Substitution of aspartic acid-217 of *Citrobacter freundii* cephalosporinase and properties of the mutant enzymes

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On the assumption that Asp-217 of a *Citrobacter freundii* cephalosporinase forms a salt-bridge with the conserved Lys-67, Asp-217 was changed to glutamic acid, threonine or lysine. The mutant enzymes retained about the same level of activity as that of the wild-type enzyme, and the participation of Asp-217 in the salt-bridge was ruled out. However, the mutations resulted in an increase in hydrolytic activity toward oxyimino-cephalosporins such as cefuroxime, cefmenoxime and ceftazidime, suggesting a possible mechanism of the bacterial resistance to the novel β-lactams by a single mutation in cephalosporinases.

β-Lactamase; Cephalosporinase; *Citrobacter freundii*; Active site; Oxyimino-cephalosporin; Site-directed mutagenesis

1. INTRODUCTION

Cephalosporinases are mainly responsible for the high levels of resistance to traditional cephalosporins of Gram-negative bacteria [1] and most of the enzymes are class C β-lactamases, according to Ambler's classification [2]. A cephalosporinase produced by *Citrobacter freundii* GN346 is a species-specific β-lactamase with the substrate profile of a typical cephalosporinase and its enzymological properties have been extensively investigated by us [3-6].

On the basis of the crystal structure of the class A β-lactamase of *Staphylococcus aureus* PC1, Herzberg and Moult [7] suggested a salt-bridge interaction between the conserved Lys-73 and Glu-166 in the class A enzyme, and the significant role of Glu-166 in the catalytic reaction was confirmed by Madgwick and Waley [8]. Lys-73 of the class A enzymes is situated at a position close to catalytic residue Ser-70 and corresponds to Lys-67 of the *C. freundii* cephalosporinase. On the basis of the comparative method proposed by Joris et al. [9], we assumed that Asp-217 of the *C. freundii* cephalosporinase corresponds to Glu-166 of the class A enzymes. We changed Asp-217 to glutamic acid, threonine and lysine in order to confirm this assumption. Although the result was contrary to our expectation, we found it an interesting fact that a single amino acid replacement at this position results in extension of the enzyme's substrate spectrum to methoxyimino-cephalosporins. In this paper, we report the properties of the mutant cephalosporinases.

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2. MATERIALS AND METHODS

2.1. *Escherichia coli strains and plasmids*

E. coli TG1 [10], a derivative of K12, was employed for DNA technology and for measuring the drug resistance of cells bearing the mutant cephalosporinase genes. *E. coli* AS226-51 is an *ampD* mutant of C600, which also has a deletion mutation in *ampC*. AS226-51 was used as a host cell for purification of the mutant enzymes. Plasmid pCFC-1 is a derivative of pHSG398, into which the wild-type cephalosporinase gene from *C. freundii* GN346 was inserted [5]. M13-CFC-1 was derived from M13mp18 [11] and also carries the wild-type enzyme gene. pHSG398 carrying the mutant cephalosporinase genes is termed pCFC-D217E, pCFC-D217T and pCFC-D217K, respectively, which are given the general name, pCFC-D217X. X denotes glutamic acid, threonine or lysine. The mutant genes are named using a one-letter amino acid code, i.e. D217E means a mutant gene in which aspartic acid-217 was changed to glutamic acid.

2.2. *Media, chemicals and enzymes*

For the transformation and transfection experiments, 2×YT broth [12] and YT agar [12] were employed. 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 (Meiji Seika Co., Tokyo, Japan); carbenicillin (Fujisawa Pharmaceutical Co., Osaka, Japan); cephalothin and cephaloridine (Shionogi Pharmaceutical Co., Osaka, Japan); cefuroxime and ceftazidime (Nihon Glaxo Co., Tokyo, Japan); cefmenoxime (Takeda Chemical Industries, Osaka, Japan); cefoxitin and imipenem (Merck Sharp and Dohme Research Laboratories, NJ, USA); aztreonam (Eisai Co., Tokyo, Japan); and chloramphenicol (Yamanouchi Pharmaceutical Co., Tokyo, Japan). [α-^{32}P]dCTP and the in vitro mutagenesis kit were purchased from Amersham Inc. (Buckinghamshire, UK). M13 sequencing kit and enzymes for DNA technology

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were purchased from Boehringer Mannheim GmbH, Takara Shuzo Co. (Kyoto, Japan), and Nippon Gene Co. (Toyama, Japan).

2.3. *Oligonucleotides and site-directed mutagenesis*

Oligonucleotides, 17- or 18-mer, were synthesized using a DNA synthesizer model 380B (Applied Biosystems, CA, USA). Site-directed mutagenesis was performed by the method of Eckstein [13]. The mutant genes were sequenced to confirm the desired exchange in the nucleotide sequence by the chain-termination method [14] using a specific oligonucleotide primer.

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

E. coli AS226-51 cells carrying pCFC-D217X were grown overnight in 2×YT medium at 37°C. The medium contained 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, the procedures established for purification of the wild-type enzyme were applied [6]. The mutant enzyme mediated by pCFC-D217X was extracted from the *E. coli* cells and purified to homogeneity by adsorption and elution on a CM-Sephadex C-50 column, and gel filtration on a Sephadex G-75 column, and its purity was confirmed by SDS gel-electrophoresis. β-Lactamase activity was assayed by a microiodometric method [15] with slight modifications, and a ultraviolet spectrophotometric method [16]. One unit of the enzyme was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate at pH 7 and 30°C. The kinetic parameters, K_m and K_i, were determined by the procedures reported previously [16].

2.5. *Antibiotic susceptibility testing*

The bacterial susceptibility to antibiotics was measured by the serial agar dilution method, according to the procedure described previously [1]. The susceptibility was expressed as the minimum inhibitory concentration of a drug (μg/ml).

3. RESULTS AND DISCUSSION

3.1. *Conversion of Asp-217 in the* C. freundii *cephalosporinase to glutamic acid, threonine and lysine, and expression of the mutant genes in* E. coli

Asp-217 of the *C. freundii* cephalosporinase was converted into glutamic acid, threonine or lysine according to Eckstein's method using M13-CFC-1 as a template. Mutant cephalosporinase genes were screened by DNA sequencing, and recloned into pHSG398. The resulting plasmids, pCFC-D217E, pCFC-D217T and pCFC-D217K, carry the mutant genes coding the mutant enzymes with Glu-217, Thr-217 and Lys-217, respectively. These plasmids and pCFC-1 were introduced into *E. coli* TG1 cells by transformation, and the transformants were examined as to their susceptibility to β-lactam antibiotics and chloramphenicol (Table I). Chloramphenicol resistance is a genetic marker from the vector plasmid, pHSG398. The transformants, contrary to our expectation, retained high levels of resistance to traditional cephalosporins such as cephalothin and cephaloridine. There were no significant decreases in the MICs of penicillins, a carbapenem (imipenem) and a monobactam (aztreonam). Moreover, the MICs of cefuroxime in the case of the cells producing the Glu-217 and Lys-217 enzymes were 4–8 times higher than that in the case of the wild-type enzyme producer. Cefuroxime, a prototype of oxyimino-cephalosporins, is known to be stable as to hydrolysis by many β-lactamases [17,18]. These results are in agreement with the β-lactamase activity found (Table I). No marked differences in the activity were observed between cells carrying the wild-type and mutant genes.

Table II

Substrate profiles of the wild and mutant cephalosporinases

E. coli strains	Substrate profiles (relative V_{max})[a]				
	CET	CER	CXM	CMX	CAZ
TG1/pCFC-1(wild)	100	928	0.006	0.001	$<3\times10^{-4}$
TG1/pCFC-D217E	100	600	0.10	0.04	0.11
TG1/pCFC-D217T	100	627	0.03	0.01	0.01
TG1/pCFC-D217K	100	1064	0.16	0.04	0.20

[a] β-Lactamase activity for cephaloridine and cephalothin as the reference was measured by the ultraviolet spectrophotometric method and the V_{max} values were determined by using the integrated Lineweaver-Burk plot analysis [19]. The relative V_{max} values for cefuroxime, cefmenoxime, ceftazidine and cephalothin as reference were determined by the microiodometric method with the saturated substrate concentrations. The relative V_{max} value is expressed as a percentage of that in the case of hydrolysis of cephalothin. The abbreviations used for cephalosporins are the same as in Table I

Table I

β-Lactamase activity of *E. coli* TG1 strains carrying the wild and mutant genes and the levels of resistance to antibiotics of the strains

E. coli strains	β-Lactamase activity[a] (units/mg protein)	MIC (μg/ml)[b]										
		CET	CER	CFX	CXM	CMX	CAZ	APC	CPC	IPM	AZT	CM
TG1/pCFC-1(wild)	1.5	1600	200	100	50	3.1	12.5	200	100	12.5	6.3	>50
TG1/pCFC-D217E	1.6	1600	100	100	200	3.1	12.5	200	200	12.5	6.3	>50
TG1/pCFC-D217T	1.7	1600	200	200	50	3.1	12.5	100	200	12.5	6.3	>50
TG1/pCFC-D217K	1.3	1600	100	100	400	3.1	12.5	100	200	12.5	6.3	>50
TG1	$<80\times10^{-3}$	6.3	3.1	1.6	<0.4	0.4	1.6	6.3	12.5	<0.2	6.3	

[a] β-Lactamase activity in sonically disrupted cells was measured by the microiodometric method with cephalothin as the substrate and the activity was expressed as units per mg of bacterial protein

[b] The antibiotics used were: CET, cephalothin; CER, cephaloridine; CFX, cefoxitin; CXM, cefuroxime; CMX, cefmenoxime; CAZ, ceftazidime; APC, ampicillin; CPC, carbenicillin; IMP, imipenem; AZT, aztreonam; CM, chloramphenicol

Table III

K_m and K_i values of β-lactams for the wild and mutant cephalosporinases

Kinetic parameters[a]	β-Lactams	Cephalosporinase			
		Wild	D217E	D217T	D217K
K_m (μM)	cephalothin	19	29	49	65
	cephaloridine	410	692	822	2180
K_i (μM)	cefuroxime	0.01	0.07	0.01	0.07
	cefmenoxime	0.02	0.08	0.02	0.3
	ceftazidime	1	7	3	28

[a] The K_m values were obtained from Lineweaver-Burk plots of the initial rate of hydrolysis, using the ultraviolet spectrophotometric method. The K_i values for inhibition by the methoxyimino-cephalosporins of the enzymes were determined with cephalothin as the substrate by the microiodometric method

3.2. *Substrate profiles of the mutant enzymes and their kinetic properties*

The oxyimino groups in the side-chain at position 7 of the cephalosporin nucleus are quite commonly employed today for new cephalosporins, because these side-chain structures confer stability to many β-lactamases on the antibiotics. Although the mutant enzymes conferring the cefuroxime resistance on the bacterial cells were obtained by means of site-directed mutagenesis in vitro, a similar evolutionary process in nature is quite possible because such a mutation can be induced by a single amino acid replacement. In order to compare the mutant enzymes with the wild-type enzyme in more detail, the 3 mutant enzymes were extracted from *E. coli* AS226-51 and purified to homogeneity. The substrate profiles of the wild-type and mutant enzymes are compared in Table II. A remarkable increase in the hydrolytic activity toward the oxyimino-cephalosporins tested was observed in the case of the mutant enzymes, particularly the Lys-217 and Glu-217 enzymes. The relative V_{max} values for cefuroxime, cefmenoxime and ceftazidime were 5–27, 10–40 and more than 33–667 times that of the wild-type enzyme, respectively. These findings, together with the results in Table I, excluded a possibility that Asp-217 participates in the hypothetical salt-bridge. The K_m or K_i values of the enzymes for two traditional cephalosporins and 3 oxyimino-cephalosporins are shown in Table III. The K_m value for cephaloridine, a zwitterionic compound, was greatly increased by the conversion of Asp-217 into a basic amino acid, lysine, suggesting an interaction between the residue and the substrate. The replacement of Asp-217 by lysine and glutamic acid also resulted in a decrease in the affinity of the enzyme for oxyimino-cephalosporins. These results indicate that an acidic amino acid residue at position 217 is not essential for the catalytic reaction, but position 217 may be located in or close to the active site pocket, and has an effect upon the substrate profile. Recently, the three-dimensional structure of a cephalosporinase from a *Citrobacter freundii* strain was reported by Ofner et al. [20]. They provided an interesting hypothesis as to the role of Ser-64, Lys-67, Tyr-150 and Lys-315 in the catalysis and the contribution of Asn-152, Ser-318 and Thr-319 to the aztreonam binding. However, it was difficult to speculate the role of the Asp-217 region from their crystallographic analysis.

In spite of increased hydrolytic activity toward ceftazidime and cefmenoxime, the MICs of the two cephalosporins for the cells producing the mutant enzymes were not altered. No probable explanation for such a result can be offered at the present time; however, this result may be attributable in part to the higher antibacterial activity of these third generation cephalosporins.

Recently, plasmid-mediated class A β-lactamases with extended substrate spectra for oxyimino-cephalosporins and aztrenam were discovered in clinical isolates resistant to these new β-lactams [21–25]. These novel class A β-lactamases were revealed to originate from SHV-1 β-lactamase or TEM-2 β-lactamase and differ from their ancestors in only one or two amino acid residues [24,26,27]. However, in the case of these class A β-lactamases, the extension of the substrate spectra was attributed to marked increase in their affinity for the new β-lactams. Our findings reported in this paper may indicate the possible development of class C β-lactamases against oxyimino-cephalosporins through chromosomal gene mutation in nature.

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