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Mutational and inhibitory analysis of a catalytic antibody. Implication for drug discovery☆

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ABSTRACT

Data on catalytic antibodies (abzymes) having critical roles in pathologies, for example in some auto-immune diseases accumulate at overwhelming pace. Nevertheless, the misunderstanding of how antibodies can mimic a catalytic activity may hamper the development of therapeutic tools. We thus investigated the structure function roles of residues of a catalytic antibody endowed with a β-lactamase activity. The catalytic antibody 9G4H9 was generated using the internal image properties of anti-idiotypic antibodies. The single-chain fragment was cloned and produced in *Escherichia coli*. Based on the structure function knowledge of β-lactamases pattern and on the tridimensional model of the scFv, five residues were selected for mutagenic analysis to learn about the contribution of putative residues in catalysis. Light chain mutants R24A, S26A, S28A, and E98A lost catalytic activity significantly. Mutant K27A retained catalytic activity but the estimated K_M was affected. Kinetic outcomes support the argument that S26 and S28 function as nucleophile and E98 as general acid/base catalyst.

We have selected a peptide Pep90 able to inhibit 9G4H9 catalytic activity. We also demonstrate the tryptophan and proline residues of Pep90 (YHFLWGP) are responsible for binding and inhibitory properties of Pep90 on 9G4H9. A three-dimensional model docked with Pep90 is therefore built in which critical residues of Pep90 are buried at the interface of CDR-L1 and CDR-L3 loops whereas other are exposed to the solvent.

1. Introduction

Apart from its natural function, the immune system represents an *in vivo* system capable of generating a biomolecular imprint of virtually any natural or synthetic compound. During the immune response, antibodies specific for idiotypic (or antigen-specific) determinants expressed within the variable regions of other antibodies are generated. This property has led (Jerne, 1974) to regard the immune system as a network of interacting idiotypes. According to the rules that govern the setting of both the idiotypic network and the relationships between successive elements of the idiotypic cascade, the idiotypic antibody (Ab1) is complementary to (i) the antigen against which it was generated and (ii) the anti-idiotypic antibody raised against Ab1 (Ab2). Ab2 thus mimics some of the antigen structural features. Hence the combining site of the anti-idiotypic antibody may carry an internal image of this antigen. This idea, later supported by experimental data (Pan et al., 1995; Fields et al., 1995) led us to the hypothesis that antigenic mimicry properties of anti-idiotypic antibodies could be used to elicit antibodies with the functional features of enzyme active sites. This proposal was further supported by the successful application in obtaining anti-idiotypic antibodies with cholinesterase (Izadyar et al., 1993), β-lactamase (Avalle et al., 1998) and protease-like (Pillet et al., 2002) activities. 9G4H9, a monoclonal IgG2bκ, was elicited by immunizing mice with a monoclonal antibody specific for the class A β-lactamase active site (Avalle et al., 1998).

Independently, this last decade highlighted emergence of catalytic antibodies involvement in some pathologies, especially in auto-immune diseases (Paul et al., 1989, 1997; Shuster et al., 1992; Gololobov et al., 1995; Pagetta et al., 2007). Thus it appears extremely challenging to understand the structural acquisition keys of catalytic activities, and also to be able to regulate these activities by elucidating molecular mechanisms of catalyst/inhibitor interactions. This notably implies to understand the catalytic mechanism of a model catalytic antibody like 9G4H9 (Avalle et al., 1998). Previous studies have shown that 9G4H9 variable domain supports the catalytic function, whereas no part of the amino acid sequence of this variable domain exhibited homology with the model enzyme

Abbreviations: IgG, immunoglobulin G; LB, Luria–Bertani; SPR, surface plasmon resonance; CDR, complementary determining region.

☆ Amino-acid residues are quoted according to the 1 letter convention.

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(Débat et al., 2001). The modification of 9G4H9 variable domain by a mechanism-based inhibitor revealed that most of the catalytic residues are located on the light chain of this antibody (Lefèvre et al., 2001; Padiolleau-Lefèvre et al., 2003). The single-chain fragment (scFv) of 9G4H9, cloned and produced in *Escherichia coli* using a chaperone system, exhibits catalytic features close to that of the whole antibody molecule (Padiolleau-Lefèvre et al., 2006). Based on known structure function relationships of β-lactamase catalytic activity (Meroueh et al., 2005) and considering that the activity of catalyst derives from the positioning of functional groups, we chose to mutate 5 positions of the scFv 9G4H9: R24, S26, K27, S28 and E98. An inhibitory cyclic heptapeptide Pep90 (YHFLWGP) was previously selected by panning on IgG 9G4H9 using phage display technology (Yribarren et al., 2003). We evaluated the interaction and inhibitory properties of Pep90 on wild-type scFv and mutants. We also identified the essential residues of Pep90 by testing different mutants. All these investigations allowed to build a homology-based 3D model of scFv 9G4H9. This model suggests the presence of a cavity within the light chain, where lay key residues. The model of Pep90 and a docked model scFv 9G4H9/Pep90 are also herein proposed.

2. Material and methods

2.1. ScFv 9G4H9 and mutant scFvs expression and cell fractionation

DNA scFv 9G4H9 was cloned into pET26b vector (Novagen) that includes an inducible IPTG T7 promoter, a kanamycin resistance gene, a pelB leader coding sequence for periplasmic expression and a poly-histidine tag grafted on the scFv C-terminal end. The DNA fragment was inserted between NcoI and NotI restriction sites. Competent Escherichia coli cells strain BL21 (DE3) (Novagen) were co-transformed by pET26b vector containing scFv 9G4H9 sequence and FkpA-pLT plasmid – kindly provided by Dr Jean-Michel Betton (Pasteur Institute, Paris) – containing chloramphenicol resistance, coding for periplasmic FkpA. Bacteria were grown in shaking culture (180 rpm, 37 °C) in 1 L LB (Luria–Bertani) broth containing 30 μg/mL kanamycin and 25 μg/mL chloramphenicol. When absorbance at 600 nm reached 0.4–0.5, protein expression was induced by adding 1 mM IPTG. Cells were incubated for 3 h at 30 °C and pelleted by centrifugation at 2500 × *g* for 10 min at 4 °C. Protein extraction was performed by osmotic shock: cells were suspended with 30 mM Tris–HCl pH 8, 0.5 M saccharose, 1 mM EDTA, incubated 15 min at 20 °C and centrifuged at 8000 × *g* for 15 min at 4 °C. Pellets were then resuspended in deionized water and centrifuged at 8500 × *g* for 15 min at 4 °C.

2.2. Site-directed mutagenesis of scFv 9G4H9

The nucleotide sequences of the variable regions V_L and V_H of IgG 9G4H9 have been assigned accession number by EMBL: aj277812 (V_L) and aj277813 (V_H).

ScFv 9G4H9 mutants were constructed by oligonucleotide mutagenesis with single-stranded pET26b DNA vector containing uracil as the template for primer extension and ligation (Kunkel, 1985). The mutagenic oligonucleotides used for the monosubstitution of codons 24, 26, 27, 28 and 98 were respectively:

24: 5′ CTT ACT AGA **AGC** GCA GGA GAT 3′;
26: 5′ AAG ACT CTT **CGC** AGA CCT GC 3′;
27: 5′ CAG AAG ACT **CGC** ACT AGA CC 3′;
28: 5′ ATG CAG AAG **CGC** CTT ACT AG 3′;
98: 5′ GAG AGG ATA **CGC** TAG ACT TTG 3′.

Bold letters indicate mutagenic codons. DNA sequences were checked by Genome-Express (Meylan, France) by priming with: 5′ CCG ATG CTG TCT TTC GCT GCT 3′.

2.3. Protein purification

The extracted periplasmic fraction was filtered on 0.45 μm membrane before fractionation by FPLC affinity chromatography on a Hi Trap chelating column (GE Healthcare). Binding buffer contained 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH 8. Elution was performed using a step gradient with elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 1 M imidazole pH 8. The flow rate was 1 mL/min. The solution was then dialyzed overnight against 2 L of 0.1 M phosphate buffer saline (pH 7.4) using a 3.5 kDa membrane (Spectrum Laboratories). Samples were concentrated by centrifugation at 4000 × *g* for 10 min at 4 °C on Amicon Ultra 10 kDa tubes (Millipore). The protein concentration was evaluated by a bicinchoninic acid assay (BCA, Pierce) using standards.

2.4. Electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12.5% polyacrylamide gels with prestained protein broad range marker (175, 83, 62, 47.5, 32.5, 25, 16.5 and 6.5 kDa, New England Biolabs) containing dithiothreitol (DTT) for scFv purity checking. After migration, the gels were Coomassie blue stained.

2.5. Fluorescence

Fluorescence measurements were performed on a Varian Cary spectrofluorimeter using a 1 cm path length cuvette. Excitation was carried out at 295 nm with excitation and emission slitwidths of 5 nm. For analysis convenience, spectra were analyzed directly after blank correction. Emission spectra were collected from 300 to 400 nm. Protein concentration was ca. 0.1 mg/mL (3 μM) in 100 mM phosphate buffer pH 7.4.

2.6. Synthetic peptides

The soluble form of cyclic heptapeptides Pep90 and Pep95 were made-to-order synthesized by Eurogentec (Belgium). They were synthesized under oxidative conditions inducing formation of a disulfide bound between the two bordering cysteines. The biotinylated form of peptides has the following sequence: $NH_2GACX_1X_2X_3X_4X_5X_6X_7CGAAGAEKCONH_2$ where X_n represents the residues of the heptapeptides at position *n*. The biotin moiety is linked to the C-terminal lysine residue.

2.7. Kinetic measurements

Hydrolysis of ampicillin was followed spectrophotometrically at 232 nm in 100 mM phosphate buffer pH 7.4 at 20 °C ($\varepsilon_M = 2800\ M^{-1}\ cm^{-1}$) with scFv at 3 μM final concentration.

For inhibition assays, scFvs were incubated for 45 min at 20 °C with various peptide concentrations. All the curves were fitted with Kaleidagraph™ software.

2.8. Phage-ELISA

Unless otherwise stated, Maxisorb microtitration plates (Nunc) wells were filled with 100 μL of indicated solutions and plates were incubated for 1 h at 37 °C. Each reaction step was followed by three washes in Tris-buffered saline (TBS: 50 mM Tris–HCl, 150 mM NaCl) pH 7.5 containing 0.1% Tween 20 (TTBS). 5 μg/well of scFv in 0.1 M carbonate buffer pH 9.3 were coated on plates. Wells were