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Recent Advances in Catalytic Antibodies

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Recent advances in catalytic antibodies

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SUMMARY

Recently the biological machinery of the immune system has been exploited with the aid of mechanistic chemistry to produce catalytic antibodies. Because antibodies can be generated that selectively bind almost any molecule of interest, this new technology offers the potential to tailor-make highly selective catalysts for applications in biology, chemistry and medicine. In addition, catalytic antibodies provide fundamental insight into important aspects of biological catalysis, including the importance of transition-state stabilization, proximity effects, general acid and base catalysts, electrophilic and nucleophilic catalysis, and strain.

1. INTRODUCTION

Chemists have become increasingly sophisticated in their ability to synthesize complex organic molecules and in their understanding of reaction mechanisms. Yet given these spectacular advances, chemistry cannot begin to match the ability of biological systems to synthesize and screen tremendous chemical diversity to produce complex molecules with interesting biological properties. One of the most remarkable examples of this is the immune system. The humoral immune system has the potential to produce on the order of 10^{12} unique receptors. A complex screening mechanism allows the immune system to select from this vast array of molecules, antibodies that bind virtually any synthetic or biomolecule with high affinity and exquisite specificity (Schultz *et al.* 1990). Hybridoma technology, which makes it possible to generate, *in vitro*, large amounts of homogenous antibody molecules, has dramatically expanded the role of antibodies in biology and medicine (Kohler & Milstein 1975). Antibodies are invaluable tools for the detection and analysis of biological materials, are key components of most diagnostic devices and hold promise as highly selective therapeutic and imaging agents.

Recently, we and others have shown that the principles and tools of organic chemistry can be used to exploit the highly evolved machinery of biology, specifically the humoral immune system, to generate a new class of bioactive molecules: catalytic antibodies. By combining the exquisite binding specificity of the immune system with our understanding of chemical and biological reaction mechanisms, antibodies have been generated that catalyse a wide array of chemical reactions from pericyclic rearrangements to peptide bond cleavage. Moreover, several strategies have been developed for generating catalytic antibodies, including the use of antibodies to stabilize negatively charged transition states and to act as entropic traps, and the generation of catalytic groups or cofactor binding sites in antibody combining sites. Because antibodies can be elicited to a vast array of biopolymers, natural products, or synthetic molecules, catalytic antibodies offer a unique approach for generating enzyme-like catalysts that differentiate complex molecules of biological, chemical and medical interest.

Catalytic antibodies might be used, for example, to develop a family of catalysts analogous to restriction enzymes that cleave proteins or sugars at a particular bond. Such antibodies would be invaluable reagents in biology and might find use as therapeutic agents to selectively hydrolyse protein or carbohydrate coats of viruses, cancer cells, or other physiological targets. Catalytic antibodies also could serve as selective catalysts for the synthesis of pharmaceuticals, fine chemicals, and novel materials. At the same time, the characterization of catalytic antibodies provides fundamental insight into important aspects of biological catalysis, including the importance of transition-state stabilization, proximity effects, general acid and base catalysis, electrophilic and nucleophilic catalysis, and strain.

Several comprehensive reviews on the generation and characterization of catalytic antibodies have been written (Schultz *et al.* 1990; Shokat & Schultz 1990*a*; Schultz 1989). This article reviews recent advances in the field out of our laboratory.

2. TRANSITION STATE STABILIZATION

The first examples of antibody-catalysed reactions were based on the notion of transition-state stabilization. Jencks first proposed over 20 years ago that antibodies raised against a transition-state analogue should selectively bind the transition state of a reaction over substrate and thereby act as a catalyst (Jencks 1969). Enzymes themselves have evolved to provide an active site that is sterically and electronically complementary to the rate-determining transition state. The

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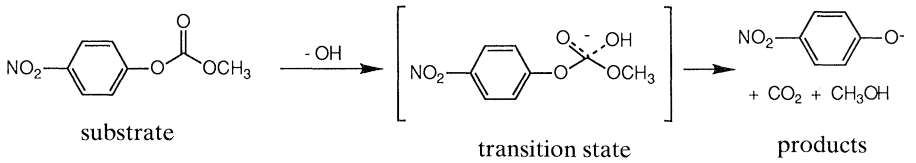
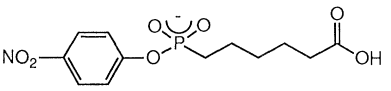
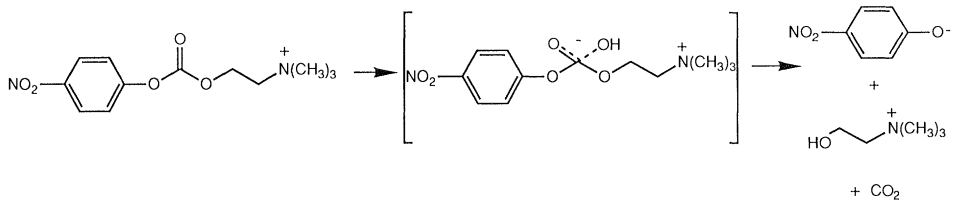


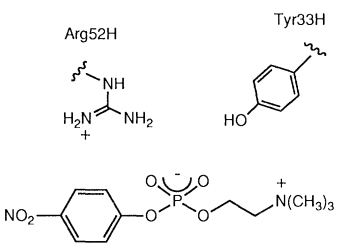
substrate transition state products



transition state analogue–hapten

Scheme 1.





Scheme 2.

first successful application of this notion to the generation of catalytic antibodies was made independently by the groups of Lerner and Schultz in 1986, when they characterized antibodies that catalyse the hydrolysis of esters and carbonates (Tramantano *et al.* 1986; Pollack *et al.* 1986). The rate-limiting step in the uncatalysed reactions is formation of the negatively charged tetrahedral transition state. A stable analogue of such a structure is formed by substitution of a tetrahedral phosphorus atom for the tetrahedral carbon (Bartlett & Marlowe 1983). As predicted, antibodies specific for these transition-state analogues selectively accelerated the hydrolysis of their respective substrates (scheme 1). The antibodies have substantially higher binding affinities for the transition-state analogues than for the reaction substrates, which suggests that they function by stabilizing the transition state. Since these first examples, over 20 acyl transfer reactions have been catalysed by antibodies including ester bond formation, lactone formation and stereospecific ester hydrolysis.

Recently we have used the technique of site-directed mutagenesis to further define the catalytic mechanism and improve the catalytic efficiency of the hydrolytic catalytic antibody S107 (Jackson *et al.* 1991). The phosphorylcholine (PC) binding antibodies provide a good starting point for investigating antibody binding and catalysis. Two phosphorylcholine binding antibodies, MOPC 167 and T15, have previously been shown to catalyse the hydrolysis of choline carbonates (Pollack *et al.* 1986; Pollack & Schultz 1987). These antibodies belong to a class of highly homologous PC binding antibodies that have been well-characterized with regard to ligand binding kinetics and specificity, biomolecular structure and genetics (Pollet *et al.* 1974; Goetze & Richards 1977; Goetze & Richards 1978; Bennett & Glaudemans 1979; Crews *et al.* 1981; Perlmutter *et al.* 1984). In addition, the three dimensional structure of one representative PC binding antibody, McPC603, has been solved by X-ray crystallography (Satow *et al.* 1986). It has been proposed that T15 and MOPC 167 preferentially stabilize the transition state in carbonate hydrolysis on the basis that phosphorylcholine diesters resemble the tetrahedral negatively charged transition state for the hydrolytic reaction. Consistent with this notion, the antibodies bind the PC transition state analogues with higher affinity than the carbonate substrates. Based on the three-dimensional structures of McPC 603, as well as previous chemical modification and kinetic studies,

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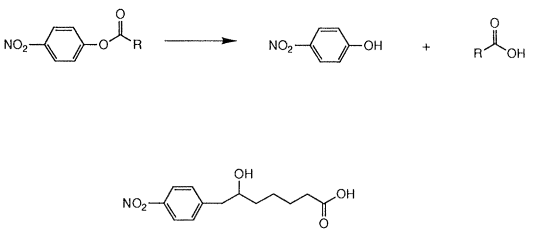
it has been argued that Tyr 33 and Arg 52 of the heavy chain play a critical role in stabilizing the rate determining transition state configuration via electrostatic and hydrogen bonding interactions. These residues are conserved in all PC specific antibodies (Perlmutter *et al.* 1984). To determine the roles of these residues in binding and catalysis, mutants of the highly homologus PC binding antibody S107 were generated.

Three Arg 52_H mutants (R52K, R52Q, R52C) and four Tyr 33_H mutants (Y33H, Y33F, Y33E, Y33D) of wild type S107 were generated by *in vitro* mutagenesis. The mutant and wild type genes were then cloned into a modified SV-2 shuttle vector containing an *Escherichia coli* xanthine-guanine phosphoribosyl transferase (gpt) selection marker (Jackson *et al.* 1991). These constructs were then used to transfect a murine myeloma cell line, and recombinants were selected on azaserine/hypoxanthine. *In vivo* expression in immunosuppressed mice and protein-A affinity chromatography resulted in the isolation of large quantities of pure antibody from ascites fluid.

Mutations at Arg 52_H which resulted in a loss of the positively charged side chain resulted in a significant decrease in k_{cat}, whereas mutants that retained the positively charged side chain (A52K) had wild type activity. In contrast, mutations at Tyr 33_H have little effect on catalytic activity. The k_{cat} value of the Y33F mutant as well as the K_m and K_i for carbonate substrate and the phosphodiester transition state analogue, respectively, are comparable to those of wild type antibody. This is somewhat surprising since the X-ray crystal structure (Satow *et al.* 1986) shows the tyrosine hydroxyl group to be within hydrogen bonding distance (2.9 Å†) of the phosphoryl oxygen. Because Tyr 33_H appears to play no role in binding or catalysis, $Y33H_H$, $Y33E_H$, and $Y33D_H$ mutants were generated in an effort to place a general base in the antibody combining site. The $Y33_H$ mutant showed a measurable increase in k_{cat} with respect to the wild type antibody, which translated into a 6700 fold overall rate acceleration when compared to the background reaction with 4-methyl imidazole. Although the precise role of the histidine residue in catalysis remains unclear, mechanistic experiments suggest that the histidine may act via a general base mechanism. This mutagenesis study points to the importance of electrostatic stabilization in acyl transfer catalysis by antibodies, and further shows that incremental improvements in antibody catalysis are readily obtained with site-directed mutagenesis.

In addition to negatively charged phosphonates, a number of uncharged transition-state analogues exist for hydrolytic enzymes. The best characterized of these is the potent pepsin inhibitor, pepstatin, which contains the novel amino acid analogue statine. The measured K_i of pepstatin for pepsin is 46 pM, making it one of the most potent enzyme inhibitors known (Rich 1986). Statine can be considered as a 'collected' substrate analogue possessing binding determinants of both the peptide substrate and the enzyme-bound water responsible for addition to the scissile amide bond. The secondary hydroxyl group is thought to mimic the tetrahedral transition state for amide bond hydrolysis.



Scheme 3.

In addition to peptidase inhibitors, uncharged transition-state analogue inhibitors of enzymes in nucleotide biosynthesis pathways have been isolated. Coformycin inhibits the enzyme adenosine deaminase and is one of the most potent inhibitors known for any enzyme with $K_i = 2.5$ pM (Frick *et al.* 1986). The enzyme converts adenosine into inosine, which involves the hydrolysis of the amidine moiety of adenine. Based on the high binding constants of these transition-state analogues, haptens with similar structural features might be expected to elicit catalytic antibodies.

Shokat *et al.* (1991) have recently shown that hydrolytic catalysts can in fact be generated by using the charge neutral statine-based peptidase hapten shown in scheme 3 (Shokat *et al.* 1991). From a panel of five monoclonal antibodies specific for the hapten, one was found that catalysed the hydrolysis of both carbonate and ester substrates. The values of k_{cat} and K_M were 0.72 min^{-1} and 3.65 mM, respectively, for the ester substrate, and 0.31 min^{-1} and 3.33 mM, respectively, for the carbonate substrate. Comparison of K_m/K_i and k_{cat}/k_{uncat} shows that the rate acceleration is between one and two orders of magnitude higher than the expected value based on the differential binding of substrate and hapten-inhibitor. This discrepancy suggests that mechanistic factors which are not clearly understood, and most likely result from the structural diversity of the humoral immune response, are important for a large degree of antibody catalysis in this system. Attempts to generate antibodies that catalyse adenosine deamination by immunizing with a coformycin derivative as a hapten failed to produce any catalytic antibodies.

The notion of transition state stabilization has also recently been applied to the metallation of porphyrins. Metallo-porphyrins represent an important class of biologically relevant cofactors involved in electron and oxygen transport as well as many oxidation reactions. Ferrochelatase, the terminal enzyme in the heme biosynthetic pathway, catalyses the insertion of iron (II) into protoporphyrin (Lavallee 1988). A potent inhibitor of this enzyme is the bent porphyrin, N-methylprotoporphyrin (Dailey & Fleming 1983). The distorted structure of the methylated porphyrin macrocycle results from steric crowding due to the internal methyl substituent, and is thought to resemble the transition state of porphyrin metallation catalysed by ferrochelatase. This distortion of the macrocyclic ring system forces the chelating nitrogen electron pairs into a position which is more accessible for binding of the

† 1 Å = 10^{-10} m = 10^{-1} nm.

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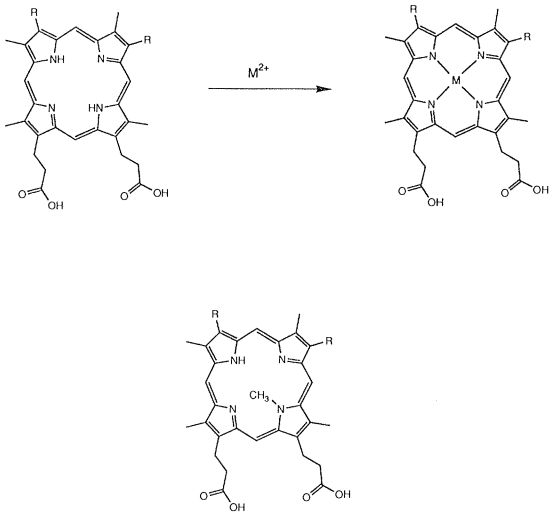


Scheme 4.

incoming metal ion. Cochran & Schultz (1990*a*) have recently shown that antibodies generated against this bent mesoporphyrin efficiently catalyse the metallation of a mesoporphyrin substrate (Cochran & Schultz 1990*a*). Antibody catalysis was observed with a variety of transition metals including Zn (II) and Cu (II). An initial rate analysis was performed for the metallation reaction with Cu (II). At 1 mM Cu (II), the antibody catalysed the metallation reaction with a K_M of 50 μM and an apparent V_{max} of 2.5 μM h^{-1}. Catalysis could be inhibited by free hapten as well as various metalloporphyrins.

The catalytic properties for this antibody are similar to those of the enzyme ferrochelatase. The reported K_M values for ferrochelatase of 10–70 μM (Levallee 1988) are comparable to the value of 50 μM determined for the antibody. Both enzyme and antibody catalysis suffer from product inhibition, and both have comparable affinities for the N-methylporphyrin. Both enzyme and antibody can insert a variety of divalent transition metals into porphyrins, and the turnover numbers (k_{cat}) are similar. A calculated value of k_{cat} for ferrochelatase with Zn (II) is 800 h^{-1}, and the experimentally determined value of k_{cat} for the antibody is 80 h^{-1}. This represents the closest correlation of turnover number achieved to date for a catalytic antibody with the analogous enzyme. In addition, this work shows the use of antibody binding energy to distort substrate conformation in a productive fashion along the reaction coordinate.

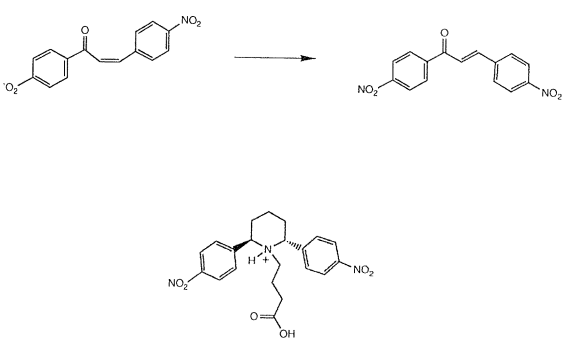
3. INTRODUCTION OF CATALYTIC GROUPS

A second important strategy for generating catalytic antibodies involves the rational generation of chemically reactive amino acid side chains in antibody combining sites. Many enzymes utilize a combination of active site residues such as nucleophiles, electrophiles, general acids, and general bases to achieve their remarkable rates. For example, staphylococcal nuclease contains both an active site glutamate residue which functions as a general base to deprotonate water, in addition to an arginine residue that stabilizes the negatively charged transition state. Shokat & Schultz have shown the viability of introducing a catalytic base into an antibody combining site for an antibody catalysed elimination reaction (Shokat & Schultz 1989). Immunization with a positively charged alkyl ammonium hapten afforded antibodies with a complementary negatively charged carboxylate residue appropriately positioned in the combining site to function as a general base for a β-elimination of hydrogen fluoride. From a panel of six monoclonal antibodies, four were able to catalyse the elimination reaction; one catalysed the reaction with a rate acceleration of approximately 10^5 over the corresponding background reaction. Chemical modification and affinity labelling experiments confirmed the presence of an active site carboxylate, and kinetic isotope effects showed that the rate-determining step involves proton abstraction from the α-carbon atom of the fluoroketone substrate (Shokat & Schultz 1990*b*). In addition, the reaction displays a pH profile pointing to an ionizable active site residue with a pKa of 6.2. This study was the first demonstration that antibody–hapten complimentarity can be exploited to obtain combining site residues capable of participating in chemical transformations.

More recently this approach has been applied to an antibody catalysed *cis–trans* isomerization reaction. Antibodies raised to the bis-nitrophenyl piperidinium hapten shown below were capable of catalysing the isomerization of the corresponding *cis* enone to the *trans* enone (Jackson & Schultz 1991). The catalysis was competitively inhibited by the free hapten, and the antibody accelerated the rate of reaction 15000-fold over the uncatalysed background reaction. A pH dependence study on k_{cat} suggests the presence of an ionizable combining site residue that, with a pKa of 5.5, participates in catalysis.

Additional evidence for a catalytic residue and corresponding covalent antibody–substrate adduct follows from chemical modification experiments including epoxide affinity labelling and treatment of the antibody with diazoacetamide, a reagent that specifically esterifies carboxylate residues.

A mechanistic model consistent with these observations is shown in scheme 6. Initial binding of the *cis* enone isomer to the antibody is followed by 1,4-nucleophilic addition of a carboxylate group leading to a covalent antibody–substrate intermediate. It was anticipated that an active site carboxylate residue



Scheme 5.

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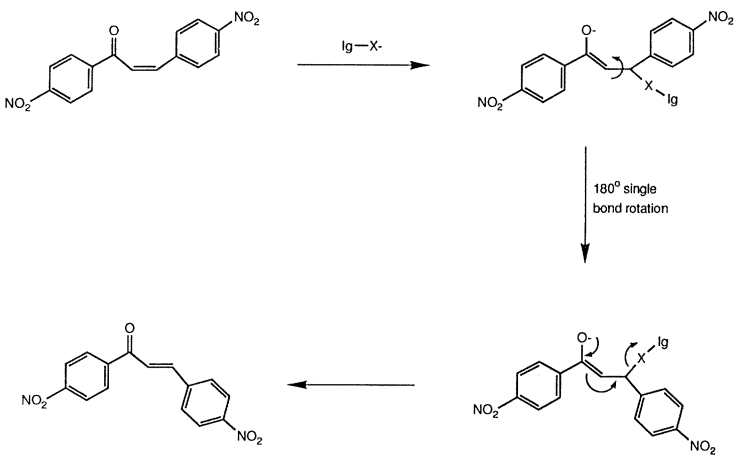
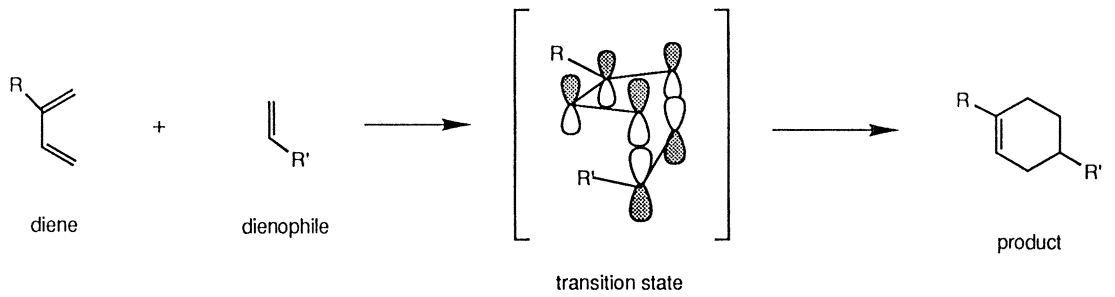


Scheme 6.



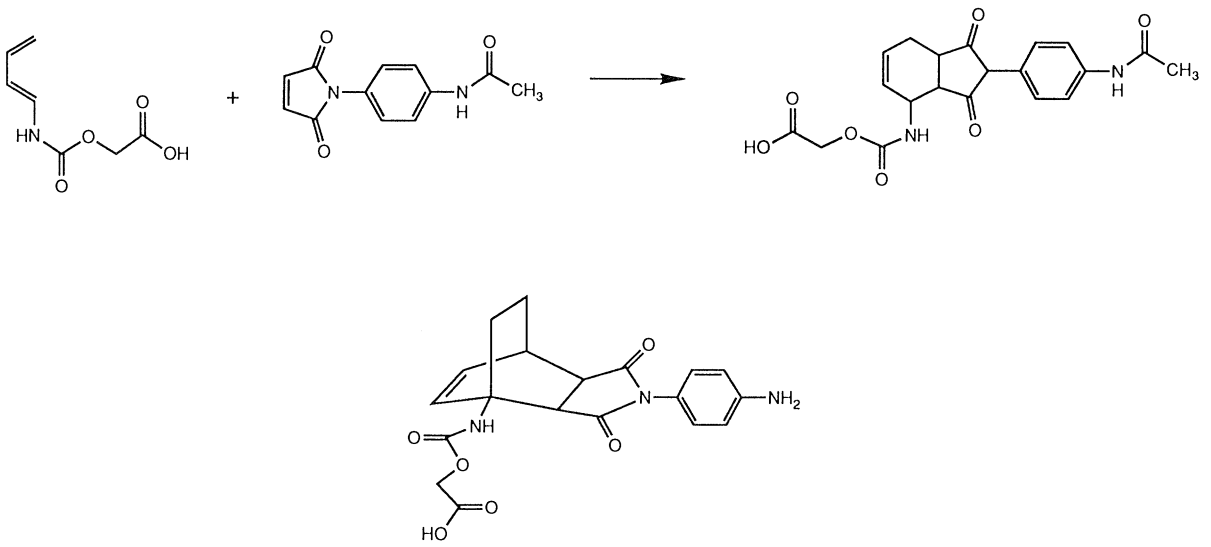
Scheme 7.

might arise in response to the ammonium ion contained in the hapten structure. The nucleophilic 1,4-addition converts the double bond of the substrate into a single bond, about which rotation is facile. Following rotation about this bond, the covalent adduct collapses by elimination, giving rise to the thermodynamically favoured *trans* enone and the regenerated antibody catalyst. For isomerization to occur, the antibody must accommodate the orthogonal transitional state for bond rotation. Computer modelling shows that the *trans* configuration of the nitrophenyl rings in the hapten do in fact mimic the geometry of a transition state in which the α, β- is rotated 90°. Consistent with this observation, only the *trans* and not the *cis* hapten afforded catalytic antibodies.

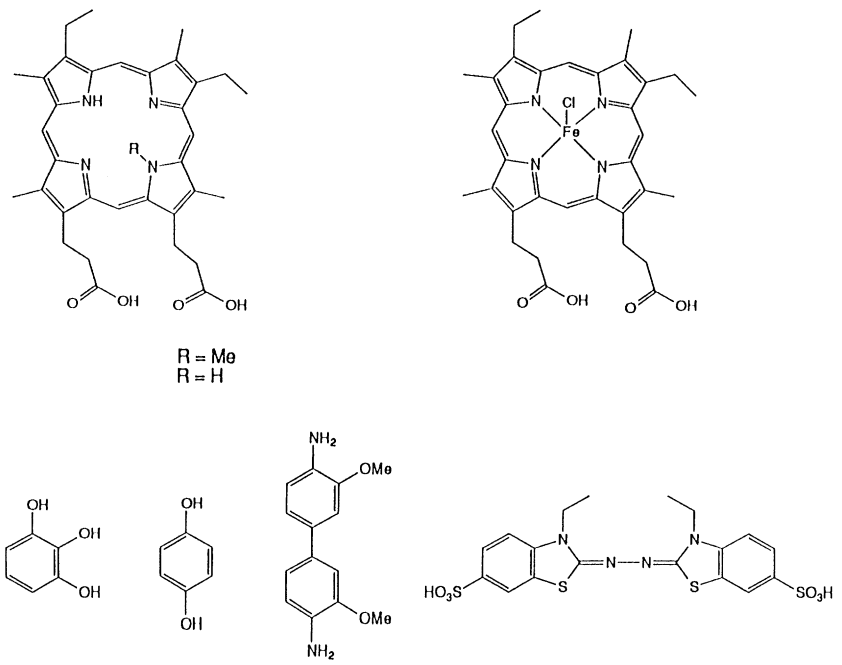
4. ANTIBODIES AS ENTROPY TRAPS

Another approach toward the design of catalytic antibodies involves the use of antibody binding energy to lower entropic barriers to reactions. Jencks & Page have argued that entropic effects can account for effective molarities of up to 10^8 M in enzyme catalysed reactions (Page & Jencks 1971). These notions have been tested in the design of antibodies that catalyse transacylation reactions (Schultz *et al.* 1990; Shokat & Schultz 1990; Schultz 1989; Napper *et al.* 1987; Janda *et al.* 1988), Diels–Alder reactions (Braisted & Schultz 1989; Hilvert *et al.* 1989) and Claisen rearrangements (Jackson *et al.* 1988; Hilvert & Nared 1988). The latter reaction involves the conversion of chorismic acid to prehenic acid. This thermal 3,3-sigmatropic rearrangement occurs through an asymmetric chairlike transition state. One might expect that an antibody combining site that is complementary to the conformationally restricted transition state would accelerate this rearrangement. In fact one antibody elicited to a bicycle transition state inhibitor of chorismate mutase increased the rate of the rearrangement 10000-fold, whereas the enzyme chorismate mutase accelerates the reactions approximately 10^6-fold over the uncatalysed background reaction (Jackson *et al.* 1988). For this antibody, mechanisms involving a cationic substituent effect or general acid catalysis were ruled out. As expected, the entropy of activation of the antibody-catalysed reaction was close to zero, compared with an entropy of activation ($\Delta S^{\ddagger}$) of −13 entropy units for the uncatalysed reaction. This antibody has recently been cloned and introduced into bacteria that lack the ability to synthesize prephenic acid. Random mutagenesis and selection will be used in an effort to increase the catalytic activity of this antibody.

Another example of the use of antibodies to act as entropic traps involves an antibody catalysed Diels–Alder reaction. The Diels–Alder reaction has long been one of the most powerful transformations in organic chemistry. This process consists of a bimolecular reaction between a diene and an alkene giving rise to a cyclohexene product. The transition state involves a highly ordered cyclic array of interacting orbitals in which carbon—carbon bonds are broken and formed in a single concerted mechanistic event (figure 7). Consequently, an unfavourable entropy of activation on the order of −30 to −40 entropy units is generally



Scheme 8.



Scheme 9.

observed. The design of a transition state analogue–hapten which would lead to catalytic antibodies for this reaction must address two fundamental issues: (i) an 'entropic sink' must be provided such that the two substrate molecules are oriented in a reactive configuration upon binding, and (ii) a mechanism for eliminating product inhibition must be incorporated, since the cyclohexene product is structurally very similar to the transition state. Hilvert and co-workers were successful in designing a system that satisfies these criteria (Hilvert *et al.* 1989). The particular Diels–Alder reaction chosen was that of tetrachlorothiophene dioxide with N-ethylmaleimide giving rise to an initial tricyclic Diels–Alder adduct which spontaneously extrudes sulphur dioxide, resulting in the dihydrophthalimide product. One of five antibodies raised to a stable [2.2.1] bicyclic transition state analogue was found to significantly accelerate the rate of reaction over background. The antibody catalysed the reaction with multiple turnovers (> 50), and catalysis could be inhibited by free hapten.

A more general strategy for Diels–Alder catalysis has recently been reported by Braisted & Schultz (1989). The hapten contains an ethano bridge that locks the cyclohexane ring into the conformation resembling the Diels–Alder transition state, and presumably makes hydrophobic binding contacts in the antibody combining site (figure 8). The cyclohexene product does not contain this hydrophobic bridge, and therefore would be bound less tightly than the hapten, and should diffuse from the combining site. The diene substrate in this system is acyclic, and is not covalently locked in the *cis–syn* reactive conformation. Since antibodies are elicited to a locked cyclohexane ring system, the reactive *cis–syn* conformation should be more favourably bound in the combining site.

From a panel of 10 monoclonal antibodies elicited to the hapten, one was found which catalysed the formation of the Diels–Alder adduct with a large rate acceleration over the background reaction. The apparent second order rate constants (k_{cat}/K_m) were found to be 900 M^{-1} s^{-1} for the diene, and 583 M^{-1} s^{-1} for the dienophile. These values can be compared to the second order rate constant for the background reaction of 1.9 M^{-1} s^{-1} in water and 2×10^{-3} M^{-1} s^{-1} in acetonitrile. The reaction is competitively inhibited by free hapten with a K_D of 126 nM, and by product with a K_D of 10 μM. This work represents the extension of antibody catalysis to a class of reactions for which no characterized enzyme exists.

5. ANTIBODIES THAT RECRUIT COFACTORS

A final strategy that has been applied to antibody catalysis involves the generation of antibodies that recruit cofactors. Many enzymes use cofactors to catalyse reactions, for example, cytochrome P450 (Fe-heme cofactor), α-ketoacid dehydrogenases (thiamine pyrophosphate cofactor), D-amino acid oxidase (flavin cofactor), and alanine racemase (pyridoxal phosphate cofactor). Strategies that allow incorporation of cofactors into antibody combining sites should expand the scope of antibody catalysis to redox reactions and energetically demanding hydrolytic reactions. The diversity of the antibody response should allow one to use both natural and unnatural cofactors.

In the past two years antibodies have been generated that bind both metal and redox active cofactors (Shokat *et al.* 1988; Iverson & Lerner 1989; Iverson *et al.* 1990). Most recently we have characterized an antibody that binds Fe(III)-mesoporphyrin IX and hydrogen peroxide and catalyses the oxidation of a number of substrates (Cochran & Schultz 1990*b*). Antibodies specific for N-methylporphyrins were subsequently found to form a stable complex with iron mesoporphyrin IX. The antibody Fe(III)–heme complex was found to catalyse the reductive breakdown of hydrogen peroxide in the presence of a variety of chromogenic electron donor substrates. The antibody–porphyrin complex remains active through at least 200 catalytic turnovers. Antibody catalysis could be completely inhibited by N-methylmesoporphyrin, and no catalysis was observed in the absence of the iron-mesoporphyrin cofactor. The catalysed peroxidation reaction followed Michaelis–Menten kinetics with respect to hydrogen peroxide reduction, with a K_M of 24 mM and k_{cat} of 394 min^{-1}. To generate a substrate binding site, in addition to the Fe(II)-heme and H_2O_2 sites, antibodies are now being generated to N-alkylporphyrins in which the alkyl group corresponds to the substrate of interest.

6. CONCLUSION

The number and diversity of reactions catalysed by antibodies continues to grow at a rapid rate. The specificity of antibody catalysed reactions is high and in some cases the rates of catalytic antibodies rival those of the corresponding enzyme catalysed reactions. The next few years will probably see an emphasis on increasing the catalytic efficiency of antibodies by generating antibodies that combine several catalytic mechanisms and by the use of genetic selections and screens. In addition, antibody catalysis is likely to be applied to reactions of biological, medical and chemical interest, for which naturally occuring enzymes do not exist.

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