Catalytic antibodies — A new window on protein chemistry
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Introduction
The relationship between structure and catalytic activity in enzymes has been well studied and this knowledge has assisted developments of the applications of enzymes in analytical, medical, and preparative chemistry. With the discovery that antibodies can catalyse chemical reactions with efficiency approaching that of enzymes, the possibility of being able to generate protein catalysts on demand for a given reaction has become a reality. Catalytic antibodies (abzymes) have been generated to catalyse a wide variety of organic reactions (for a review see [1]) including acyl transfer and electrocyclic reactions. Some of these reactions are not known in nature as enzyme-catalysed reactions. Also, the structure of the binding regions of antibodies differs significantly from the typical structure of active sites of enzymes; catalysis by proteins can therefore be studied within a new molecular framework. For both of these reasons, catalytic antibodies offer a new window on protein chemistry. This paper summarizes what we have seen through this window in our work.

Before describing our results it is necessary to outline the normal methods of generating abzymes. The concept of catalysis by enzymes and abzymes rests upon their binding the transition state for the reaction thereby strongly decreasing the activation energy for the rate-determining step and increasing the rate of reaction. Thus to obtain a new abzyme, it is necessary to have a knowledge of the molecular mechanism of the reaction and to be able to postulate a structure for the transition state. Transition states themselves have only a fleeting existence; to provide the template for the binding of the antibody, it is therefore necessary to employ stable analogues of the transition state. The most exploited example of this is the use of tetrahedral phosphorus (V) anions to mimic the tetrahedral intermediate and its related transition state in acyl transfer reactions. To raise the antibodies, the transition-state analogue is coupled to an immunogenic protein and normal...
hybridoma technology employed. The resulting clones are screened for their ability to bind the transition-state analogue and to catalyse the required reaction. Although other methods of generating antibodies are being developed [2, 3], the key to identifying catalytic antibodies remains a rapid and reliable screen for catalytic activity.

Antibody catalysis of the Diels–Alder reaction
Our chief interest is in the generation of antibodies with the potential to catalyse reactions of significance in preparative organic chemistry. One of the benefits of enzyme-catalysed reactions in organic synthesis is their ability to mediate stereoselective reactions leading to the production of optically pure homochiral building blocks [4]. However, no enzymes that catalyse the Diels–Alder reaction, one of the most important for the synthesis of complex organic compounds (for leading historical examples see [5, 6]), have been discovered. There is thus a niche for the discovery of abzymes. This niche has also been recognized by the groups of Schultz and Hilvert in the United States [7, 8].

The mechanism of electrocyclic reactions such as the Diels–Alder reaction is understood to proceed through a cyclic transition state that is similar to the product of the reaction [9]. The design of a transition-state analogue is therefore straightforward and is modelled on the product. However, this leads to a potential problem; since the abzyme is designed to bind a product analogue, there is a real risk that the product when it is formed will bind to the abzyme preventing the access of further reactants and hence inhibiting the reaction. Schultz and Hilvert devised ingenious strategies for preventing product inhibition (Fig. 1 a & b) and were rewarded by discovering antibodies that catalysed Diels–Alder reactions of dienes and N-substituted maleimides with significant effective molarities (100–1000). We also chose maleimides as our dienophile but we relied upon differences between the N-alkyl substituent in the hapten and the substrate to prevent substrate inhibition [10].

![Fig. 1](image)

Diels–Alder reactions catalysed by antibodies

(d)  

![Chemical structure 7](image)

(b)  

![Chemical structure 8](image)

(c)  

![Chemical structure 10](image)
With regard to the synthetic potential of our product, we also included an acetate ester that, on hydrolysis, would afford an allylic alcohol (Fig. 1c).

We isolated four clones of antibodies that bound the hapten strongly and all have been found to be catalytic. The most studied of these, designated H11, displays an effective molarity of only 1 M using acetoxybutadiene and N-ethyl maleimide as substrates. This is a low figure as is the value of \( k_{cat}/K_m \) for acetoxybutadiene (6.6 M\(^{-1}\) s\(^{-1}\)); in experimental terms, it corresponds to reducing the time of a typical reaction from about 24 to 6 h. Expressed differently, comparing the pseudo-first-order rate constant for the uncatalysed reaction with the rate constant for the catalysed reaction, a rate enhancement of 1700 is observed. Catalysis is therefore significant. Furthermore, we have not observed any product inhibition in the millimolar concentration range used in these experiments. It is worth noting in passing that these catalytic properties would not be suitable for large scale preparative chemistry; both the concentrations of reactants and rates are too slow. The properties of H11 would need to be developed and improved for such an application to be practical. However, the results so far are a satisfactory start and further indications of the potential of antibodies like H11 have come from more detailed studies of its properties.

A useful catalyst for a biotransformation should have high enantioselectivity but accept a range of similar substrates. The enantioselectivity of H11 has not yet been determined but we have found that it will accept N-ethyl or N-benzylmaleimide equally. In contrast, it shows clear selectivity for the diene: whereas 1-acetoxybutadiene is a substrate, neither 1-methoxybutadiene or pent-1,3-diene are substrates. This order of reactivity is significant because in control reactions, 1-methoxybutadiene is a substrate, neither 1-methoxybutadiene or pent-1,3-diene are substrates. This order of reactivity is significant because in control reactions, 1-methoxybutadiene is the most reactive, as would be expected. With regard to the potential application of H11 on a large scale, we have shown that the Fab fragment derived from H11 by cleavage with immobilized papain is essentially identical in its catalytic behaviour to the complete H11 antibody. This means that it will be sufficient to clone and express the gene for the Fab fragment to obtain large quantities of a catalytically active protein. The cycloaddition reaction catalysed by H11 was found to be pH dependent. At pH 4, the reaction occurred no faster than the control experiment but the rate increased rapidly between pH 7 and 8 reaching a maximum at pH 8.5–9.0. The mechanism of cycloaddition would not lead one to expect a pH dependence unless the diene were protonated by an acid at the active site thereby reducing its activity. Alternatively, H11 may exist in an unfavourable conformation at low pH. We are currently investigating this question.

One significant and surprising result from the study of H11 was that when N-benzylmaleimide and acetoxybutadiene were used as substrates, instead of the expected acetate, the corresponding alcohol was isolated as product. It was possible to show that the formation of the alcohol was catalysed by H11 subsequent to formation of the expected Diels–Alder adduct; the rates of cycloaddition and hydrolysis were similar. Thus H11 has the surprising property of catalysing two successive reactions of quite different character. To our knowledge, no enzyme with similar properties is known. Our current work is designed to establish the molecular details of the mechanism of action of H11, to explore its substrate selectivity with respect to the esters, and to determine its enantioselectivity.

**A second unexpected esterase**

In connection with our interest in reactions with no enzymic counterpart, we began an investigation into aromatic substitution catalysed by antibodies [11]. An amine was used as hapten in an attempt to obtain an antibody that would stabilize a positively charged transition state expected in an electrophilic substitution (Fig. 2a). Of many clones of antibodies isolated to this hapten, two have been examined in some detail. Neither of the two catalysed aromatic substitution but one, designated C3, catalysed the hydrolysis of a 4-nitrophenyl ester that we have used as a potential substrate for the aromatic substitution reaction (Fig. 2b). Although we tested several other esters as substrates (ethyl, thioethyl, phenyl, 4-chlorophenyl), only the 4-nitrophenyl ester appears to react at a significant rate. The other antibody, designated C5, appears to catalyse no related reactions.

In an attempt to understand the differences between the two antibodies, we have investigated their ability to bind a series of molecules of closely related structure. Catalytic activity in C3 is paralleled by binding to those molecules that have a positive charge; neutral molecules do not bind strongly to C3. C5 exhibits complementary behaviour; it binds strongly to the neutral but polar molecules that C3 rejects. It is tempting to suggest that the cell that produced C3 recognized the protonated form of the N-group in the hapten (pK\(_a\) approx. 5) and responded with an anionic group [11]. The parallel behaviour with C5 would imply that the neutral form was recognized.
Fig. 2

Hydrolytic and non-hydrolytic antibodies binding an amine-containing hapten

(a) Affinity of some analogues of the hapten for C3 and C5 determined by competitive e.l.i.s.a. measurements. (b) Substrates tested and the reaction catalysed by C3.

<table>
<thead>
<tr>
<th>Substrate Tested</th>
<th>Reaction Catalysed by C3</th>
</tr>
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<tbody>
<tr>
<td>R = H</td>
<td>100%</td>
</tr>
<tr>
<td>R = OH, OEt, SPh, 4-CIC6H5O</td>
<td>No reactions detected</td>
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We have made some efforts to identify the catalytically active group in C3. Reagents directed at carboxylate, histidine, and thiol have no effect on the reaction. Unlike the elegant elimination reactions studied by Shokat et al. [12], C3 did not catalyse the elimination of HCl or HF nor did it promote exchange of hydrogen for deuterium in the α-positions of ketone-containing substrates. Nevertheless, the rate of hydrolysis was strongly pH dependent, showing a plateau maximum above pH 8.5. It is particularly notable that for 4-nitrophenyl esters that bind, C3 is a remarkably effective catalyst; the rate enhancement is $3.5 \times 10^4$ compared with the background rate under the same conditions. For this antibody, $k_{cat}/K_M$ is more significant ($5 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$) than for H11.

The absence of a readily identifiable, catalytically active group and the pH dependence of hydrolysis require some explanation. What follows is conjecture. The binding site of antibodies is on the surface of the protein. Inspection of the surfaces of all water-soluble globular proteins reveals a large number of charged and polar residues as required for solvation. There is a high probability, therefore,
that there will be at least one such charged group in the vicinity of the binding site of the antibody. Any of the charged residues could in principle act as a general base in a hydrolysis reaction (Fig. 3). Thus if binding is strong enough, hydrolysis of suitably reactive substrates could easily ensue, as we found with the Diels–Alder reaction. Similarly hydrolysis of the 4-nitrophenyl ester (Fig. 2b) is understandable through binding and general base catalysis. Since other esters failed to react, it is possible that there is a favourable binding interaction with the nitrophenyl group also, or that it alone of the esters tested is sufficiently electrophilic to be hydrolysed by antibody C3. The discovery of hydrolysis by antibodies should therefore be relatively common. Indeed Paul et al. [13] has recently reported observations to suggest that some human autoantibodies to vasointestinal peptide catalyse its hydrolysis with some selectivity; similar observations that antibodies catalyse hydrolysis reactions had also been made ten years ago [14, 15] but their significance was not clear. These observations of hydrolysis, and ours too, have little to do with the original concept of transition-state analogues promoting the formation of a catalytically active site in an abzyme; if this hypothesis is correct, it will be an intrinsic property of antibodies, and indeed all proteins, provided that there is sufficiently potent binding.

**Conclusion**

In a sense, the discussion on hydrolysis is a diversion from the main thrust of work on catalytic antibodies. It does, however, underline the title of this article by adding to the unexpected results that are emerging in this field. The main direction of our work is to progress selected antibodies to the stage at which they become potentially useful catalysts in preparative chemistry. This will require a continuing contribution from chemistry, immunology, and molecular biology.

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