[73] 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.[72] 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. Traditional polyethyleneglycol (PEG) fusions of splenocytes with the SP2/0 myeloma cell line have been used.[71,74] 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

[68] S. J. Pollack, G. R. Nakayama, and P. G. Schultz, this series, Vol. 178, p. 551.

[69] G. Kohler and C. Milstein, *Nature (London)* **256**, 495 (1975).

[69a] G. Galfrè, and C. Milstein, this series Vol. 73, p. 3. See also several chapters in Vols. 92 and 121. [Ed.]

[70] J. W. Goding, "Monoclonal Antibodies: Principles and Practice." Academic Press, New York, 1986.

[71] E. Engvall and P. Perlmann, *J. Immunol.* **109**, 129 (1972).

[72] T. Nishima, A. Tsuji, and D. K. Fukushima, *Steriods* **24**, 861 (1974).

[73] B. F. Erlanger, this series, Vol. 70, p. 85.

[74] R. Sugasawara, C. Prato, and J. Sippel, *Infect. Immun.* **42**, 863 (1983).

characterized. In our experience only IgG and IgA antibodies have been proved to function as catalysts, even though a large number of IgM antibodies have been tested. Either protein A affinity or ion-exchange chromatography methods have been used to purify antibodies to homogeneity.[75]

Characterization of the kinetic parameters, specificity, mechanism, and structural properties of catalytic antibodies is complicated and difficult to interpret unless a monoclonal antibody is generated. Moreover, reproducibility becomes an important concern when a polyclonal antibody preparation is generated. It should also be noted that over 15 years ago Raso and Stollar attempted to use pyridoxamine binding antibodies to catalyze Schiff base formation and transmination reactions.[76] 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 $3 \times 10^4 min^{-1}$, contamination of the antibody with 6×10^{-5}% (on a mole/mole basis) of the natural enzyme would lead one to believe that the antibody is catalytic when in fact 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 polyacrylamide gel electrophoresis (PAGE) to be homogeneous antibody.

Prospects

The rapid development of diverse strategies to elicit catalytic antibodies has led to the catalysis of a wide variety of reactions (see Table I). The concerted use of several of these strategies will be necessary in order to fully exploit the usefulness of catalytic antibodies.

Acknowledgements

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[76] V. Raso and B. Stollar, *Biochemistry* **14**, 584 (1975).