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# Detection strategies for catalytic antibodies

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**Abstract**

This paper discusses the detection of antibody catalysis using soluble test substrates. Antibodies raised against a transition state analog of a chemical reaction typically show dissociation constants for the antibody–hapten complexes in the range of $10^{-9}$–$10^{-7}$ M. If hapten binding is transferred to catalysis as measured by the transition-state dissociation constant $K_{TS}=K_M/(k_{cat}/k_{uncat})=k_{uncat}/(k_{cat}/K_M)$, this corresponds to the concentration of antibody necessary to double the apparent rate of the reaction. This sets a lower limit for the detection of catalysis if no additional effects are present to induce catalysis. The concentration of antibodies in hybridoma cell culture supernatants (5–50 μg/ml) meets this requirement. The use of high-throughput screening (HTS) for catalysis together with ELISA to select hybridomas leads to the isolation of not only one, but also many catalytic antibodies. As examples, the application of HTS for catalysis using fluorogenic reactions to isolate retro-Diels-Alderase and pivalase catalytic antibodies useful for prodrug activation chemistry are discussed.
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*Keywords:* Antibody catalysis; Soluble test substrates; Transition-state analog

## 1. Binding, catalysis and the immune repertoire

Immunoglobulins are proteins of the immune system. Their genetic diversity, which is concentrated mainly in the complementarity-determining regions (CDR) that make up the antigen-combining site, is given by the recombination of gene sequences during B-cell differentiation. Although this diversity may be augmented by the occurrence of random somatic mutations during antibody maturation, the total theoretical diversity available is probably not larger that $10^{12}$ structures. The work on catalytic antibodies has been primarily to ask whether a particular catalytic activity can be found within this set of proteins, and the subject has been excellently reviewed (Schultz and Lerner, 1995; Stevenson and Thomas, 2000). The complete set of antibodies cannot be accessed practically. The search for catalytic antibodies therefore starts with the design of a hapten, usually a stable transition-state analog of a reaction, or a reactive compound, that is selected in order to challenge the immune system of an experimental animal to produce a subset of antibodies that can be expected to contain the expected catalyst, if it should actually exist within the repertoire of possible antibodies.

How should one choose a reaction for antibody catalysis? The first consideration deals with binding

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energies. Catalytic antibodies are typically raised against small molecule haptens that are transition-state analogs of chemical reactions. In the best cases, one might expect that all the binding energy available from the antibody–antigen interaction will be converted into transition state stabilization, expressed in the quantity $\Delta\Delta G^*$, which relates directly to the transition state dissociation constant $K_{TS}$ (Fig. 1). Thus, a typical antibody–antigen complex with a dissociation constant in the nanomolar range ($K_D = 10^{-9}$ M) would translate into a dissociation constant for the reaction's transition state of $K_{TS} = 10^{-9}$ M. For a one-substrate reaction, with a typical Michaelis–Menten constant of $K_M = 10^{-3}$ M, the specific rate acceleration that can be expected should then be $k_{cat}/k_{uncat} = K_M/K_{TS} = 10^6$, corresponding to lowering the transition state energy by approximately 8.4 kcal/mol. For the antibody-catalyzed reaction to be observable, one must set a lower limit to $k_{cat}$, which can be for example $k_{cat} = 1$ $min^{-1}$. From this results a lower limit of $k_{uncat} = 10^{-6}$ $min^{-1}$ for the spontaneous reaction to be studied. Thus, one is better served by looking at relatively reactive systems for catalysis.

The second consideration for targeting an 'antibody catalyzable' reaction concerns the actual structure of the compounds to be used as haptens. Antibodies from the immune repertoire are accessed in practice either using monoclonal antibody technology, or using phage display technologies starting either from an immunized animal, or from artificial immunizations. In all cases, the immune system operates by binding affinity in an aqueous environment, and this works best when driven by hydrophobic forces, which are strongest with aliphatic and aromatic substituents in molecules. It is both simplest and most efficient to design haptens, and the corresponding substrates in the cases of transition-state analogs of reactions, that carry aromatic groups. Indeed aromatic groups in the substrates also facilitate kinetic analysis of the reaction products, which is most often done by UV or fluorescence.

## 2. Screening and the theoretical detection limits

There are many more antibodies that will show binding affinity to a given transition-state analog than antibodies that will display actual catalytic activity for the target reaction. The impression that every single antibody binding to a given TSA tightly should also be a catalyst results from the simplification of catalysis as transition state binding along the one-dimensional reaction coordinate (Fig. 1). This view is correct in quantitative terms, but ignores the details

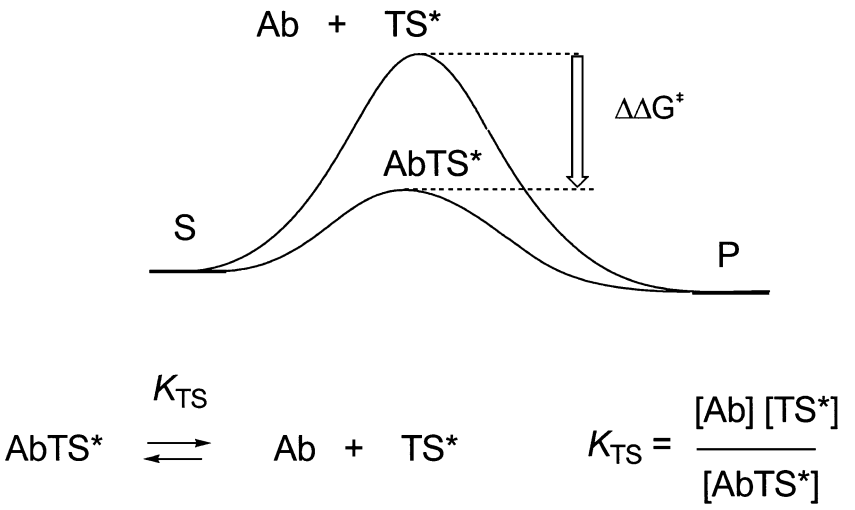



Fig. 1. Energy diagram for an antibody-catalyzed and uncatalyzed transformation of a substrate S to a product P proceeding via a transition state TS* or an antibody-bound transition state Ab.TS*. The catalytic effect of the antibody is given by $\Delta\Delta G^*$, which corresponds to the transition state dissociation constant $K_{TS}$.

and complexity of both ligand recognition and chemical catalysis. Having selected a reactive system, a suitable hapten structure, and an immunization technique, the question is therefore how to find the catalytic antibodies among the many hapten-binding antibodies. As for finding a needle in a haystack, *efficiency, sensitivity and selectivity are of decisive importance*. Here we discuss the problem of direct detection of antibody catalysis using soluble test substrates. The issue of catalyst detection sensitivity is considered first.

### 2.1. Highest sensitivity at lowest substrate concentration

As mentioned above, catalytic antibodies are most often set to operate with reactive systems, which show a certain level of noncatalyzed, background reaction. With the reasonable expectation that the target antibodies will display substrate saturation kinetics following the Michaelis–Menten model, the ratio of catalyzed to uncatalyzed reaction increases to a maximum for the substrate concentration approaching zero (Fig. 2). At the same time, however, the absolute rate of the reaction tends towards zero. Generally, antibody catalysis will thus be best assayed at the lowest possible substrate concentration where product formation is still detectable. This is somewhat counterintuitive, as one would expect that using high substrate concentrations might be necessary to saturate substrate-binding sites. The saturation of binding sites only makes sense for reactions that do not show any uncatalyzed components.

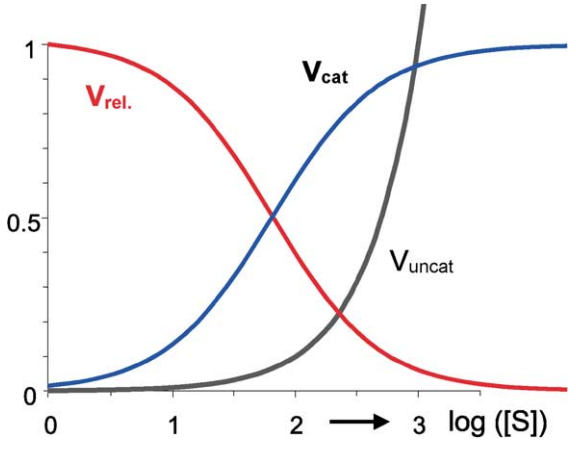



Fig. 2. Logarithmic scale representation of the catalytic rate $V_{cat}$, the uncatalyzed background reaction $k_{uncat}$, and the ratio of both $V_{rel}$, as a function of substrate concentration, for a one-substrate reaction following the Michaelis–Menten preequilibrium binding model.

### 2.2. Catalyst concentration and observed reaction rates

The second aspect of catalyst detection sensitivity concerns the concentration of potential catalyst available in solution for testing. The observed reaction rate under optimal conditions with the substrate concentration below $K_M$, is given by

$$V_{obs} = V_{uncat} + V_{cat} = k_{uncat} \times [S] + (k_{cat}/K_M) \times [Ab] \times [S] (\text{for } [S] \ll K_M)$$

$$V_{cat}/V_{uncat} = ((k_{cat}/k_{uncat})/K_M) \times [Ab] = [Ab]/K_{TS}$$

If the detection technique is precise and sensitive, one can expect to reliably detect as little as a doubling of the reaction rate, which sets a lower limit for the catalyst concentration in solution.

detection limit: $V_{obs} = 2 \times V_{uncat}$, thus $V_{cat} = V_{uncat}$

lowest concentration of catalyst: $[Ab] = K_{TS}$

Inspection of published kinetic data for catalytic antibodies shows that many catalytic antibodies have transition-state dissociation constants $K_{TS}$ around $10^{-7}$ M, which corresponds to specific rate enhancements of $k_{cat}/k_{uncat} = 10^3$ (Reymond and Chen, 1996). For antibodies having a molecular weight of approximately 70 kDa per binding site, a minimum screening concentration of 2 μg/ml = 2 mg/l is needed to detect that level of activity. Interestingly, the value of $K_M$ does not enter into this consideration as long as it is larger than the substrate concentration used, which usually will itself be larger than the amount of catalyst present. The concentration of monoclonal antibodies in hybridoma cell culture (5–50 μg/ml) will generally be suitable for screening catalysis. Recombinant expression systems, by contrast, usually do not yield such concentration for antibodies under nonoptimized conditions.

## 3. The proof of selective antibody catalysis

The issue of *selectivity* in screening concerns the problem of knowing whether an observed effect on the rate of a reaction has anything to do with the desired catalysis. The observation of a rate enhancement in a given antibody sample over the uncatalyzed reaction in an identical sample lacking the antibody does not imply that the observed effect actually stems from the combining site of the antibody, let alone from the antibody itself. Fortunately in the case of anti-hapten antibodies, a tight-binding inhibitor of the hypothetical catalytic site is available in the form of the hapten used for immunization. The observed catalytic effect *must* be inhibited by the hapten, and this can be tested immediately during screening. This is a simple, necessary condition to establish the origin of catalysis. Catalysis should also be verified in terms of multiple turnovers, one of the key problems encountered with antibodies being product inhibition.

The observation of an efficient catalysis (low $K_{TS}$) should generally be matched by a correspondingly high affinity for the hapten (low $K_i$). The correlation between catalytic efficiency and hapten binding has been observed in many catalytic antibodies (Stewart and Benkovic, 1995). Claims of catalysis far in excess of observed hapten-binding affinities are not ruled out a priori, but have to be analyzed with caution and traced back to precise chemical catalysis effects.

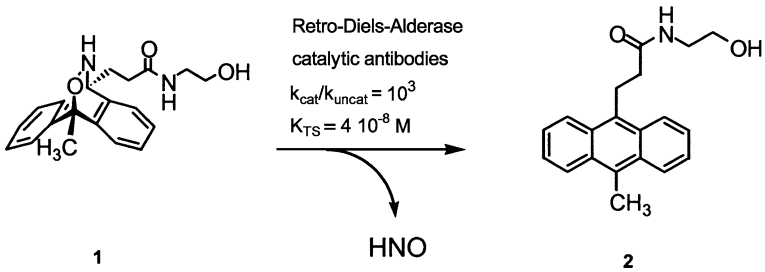
The observation of a tight-binding hapten further facilitates the proof of antibody catalysis once the antibody has been produced and purified since the catalytic activity can be cleanly titrated to the expected number of antibody combining sites. The quantitative inhibition is particularly useful in ruling out the effect of possible enzyme contaminants. Indeed, an enzyme catalyzing the reaction under study would also be expected to be inhibited quite well by the transition-state analog used as hapten, but would then possibly require much less than one equivalent of hapten relative to antibody binding sites for inhibition. The danger of enzyme contaminant is usually low when studying typical "abiological" reactions, which are also the most interesting from the point of view of enzyme design. However, enzyme contamination can be very problematic when studying typical enzyme-like reactions. The last proof of antibody catalysis is finally that the activity must also be observed when the sample is grown again and purified, either from the hybridoma cell line, or in recombinant form as a Fab-fragment.

## 4. Practical screening with antibodies from hybridoma

While the issues of detection sensitivity and selectivity can be dealt with generally to set the parameters necessary for finding catalytic antibodies, the issue of *efficiency of screening* for catalytic antibodies can only be approached in particular examples. We have concentrated our effort on screening antibodies produced in hybridoma using fluorogenic and chromogenic reactions. In principle, such reactions allow catalysis to be assayed rapidly and simply on almost any number of samples (Wahler and Reymond, 2001a,b).

### 4.1. Retro-Diels-Alderase antibodies

The retro-Diels-Alder reaction of bicyclic prodrug **1** releases nitroxyl, a precursor of the regulatory molecule nitric oxide, and the anthracene product **2** (Scheme 1) (Bahr et al., 1996). The reaction can be



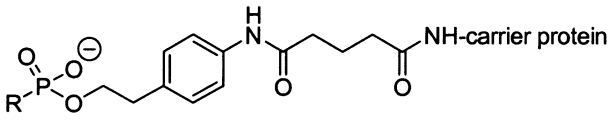

Scheme 1. Antibody-catalyzed retro-Diels-Alder reaction.

followed readily by fluorescence since the substrate is nonfluorescent, while the product **2** strongly fluoresces in the blue. This abiological retro-Diels-Alder reaction appears to be independent of pH between pH 4 and pH 10, as well as completely insensitive to buffer or co-solvent effects. At the same time, the reaction has a measurable spontaneous rate of $k_{uncat} = 10^{-5}$ $s^{-1}$. The reaction thus falls within the useful parameters for antibody catalysis. More importantly, the reaction is not catalyzed nonspecifically by proteins such as albumins, antibodies, or enzymes, and can therefore be measured directly in cell culture.

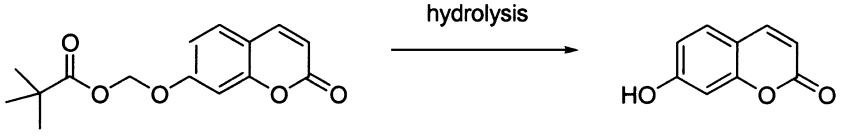
Using high-throughput screening by fluorescence, we were able to screen a total of more than 14,000 hybridoma cell culture samples generated from mice immunized against transition state analogs of the reaction, and isolate a total of eight catalytic antibodies for the reaction showing rate enhancement $k_{cat}/k_{uncat} = 10^2–10^3$ (Bensel et al., 1999). These rate enhancements fall within the typical range for catalytic antibodies, with $K_{TS}$ values ranging between 0.1 and 0.9 μM. One of the most surprising lessons from this high-throughput screening exercise was that even weakly active antibodies with $k_{cat}/k_{uncat} = 100$ were readily detected in cell culture. The most active antibodies have been studied in detail and their mechanism of action established on the basis of an X-ray crystal structure (Hugot et al., 2002).

### 4.2. Pivalase catalytic antibodies

Screening directly in cell culture is feasible as long as the reaction under study is not or cannot be catalyzed by contaminant enzymes or nonspecific proteins. We have recently isolated esterolytic antibodies cleaving pivaloyloxymethyl (POM) protected phenols, where this condition was not fulfilled (Bensel et al., 2001). Indeed, esterases may be present in cell culture samples, and be responsible for the occurrence of an observed catalytic effect. We undertook immunizations against phosphonate haptens **3**–**5** using the fluorogenic substrate **6** as test substrate, which releases the strongly fluorescent umbelliferone **7** upon hydrolysis (Scheme 2). Although the POM derivative **6** shows very little nonspecific hydrolysis in cell culture, it is potentially a substrate for esterases. Nevertheless, we undertook a first round of immunization and screening using direct detection of activity in cell culture, doubled with a requirement for strong hapten binding as detected by ELISA, and the condition of inhibition of catalysis by added hapten. This experiment led to the isolation of five monoclonal antibody cell lines showing specific, hapten-inhibited




**3** (R = t-Bu)
**4** (R = $CH_2Cl$)
**5** (R = $C_6H_5$)




**6** **7**

Scheme 2. Antibody-catalyzed hydrolysis of pivaloyloxymethyl (POM) ethers.

catalysis. However, purification of the antibodies showed that the activity did not bind to the protein-G antibody-affinity columns, and must have been caused by contaminant esterases. The inhibition of catalysis by the hapten was rather weak ($10^{-5}$ M) and did not match the strong affinity of the antibodies for the hapten as detected by ELISA, providing further evidence that the catalytic activity did not originate from the antibodies.

In the light of these experiments, we decided to establish a pre-purification procedure for antibodies that we could apply during the screening of hybridoma cell culture samples. Immunoglobulins present in 0.5–5 ml cell culture samples can be selectively bound by passing the sample through a very small amount of protein-G gel, which is simply filled in a Pasteur pipette (Fig. 3). The gel is washed with neutral buffer, and the antibody then selectively eluted using a small volume of acidic buffer. The eluted sample is neutralized and can then be assayed for specific, hapten-inhibited catalysis by comparing the rate of reaction of the fluorogenic substrate **6** in the present or absence of the haptens.

The protein-G pre-purification procedure can be used to manually purify up to 40 samples within 2 h. We used it to assay approximately 500 pre-clonal hybridoma samples generated from the different immunizations. Catalysis screening was done on hybridoma samples at a cell culture volume of approximately 5 ml, just before the first sub-cloning event. Sixteen cell lines showing selective, hapten-inhibited catalysis were detected and cultured further. All the antibodies were successfully sub-cloned twice. Eleven showed confirmed, hapten-inhibited catalysis after expression and purification. Interestingly, we found that it was necessary to test for catalysis not only after the first identification of catalysis, but also after the first and second sub-cloning event. Indeed sub-clones from an initial catalytic hybridoma were often found positive on ELISA, but negative on catalysis, while others were positive both on ELISA and on catalysis. The "pivalase" catalytic antibodies isolated are the

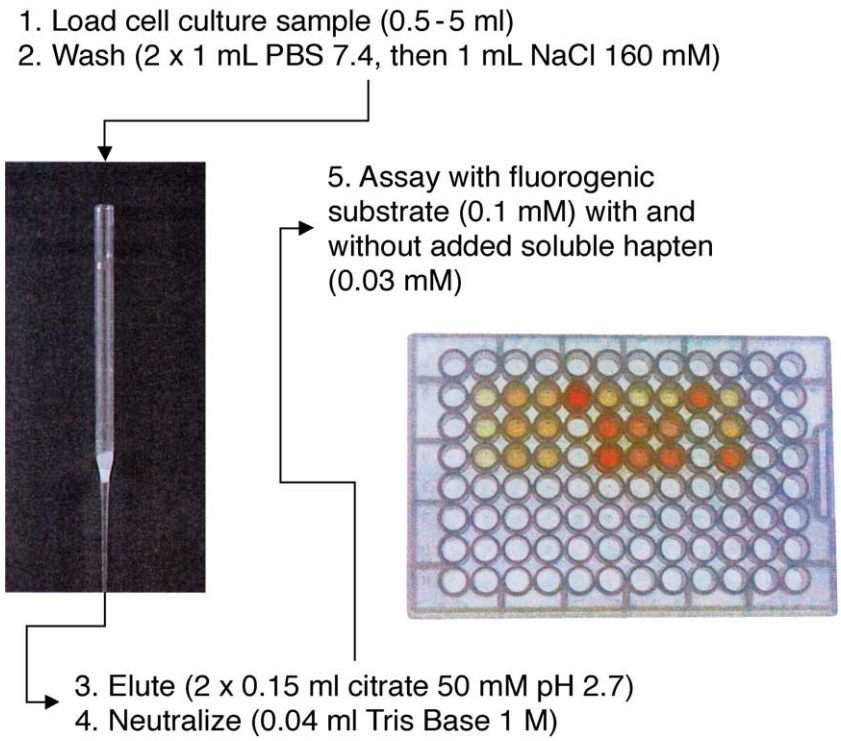



Fig. 3. Antibody prepurification procedure for assaying hybridoma cell culture samples.

first antibodies capable of hydrolyzing pivalate esters, which are sterically demanding substrates. The antibodies all show rate enhancements in the range of $k_{cat}/k_{uncat} = 10^3$. In these cases, inhibition by the soluble hapten is strong and quantitative, and matches perfectly the hapten affinity as measured by ELISA.

## 5. Conclusions and outlook

Once a catalytic antibody for a given reaction has been isolated, the screening method that has been used to find it becomes irrelevant. Many catalytic antibodies have been found for reactions that are rather difficult to assay and require HPLC separation of products. Here, we have discussed the use of fluorogenic assays for detecting catalysis, assuming that catalysis should arise to the extent of the hapten-binding affinity. The occurrence of catalysis beyond the hapten-binding effect can be observed in some cases, for example by using reactive immunization (Wirshing et al., 1995; Wagner et al., 1995).

In both experiments discussed above, we used the potential of high-throughput screening from the start by generating antibodies not only from the immunized mice showing the strongest immune titers, but from all mice utilized from immunization, which represented between 8 and 12 animals. The benefits of this approach are not to be underestimated. For example, the pivalase catalytic antibodies were all isolated from 1 out of the 12 mice used for screening. Thus, the HTS approach allowed us to overcome the risk present in the variability of the biological part of the experiment.

Furthermore, we were able to find not only one, but several catalytic antibodies of comparable potency. Clearly, fruitful combinations of haptens and reactions, when they occur, can be detected by combining catalysis screening with ELISA as a criterion for selecting hybridoma. The implementation of this combined approach requires simply a good coordination between the immunologists and the chemists involved in the project, and succeeds best when both activities are carried out in the same laboratory.

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