A suicide-substrate mechanism for hydrolysis of β-lactams by an anti-idiotypic catalytic antibody

Séverine Lefevre, Hélène Debat, Daniel Thomas, Alain Friboulet, Bérangère Avalle*

Génie Enzymatique et Cellulaire, UMR 6022 CNRS, Université de Technologie de Compiègne, P.O. Box 20529, 60205 Compiegne Cedex, France

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Abstract The catalytic mechanism of an anti-idiotypic antibody, 9G4H9, displaying a β-lactamase activity was investigated. Kinetics experiments suggest that some penicillinic derivatives behave both as substrates and inactivators. Biochemical and immunological experiments strongly indicate that ampicillin may be regarded as a suicide substrate for hydrolysis by 9G4H9. The anti-idiotypic network appears as a way to create enzyme mimics with modified catalytic activities.

Key words: Suicide substrate; β-Lactamase; Anti-idiotypic antibody; Catalytic antibody; Abzyme

1. Introduction

Complementarity rules that govern antigen–antibody interactions were extensively exploited to elicit antibodies with desired properties. In 1974, N. Jerne has suggested that anti-idiotypic antibodies possessing internal image properties may regulate the immune response [1]. This property has led to consider anti-idiotypic antibodies as surrogate antigens. Therefore, many molecular functions were shown to be mimicked by anti-idiotypic antibodies. In particular, we have previously demonstrated that catalytic anti-idiotypic antibodies can be obtained [2]. Recently, an anti-idiotypic monoclonal IgG, 9G4H9, generated against an antibody recognizing the active site of β-lactamase was obtained that displays a β-lactamase-like activity [3]. β-Lactamases are key elements of bacterial resistance to antibiotics. They catalyze the hydrolysis of molecules owing a β-lactam cycle by cleaving their endocyclic amide bond. Class A β-lactamases function with an essential serine residue. The role of some other amino acid residues is still dealt with controversy, but the hydrolytic mechanism of antibiotics by β-lactamases is well known (for review, see [4]). The catalytic mechanism of 9G4H9 monoclonal antibody was thus investigated to elucidate the extent of functional similarities between the original enzyme and its mimicking counterpart anti-idiotypic abzyme.

*Corresponding author. Fax: (33)-3-44 20 39 10.
E-mail: avalle@utc.fr

Abbreviations: PBS, phosphate-buffered saline; BCA, bicinchoninic acid; ELISA, enzyme-linked immunosorbent assay; ABTS, azino-bis(ethylbenzthiazoline sulfonate); TBS, tris(hydroxymethyl)aminomethane-buffered saline; PBP, penicillin-binding protein

2. Materials and methods

2.1. Preparation of antibody

Ascitic fluids from mice injected with hybridoma cells were fractionated by FPLC affinity chromatography on HiTrap rProtein A (Pharmacia Biotech). Binding and elution buffers were ready made and used according to commercial instructions (Bio-Rad). The solutions were dialyzed overnight against phosphate-buffered saline (PBS: 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 140 mM NaCl), and centrifuged in MicrosepTM tubes (Pall Filtron – 30 kDa) at 8000 rpm for 35 min. The antibody concentration was evaluated by a bicinchoninic acid test (BCA-Sigma) as detailed by the manufacturer's instructions.

2.2. Kinetic measurements

Hydrolysis of ampicillin (Sigma) by IgG 9G4H9 or by β-lactamase A was spectrophotometrically followed at 232 nm in 100 mM phosphate buffer pH 7.4. For inhibition experiments, the product of hydrolysis was obtained after heat treatment of 10 mM penicillin G at 70°C during 3 h. The composition of the resulting product was controlled by electrospray ionization (ESI) mass spectroscopy (Finnigan SSQ 710, San Jose, CA, USA).

2.3. Recognition of penicillin by 9G4H9

Binding of biotinylated penicillin to 9G4H9 was evaluated by enzyme-linked immunosorbent assay (ELISA). Each step was followed by three washes in 0.05% Tween–PBS. Microtitration plates were coated with 160 μg/ml of avidin during 1 h at 37°C in 50 mM carbonate buffer pH 9.6. Antibodies were incubated during 1 h at 37°C. Dilutions of antibodies were performed in PBS. The revealation of bound antibodies was carried out using peroxidase-conjugated goat anti-mouse IgG, incubated 1 h at 37°C, and followed by the addition of 0.55 mM azino-bis(ethylbenzthiazoline sulfonate) (ABTS) in 25 mM citrate buffer pH 5.0 containing 0.01% H_2O_2. The resulting absorbance was measured at 405 nm.

2.4. Western blotting

A Bio-Rad Mini-Trans-Blot apparatus with microcomputer electrophoresis power supply (Bioblock E752) was used for these experiments. The migration of samples in Laemmli buffer [5], in a 10% polyacrylamide gel electrophoresis (PAGE) was carried out in two steps: at 90 V in the stacking gel and at 150 V in the separating gel. The transfer on nitrocellulose membrane was done at 100 V and 250 mA for 1 h. For blocking, the membrane was twice incubated in 5% Milk–tris-buffered saline (TBS: 50 mM tris(hydroxymethyl)-aminomethane, 150 mM NaCl) during 30 min. After three washes in 0.05% Tween–TBS, the membrane was incubated with 1 μg/ml peroxidase-conjugated avidin during 1 h. Blots were revealed, after three washes, by incubating with 0.55 mM ABTS.

3. Results

3.1. Hydrolysis of β-lactamase substrates by 9G4H9

Monoclonal anti-idiotypic IgG 9G4H9 was previously described to efficiently catalyze the hydrolysis of penicillin and cephalosporin compounds [3]. Catalytic parameters measured

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for ampicillin hydrolysis $k_{cat} = 0.9$ min^{-1} and $K_M = 2$ mM clearly show a loss in efficiency when compared with the values measured with β-lactamase from *Bacillus cereus* (EC 3.5.2.6), $k_{cat} = 2.8 \times 10^5$ min^{-1} and $K_M = 0.2$ mM. However the catalytic efficiency measured with 9G4H9 is one of the highest when compared to elicited abzymes previously described to catalyze amide bond hydrolysis [6,7]. On the other hand, 9G4H9 clearly demonstrated to be a true anti-idiotypic antibody since polyclonal anti-9G4H9 antibodies (Ab3) were shown to recognize *B. cereus* β-lactamase [3].

Surprisingly, ampicillin and penicillin hydrolysis by 9G4H9 exhibits a decrease in activity during the course of the hydrolytic reaction, and is characterized by a multiple turnover pre-steady state burst before reaching an apparent steady state. This behavior could be explained either as a denaturation process of the antibody during the reaction or as a progressive inactivation of the catalytic activity by the substrate itself or even by the product of the reaction. However, no loss in activity was observed for 9G4H9 in the absence of catalytic reaction. When 3.3 μM 9G4H9 were preincubated with 1 mM ampicillin and then tested for catalytic activity, the burst observed for initial velocity of hydrolysis decreased with incubation time (Fig. 1). Moreover, the addition of substrate during the steady state does not induce a new burst in hydrolysis velocity. On the other hand, when 9G4H9 was preincubated with the product of hydrolysis of penicillin G, a very slight decrease in velocity was observed that could not explain the observed phenomenon. We have checked by mass spectroscopy analysis the purity of penicillinoic acid, the product of hydrolysis obtained by heat treatment. Taken together, these observations do not allow to explain the observed catalytic behavior of 9G4H9 by the denaturation of the abzyme during the reaction, neither by a reversible inhibition of the catalyst by the product of hydrolysis of the substrate.

These results suggest that ampicillin and penicillin G could act both as a substrate and as an inactivator of the abzyme like other suicide substrates. The observed behavior could thus be explained by a substrate-suicide mechanism involving concurrently the hydrolysis of the substrate or its covalent binding to the catalyst.

3.2. Characterization of an irreversible complex between 9G4H9 and a penicillin derivative

The hypothesis of a mechanism-based inactivation of 9G4H9 by some penicillinic substrates was tested by reacting

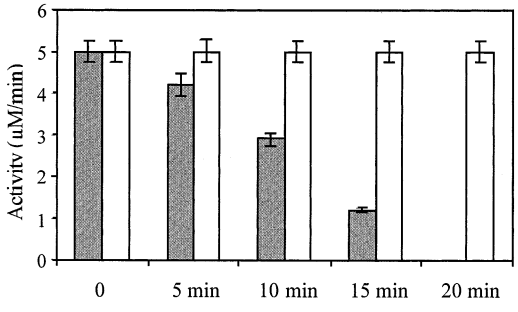


Fig. 1. Hydrolytic activity of 3.3 μM 9G4H9 pre-incubated with 1 mM ampicillin for different times (■). The control experiments (□) correspond to antibody incubated in 100 mM phosphate buffer pH 7.4. The value of the residual activity, measured in the presence of 850 μM penicillin G, is the mean of four independent determinations (± standard deviation).

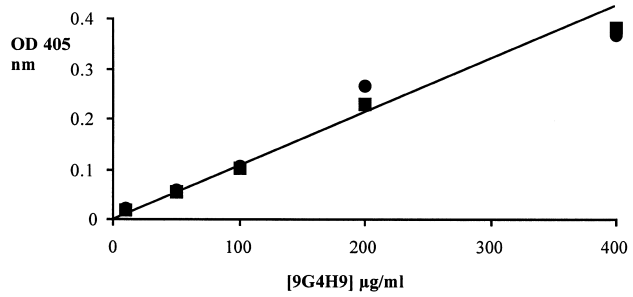


Fig. 2. Binding of 9G4H9 complexed with biotinylated penicillin on 160 μg/ml avidin. Various amounts of antibody incubated with 90 μM biotinylated penicillin were tested for their ability to bind avidin without (●) or with (■) competition by 1 mM non-biotinylated penicillin G. The values are the means of three independent determinations.

the abzyme with biotinylated penicillin. Increasing 9G4H9 concentrations were incubated with 90 μM biotinylated penicillin for 1 h at 20°C to allow irreversible inactivation to occur. The reacting medium was then transferred into microtitration wells previously coated with avidin in order to insolubilize all the species linked to biotin. The presence of insolubilized penicillin complexed to antibody was revealed using peroxidase-labeled anti-IgG antibodies (Fig. 2). In our experimental conditions, unspecific binding of 9G4H9 to avidin was never observed. On the other hand, by adding 1 mM non-biotinylated penicillin G for 15 min at 20°C to insolubilized 9G4H9, we never observed any release of the antibody. Consequently, no competition between biotinylated and non-biotinylated penicillin could be measured. This suggests that the 9G4H9 reaction with biotinylated penicillin forms either an irreversible or a tight binding complex.

To further characterize the irreversible complex formed by the catalytic reaction, Western blot experiments were performed. 9G4H9 was incubated with biotinylated penicillin for 1h and then tested on SDS–PAGE under reducing and non-reducing conditions. After transfer on nitrocellulose membrane, labeling with avidin-peroxidase clearly indicates that 9G4H9 forms an irreversible complex with biotinylated penicillin (Fig. 3). Electrophoresis under reducing conditions demonstrates that the residues involved in binding with penicillin are located on the light chain of the antibody. Once

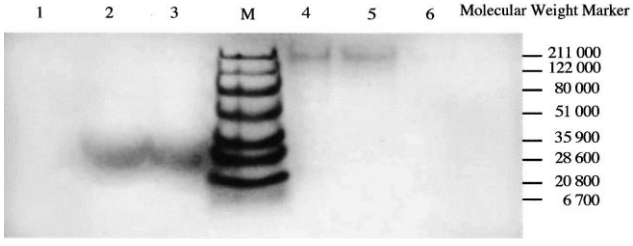
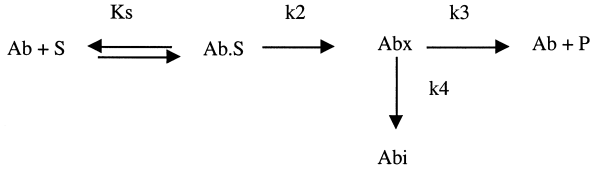


Fig. 3. Electrophoretic migration of 2.3 μM 9G4H9 under reducing (lanes 1–3) and non-reducing conditions (lanes 4–6). M = Molecular Weight Marker, lane 1: antibody incubated 1 h in phosphate buffer, 100 mM pH 7.4; lane 2: antibody incubated for 1 h with 36 μM biotinylated penicillin; lane 3: antibody incubated for 45 min with 36 μM biotinylated penicillin and for 15 min with 10 mM penicillin G; lane 4: antibody incubated with 130 μM biotinylated penicillin for 1 h; lane 5: antibody incubated for 45 min with 130 μM biotinylated penicillin and for 15 min with 10 mM penicillin G; lane 6: antibody incubated 1 h in phosphate buffer, 100 mM pH 7.4.

again, by adding 10 mM penicillin G to the labeled antibody, no release of the biotinylated marker was observed. These results reinforce the suicide substrate mechanism proposed for the hydrolysis of penicillin substrates by 9G4H9. The substrate hydrolysis could either form the product of the reaction or undergo an alternative mechanism that results in irreversible binding to the abzyme and thus inactivation.

3.3. Inactivation mechanism

The suicide substrate mechanism proposed for ampicillin interaction with 9G4H9 can be described by Scheme 1:



Scheme 1.

where Ab and Abi are the abzyme and inactivated abzyme respectively, S is the substrate and P the product of hydrolysis. Ab.S is the abzyme–substrate complex and Abx the acyl–abzyme intermediate.

According to Waley [8,9] the progress curve for the relative concentration of inactivated abzyme can be expressed as:

$$t = N \ln(1-M \cdot z) - N' \ln(1-z) \quad (1)$$

where:

$z = [\mathrm{Ab_i}]/[\mathrm{Ab}]_0$

$N = K'/(k_{\mathrm{inact}}(1-M)\ [\mathrm{S}]_0)$

$N' = (1/k_{\mathrm{inact}}) + K'/(k_{\mathrm{inact}}(1-M)[\mathrm{S}]_0)$

with:

$$K' = \left(\frac{k_{-1}+k_2}{k_1}\right)\left(\frac{k_3+k_4}{k_2+k_3+k_4}\right)$$

$k_{\mathrm{inact}} = k_2\ k_4/(k_2+k_3+k_4)$

and

$M = (1 + k_3/k_4)\ ([\mathrm{Ab}]_0/[\mathrm{S}]_0)$

The time for 50% inactivation ($t_{1/2}$) is obtained experimentally and Eq. 1 can be rearranged as:

$$[\mathrm{S}]_0\ t_{1/2} = \frac{\ln(2-M)}{1-M}\frac{K'}{k_{\mathrm{inact}}} + \frac{\ln 2}{k_{\mathrm{inact}}}[\mathrm{S}]_0 \quad (2)$$

By plotting $[\mathrm{S}]_0\ t_{1/2}$ against $[\mathrm{S}]_0$ for a series of $[\mathrm{S}]_0$ concentrations with keeping the ratio $[\mathrm{Ab}]_0/[\mathrm{S}]_0$ constant, the values of k_{inact} and K' can be calculated from the slope and intercept respectively [10]. We found that the catalytic constant for inactivation $k_{\mathrm{inact}} = 0.024\ \mathrm{min}^{-1}$ while K', the Michaelis constant for inactivation, was 2.5 mM.

4. Discussion

Class A β-lactamases function with a mechanism similar to that of other enzymes, the DD-peptidases [11–13] that play a role in the synthesis of the bacterial wall by cross-linking polysaccharide linear chains. DD-peptidases are inactivated by β-lactam antibiotics and therefore represent the major target in antibiotherapies. Penicillin derivatives covalently bind to DD-peptidases by forming an acyl–enzyme complex much more stable than the complex formed by the natural substrate of the enzyme [14]. DD-peptidases and β-lactamases are both serine enzymes involving an acyl–enzyme intermediate with β-lactam substrates. However, the penicilloyl–enzyme intermediate involved in β-lactam ring opening by β-lactamase rapidly deacylates [15].

When compared with the catalytic properties measured with class A β-lactamase, the catalytic efficiency of 9G4H9 antibody for β-lactam substrates is largely reduced. However some similarities in the catalytic machinery seem to appear between both catalysts. A nucleophilic residue is thus suggested to play a key role by forming a covalent intermediate during the catalytic process.

Surprisingly, the initial velocity of ampicillin hydrolysis by 9G4H9 rapidly deviates from linearity at low and even at high substrate concentrations ($> 20 \times K_{\mathrm{M}}$). A multiple-turnover pre-steady-state burst was observed before the reaction reached steady state. The addition of more ampicillin during the steady state does not induce a new burst, indicating that this phenomenon is not due to a decrease in substrate concentration. We have checked that this result could not be explained by a denaturation of the catalyst during the time course of the reaction, nor by an inhibition of hydrolysis by the product formed during ampicillin hydrolysis. Such a behavior was previously observed for the hydrolysis of phenyl acetate by a monoclonal catalytic antibody 20G9 elicited against a transition state analog [16]. The authors have proposed a suicide-substrate mechanism to explain their results. This mechanism involves turnover and inactivation, both occurring via a Michaelis complex.

To check the occurrence of such a mechanism in ampicillin and penicillin G hydrolysis by 9G4H9, the formation of an irreversible complex during the reaction was tested using biotinylated penicillin. The addition of high concentrations of unlabeled penicillin was never found to dissociate the formed complex. Moreover this stable complex was found to involve amino acid residues present on the light chain of the antibody. On the other hand, we observed that β-lactam derivatives, cephalexin and nafcillin behave as reversible inhibitors of β-lactamase TEM-1 from *Escherichia coli*, with $K_{\mathrm{i}} = 400\ \mu\mathrm{M}$ for cephalexin and $K_{\mathrm{i}} = 20\ \mu\mathrm{M}$ for nafcillin. These two molecules were found to reversibly inhibit ampicillin hydrolysis by 9G4H9, but were never found to form any dead-end complex with the abzyme (data not shown). These latter results strongly suggest that an intermediary penicilloyl–abzyme intermediate is necessary to allow the formation of the irreversible complex to occur.

All these results lead us to propose a suicide-substrate mechanism for hydrolysis of ampicillin and penicillin G substrates by 9G4H9. The proposed mechanism states that 9G4H9 can either hydrolyze the substrates or follow a mechanism-based inactivation with the same compounds, both via a Michaelis complex.

The anti-idiotypic approach has allowed us to obtain a catalyst that could behave as a true β-lactamase for penicillinic substrate hydrolysis and as a penicillin-binding protein (PBP) for forming a stable penicilloyl complex. While β-lactamases and PBPs share primary sequence similarities [17] and were suggested to be issued from the common ancestral gene [18] allowing their possible interconversion by mutation [19,20], the primary sequence of 9G4H9 has no similarity in primary sequence with both enzymes [21]. The mimicry-based anti-idiotypic approach could be able to generate three-dimensional structures that resemble that of model enzymes, with the correct orientation of catalytic residues necessary for catalysis but with a different linear amino acid sequence and with some differences in catalytic machinery. The precise characterization of the molecular events involved in substrate interaction with 9G4H9, together with the selection of variants of this antibody will bring new insights both in understanding the structure–activity relationships between enzymes and abzymes and in understanding the extent of structural and functional mimicry by anti-idiotypic antibodies.

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