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Function of the conserved triad residues in the class C β-lactamase from *Citrobacter freundii* GN346

Kikuo Tsukamoto, Naoko Nishida, Miyuki Tsuruoka and Tetsuo Sawai

Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 260, Japan

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The conserved KTG triad in the class C β-lactamase from *Citrobacter freundii* GN346 was examined as to its function by means of site-directed mutagenesis. The following conversions were performed; Lys-315 to arginine, alanine or glutamic acid, Thr-316 to valine, and Gly-317 to alanine, proline or isoleucine. The resultant mutant enzymes revealed that a basic amino acid at position 315 and a small uncharged residue at position 317 are essential for the enzyme activity, but a hydroxyl group at residue 316 is not required for the enzymatic catalysis. The kinetic properties of the purified Arg-315 and Val-316 enzymes provided information on the function of these residues.

β-Lactamase; Cephalosporinase; Active site; Conserved residue; Site-directed mutagenesis; *Citrobacter freundii*

1. INTRODUCTION

β-Lactamases comprise many enzymes with a variety of substrate profiles, and can be divided into three groups, i.e., classes A to C, according to the amino acid sequence around the active site [1]. Cephalosporinases, a group of β-lactamases preferring cephalosporins as substrates, are mainly class C β-lactamases and are responsible for the high levels of the β-lactams resistance of Gram-negative pathogens [2]. β-Lactamases, other than the class B enzymes, are active-site-serine enzymes and are believed to be members of a single superfamily including penicillin-binding proteins. There is no significant overall amino acid sequence homology between class A β-lactamases, class C β-lactamases and penicillin-binding proteins; however, a considerable number of amino acid residues in them have been recognized to be conserved. Joris et al. [3] have demonstrated seven conserved boxes found in the amino acid sequences of all the superfamily enzymes. Among them, box VII is an outstanding region with three successive conserved residues (Lys-Thr/Ser-Gly; the triad residues), located at a distance from the active-site serine in the primary sequence. Recently, Oefner et al. [4] reported the three-dimensional structure of the cephalosporinase from *Citrobacter freundii* 1203 and determined the position of the triad residues (Lys-315, Thr-316 and Gly-317), which are three-dimensionally close to the active-site Ser-64. They assumed that Lys-315 forms a hydrogen-bonding network together with Ser-64, Lys-67, Tyr-150 and Asn-152, and proposed the functioning of Lys-315 in aztreonam hydrolysis.

In preceding studies [5–9], we selected the cephalosporinase from *C. freundii* GN346 for a structure–function study on the active site of a typical cephalosporinase and examined the essential role of Lys-67, a conserved residue located at three positions downstream of the active-site serine, by means of site-directed mutagenesis [7]. The present investigation was undertaken as an extension of the previous studies, we examined the role of the triad residues from the enzymological aspect.

2. MATERIALS AND METHODS

2.1. Escherichia coli *strains and plasmids*

As bacterial cells bearing the cephalosporinase gene, *E. coli* AS226-51, a derivative of C600, was employed. Strain AS226-51 is an *ampD* mutant and has a deletion mutation in *ampC* [7]. Plasmid pCFC-1 is a derivative of pHSG398, into which the wild-type cephalosporinase gene from *C. freundii* GN346 was inserted [7]. pHSG398 carrying a mutant cephalosporinase gene is called, for example, pCFC-K315R. A one-letter amino acid code is used to express the mutant gene, i.e., K315R means the mutant gene in which Lys-315 has been changed to arginine.

2.2. *Media, chemicals and enzymes*

For the transformation and transfection experiments, 2 × YT broth [10] and YT agar [10] were employed, respectively. Heart infusion agar (Eiken Chemical Co., Tokyo, Japan) was used for measuring the bacterial susceptibility to antibiotics. For enzyme preparation, bacteria were grown in 2 × YT broth. The antibiotics used in this study were kindly provided by the following pharmaceutical companies: benzylpenicillin, ampicillin and cloxacillin, Meiji Seika Kaisha Ltd., Tokyo, Japan; carbenicillin, Fujisawa Pharmaceutical Co., Osaka, Japan; cephalothin and cephaloridine, Shionogi & Co., Osaka, Japan; cefuroxime, Nippon Glaxo Co., Ltd., Tokyo, Japan;

Correspondence address: T. Sawai, Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi-cho, Chiba 260, Japan

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cefoxitin, Merck Sharp and Dohme Research Laboratories, NJ, USA; aztreonam, Eisai Co., Tokyo, Japan; carumonam, Takeda Chemical Industries Co., Osaka, Japan; and chloramphenicol, Yamanouchi Pharmaceutical Co., Tokyo, Japan. [α-^{32}P]dCTP and the in vitro mutagenesis kit were purchased from Amersham International, UK. The M13 sequencing kit and enzymes for DNA technology were purchased from Boehringer-Mannheim (Mannheim, FRG), Takara Shuzo Co., Kyoto, Japan, and Nippon Gene Co., Toyama, Japan.

2.3. *Oligonucleotides and site-directed mutagenesis*

Oligonucleotides, 17- and 20-mers, were synthesized using a DNA synthesizer model 380B (Applied Biosystems, CA, USA). Site-directed mutagenesis was performed by the method of Eckstein [11]. The mutant genes were sequenced to confirm the desired exchange in the nucleotide sequence by the chain-termination method [12] using a specific oligonucleotide primer.

2.4. *β-Lactamase purification, β-lactamase assay and kinetic parameters*

E. coli AS226-51 cells carrying the wild or a mutant cephalosporinase gene were grown overnight in 2 × YT medium at 37°C. The medium contained sublethal concentrations of chloramphenicol (30 μg/ml) and cephalothin (50 μg/ml) to ensure that the plasmid would not be lost. The preculture was then diluted with a 40-fold volume of fresh medium, followed by growth at the same temperature under aeration until the middle of the logarithmic phase. β-Lactamase activity in the bacterial cells was measured after sonic treatment of the cells. For further purification of the Arg-315 and Val-316 enzymes, the procedures established for purification of the wild-type enzyme were used [13], their purity being confirmed by SDS gel-electrophoresis. β-Lactamase activity was assayed by a microiodometric method [14], with slight modifications, and an ultraviolet spectrophotometric method [15]. One unit of the enzyme was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per minute at pH 7.0 and 30°C. The kinetic parameters, K_m and K_i, were determined by the procedures reported previously [15].

2.5. *Antibiotic susceptibility testing*

The bacterial susceptibility to β-lactams was measured by the serial agar dilution method, according to the procedure described previously [2]. The susceptibility was expressed as the minimum inhibitory concentration of a drug (μg/ml).

3. RESULTS AND DISCUSSION

3.1. *Conversion of the triad residues (Lys-315, Thr-316 and Gly-317) to Arg-315, Ala-315, Glu-315, Val-316, Ala-317, Pro-317 and Ile-317, and expression of the mutant genes in* E. coli

On the assumption that the significance of the triad residues lies in a positive charge at position 315, a hydroxyl group at position 316 and a small uncharged residue at position 317, Lys-315 was converted to arginine, alanine or glutamic acid, Thr-316 was converted to valine, and Gly-317 was converted to alanine, proline and isoleucine. The plasmids carrying the mutant genes and that carrying the wild gene (pCFC-1) were introduced into *E. coli* AS226-51 cells by transformation, and then the transformants were examined as to their susceptibility to seven β-lactams and their β-lactamase activity (Table I).

The *E. coli* cells harbouring pCFC-K315E almost completely lost their β-lactamase activity, the MIC levels being about the same as those in the case of a sensitive strain, *E. coli* AS226-51. The enzyme activity in the cells harbouring pCFC-K315A was estimated to amount to about 0.1% of that in the cells harbouring pCFC-1, the remaining activity of the mutant enzyme being confirmed by that the MIC levels were somewhat higher than those in the case of the sensitive strain. On the other hand, the cells carrying pCFC-K315R showed moderate resistance to all the β-lactams except cefuroxime and cefoxitin, and had a considerable level of the enzyme activity. These results suggest that a basic amino acid residue at position 315 greatly enhances the enzyme reaction. The cells harbouring pCFC-T316V retained more than half the activity in the cells harbouring the wild gene, indicating that a hydroxyl group at residue 316 is not required for β-lactam hydrolysis. The conversion of Gly-317 to isoleucine reduced the enzyme's activity to about 0.1% of that of the wild-type enzyme, but the cells producing the Ala-317 enzyme showed moderate resistance to β-lactams and retained a significant level of the activity. These results suggested the requirement for a smaller residue than alanine at position 317.

3.2. *Kinetic parameters of the Arg-315 and Val-316 enzymes and their comparison with those of the wild-type enzyme*

In order to obtain information as to the function of the triad residues, the mutant enzymes retaining a significant level of activity were extracted from *E. coli* AS226-51 cells carrying pCFC-K315R or pCFC-T316V and purified. The affinities of these mutant enzymes for nine β-lactams were measured and expressed as the K_m or K_i value, and the catalytic activity, k_{cat}, was also determined for four typical β-lactams, i.e., cephalothin, cephaloridine, benzylpenicillin and ampicillin. These kinetic parameters were compared with those of the wild-type enzyme (Table II). The conversion of Thr-316 to valine resulted in lowering of the affinities for cephalothin and cephaloridine, the K_m values for these favorable substrates being about 4-times that of the wild-type enzyme. The k_{cat} values of the Val-316 enzyme for the favorable substrates, however, were in the range of 0.5- to 1.0-times those of the wild-type enzyme. These results confirmed that the hydroxyl group of Thr-316 has no functional importance in the enzyme reaction. On the other hand, the Arg-315 enzyme showed greatly reduced affinity for cephaloridine, a zwitterionic compound, though its affinity for cephalothin, a monoanionic compound, was not affected by the conversion. The k_{cat} values of the Arg-315 enzyme for cephalothin and cephaloridine were 8 and 29% those of the wild-type enzyme, respectively. It should be emphasized, here, that cephaloridine has the same chemical structure as cephalothin except for a side-chain with a positive charge at the 3 position of the cephalosporin nucleus. These observations suggest that Lys-315 comes into

Table I

β-Lactamase activity and susceptibility to cephalosporins and penicillins of *E. coli* strains carrying the wild-type and mutant β-lactamase genes

E. coli strains	β-Lactamase activity (units/mg protein)	MIC (μg/ml)						
		CET	CER	CXM	CFX	PCG	APC	CPC
AS226-51/pCF-1(wild)	1.9	800	200	50	25	1600	200	50
AS226-51/pCFC-K315R	0.12	200	25	6.3	6.3	200	25	50
AS226-51/pCFC-K315A	2×10^{-3}	6.3	3.1	6.3	3.1	25	1.6	3.1
AS226-51/pCFC-K315E	1×10^{-4}	3.1	<1.6	<1.6	<1.6	12.5	<1.6	<1.6
AS226-51/pCFC-T316V	1.0	200	12.5	25	6.3	1600	100	12.5
AS226-51/pCFC-G317A	5×10^{-2}	100	6.3	6.3	6.3	400	25	12.5
AS226-51/pCFC-G317P	$<10^{-5}$	1.6	1.6	1.6	3.1	6.3	1.6	3.1
AS226-51/pCFC-G317I	2×10^{-3}	3.1	1.6	3.1	3.1	25	<1.6	3.1
AS226-51	$<10^{-5}$	<1.6	<1.6	<1.6	<1.6	6.3	<1.6	<1.6

β-Lactamase activity in disrupted bacterial cells was measured by the microiodometric method, with cephalothin as the substrate. The antibiotics used were as follows: CER, cephaloridine; CET, cephalothin; CXM, cefuroxime; CFX, cefoxitin; PCG, benzylpenicillin; APC, ampicillin; CPC, carbenicillin

contact with a substrate through an ionic interaction and they are consistent with the model proposed by Oefner et al., i.e., an ionic interaction of the ε-amino group of Lys-315 with the sulphonate of aztreonam [4].

The two mutant enzymes showed higher k_{cat} values for benzylpenicillin and ampicillin than the wild-type enzyme except for the case of Val-316 enzyme to ampicillin, though the conversion caused a decrease in the affinity of the mutant enzymes for most of the undesirable substrates, i.e., penicillins, cephamycin and monobactams. However, the higher k_{cat} values of the Arg-315 enzyme for benzylpenicillin and ampicillin disagree with the MICs of the two penicillins to the cells harbouring pCFC-K315R, that is, the MIC levels are evidently lower than those to the cells harbouring pCFC-1 (Table I). We could not detect a difference in the thermostability between the mutant and wild-type enzymes, and this discrepancy has remained unsettled.

During kinetic analysis of the Arg-315 enzyme, we observed an interesting phenomenon as to the effect of pH on the enzyme activity. As shown in Fig. 1, the pH–activity curve for the Arg-315 enzyme shifted to the alkaline side and the optimum pH was higher than that in the case of the wild-type enzyme, the difference from that of the wild-type enzyme being about 1 pH unit. This deviation may be due to the difference between the pKa values for the ε-amino group of lysine and the guanidino group of arginine. A particularly interesting fact is that the Arg-315 enzyme retains activity in the alkaline pH range higher than 10. This can be attributed to that the arginine residue is able to undergo an ionic interaction in the higher pH. On the basis of the model for the β-lactam hydrolysis proposed by Oefner et al. [4], an explanation for this phenomenon may be possible, that is, the guanidino group at position 315 of the mutant enzyme can interact with

Table II

Kinetic constants for β-lactams of the wild-type and mutant enzymes

β-Lactams	K_m (μM)			K_i (μM)			k_{cat} (min^{-1})		
	Wild-type	Arg-315	Val-316	Wild-type	Arg-315	Val-316	Wild-type	Arg-315	Val-316
Cephalothin	19.0	18.5	73.0				102	8.2	106
Cephaloridine	410	5450	1430				947	278	478
Benzylpenicillin	5.4	22.9	0.95				0.47	1.96	1.10
Ampicillin	8.5	5.1	ND				0.074	0.198	0.068
Carbenicillin				0.016	1.3	0.04			
Cloxacillin				0.003	0.27	0.0046			
Cefoxitin				0.49	1.3	17.0			
Aztreonam				0.046	0.081	0.024			
Carumonam				0.16	1.8	0.108			

The K_m and k_{cat} values were determined by the ultraviolet spectrophotometric method, and the K_i value was measured by the microiodometric method, with cephalothin as the substrate. The values for the wild-type enzyme (Lys-315 enzyme) were taken from previous papers [8,9]. ND, could not be determined

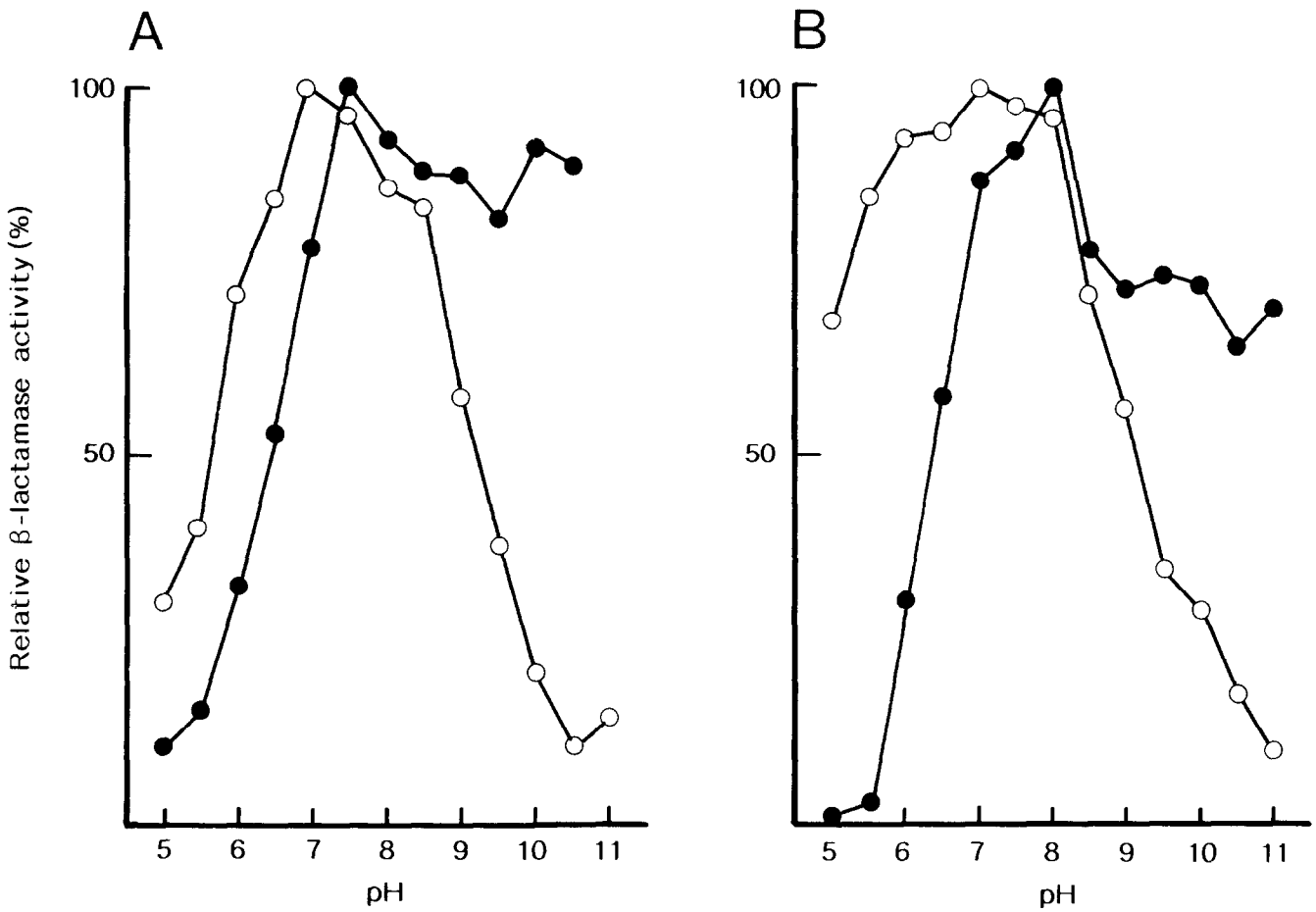

Fig. 1. Effect of pH on the activity of the wild-type enzyme (Lys-315) and that of the Arg-315 mutant enzyme. The following buffers (50 mM) were employed for the assay; citric acid/sodium citrate (pH 5.0–6.0), sodium phosphate (pH 6.0–8.5), borate/NaOH (pH 7.0–10.5) and glycine/NaOH (pH 7.5–11.0). The activity was measured by the ultraviolet spectrophotometric method with cephalothin (A) and benzylpenicillin (B) as substrates. (○) The wild-type enzyme; (●) the Arg-315 mutant enzyme.

Tyr-150 in the higher pH range and this interaction is essential for deacylation of the acyl intermediate by the water attack. The optimum pH for the wild-type enzyme was between 7.0 and 7.5 with cephalothin as the substrate, but a broad optimum pH range was seen with benzylpenicillin as the substrate. However, it should be pointed out that this difference in the pH-activity curve disappeared with the conversion of Lys-315 to arginine. Position 315 may be one of the sites that distinguish between the cephalosporin and penicillin nuclei, and a similar assumption may be possible as to position 316 from a remarkable decrease in the affinity of the Val-316 enzyme for cefoxitin with the cephamycin nucleus.

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REFERENCES

[1] Ambler, R.P. (1980) Phil. Trans. R. Soc. Ser. B 289, 321–331.
[2] Sawai, T., Yoshida, T., Tsukamoto, K. and Yamagishi, S. (1981) J. Antibiot. 34, 1318–1326.
[3] Joris, B., Ghuysen, J.M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frere, J.M., Kelly, J.A., Boyingtonm, J.C., Moews, P.C. and Knox, J.R. (1988) Biochem. J. 250, 313–324.
[4] Oefner, C., D'Arcy, A., Daly, J.J., Gubernator, K., Charnas, R.L., Heinze, I., Hubschwerlen, C. and Winkler, F.K. (1990) Nature 343, 284–288.
[5] Yamaguchi, A., Adachi, H. and Sawai, T. (1987) FEBS Lett. 218, 126–130.
[6] Sawai, T., Yamaguchi, A. and Tsukamoto, K. (1988) Rev. Infect. Dis. 10, 721–725.
[7] Tsukamoto, K., Tachibana, K., Yamazaki, N., Ishii, Y., Ujiie, K., Nishida, N. and Sawai, T. (1990) Eur. J. Biochem. 188, 15–22.
[8] Tsukamoto, K., Kikura, R., Ohno, R. and Sawai, T. (1990) FEBS Lett. (in press).
[9] Tsukamoto, K., Ohno, R. and Sawai, T. (1990) J. Bacteriol. (August issue).
[10] Miller, J. (1972) in: Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, New York.
[11] Taylor, J.W., Schmidt, W., Cosstick, R., Okruszek, A. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8749–8764.
[12] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[13] Sawai, T., Kanno, M. and Tsukamoto, K. (1982) J. Bacteriol. 152, 567–571.
[14] Novick, R.P. (1962) Biochem. J. 83, 236–240.
[15] Yamaguchi, A., Hirata, T. and Sawai, T. (1983) Antimicrob. Agents Chemother. 24, 23–30.