**Abzymes**

Molecular Enzymology Group/Industrial Biochemistry and Biotechnology Group Colloquium Organized and Edited by R. O’Kennedy (Dublin City University). 647th Meeting held at the University of Sheffield, 20–23 July 1993.

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**Polyclonal catalytic antibodies**

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**Introduction**

In 1969, Jencks indicated [1] that it should be possible to generate a catalyst by using a hapten that resembles a transition state of a reaction to elicit antibodies. Shortly after this, Raso and Stollar began to communicate investigations into the generation of catalytic antibodies. They initially reported [2] the synthesis of N-(5-phosphopyridoxy1)-3-amino-1.-tyrosine (I, see Figure 1), an analogue of the various coenzyme-substrate complexes involved in pyridoxal-phosphate-dependent enzymic transamination of tyrosine. Later, they described [3] the production and characterization of rabbit polyclonal antibodies elicited by an immunogen prepared from the analogue (I) coupled to a carrier protein through the 3-amino group. It was argued that the analogue (I) resembles the transition state for the formation of a Schiff base between tyrosine (II) and pyridoxal phosphate (III), because C-4 is tetrahedral, as it is in the intermediate hemiaminal. The antibodies did not accelerate Schiff base formation, and this disappointing conclusion to a long and conscientious programme may have discouraged others from investigating polyclonal antibodies for catalytic activity. It ought to be recorded, however, that Raso and Stollar found that transamination of tyrosine by pyridoxal phosphate was accelerated by their antibodies. The effect was small and it was suggested that the antibodies may accelerate a step after Schiff base formation.

In 1986, two groups (Tramontano et al. and Pollack et al.) independently described [4, 5] monoclonal catalytic antibodies. In each case monoclonal antibodies that bound to charged phosphorous transition-state analogues, catalysed the hydrolysis of corresponding O-acyl compounds. These exciting results stimulated extensive work in this new area. We decided to exploit expertise in the synthesis of immunogens and the production of polyclonal antibodies and in mechanistic enzymology that existed within the Medical College of St. Bartholomew’s Hospital to investigate the possibility of generating and characterizing polyclonal catalytic antibodies. Exploitation of existing expertise was one reason for our interest in polyclonal catalytic antibodies, other reasons were as follows. (1) Much discussion [6–9] of catalytic antibodies conveyed the importance of monoclonal antibodies for success. It seemed unreasonable to us that monoclonal antibodies would possess properties denied to polyclonal antibodies. (2) Nature uses polyclonal antibodies. (3) Polyclonal antibodies are simple and inexpensive to obtain, and are available to many more investigators than have access to monoclonal antibodies.

**Results**

The carbonate (IV) was designed [10] as a potential substrate for an antibody-catalysed hydrolysis reaction, and the corresponding phosphate immunogen (V) was designed as the charged tetrahedral transition-state analogue required for the generation of the catalytic antibodies. The nitrophenyl moiety provides a chromogenic leaving group during the hydrolysis of the carbonate and also an antigenic group in the immunogen. The second functionalized phenyl moiety was designed to maximize binding interactions particularly between the antibody and substrate. The phenyl ring was expected to provide strong hydrophobic interactions, and the amide to provide hydrogen-bonding interactions, with complementary groups in the antibody paratope. By using this strategy it was expected that binding interactions would be symmetrical across the target carbonate group, and would encourage binding of carbonate such that tetrahedral geometry
Structures of I Raso and Stollar’s analogue of the transition state for the formation of the Schiff base between tyrosine and pyridoxal phosphate, II tyrosine, III pyridoxal phosphate, IV the carbonate substrate used in our investigations, V the transition-state analogue haptenic determinant used in our investigations (KLH, keyhole limpet haemocyanin), VI the immunogen used by Iverson to elicit rabbit polyclonal catalytic antibodies, and VII the substrate used by Iverson

at the carbonyl carbon was preferred. The amide in the immunogen results from the condensation of a carboxyl from a precursor phosphate and an amino group from a carrier protein.

Three sheep (numbered 270, 271, and 272) were immunized with the phosphate immunogen (V), and samples of antisera were collected at intervals over a two year period. Sheep were used because they provide large amounts of antisera, typically a sample of 300 ml may be collected, which provides sufficient IgG for 60 000 initial rate experiments. Cross-bred sheep were used because they possess hybrid vigour and give a good immune response. One sample of antiserum (from sheep 270, collected 22 weeks after commencing immunization) was selected for detailed characterization and was designated PCA 270-22. IgG was isolated by sodium sulphate precipitation followed by affinity chromatography on Protein G-Sepharose. The IgG was found [10] to catalyse the hydrolysis of the carbonate (IV). The catalysis, at pH 8 and 25°, obeyed Michaelis–Menten kinetics with a $K_{\text{m}}$ value of 3.34 µM and a lower limit for the value of $k_{\text{cat}}$ of 0.029 s$^{-1}$. This value for $k_{\text{cat}}$ assumes that the concentration of catalytic IgG is equal to the concentration of IgG. Since only a fraction of the IgG will be anti-phosphate IgG, and only a fraction of this will be catalytic IgG, a more realistic estimate of $k_{\text{cat}}$ might be 2.9 s$^{-1}$. The intrinsic catalytic activity of the polyclonal catalytic antibodies is considerable and surpasses most monoclonal antibodies that catalyse similar reactions. It seems likely...
that using polyclonal antibodies is a particularly effective way of surveying the immune response.

Enzyme contamination is a continuing anxiety when dealing with catalytic antibodies. This is so whether the antibodies are monoclonal or polyclonal. However, the above results were the first demonstration of Michaelian catalytic activity in a polyclonal antibody preparation, and additional experiments were conducted to determine whether the catalysis was indeed antibody-mediated or was the result of a contaminant enzyme. Catalytic activity has not been found in our laboratories in IgG isolated from non-immunized sheep nor in IgG isolated from sheep that have been immunized with a sulphone or a sulphone analogue of the phosphate immunogen. The catalytic activity of the IgG from sheep 270 is inhibited by the phosphate hapten that was used to generate the antibodies, and its catalytic specificity is typical of antibody specificity and not of enzyme specificity. Thus enzymes, including sheep serum enzymes, catalyse the hydrolysis both of the 4-nitrophenyl carbonate (IV) and of the isomeric 2-nitrophenyl carbonate. The IgG preparation catalyses the hydrolysis only of the hapten-congruent 4-nitrophenyl isomer. Each of the above results indicate that catalysis is antibody-mediated and, taken together, they provide convincing evidence that the observed catalysis is due to antibody and not to contaminant enzyme.

Samples of antisera (13) collected at intervals from each of the three sheep immunized have been assessed for their effect on the hydrolysis of the carbonate (IV). IgG isolated from every sample was found [11] to catalyse the hydrolysis and the catalytic parameters $k_{cat}$ and $K_m$ are shown in Table 1. These results have ameliorated earlier concern [12] that polyclonal catalytic antibody responses might be erratic. The variation in catalytic parameters in the above experiments is small – even between samples of IgG from different sheep. Moreover, the nature of the small variations is interesting. A plot of $k_{cat}$ against $K_m$ shows that for 11 of the 13 samples there is a correlation between $K_m$ and $k_{cat}$, such that $k_{cat}$ increases as $K_m$ increases (correlation coefficient, $r=0.87$). An increase in $K_m$ is indicative of the destabilization of antibody-substrate interactions, and this is correlated with an increase in the rate of catalysis. For the same 11 samples, the second-order rate constant $k_{cat}/K_m$ increases as $K_m$ increases with a correlation coefficient, $r=0.69$. An increase in the second-order rate constant is indicative of the stabilization of the transition state for the catalysed reaction, and therefore stabilization of antibody-transition-state interactions. Hence, for this subset of catalytic IgG, transition-state binding and catalysis improves as substrate-binding weakens. This is in agreement with the theoretical foundations of antibody catalysis – that catalysis results from differential binding of substrate and transition state.

Other investigators are beginning to be convinced of the merits of investigating polyclonal catalytic antibodies. Iverson recently communicated [13] the generation of rabbit polyclonal catalytic antibodies that were elicited by the phosphonium transition-state analogue (VI). These antibodies catalyse hydrolysis of the triaryl ether (VII).

**Conclusion**

In conclusion, our group has demonstrated [10] that it is possible to generate polyclonal catalytic antibodies, and that the characterization is straightforward. We have reported further [11] catalytic activity in every anti-phosphate IgG investigated so far, and this established the feasibility of generating a polyclonal catalytic antibody response routinely. Our results suggest that (1) a catalytic antibody response may be generated for therapeutic purposes by active immunization, and (2) polyclonal antibodies are a valuable resource for evaluating immunogens for their ability to generate catalytic antibodies.
The research outlined above resulted from a collaborative effort with Professor K. Brocklehurst at Queen Mary and Westfield College, University of London. I thank Professor Brocklehurst, Dr. Caroline S. Jackson, Dr. Mark Searcey, and other members of our research groups involved in the above investigations. I also thank Therapeutic Antibodies Inc. and the Wellcome Trust for financial support.


Received 2 August 1993

Catalytic antibodies: the rerouting of chemical reactions
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A considerable amount of effort has been focused on the development of efficient catalysts for the synthesis or modification of complex molecules. Two methodologies have, in general, been applied in these endeavours: protein- and synthetic ligand-binding catalysts. To attack such problems, we have recruited antigen-induced catalysts from the immune system [1]. These catalytic antibodies or 'abzymes' as they have been termed have generated an avalanche of 'forecasts and predictions' about their future applications, because, in principle, their only limitations are the designer's creativity and one's ability to access the repertoire of the entire immune system. Keeping these so-called predictions in mind, we have sought out a truly formidable test for our de novo catalyst programme: the creation of catalytic antibodies that have no enzymic or synthetic catalyst equivalent (see [2] for an account of our previously described work).

Catalysts for ring-forming reactions
Ring-forming reactions are important and common processes in organic chemistry. Almost 20 years ago, J. E. Baldwin supplied a specific set of 'favoured and disfavoured' rules for certain closings of 3–7-membered rings [3–5]. The physical basis of these guidelines was formulated based on the stereochemical requirements of the transition states for the various ring-closure processes, with favoured pathways being those in which the length and nature of the linking chain enable the terminal atoms to achieve the proper geometry for reaction, and disfavoured cases requiring severe distortion of bond angles. Many cases in the literature are in substantial accord with Baldwin's rules, both experimentally and theoretically [3–6].

The skeletal structures of a number of bioactive marine natural products are found to contain O-heterocyclic rings [7–9]. Of particular importance are the ubiquitous tetrahydropyrans, towards the synthesis of which much work has been done [10–12]. One attractive strategy reduces to a regioselective 6-endo-tet ring opening of an epoxide by an internal nucleophilic oxygen (Scheme 1). However, conventional considerations (Baldwin's rules) suggest that the 5-exo-tet mode of cyclization is the preferred pathway (Scheme 1). Indeed, experimental evidence confirms this, since the tetrahydrofuran system is the product that is observed exclusively. A specifically tailored catalytic antibody could provide a means for obtaining the desired, yet disfavoured 6-endo-tet adduct. Hence, a catalytic