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Catalytic Mechanism of an Abzyme Displaying a Beta-Lactamase-Like Activity

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Abstract

A catalytic IgG (Ab2) displaying a beta-lactamase-like activity was previously obtained by using the antiidiotypic pathway: the particularity of this antibody is that it is a true antiidiotype of the beta-lactamase active site. We have previously demonstrated that this IgG has retained some of the structural information displayed by the beta-lactamase active site, evident from data that polyclonal anti-Ab2 antibodies (Ab3) recognize beta-lactamase. In this article, we investigated the catalytic mechanism of the abzyme compared to that of the enzyme. The experimental data, allow us to draw hypothesize the catalytic residues required for catalysis.

Index Entries: Beta-lactamase; antiidiotypic antibody; abzyme; catalytic antibody; suicide substrate.

Introduction

The development of highly efficient catalysts by rational design represents one of the most crucial efforts of chemistry today. Since Linus Pauling's postulate *(1)*, the tremendous potential of the immune system—especially in regard to diversity—has been exploited to reach this goal *(2)*. The idea that antibody-binding sites possess a virtually endless repertoire and may consequently be catalytic derives from data concerning the variability of the immune response. The pool of 10^{10}–10^{12} antibodies provided by the immunization process offers many possibilities for the selection of efficient and highly selective catalysts for a broad range of reactions. Today, the main consideration of chemists is the geometric or electrostatic features of the hapten used to induce these catalysts *(3)*. Since the first attempts *(4)* and successes *(5,6)*, considerable progress has been made in various experimental systems, leading to more catalytically efficient molecules.

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Hundreds of reactions were thus performed. At this stage of the development of the substrate-based approaches, the question arises about of how antibodies, which are naturally made for achieving high-affinity binding during the short time of immunization, could reach the efficiency of enzymes, which are selected for catalysis over billions of years of evolution. Many solutions were proposed: reactive immunization *(7)*, engineering of binding sites *(8)*, emergence of selection or screening methods *(9)*, and development of an enzyme-based approach *(10)*.

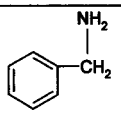
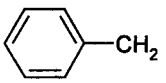
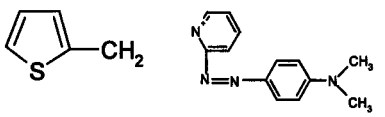
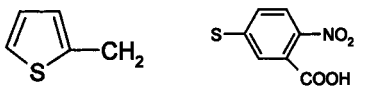
This latter approach is an emanation of Jerne's theory *(11)* concerning the possibility of producing second-generation antibodies (antiidiotypic antibodies) resembling the inducing antigen. This concept was applied to obtain antiidiotypic antibodies that mimic the enzymatic function. In this way, we have obtained an antiidiotypic IgG displaying a beta-lactamase-like activity *(12)* with hydrolytic activity that is decreased 10^5 compared with the initial enzyme. This article aims to investigate the catalytic properties and mechanism of the abzyme through reference to beta-lactamase and to discuss the possibilities of improving or modifing its activity.

Features of Beta-Lactamase

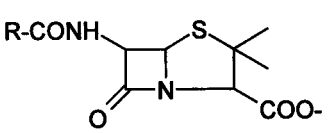
Beta-lactamase from *Bacillus cereus* was chosen as the model enzyme for the generation of antiidiotypic catalytic antibodies. This enzyme is the key element in bacterial resistance to antibiotic treatments in infectious diseases. In the early 1940s, pioneering observations indicated that adding penicillin to a bacterial culture caused irreversible damage to the external membranes of bacteria. Further investigations showed that penicillin is an inhibitor of cell-wall synthesis. Beta-lactamase catalyzes the hydrolysis of penicillins and other molecules containing a beta-lactam structure by cleaving the endocyclic amide bond. Class A beta-lactamases contain an essential active-site serine. Hydrolysis of beta-lactam antibiotics involves the formation of an acyl-enzyme intermediate. A mechanistic scheme based on the three-dimensional structure of beta-lactamase from *Streptomyces albus* G was proposed in 1991 *(13)*. According to this model, the active serine and an alanine residue are involved in the polarization of the carbonyl bond, as well as in the stabilization of the transition states, thus forming an oxyanion hole comparable to that observed in proteases. The substrate hydrolysis is suspected to be performed by four residues (Ser 70, Lys 73, Ser 130, Glu 166) and two water molecules. The formation of the acyl-enzyme intermediate results from both the cleavage of the amide bond and the transfer of the proton of Glu 166 toward the nitrogen atom of the beta-lactam.

We studied the beta-lactamase activity on four substrates: two penicillins (benzylpenicillin and ampicillin) and two cephalosporins (PADAC and CENTA). Results are shown in Table 1. As for many other enzymes, catalysis by beta-lactamase is influenced by the ionization state of essential amino-acid residues. The variation of K_m and k_{cat} values as a function of pH is shown on Fig. 1.

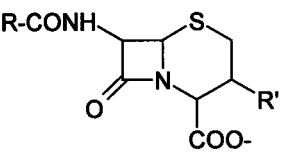
Table 1
Catalytic Parameters of Beta-Lactamase from *B. cereus*[a]

Substrate	R	R'	K_m (μ*M*)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}/min)
Ampicillin		-	200	282 000	1410
Benzylpenicillin		-	121	44 000	365
PADAC			22	753	34
CENTA			25	2153	86

Penicillins



Cephalosporins



[a]Hydrolysis of benzylpenicillin and ampicillin was followed at 240 nm, while the hydrolysis of PADAC and CENTA was measured at 566 nm and 340 nm respectively. In all cases, measurements were performed in 100 m*M* phosphate buffer pH 7.4, 20°C, in the presence of 2.3 n*M* beta-lactamase.

The activity of the enzyme is clearly influenced by pH. This aspect of catalysis by beta-lactamases is related to two essential residues involved in catalysis *(14,15)*: Glu 166 and Lys 234.

Catalytic Properties of IgG 9G4H9

Our goal was to generate antibodies displaying a beta-lactamase-like activity by antiidiotypic reaction. To accomplish this, we first immunized BALB/c mice with 40 μg of beta-lactamase, and then screened for specific antibodies through ELISA tests. Finally, we selected an antibody to recognize the active site of the enzyme specifically (IgG 7AF9). IgG 7AF9 turned out to inhibit the enzyme activity in molar excess conditions. At a ratio IgG: enzyme = 200:1, the inhibition reached 60%. Several competition assays were performed to determine whether IgG 7AF9 recognizes the

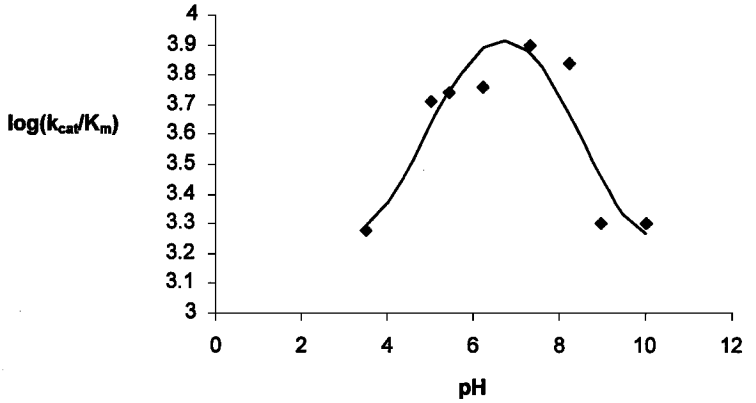


Fig. 1. Kinetic behavior of beta-lactamase from *B. cereus* as a function of pH in the presence 0.5 m*M* benzylpenicillin in 100 m*M* phosphate buffer. Final concentration of enzyme is fixed at 2.3 n*M*.

Table 2
Catalytic Parameters of Hydrolysis of Ampicillin and PADAC by IgG 9G4H9[a]

Substrate	K_m (μ*M*)	k_{cat}/K_m (μM^{-1}/min)	k_{cat} (min^{-1})	k_{cat}/k_{uncat}
Ampicillin	2000	0.5×10^{-3}	0.9	16,000
PADAC	10	2.3×10^{-4}	2.3×10^{-3}	20

[a]Hydrolysis was followed spectrophotometrically at 240 and 566 nm, respectively, in the presence of 100 m*M* phosphate buffer pH 7.4 at 20°C, using 0.38 mg/mL IgG and 1.2 mg/mL IgG, respectively.

substrate-binding site of the enzyme. These tests (RESIA, DIBCO, and RESSU) (described in *16*) confirmed that substrates and IgG 7AF9 compete for binding of at least a part of a common site in beta-lactamase.

For these reasons, we chose IgG 7AF9 as the idiotype to induce the production of antiidiotypic antibodies (Ab2) displaying the beta-lactamase activity. Biozzi mice were immunized with 200 μg of 7AF9 according to standard procedures. Monoclonal antibodies (MAbs) were produced and screened for both 7AF9 recognition and benzylpenicillin hydrolysis ability.

One IgG turned out to be catalytic: IgG2bk 9G4H9. Kinetic parameters are summarized in Table 2.

In order to compare the catalytic activity of the antibody with that of the enzyme, and to define the residues presumably involved in catalysis, we studied the variation of activity as a function of pH (*see* Fig. 2). The pH data are consistent with, but do not prove the involvement of, an acidic residue in the catalytic mechanism. The catalytic parameters of antibody β-lactamase activity could not be accurately determined in alkaline con-

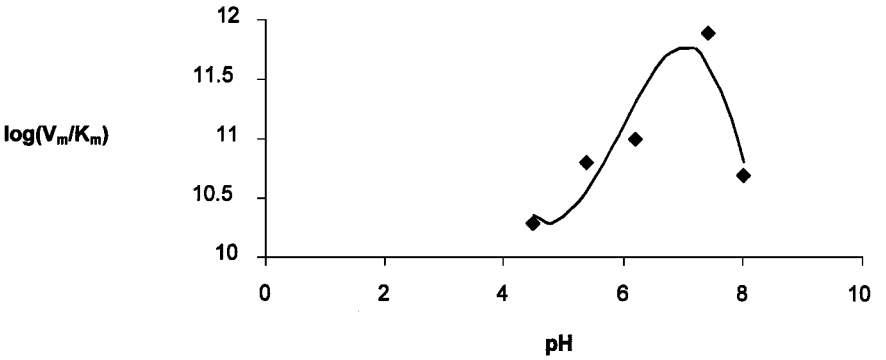
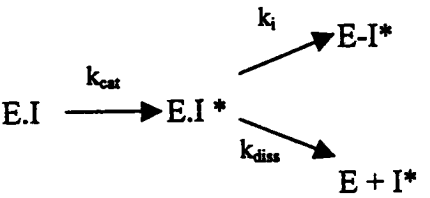


Fig. 2. pH-dependence of ampicillin (0.5 m*M*) hydrolysis by the abzyme in 100 m*M* phosphate buffer at 20°C. The abzyme concentration was fixed at 0.38 mg/mL.

ditions, since a slight precipitation of the antibody preparation occurred at pH higher than 9.0.

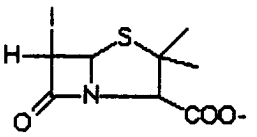
Mechanistic Investigations

As we studied more precisely the kinetic behavior of the abzyme, we observed that the activity plots presented two phases: the first one is rapid and short, and the second one is slow and long. This kind of plot is typical for a mechanism-based irreversible inhibition, in which the substrate reacts with the antibody according to the following scheme:



Ampicillin may be thus considered as a substrate that deacylates slowly, i.e., a suicide inhibitor. According to this statement, ampicillin may react with the antibody-binding site by forming a covalent bond with a potential nucleophilic group. No change of the antibody activity was observed when 400 μg/mL of antibody was preincubated with 1 m*M* iodoacetamide for 30 min, arguing against the involvement of a Cys in the mechanism. If a nucleophilic mechanism is involved, it is possible that a serine hydroxyl is a part of the reactive center, in view of the participation of this group in other acyl hydrolases.

Attempts to inhibit the abzyme with by β-iodopenicillanate **1** were carried out. This molecule is known to inhibit class A β-lactamases *(17)*. No difference was observed between the activity of the abzyme with or without 0.1 m*M* of β-iodopenicillanate.



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Discussion

Many studies have shown that the idiotypic network offers the possibility to make a molecular imprint of a specific region of a protein *(18)*. We have previously demonstrated that a functionality as complex as catalysis may be mimicked by this pathway *(19)*. Comparison of the catalytic properties of the enzyme and the abzyme has allowed us to obtain better knowledge about the relationship between structure and function of the catalytic site. Data collected with the antibody 9G4H9 are consistent with the involvement of an acidic residue in catalytic mechanism. These results—together with the possible involvement of a serine hydroxyl as part of a nucleophilic mechanism—suggest that although the abzyme mechanism differs from that of the enzyme, it could involve at least two residues required for the enzymatic activity: an acidic and a nucleophilic residue. The cloning and sequencing of the antibody should allow us to define to what extent the structural information in the enzyme was transferred to the abzyme, either as primary-sequence identity or as conformational mimicry.

Attempting to improve the catalytic activity of 9G4H9 should fulfill the challenge presented by our results. Several routes of random mutagenesis can be foreseen to this end—e.g., random mutagenesis by error-prone PCR *(20)* to introduce mutations throughout the cloned gene, or DNA shuffling to get a larger size of library *(21)* and generate a diverse repertoire of mutants. Phage display will allow us to efficiently select variants with new β-lactamase activity and substrate specificity. These studies, complemented by crystallographic data and chemical modifications, will improve the knowledge of molecular parameters involved in β-lactam recognition and in β-lactamase activity.

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Discussion

Koengten: How can your approach be used to get catalytic antibodies for which enzymes already exist? How would you get a proteolytic antibody if there is no corresponding enzyme?

Thomas: This may appear a limitation when compared with the approach using transition-state analogs. But not only can you produce antibodies against enzymes, you can also produce antibodies against chemically modified enzymes. For instance, we made antibodies to a chemically modified lactamase, and we got some activity that is not similar to what exists in nature.

Vijayalakshmi: We are also trying to use metal-chelates as antigens and to produce metal-chelating antibodies and then try to create metalloenzyme active-site structures.

Rodkey: It was not clear to me that your Ab2 showed some activity in serum. Have you cloned an Ab2 that is catalytic?

Thomas: For acetylcholinesterase we made polyclonal antibody and monoclonal antibody. Sasha Gabibov is preparing recombinants. For me, a critical point after cloning is that the separate heavy chains and light chains have no activity, but after reassociation of the Fab, there is activity.

Rodkey: Did you try to reassociate the light chains with a different heavy chain or the heavy chain with a different light chain?

Thomas: We did not try this, but we are now working with Sasha on these experiments.

Paul: I think your approach has the potential of becoming a major route to catalytic antibodies. I want to ask about the concepts underlying the approach. You spoke about the new kinetic parameters and new specificities. If I understand correctly, you create a replica of the enzyme-active site in the antibody. Any new properties that the antibody acquires which are different from the enzyme must be caused by errors, because the replica is not exactly like the antibody. How then can you expect to get specificities or activities greater than the enzyme? A lower specificity than the enzyme itself will be understandable, on the other hand.

Thomas: I think you are wrong in calling it an error-based approach. When you raise an antiidiotype and it elicits a second antiidiotype, sometimes the second antiidiotype will resemble the enzymes, and sometimes it will contain only parts of the active site or related active sites. This does not necessarily mean lower specificity or activity. In fact, there is a very big variabililty when you produce Ab1 and Ab2, and the key point is how you screen. Once we get an Ab1 with a good inhibitory effect, it is possible to generate many different Ab2 variants. You can get Ab2 with no similarity of structure to the enzyme, and other Ab2 molecules had no similarity to the enzyme structure. There is a distribution, and the outcome is a result of the screening that is applied.

Paul: Perhaps I should define the term "error." It is sequence and a three-dimensional structure different from the enzyme. Now let's go one step further. If you ask for an improvement in the catalytic function or a superior specificity, it is necessary to apply an immunological pressure to improve the function. Otherwise, you leave it to pure chance. Yes, I agree with you that there will be a whole lot of antibodies, and most will be poorer—and the rare one may be better than the enzyme.

Thomas: I can only state that what is positive or not is a question of definition. As far as were concerned, we were looking for less specificity. For people in the enzyme technology field, the enzymes existing in nature are too specific.

Kohler: I just wanted to comment about the technological uses. I always thought one of the most interesting uses of catalytic antibodies is to accelerate reactions for which there are no enzymes. Sudhir, trying to answer your concern, I think from antibody crystal structures we can know that

idiotypic mimicry depends on the similarity of side-chain contacts and not shapes or chemical similarity. So the kind of contacts between the enzyme and Ab1 are mimicked by the Ab2-Ab1 complex. I think in one published study there were 16 or 18 contacts originally in the antigen–Ab1 complex, and the Ab2 made 14 contacts. So there are differences. Concerning the idea of using catalytic antibodies for therapy, I think we should be very careful, because we are opening a new field of potential toxicity. When you deal with catalytic antibodies, you have to control for two potential toxicities. One is toxicity due to the classical antibody binding. Second, you have the potential for toxicity because of the catalytic activity. So, we have to control two biological activities to avoid toxicity.

Thomas: As far as safety and regulation are concerned, it is simpler to inject an antibody than to inject transition-state analog. People are already using antibodies for clinical application. It's a new application, so of course we have to check the efficacies and toxicities.

Kohler: Yes, but Sudhir will tell you that naturally occurring catalytic antibodies are correlated with disease. That may already tell you something—these antibodies are very dangerous.

Gololobov: I think the antiidiotypic approach has some similarity to the transition-state analog approach. There are certain limits in the design of transition-state analog so you cannot get rate accelerations greater than a certain limit. A limit is also imposed on the antiidiotypic approach because you presumably cannot do better than the enzyme you are trying to mimic. When a better catalyst is found, it is probably something that originates from the antibody genes. We call it an unexpected activity when the expected rate enhancement is 10^3, but the observed enhancement is 10^7. Then it emerges that the antibody has a serine-protease type of active site, which is completely against the design of the immunogen. Even in the antiidiotypic approach, when you find a good catalyst, the structural elements may already preexist in antibodies.

Thomas: What you say applies nicely to the innate aspect of antibody catalysis. But I think in the antiidiotypic approach, unlike the transition-state analog approach, we are trying to introduce into the structure of the antibody mechanisms discovered over evolution by the enzyme. The relationship between an enzyme and antibody is closer in our approach than in the classical approach. But I agree fully with Professor Sela—that the main goal is to obtain something not existing in nature.