



Pergamon

Tetrahedron Letters 40 (1999) 8063–8066

TETRAHEDRON  
LETTERS

# Catalytic single-chain antibodies possessing $\beta$ -lactamase activity selected from a phage displayed combinatorial library using a mechanism-based inhibitor

Fujie Tanaka, Helena Almer, Richard A. Lerner\* and Carlos F. Barbas III\*

*The Skaggs Institute for Chemical Biology and the Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA*

Received 19 August 1999; accepted 1 September 1999

## Abstract

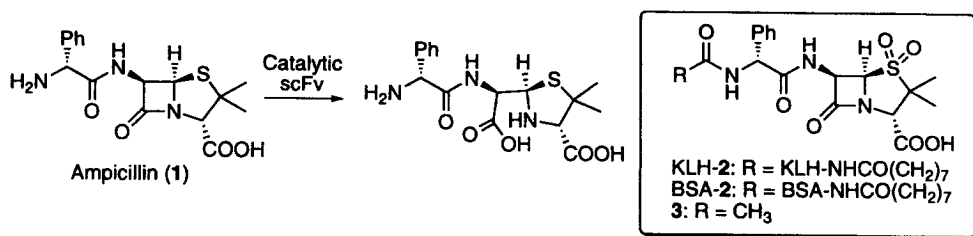
Catalytic single-chain antibodies (scFvs) possessing  $\beta$ -lactamase activity were selected from a phage displayed combinatorial antibody library using a penam sulfone mechanism-based inhibitor of  $\beta$ -lactamase. The scFvs FT6 and FT12 catalyzed the hydrolysis of ampicillin with rate accelerations ( $k_{\text{cat}}/k_{\text{uncat}}$ ) of 5200 and 320. © 1999 Elsevier Science Ltd. All rights reserved.

*Keywords:* antibodies; azetidinones; catalysis; phage display.

Immunization with designed reactive compounds has been reported to be an effective strategy to obtain antibody catalysts.<sup>1</sup> Phage display systems<sup>2</sup> should facilitate this approach since phage that display the desired catalytic antibody can be directly trapped after reaction with a mechanism-based inhibitor allowing for effective searches through large combinatorial libraries.<sup>3</sup> Since mechanism-based inhibitors<sup>4</sup> react to form covalent adducts with the enzymes that process them along a defined reaction pathway, they should allow for the direct selection of catalytic antibodies that utilize particular features of a designed mechanism. According to these ideas, we selected single-chain antibodies (scFvs) that catalyze the hydrolysis of ampicillin (**1**) from a phage displayed combinatorial antibody library using a penam sulfone derivative (Scheme 1).<sup>4,5</sup> Penam sulfones have been shown to be potent mechanism-based inhibitors of  $\beta$ -lactamase enzymes that act to trap the acyl-enzyme intermediate formed during the reaction.

To produce antibodies that would mimic the catalytic activity of nature's  $\beta$ -lactamase, mice were immunized with a keyhole limpet hemocyanin (KLH) conjugate of a penam sulfone, KLH-2. RNA from the spleen of an immune animal was prepared. Following preparation of cDNA, combinatorial libraries consisting of the genes of the immunoglobulin light ( $\kappa$ ) and heavy chain variable domains ( $V_L$  and  $V_H$ ) were generated by PCR.<sup>6</sup> The single-chain antibody variable region (scFv) gene library,

\* Corresponding authors.



Scheme 1.

in which  $V_L$  and  $V_H$  genes are connected with DNA coding for an 18-amino acid polypeptide linker (SSGGGGSGGGGGSSRSS) region, was prepared by overlap fusion PCR. To avoid degradation of the penam sulfone by  $\beta$ -lactamase enzyme that is typically used as a vector selection marker, we constructed a novel vector, pNPC3zeo, with a zeocin resistance gene. The scFv gene library was ligated into the *Sfi*I sites of pNPC3zeo.<sup>7</sup> Transformation into *Escherichia coli* (*E. coli*) ER2537 cells produced a library of  $6 \times 10^6$  members. The phage library was prepared using helper phage VCS-M13 and isolated according to standard protocols.<sup>6</sup> In this approach, the scFv proteins are displayed on the surface of the phage as a fusion protein with the phage gene III protein.

Phage panning was initiated by incubating  $4.8 \times 10^{10}$  phage particles in a microtiter plate coated with bovine serum albumin (BSA) conjugate, BSA-2. The unbound phage were removed by washing 10 times with 0.5% Tween 20/TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) (TBST) and then bound phage were eluted by trypsin digestion and used to infect ER2537 cells. In the next three rounds of selection, the wells were washed 10 times with TBST and once with 50 mM citric acid (pH 2.5) to remove all noncovalently bound phage. To exclude phage that bind to BSA alone, for the third and fourth rounds of selection, phage were added first to the BSA-coated wells, and then transferred to BSA-2-coated wells. After the fourth round of panning, plasmid DNA was isolated and the selected scFv encoding genes were moved to an expression vector, pZerH6HA<sup>8</sup> that expresses the scFv with an amino terminal histidine tag (H6) and a carboxy terminal hemagglutinin decapeptide tag (HA). A panel of 12 individual clones was assayed by ELISA for the production of soluble scFv capable of binding to BSA-2. Citric acid washes were used to remove proteins that bound noncovalently. The binding of eight antibodies was insensitive this acid treatment.

To study catalytic activity, the scFv proteins were produced using pZerH6HA and pAraH6HA<sup>9</sup> vectors in *E. coli* XL1-Blue cells and were purified by H6 affinity purification followed by FPLC cation-exchange chromatography. The hydrolysis of **1** (200  $\mu$ M) in 10% DMSO/PBS (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, pH 7.4) at 24°C was followed by monitoring the decrease of **1** by HPLC. The most active antibodies, FT6 and FT12, were characterized in detail. For large scale protein production, pAraH6HA was used for scFv FT6 and pGAPZ $\alpha$ A vector in yeast *Pichia pastoris* was used for scFv FT12.<sup>10</sup> The highly purified scFvs FT6 and FT12 were also positive in the ELISA against BSA-2 using the acid washing condition. Since antibody-antigen interaction are typically disrupted at low pH, this result suggests that these antibodies bind BSA-2 covalently.

Antibodies FT6 and FT12 displayed saturation kinetics in their hydrolysis of **1**. Kinetic data fit the Michaelis-Menten equation. The kinetic parameters of FT6 and FT12 are shown in Table 1. The rate accelerations above background hydrolysis ( $k_{cat}/k_{uncat}$ ) with FT6 and FT12 were 5200- and 320-fold, respectively. Burst-type kinetic behavior was not detected in these reactions. Both antibody catalyzed hydrolysis reactions of **1** were inhibited by the addition of sulfone **3**. When antibody FT12 was mixed with **3** and the hydrolysis of **1** was measured immediately, FT12 catalyzed the hydrolysis with partial inhibition by **3**. However, when FT12 (10  $\mu$ M) was mixed with **3** (50  $\mu$ M) and the mixture was left

Table 1  
Kinetic parameters of the scFv-catalyzed hydrolysis of Ampicillin (1)<sup>a</sup>

scFv	$K_m$	$k_{cat}$	$k_{cat}/k_{uncat}^b$	$K_i$ (inhibitor 3)
FT6	560 $\mu$ M	$2.9 \times 10^{-1} \text{ min}^{-1}$	5200	ND <sup>c</sup>
FT12	162 $\mu$ M	$1.8 \times 10^{-2} \text{ min}^{-1}$	320	26 $\mu$ M <sup>d</sup>

<sup>a</sup> Reaction conditions: 10% DMSO/PBS, pH 7.4, 24 °C. <sup>b</sup> The first order kinetic constant of the background reaction ( $k_{uncat}$ ) was  $5.6 \times 10^{-5} \text{ min}^{-1}$ . <sup>c</sup> Not determined. See text. <sup>d</sup>  $K_i$  was determined by Dixon plots.

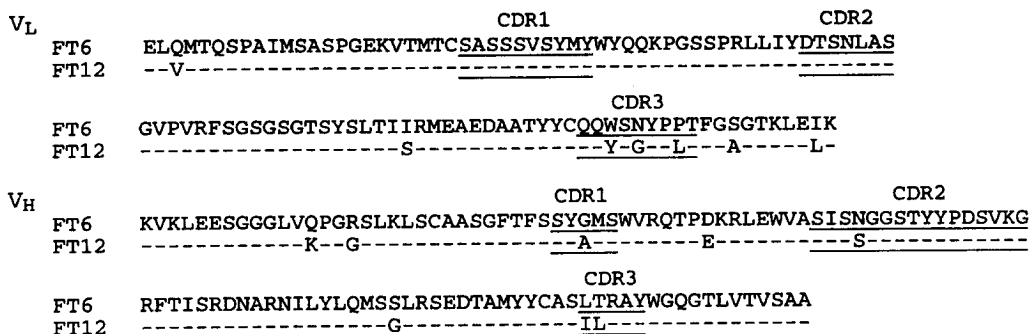


Figure 1. The deduced amino acid sequences of V<sub>L</sub> and V<sub>H</sub> of FT6 and FT12. Dashes designate identity. CDR (underlined) designation is according to the consensus described by Kabat et al.<sup>12</sup>

for 1 day, the catalytic activity towards the hydrolysis of **1** (200  $\mu$ M) was not detected. The complete modification of the antigen-combining site of the scFv with sulfone **3** occurred slowly and this time-dependent inactivation suggests an irreversible covalent modification of the antigen-combining site.<sup>4,11</sup> Antibody FT12 did not catalyze the decomposition of sulfone **3**. In the case of scFv FT6, the hydrolytic decomposition of sulfone **3** was also catalyzed:  $k_{cat}=1.3 \times 10^{-1} \text{ min}^{-1}$ ,  $K_m=380 \mu\text{M}$ , and  $k_{cat}/k_{uncat}=990$  ( $k_{uncat}=1.3 \times 10^{-4} \text{ min}^{-1}$ ). A time-dependent inactivation was also observed in the FT6-catalyzed reaction.

The deduced sequences of scFvs FT6 and FT12 are shown in Fig. 1. The sequences of FT6 and FT12 are highly homologous. Although hydrolytic antibodies generated against phosphonates and phosphoramidates often share common structural features key to their catalytic mechanism,<sup>13</sup> FT6 and FT12 did not have a significant homology to those hydrolytic antibodies that have been described in the literature.

In conclusion, antibodies that catalyze the hydrolysis of  $\beta$ -lactams were obtained from a phage displayed combinatorial library using a mechanism-based inhibitor of  $\beta$ -lactamase. These results suggest that mechanism-based inhibitors provide an effective means for the direct selection of catalytic antibodies from phage display libraries.

## References

- (a) Wagner, J.; Lerner, R. A.; Barbas III, C. F. *Science* **1995**, *270*, 1797. (b) Wirsching, P.; Ashley, J. A.; Lo, C.-H.; Janda, K. D.; Lerner, R. A. *Science* **1995**, *270*, 1775. (c) Barbas III, C. F.; Heine, A.; Zhong, G.; Hoffmann, T.; Gramatikova, S.; Bjornestedt, R.; List, B.; Anderson, J.; Stura, E. A.; Wilson, I. A.; Lerner, R. A. *Science* **1997**, *278*, 2085. (d) Zhong, G.; Lerner, R. A.; Barbas III, C. F. *Angew. Chem., Int. Ed. Engl.* **1999**, in press.
- (a) Rader, C.; Barbas III, C. F. *Curr. Opin. Biotechnol.* **1997**, *8*, 503. (b) Burton, D. R.; Barbas III, C. F. *Advances in Immunology* **1994**, *57*, 191.

3. (a) Janda, K. D.; Lo, C.-H. L.; Li, T.; Barbas III, C. F.; Wirsching, P.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2532. (b) Janda, K. D.; Lo, L.-C.; Lo, C.-H. L.; Sim, M.-M.; Wang, R.; Wong, C.-H.; Lerner, R. A. *Science* **1997**, *275*, 945. (c) Lo, C.-H. L.; Wentworth, P.; Jung, K. W.; Yoon, J.; Ashley, J. A.; Janda, K. D. *J. Am. Chem. Soc.* **1997**, *119*, 10251.
4. (a) Silverman, R. B. *Mechanism-based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press: 1988; Vol. 1. (b) Fisher, J.; Charnas, R. L.; Bradley, S. M.; Knowles, J. R. *Biochemistry* **1981**, *20*, 2726. (c) Brenner, D. G.; Knowles, J. R. *Biochemistry* **1981**, *20*, 3690.
5. Trapping of phage displayed  $\beta$ -lactamase enzyme by a penam sulfone derivative has been reported: Soumillion, P.; Jespers, L.; Bouchet, M.; Marchand-Brynaert, J.; Winter, G.; Fastrez, J. *J. Mol. Biol.* **1994**, *237*, 415.
6. *Phage Display of Proteins and Peptides: A Laboratory Manual*; Barbas III, C. F.; Burton, D.; Silverman, G.; Scott, J., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 2000, in press.
7. NPC3zeo was constructed from pComb3H<sup>2</sup> by insertion of the zeocin resistance gene into the middle of the  $\beta$ -lactamase coding region. The zeocin resistance gene was taken from pPICZ $\alpha$ A vector (Invitrogen) by PCR.
8. pZErH6HA vector was constructed from pZErO-1 (Invitrogen).
9. Barbas III, C. F.; Wagner J. *Methods: a Companion to Methods in Enzymology* **1995**, *8*, 94.
10. The scFv protein concentration was determined by Bradford analysis.
11. Tanaka, F.; Lerner, R. A.; Barbas III, C. F. *Chem. Commun.* **1999**, 1383.
12. Kabat, E. A.; Wu, T. T.; Perry, H. M.; Gottesman, K. S.; Foeller, C. *Sequences of Proteins of Immunological Interest*, 5th Ed.; US Public Health Service, National Institute of Health: Bethesda, MD, 1991.
13. MacBeath, G.; Hilvert, D. *Chemistry and Biology* **1996**, *3*, 433.