Exploring Biocatalysis Using Catalytic Antibodies

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Abstract: Antibodies with catalytic properties can be obtained using stable transition state analogs (TSA) of chemical reactions as immunogens. However only a few of the antibodies generated against a given TSA turn out to be catalytic. Identifying these catalytic antibodies requires an efficient screening protocol for catalysis. Many of the screening methods developed for assaying catalytic antibodies turn out to be generally useful for assaying enzymatic activities with high-throughput.

Keywords: Catalytic antibodies · Fluorescence assay · High-throughput screening · Transition state analogs

Introduction

Enzymes are enjoying increasing popularity in the chemical industry as environmentally friendly, economical, and clean catalysts in applications ranging from laundry detergents and paper processing to fine chemical synthesis and diagnostic/research reagents. In all of these applications there is a strong demand for improving existing enzymes or for finding new ones [1]. At the same time the science of enzymology is addressing the fundamental problem of creating new enzymes. The goal here is to understand how these sophisticated catalysts might have appeared during evolution.

Massive screening of candidate enzyme libraries is perceived today as the most efficient way to find new enzymes. Screening in silico has an almost unlimited throughput. While it is well suited for identifying sequences coding for known enzyme types in genetic databases, this approach is currently impractical for de novo design due the lack of predictive value in current theories of enzyme structure and activity. Screening in vitro has the advantage of dealing with the real thing, but is limited to a much more modest throughput. As a consequence, diversity must be reduced and suitably focused by making appropriate choices at the outset of the experiment. These choices include the structural framework on which to build structural diversity, the selection pressure to apply to trigger the appearance of an active catalyst, and the assay to employ for identifying it.

In my laboratory at the University of Bern we are addressing the enzyme design problem by means of catalytic antibodies. Antibodies are proteins of the immune system. Antibodies have a common Y-shaped basic architecture supporting two identical antigen binding sites similar in size and shape to the typical active site of an enzyme, and which therefore provides a convenient framework on which to build a catalytically active protein (Fig.). Genes for antibodies have the ability to undergo genetic re-combinations, which can give rise to $10^{10}$–$10^{12}$ different antibodies differing in the structure of their antigen binding sites. This represents a significant source of structural diversity from which one can hope to derive a desired catalytic function.

The natural function of antibodies is to bind specific molecules, called anti-
gens, which are usually part of infectious agents. The so-called immune reaction is a small scale evolution/selection process by which B-cells (a type of cell of the immune system which makes antibodies) produce antibodies that bind an antigen are selected and amplified [2]. A selection pressure towards catalytically active antibodies can be exerted by using a stable transition state analog of a chemical reaction as immunogen. The idea follows the principle originally formulated by Linus Pauling that enzymes bind transition states while antibodies bind ground state molecules [3]. In generating antibodies that bind to stable structural analogs of transition states, one hopes that some will also bind the actual reaction's transition state, and thus catalyze the reaction.

In practice, the experiment proceeds as follows: For a given reaction, a transition state analog and a high-throughput screening protocol are first designed and the corresponding molecules synthesized. Laboratory mice are then immunized with a protein conjugate of the transition state analog following standard procedures. The antibody-producing spleen cells of the mice are isolated and fused with myeloma cells, which are cancerous immune cells with the ability to grow indefinitely in culture (immortal). The antibodies are the products of the resulting hybridoma cell lines, which are hybrid cells possessing both a gene for an antibody and the immortality of the myeloma cell line [4]. Binding of the antibodies to the transition state analog (TSA) can be verified using the standard ELISA, or Enzyme Linked Immunosorbent Assay [5], as used by all immunologists. In the context of catalytic antibodies, a high-throughput screen (HTS) for catalysis now enables the identification of the cell lines producing antibodies which display, in addition to the binding affinity to the TSA, the desired catalytic activity. The approach succeeds through a well-balanced combination of organic synthetic and biological expertise following the outline in Scheme 1. Catalytic antibody technology is quite efficient in delivering new catalysts, as evidenced by the many examples reported over the last 15 years [6]. Given the fact that one has to design and synthesize a stable transition state analog of a reaction to generate a catalytic antibody, the overall exercise may be considered as an enzyme design experiment.

### Nitroxyl Synthase Catalytic Antibodies

In a recent series of experiments, we were able to isolate several 'nitroxyl synthase' catalytic antibodies following immunizations using acridine derivatives 1–3 as antigens (Scheme 2) [7]. Our antibodies catalyze the release of nitroxyl from the heterobicyclic prodrug 4 by means of a retro-Diels-Alder reaction. The nitroxyl released can be converted in situ to nitric oxide, an important biological messenger, by reaction with the enzyme superoxide dismutase (SOD). We envisage that these antibodies could be used as research tools in biology as alternate sources for nitric oxide.

Catalysis of the retro-Diels-Alder reaction is thought to arise by shape complementarity between the reaction's transition state and the catalytic antibody's binding pocket, which is optimized by the immune reaction to recognize the transition-state analogs 1–3. Indeed the transition state geometry with regard to the dihedral angle $\alpha$ between the two aromatic rings flanking the reacting heterobicyclic core is at an intermediate angle between 109° in the substrate and 180° in the product. The corresponding angle in the transition state analogs 1–3 is around 140°, which is very close to the transition state value. It should also be noted that the reaction is 'abiological' and does not respond to any known biological or chemical catalyst apart from our catalytic antibodies. This illustrates that catalytic antibodies can provide catalytic solutions for new and yet unsolved catalytic problems.

In the course of these experiments we took advantage of the fluorescence of the second reaction product, anthracene 5, to test catalysis of the antibodies directly in cell culture. Catalysis testing is performed very early in the course of subcloning, a lengthy and costly procedure necessary to stabilize and purify the antibody-producing hybridoma to single, monoclonal cell lines. Most remarkably, the statistics and history of the experiment (only 0.1% of all identified cell lines turned out to produce catalytic antibodies) show that we would not have found any catalytic antibodies if we had followed the classical procedure of stabilizing about 20 monoclonal cell lines prior to testing catalysis. This series of experiments has convinced us that an efficient search for catalytic antibodies will

![Scheme 1. Experimental procedure for preparing catalytic antibodies.](image-url)
generally be possible for any given reaction only if a simple high-throughput catalysis assay is available, and has led us to address the problem of catalysis assays in the general context of enzyme screening.

Fluorogenic Substrates Based on Umbelliferone

Our current effort in the development of catalysis assays focuses on fluorogenic and chromogenic substrates, which are the easiest to use in a high-throughput format. We have developed a series of fluorogenic substrates in which umbelliferone is released by a β-elimination that occurs as a secondary process following the reaction being assayed. The secondary β-elimination is catalyzed by bovine serum albumin (BSA), an inexpensive protein. The first and prototypical example of this technique was an enantioselective assay for alcohol dehydrogenases using the oxidation alcohol 6, which leads to ketone 7 and umbelliferone 8 (Scheme 3) [8]. By using a pair of enantiomeric substrates, it was possible to assess the enantioselectivity of an enzyme for the oxidation step. By virtue of microscopic reversibility, the assay also predicts the stereoselectivity of the reverse reduction reaction.

Structurally related stereochemically pure fluorogenic polypropionate fragments allow us to assay aldolases following a similar scheme (Scheme 4) [9]. For example, substrate 9 is a fluorogenic substrate for the aldolase antibody 38C2 [10].

Hydrolactic enzymes are among the most abundant and the most desired catalysts for industrial applications. We have found that a variety of hydrolactic enzymes can be detected by fluorescence by using esters or amides of 1,2-diol 14 or 1,2-aminoalcohol 15 as test substrates (Scheme 5) [11]. Once 14 or 15 have been released by enzymatic hydrolysis, they can be oxidized in situ by chemical oxidation using sodium periodate. The oxidation reaction gives aldehyde 16, which then undergoes β-elimination to liberate the fluorescent product umbelliferone 8 as above. For example, penicillin G acylase catalyzes the hydrolysis of amide 10, lipases and esterases react with acetate ester 11, epoxide hydrolases cleave epoxide 12, and phosphatases hydrolyse bis-phosphate 13.
The key feature of these fluorogenic substrates is that the functional groups undergoing the catalytic reaction are unactivated and tend not to react spontaneously. They can be used without any problems at high temperature, extreme pH values or high buffer or protein concentration. This represents their main advantage in comparison to the usual carboxylic or phosphoric esters of umbelliferone or nitrophenol, which are sold as fluorogenic substrates but are highly reactive, quite unstable, and extremely prone to artifacts. Thus our substrates represent the first series of reliable fluorogenic probes to assay enzymes from extremophiles. They are also the first reliable probes to search for designed biocatalysts capable of cleaving chemically non-activated functional groups, as natural enzymes do.

Conclusion

Creating enzymes de novo represents the 'holy grail' for enzymologists. Meeting that goal not only requires an understanding of the working principles underlying molecular Darwinian evolution, but also an ability to translate these into practical experiments. Our work with catalytic antibodies and high-throughput screening methodologies contributes to the general effort towards de novo enzyme design, an important area of chemical biology.

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