Effect of an Amino Acid Insertion into the Omega Loop Region of a Class C β-Lactamase on Its Substrate Specificity†

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ABSTRACT: The extended-substrate specificity of *Enterobacter cloacae* GC1 β-lactamase is entirely due to a three amino acid insertion after position 207. To clarify the reason for the extended-substrate specificity, Ala, Ala-Ala, Ala-Ala-Ala, and Ala-Ala-Ala-Ala were inserted after position 207 on the basis of the class C β-lactamase from *E. cloacae* P99, respectively. The k_{cat} and K_m values of all the mutant enzymes for cephalothin, benzylpenicillin and ampicillin were almost the same as those of the wild-type enzyme, except for those of P99-210-4A which were decreased 4−15-fold. On the other hand, the k_{cat} and K_m values for oxyimino β-lactams such as cefuroxime, ceftazidime, and aztreonam increased with increasing numbers of inserted alanines. The k_{cat} values of the mutant enzymes for cefroxime increased 140−7400-fold compared with that of the wild-type. The K_m values also increased with almost the same magnitude, resulting in about the same k_{cat}/K_m values as that of the wild-type. On progressive inhibition analysis of aztreonam of the mutant enzymes, two kinds of inactive acyl-enzyme with distinct stabilities were observed, and the proportion of the less stable inactive enzyme increased with increasing numbers of inserted alanines. This suggests that the extension of the substrate specificity is due to instability of the acyl-intermediate caused by an increased deacylation rate in the reaction process.

Most third-generation β-lactams which are resistant to class A and class C β-lactamases have a bulky and plane group such as an aminothiazolemethoxyimino or aminothiazole-carboxypropylozyimino side chain at position 7 of their cephalosporin nucleus or at position 2 of their monobactam nucleus, and they are named oxyimino β-lactams. With the extensive use of oxyimino β-lactams, extended spectrum β-lactamases capable of hydrolyzing oxyimino β-lactams have been found in the natural environments. These β-lactamases contain one to three amino acid substitutions compared to their prototypes.

Most known extended spectrum β-lactamases originated from plasmid-mediated TEM or SHV type β-lactamases. In the case of TEM type β-lactamases, five positions for effective amino acid substitutions were reported. Since most of these residues are located at the border of the active site cavity, alteration of the forms of the substrates binding to the active site is speculated. In the case of class C β-lactamases, the only extended spectrum β-lactamases reported have been one from a clinically isolated strain and two in vitro mutant β-lactamases. In these examples, an omega loop region [positions 185−225; assigned by Knox (*1*)] of class C β-lactamases is the hot spot for mutation to obtain oxyimino β-lactam hydrolysis activity. The effective substitution positions were Glu219 (*2, 3*) and Asp217 (*4*), which exist in the omega loop of *Citrobacter freundii* GN346 class C β-lactamase, resulting in extension of the substrate specificity to oxyimino β-lactams. The X-ray crystal structures of class C β-lactamases suggest that Glu 219 and Asp217 cannot interact in substrate binding since their side chains point away from the substrate binding site (*5, 6*). Another case is the β-lactamase of GC1, which is a clinical isolate highly resistant to third-generation β-lactams. The GC1 β-lactamase was confirmed to have an effective mutation of a three amino acid insertion in the omega loop region (Figure 1). Because the three amino acid insertion site is located far from the active site, no direct interaction between the substrate and the inserted amino acids is expected. These mutant β-lactamases could hydrolyze oxyimino β-lactams up to 1000-fold faster than wild-type β-lactamases without an effect on the good substrate hydrolysis.

In this study, to examine the effect of a mutation in an area far from the substrate binding site on the extension of substrate specificity, a series of mutant β-lactamases, which have an insertion of one to four alanine(s) after Arg210 of class C β-lactamases, was constructed, and their kinetic characters were examined.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmid. E. coli TG1 [Δ*(lac-pro), supE, thi, hsdD5/FtraD36, pro A+B+, lacI*^{q}, lacZΔ*M15*], a derivative of K12, was employed for DNA technology. *E. coli* AS226-51 (*7*), an *ampD* mutant of C600, which also has a deletion mutation in *ampC*, was used to measure the

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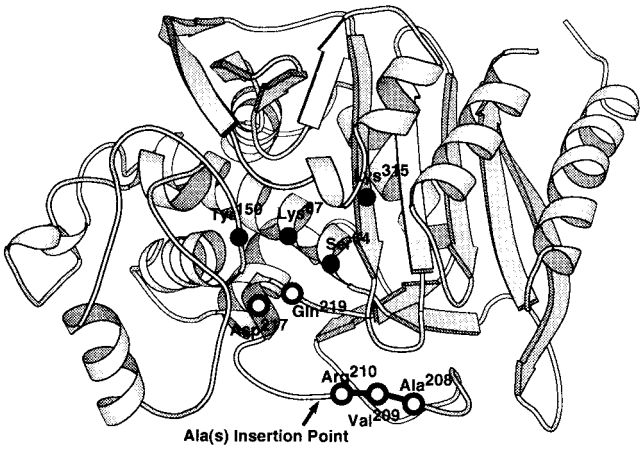


FIGURE 1: 3D structure of class C β-lactamase from *E. cloacae* P99 (*6*). Catalytic residues Ser64, Lys67, Tyr150, and Lys315 of class C β-lactamase are indicated by (●). Asp217, Gln219 (Glu219 of C. freundii class C β-lactamase), and Ala208-Val209-Arg210, mutations known to cause extended specificity to oxyimino β-lactams, are indicated by (○). This figure was drawn with the aid of MOLSCRIPT (*23*).

Table 1: Amino Acid Sequences of Mutant β-Lactamases

Enzyme	Plasmid	Amino Acid Sequence	
P99	pCS900	^{202}GYRDGKAVR	^{211}VSPGML
P99-210-1A	pCS9-210-1A	^{202}GYRDGKAVR ^{211}A	^{212}VSPGML
P99-210-2A	pCS9-210-2A	^{202}GYRDGKAVR ^{211}AA	^{213}VSPGML
P99-210-3A	pCS9-210-3A	^{202}GYRDGKAVR ^{211}AAA	^{214}VSPGML
P99-210-4A	pCS9-210-4A	^{202}GYRDGKAVR ^{211}AAAA	^{215}VSPGML
GC1	pCS100	^{202}GYRDGKAVR ^{211}AVR	^{214}VSPGML

β-lactam susceptibility of cells bearing the mutant β-lactamase genes, and as host cells for enzyme preparation in order to avoid contamination by the *ampC* β-lactamase of *E. coli*. pCS100 and pCS900 are the derivatives of pHSG398 into which a 1.55 kb fragment carrying the GC1 and P99 β-lactamase genes has been inserted. The names of the mutant β-lactamase genes and the amino acid sequences around the mutation point are summarized in Table 1. pTTQ18K is a derivative of pTTQ18 in which the original ampicillin resistance marker is replaced with the kanamycin resistance gene (*8*).

Media, Chemicals, and Enzymes. For the transformation and transfection experiments, 2× yeast extract/tryptone (2×YT) broth and yeast extract/tryptone (YT) agar (*9*) were employed. For β-lactamase preparation, the bacteria were grown in nutrient broth (Eiken Chemical Co., Tokyo, Japan). Heart infusion agar (Eiken Chemical Co.) was used for measuring the bacterial susceptibility to β-lactams.

Enzymes and enzyme kits for DNA technology were purchased from Takara Shuzo Co. (Kyoto, Japan), Toyobo Co. (Osaka, Japan), and Wako Nippon Gene Co. (Tokyo, Japan). [α-^{32}P]dCTP was purchased from Amersham Co. The antibiotics used in this study (Figure 2) were kindly provided by the following pharmaceutical companies: benzylpenicillin, ampicillin, and kanamycin from Meiji Seika Kaisha Ltd., Tokyo, Japan; cephalothin from Shionoge and Co., Ltd., Osaka, Japan; cefuroxime and ceftazidime from Nippon Glaxo Ltd., Tokyo, Japan; aztreonam from Eizai Co., Tokyo, Japan; sulbactam from Pfizer Seiyaku, Tokyo, Japan; and chloramphenicol from Yamanouchi Pharmaceutical Co., Tokyo, Japan.

Site-Directed Mutagenesis. The mutant β-lactamase genes constructed in this study are summarized in Table 1. Site-directed mutagenesis was performed by the modification-of-restriction-site method using the PCR reaction described by Ito et al. (*10*) The primers used in this study are summarized in Table 2. First and second PCR reactions were performed as described previously (*8*). The amplified DNA fragments were separated by agarose gel electrophoresis and identified by ethidium bromide staining. A slice of an agarose gel containing the fragment of interest was excised, and DNA was recovered using a QIAEX II Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany). The final amplified fragment was digested with *Afl*II to select the mutated fragment. The 260 bp *Afl*II fragment including the mutation was used to be replaced with the corresponding region of the P99 β-lactamase gene on pCS900. The region was entirely sequenced to ensure that the desired mutation was introduced and that no unwanted mutation was present.

Purification Procedure and β-Lactamase Assay. E. coli AS226-51 cells carrying pTTQ18K with the β-lactamase gene under the control of the *tac* promoter were cultivated in 5 L of nutrient broth under the same conditions as described previously (*8*). Crude β-lactamases were prepared by disruption of the cells with a French press in 50 mM sodium phosphate buffer, pH 7.0, containing 10 μg/mL DNase II, followed by centrifugation for 10 min at 1400*g* and 4 °C to remove the cell debris. The supernatant was centrifuged again for 1 h at 40000*g* and 4 °C. The crude enzymes were purified as follows: they were dialyzed against three changes of 10 mM triethanolamine hydrochloride buffer, pH 7.0. A sample of each was then applied to a column of CM-Sephadex C-50 equilibrated with the same buffer. The β-lactamase was eluted with a 0−0.6 M linear NaCl gradient. The fractions containing the activity were applied directly to a column of *m*-aminophenylboronic acid coupled to Affigel 10 (Bio-Rad) equilibrated with 20 mM triethanolamine hydrochloride buffer, pH 7.0, and eluted with a 0−0.6 M linear sodium borate gradient (*11*). The purified enzyme was desalted on a column of Sephadex G-25

Table 2: Chemically Synthesized Mutant Primers

primer	sequence	purpose
ECGCPR−F	5′-CGAAGGGATCCAGCATTCCGGCTAT-3′	commonly used to amplify the β-lactamase gene
ECGCPR−R	5′-CCCGGCAATGTTTTACTGTAGCGCC-3′	commonly used to amplify the β-lactamase gene
Mutafl	5′-GACGCGGGTCCTTTAGCCGCTCAAG-3′	destroy *Afl*II site of base position 994
Ins-1A	5′-CAGCATACCCGGCGAAACAGCGCGCACCGCTTTACCGTC-3′	insertion of Ala at position 210
Ins-2A	5′-CAGCATACCCGGCGAAACAGCTGCGCGCACCGCTTTACCGTC-3′	insertion of Ala-Ala at position 210
Ins-4A	5′-CAGCATACCCGGCGAAACTGCTGCTGCAGCGCGCACCGCCT-3′	insertion of Ala-Ala-Ala-Ala at position 210
Ins-4AF	5′-AGCGGTGCGCGCTGCAGCAGCAGTTTCGCCGGGTATGCTGGAT-3′	insertion of Ala-Ala-Ala-Ala at position 210

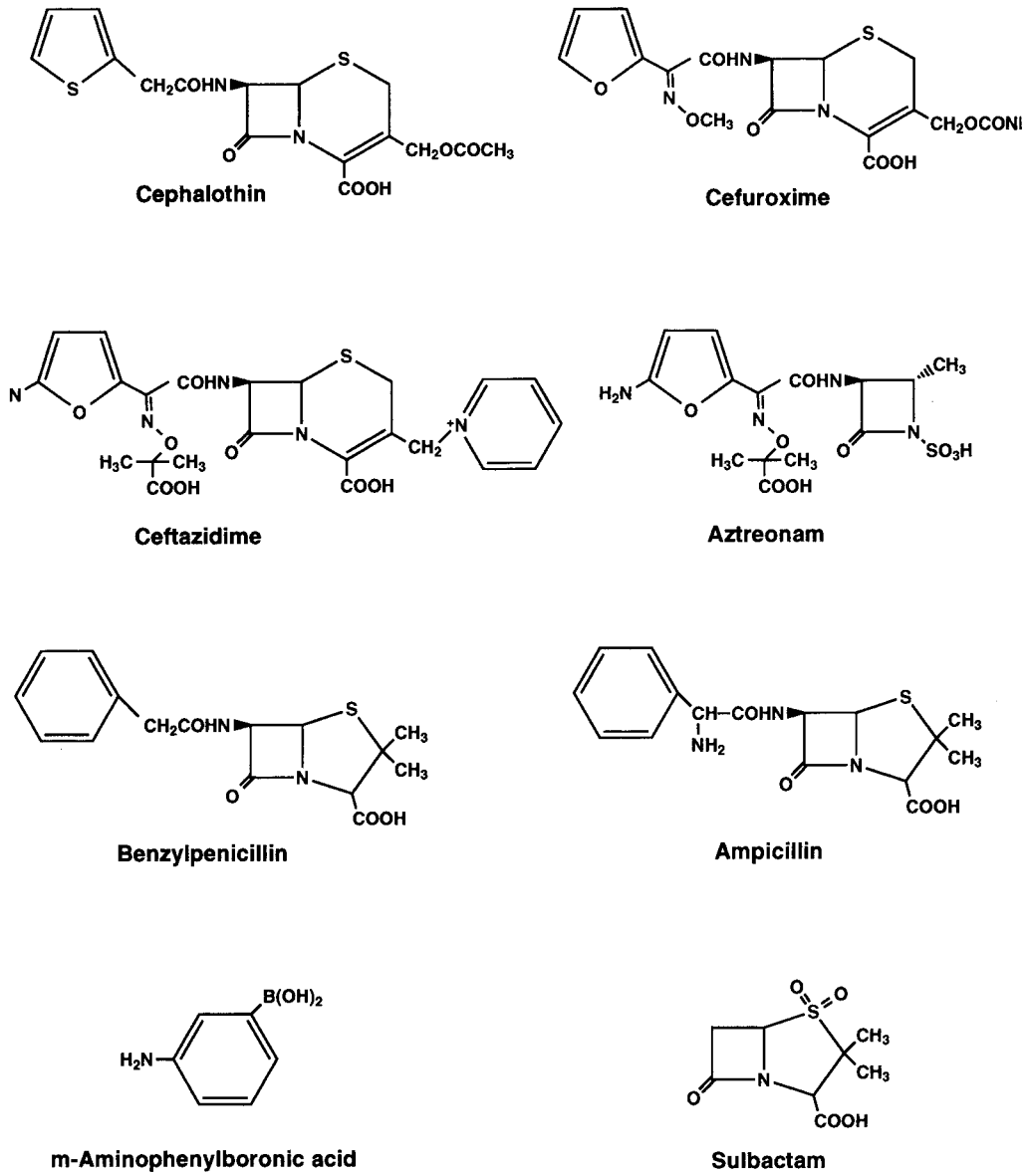


FIGURE 2: Structures of β-lactams and β-lactamase inhibitors used in this study.

equilibrated with 100 mM sodium phosphate buffer, pH 7.0. All the enzymes were purified to more than 95% homogeneity, as judged on SDS−PAGE.

Kinetics. The β-lactamase reaction was assayed in 50 mM sodium phosphate buffer, pH 7.0, at 30 °C by the UV spectrophotometric method. Substrate hydrolysis was measured as the change in A_{265} or A_{290} for cephalothin, A_{260} and A_{330} for cefuroxime, A_{260} for ceftazidime, A_{318} for aztreonam, A_{235} for benzylpenicillin, or A_{230} for ampicillin with a temperature-controlled 220A spectrophotometer (Hitachi). The k_{cat} and K_m values were determined from the reaction curve by fitting to the integrated form of the Lineweaver-Burk plot (*12*). Because the K_m values of some mutants for ceftazidime and cefuroxime were so high that the reaction could not be saturated, 100 μM ceftazidime and 500 μM cefuroxime were used to determine the k_{cat} and K_m values, and the k_{cat}/K_m ratio was determined from measurements of the first-order rate of hydrolysis with substrate concentrations much less than the K_m values deduced with the on-line method. The K_i values were determined by competition with cephalothin as the reporter substrate. Kinetic parameters were determined at least 3 times.

Progressive inhibition was examined as described previously (*13*). 2 μM β-lactamase was incubated at 30 °C with 10 μM aztreonam ($I/E = 5$) in 1 mL of 50 mM sodium phosphate buffer, pH 7.0. At different times, 20 μL of the reaction mixture was withdrawn and mixed with excess substrate (500 μM cephalothin), and then the residual enzyme activity was determined by the UV spectrophotometric method. The reactivation rate constant, k_{react}, was determined from a semilogarithmic plot of the recovered activity versus the incubation time.

Antibiotic Susceptibility Testing. The bacterial susceptibility to antibiotics was measured by the serial agar dilution method, according to the procedure described previously (*14*). The susceptibility was expressed as the minimum inhibitory concentration of a drug (μg/mL).

RESULTS

Construction of the Mutant β-Lactamase Genes and Their Phenotype. pCS9-210-3A was constructed in a previous work (*14*). All the other mutant genes were constructed by site-directed mutagenesis using PCR. pCS9-210-1A and

Table 3: MIC Values of β-Lactams for *E. coli* AS226-51 Carrying Mutant β-Lactamase Genes

	plasmid					
β-lactams	pCS900	pCS9-210-1A	pCS9-210-2A	pCS9-210-3A	pCS9-210-4A	pCS100
cephalothin	800	800	400	400	200	800
cefuroxime	25	400	400	400	200	400
ceftazidime	3.1	6.3	12.5	25	25	25
aztreonam	1.6	3.1	6.3	12.5	1.6	6.3
ampicillin	100	100	100	100	25	100
benzylpenicillin	1600	800	800	800	200	400

Table 4: Thermal Stability of Mutant Enzymes

enzyme	$t_{1/2}$ at 50 °C (min)
P99	93.3
P99-210-1A	84.6
P99-210-2A	90.2
P99-210-3A	64.1
P99-210-4A	56.7
GC1	68.8

pCS9-210-2A were constructed by the modification-of-restriction-site method (*10*). To construct pCS9-210-4A, a mutation, codon GAT of Asp217 changed to GTT, was introduced reproducibly. To avoid this unwanted mutation, primer Ins-4AF was employed for the construction of pCS9-210-4A by means of the overlap extension method (*15*).

The β-lactam resistance levels of *E. coli* AS226-51 carrying the mutant β-lactamase genes were evaluated as MICs and are summarized in Table 3. The MICs of classical β-lactams such as cephalothin, benzylpenicillin, and ampicillin of *E. coli* carrying the mutant genes with a one to three alanine residue(s) insertion were almost the same as those of *E. coli* carrying the wild-type gene. The MICs of classical β-lactams of *E. coli* with pCS9-210-4A were decreased 4−8-fold compared with those of the wild-type. On the other hand, the MICs of oxyimino β-lactams such as cefuroxime, ceftazidime, and aztreonam of the *E. coli* strains carrying the mutant genes tended to increase with increasing number of Ala residues. The highest effect on the MICs of oxyimino β-lactams of the mutation was observed in the case of *E. coli* with pCS9-210-3A. On the other hand, the MICs of oxyimino β-lactams of *E. coli* with pCS9-210-4A were decreased compared with those of the cells carrying pCS9-210-3A.

Thermal Stability of the Mutant Enzymes. In a previous study on the mutants to position 219 of *C. freundii* class C β-lactamase, it was found that a lowering of the thermal stability was closely related to an increase in the cefuroxime hydrolysis activity. This suggests that structural instability of the omega loop results in an increase in oxyimino β-lactam hydrolysis. To confirm the effects of alanine insertions on the structural stability, the thermal stability of the mutant enzymes was examined at 50 °C and compared with that of the wild-type enzyme (Table 4). Although a marked decrease in the thermal stability of the mutant enzymes was not observed, the alanine insertions tended to decrease the thermal stability with increasing numbers of alanines. In the case of the most labile mutant, the P99-210-4A enzyme, the half-life period of its activity at 50 °C was only half that of the wild-type enzyme.

Kinetic Properties of the Mutant Enzymes. Kinetic parameters were determined for one traditional cephalosporin (cephalothin), two traditional penicillins (benzylpenicillin and ampicillin), two oxyimino cephalosporins (cefuroxime and ceftazidime), and one monobactam, which also contains an oxyimino side chain (aztreonam). As shown in Table 5, the Ala insertions did not affect the k_{cat} values for cephalothin, benzylpenicillin, and ampicillin. The K_m values were not influenced by the alanine insertions until three alanines. On the other hand, the K_m values of P99-210-4A for the favorite substrates were increased 4−15-fold compared with those of the wild-type enzyme, suggesting a structural alteration in the active site space.

Table 5: Kinetic Parameters of Mutant β-Lactamases for Favorable Substrates[a]

substrate	enzyme	k_{cat} (s^{-1})	K_m (μM)	K_i (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
cephalothin	P99	84.8	10		8.2
	P99-210-1A	124.0	39		3.2
	P99-210-2A	59.3	37		1.6
	P99-210-3A	94.0	34		2.8
	P99-210-4A	83.6	148		0.56
	GC1	74.8	31		2.4
benzylpenicillin	P99	10.1	3.1	1.6	3.3
	P99-210-1A	6.3	2.9	5.7	2.2
	P99-210-2A	3.7	3.0	5.0	1.2
	P99-210-3A	5.5	3.4	3.7	1.6
	P99-210-4A	8.6	25.0		0.34
	GC1	5.7	3.1	4.7	1.8
ampicillin	P99	0.36	0.97	0.82	0.37
	P99-210-1A	0.25	1.0	1.3	0.25
	P99-210-2A	0.16	0.99	1.7	0.16
	P99-210-3A	0.60	2.5	1.9	0.24
	P99-210-4A	0.73	8.0	20	0.09
	GC1	0.52	4.3	4.7	0.12

[a] The k_{cat} and K_m values were determined by the UV spectrophotometric method, and the K_i values were measured by the UV spectrophotometric method with cephalothin as the substrate.

The steady-state kinetics of the oxyimino β-lactams, i.e., cefuroxime, ceftazidime, and aztreonam, were significantly affected by the Ala insertions (Table 6). The k_{cat} values of the mutants increased with increasing numbers of alanine(s) inserted. The most striking difference was observed in the case of cefuroxime, the k_{cat} values of the mutants being 140−7400-fold those of the wild-type enzyme. The increasing k_{cat} values for oxyimino β-lactams were accompanied by increases in the K_m or K_i values. The increases in the k_{cat}, K_m, and K_i values were all about the same, and the k_{cat}/K_m values were less affected by the insertions. The most remarkable change in the k_{cat}/K_m value was observed in the case of cefuroxime and P99-210-3A, there being a 3-fold increase compared with that of the wild-type enzyme.

Susceptibility of the Mutants to β-Lactamase Inhibitors. The inhibition constants (K_i) of the wild-type and mutant enzymes for two β-lactamase inhibitors, i.e., and *m*-aminophenylboronic acid, were measured. The chemical struc-

Table 6: Kinetic Parameters of Mutant β-Lactamases for Oxyimino β-Lactams[a]

substrate	enzyme	k_{cat} (s^{-1})	K_m (μM)	K_i (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
cefuroxime	P99	0.024	0.07	0.01	0.33
	P99-210-1A	3.4	3.3	4.7	1.0
	P99-210-2A	19.4	32		0.6
	P99-210-3A	50.4	46		1.1
	P99-210-4A[b]	178	912		0.19[c]
	GC1	24.1	26		0.92
ceftazidime	P99	0.007		11	0.0033
	P99-210-1A[b]	2.7	441		0.0033[c]
	P99-210-2A[b]	3.1	614		0.0044[c]
	P99-210-3A[b]	6.5	575		0.0072[c]
	P99-210-4A[b]	12.1	821		0.0080[c]
	GC1	11.0	919		0.0012[b]
aztreonam	P99	<0.0002		0.052	
	P99-210-1A	0.0006		0.92	
	P99-210-2A	0.0056		0.85	
	P99-210-3A	0.009		0.59	
	P99-210-4A	0.05		5.1	
	GC1	0.011		1.1	

[a] The k_{cat} and K_m values were determined by the UV spectrophotometric method, and the K_i values were measured by the UV spectrophotometric method with cephalothin as the substrate. [b] The substrate concentrations (500 μM cefuroxime and 100 μM ceftazidime) are unsaturated conditions for the enzyme reaction, and the k_{cat} and K_m values were estimated by extrapolation to the Hanes−Woolf plot. [c] The k_{cat}/K_m values were determined using the measurements of the first-order rates of hydrolysis at the substrate concentrations much less than K_m values.

Table 7: K_i Values of Mutant β-Lactamases for Inhibitors[a]

enzyme	K_i (μM)	
	sulbactam	*m*-aminophenyl-boronic acid
P99	707	109
P99-210-1A	1960	152
P99-210-2A	1400	242
P99-210-3A	625	190
P99-210-4A	640	780
GC1	860	155

[a] The K_i values were determined by the UV spectrophotometric method with cephalothin as the substrate.

Table 8: k_{react} Values and Fractions of More Stable Inactive Forms of the Wild-Type and Mutant β-lactamases

enzyme	k_{react}[a] (s^{-1})	fraction of more stable enzyme (%)[b]
P99	<0.0002[c]	94
P99-210-1A	0.0005	59
P99-210-2A	0.0024	32
P99-210-3A	0.0050	16
P99-210-4A	ND	<10
GC1	0.0049	16

[a] The k_{react} values were determined from a semilogarithmic plot of the recovered activity versus the incubation time in Figure 3b. [b] The fractions of the more stable inactive enzymes were calculated from the difference in rate between the steady state and without aztreonam treatment. [c] Deduced from the k_{cat} values.

tures of the inhibitors are shown in Figure 2. Sulbactam, a penam sulfone, is known to inhibit class C β-lactamases pseudoirreversibly (*16*). Sulbactam, lacking an acyl amino side chain, may be useful for examining an alteration in the active site space. The K_i values of P99-210-1A and P99-210-2A for sulbactam were about 3 times that of the wild-type enzyme, and evidently higher than those of the other mutant enzymes. In the cases of P99-210-3A and P99-210-4A, the K_i values were about the same as that of the wild-type enzyme. An appropriate configuration for binding may be restored on further addition of Ala.

Boronic acid derivatives are known to bind to the active site serine in a manner similar to a tetrahedral intermediate (*17, 18*). We used *m*-aminophenylboronic acid as a ligand for affinity chromatography for the β-lactamase purification. P99-210-1A, P99-210-2A, P99-210-3A, and GC1 β-lactamases bound to the ligand equilibrated with 20 mM triethanolamine hydrochloride buffer, pH 7.0, with 0.5 M NaCl. However, P99-210-4A could not bind to the ligand in the presence of 0.5 M NaCl, but bound to it in the NaCl-free buffer. This suggested the possibility that excess alanine insertion caused perturbation of the active site configuration. The K_i values of the wild-type and mutant enzymes for *m*-aminophenylboronic acid are shown in Table 7. Although there was no significant difference between GC1, P99-210-1A, P99-210-2A, and P99-210-3A, P99-210-4A showed an about 7-fold increase in K_i, indicating lower binding ability as compared to *m*-aminophenylboronic acid.

Progressive Inactivation of the Mutants by Aztreonam. The progressive inhibition profiles of the reaction between class C β-lactamases and oxyimino β-lactams are biphasic. The enzymes are completely inactivated in the initial period, and then the activity is completely recovered (*13*). The progressive inhibition profiles of the wild-type and mutant enzymes with aztreonam at an *I*/*E* ratio 5 are shown in Figure 3a. All the enzymes except P99-210-4A were fully inactivated by aztreonam within 1 min. The inactivation of P99-210-4A could not be observed because of its high k_{cat} value for aztreonam. The wild-type could not recover its original activity within the 2 h reaction time because of a significantly stable acyl intermediate. The phase of the recovery obeyed first-order kinetics (Figure 3b), and the rate constants were represented by k_{react}. The k_{react} values were almost the same as the k_{cat} values, and increased with increasing numbers of inserted Ala residues (Table 8).

Two inactive forms differing in stability, i.e., a less stable form and a more stable form, have been reported for an aztreonam-treated class C β-lactamase (*13*), and were observed in the cases of the mutant, except for P99-210-4A, and wild-type enzymes (Figure 4). The proportion of the more stable inactive form can be calculated from the difference in activity between the steady-state form and the untreated aztreonam form. The proportion of the less stable inactive form was also calculated from the difference in activity between the initial and steady states. The proportion of the more stable inactive enzymes decreased with increasing k_{react} and k_{cat} values (8). This suggested that the inserted mutations disturbed the formation of the more stable inactive enzyme.

DISCUSSION

The P99-210-4A mutant showed a significant decrease in affinity to its favorite substrates, oxyimino β-lactams and *m*-aminophenylboronic acid, suggesting that the 4 Ala insertion had an unfavorable effect on both the initial binding and the transition state. This mutational effect on the kinetic

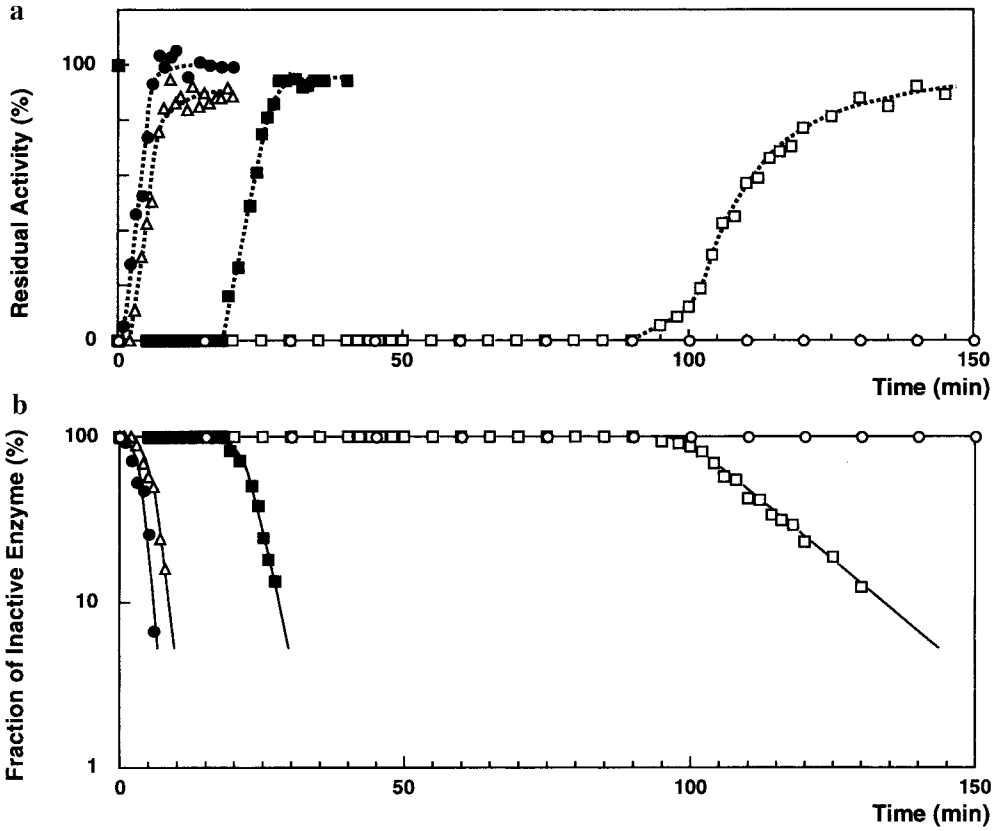
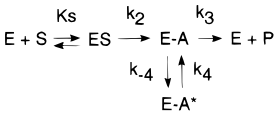


FIGURE 3: Progressive inactivation (a) and reactivation of the wild-type and mutant β-lactamases by aztreonam. Wild-type (○); P99-210-1A (□); P99-210-2A, (■); P99-210-3A (△); GC1 (●). (a) After 2 μM enzyme had been preincubated with 10 μM aztreonam ($I/E = 5$), the residual activity, evaluated as the initial rate of cephalothin hydrolysis, was measured as described in the text, and plotted against the preincubation period. The untreated enzyme activity was taken as 100%. (b) Logarithm of the inactivated fraction of enzyme treated with aztreonam was calculated from the phase of reactivation in (a) and plotted against the incubation time.

parameters is similar to the case of the mutants for Glu219 of *C. freundii* class C β-lactamase (*8*), suggesting that acceleration of the deacylation step is responsible for the higher hydrolytic activity toward oxyimino β-lactams. There was no relationship between the Ala insertions and the observed variation in the K_i values for two inhibitors, i.e., and *m*-aminophenylboronic acid. Sulbactam has a small side chain at position 6 of its nucleus, and *m*-aminophenylboronic acid is an analogue of the transition state. This observation may suggest that a structural modification of the omega loop structure affects the recognition of the side chain structure at position 6 or 7 of the β-lactam nucleus.

The kinetic process of oxyimino β-lactam hydrolysis by extended spectrum class C β-lactamases is different from that of class A β-lactamases. In general, the k_{cat}/K_m value of class A and C β-lactamases corresponds to an apparent second-order rate constant for acyl-enzyme formation, and the value is independent of the deacylation rate. Class A β-lactamase show low k_{cat} and high K_m values similar to oxyimino β-lactams, resulting in a low k_{cat}/K_m value. The acylation process is the rate-limiting step in the reaction sequence. The extended spectrum β-lactamase derived from the TEM type class A enzyme shows an increased k_{cat} value and a decreased K_m value, resulting in an increased k_{cat}/K_m value, showing the accelerated acylation reaction in the reaction sequence. On the other hand, class C β-lactamases usually show high affinity (low K_m or K_i value) and low k_{cat} values for oxyimino β-lactams. The deacylation process is a rate-limiting step in the reaction sequence. The increased

Scheme 1



k_{cat} values of mutant enzymes for oxyimino β-lactams hydrolysis suggest the accelerated deacylation step. As the unvaried k_{cat}/K_m values of mutant enzymes for oxyimino β-lactams hydrolysis, the mutations were less effective for the acylation reaction.

Two types of inactive enzymes were observed in the oxyimino β-lactam hydrolysis reaction of class C β-lactamases, and a reaction mechanism with a branched pathway was proposed in a previous study (*13*) (Scheme 1). In this study, two inactive intermediates were also observed in the reaction of the Ala-inserted mutants with aztreonam. According to an increase in the number of inserted Ala, the proportion of the more stable intermediate (E−A*) in the inactive enzyme fraction evidently decreased. This fact indicates that an amino acid insertion in the omega loop has an effect on the acyl-enzyme conformation which results in an increase in the amount of the less stable inactive enzyme (E−A), which easily undergoes the deacylation reaction.

Recent X-ray crystallographic and computer modeling studies have enabled us to rationalize how a mutation might alter the substrate specificity of class A β-lactamases. On the other hand, class C β-lactamases have been scarcely analyzed by an analogous manner. It is difficult to apply such a computer modeling technique to the identification of

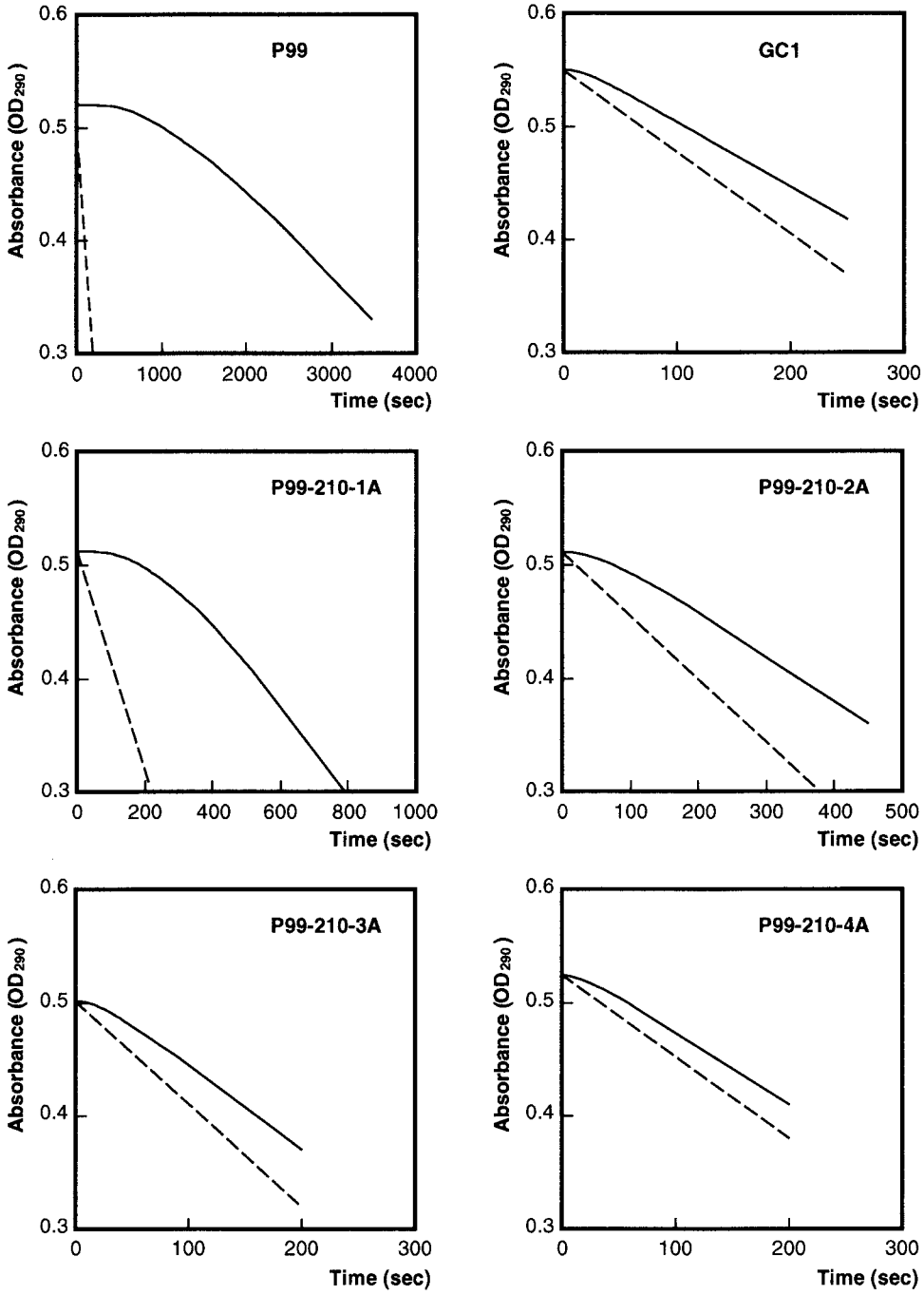


FIGURE 4: Absorption changes in the reaction mixtures of aztreonam-treated wild-type and mutant β-lactamases with cephalothin as the substrate. The β-lactamases (final concentration 2 μM) were incubated with 10 μM aztreonam in 50 mM sodium phosphate buffer (pH 7.0) for 1 min at 30 °C. A portion (20 μL) was mixed with 3 mL of 500 μM cephalothin in the buffer, and then the time course of A_{290} was monitored (solid line). The dashed line indicates the absorption change with the same amount of untreated enzyme as treated enzyme.

a structural modification of the loop structure as a cause for the extended spectrum phenomenon, because the highly free rotation of the loop is deduced. The omega loop region of class C β-lactamases is different from that of class A β-lactamases. The twisting polypeptide chain of this region in class C β-lactamases proceeds in the reverse direction relative to that in the class A β-lactamases (*6*). Moreover, class C β-lactamases lack a catalytic residue in the loop corresponding to Glu166 of the class A β-lactamases (*19*).

The extended substrate phenomenon induced by a mutation in the loop may be attributed to an indirect effect on the active site space, since the three amino acid insertion is located too far away from the active site, and each residue cannot interact with the oxyimino β-lactams directly. We propose two possible interpretations of the structural change. One is a possibility that the mutant enzymes acquire extra space for the large oxyimino side chain in the active site. The region around Tyr203 in the loop is located in the vicinity of Gly321 of the β domain, and a mutation in the loop might result in enlargement of the oxyimino side chain binding site through a shift of the β3 strand. The movement of the β3 strand might add more space to permit another conformation of the acyl intermediate (E−A*) with oxyimino β-lactams. This assumption reminds us of the extended spectrum class A β-lactamases, which include mutations at positions 238 and 69 with a buried side chain. Huletsky et

al. (*20*) suggested that the replacement of each of the residues at positions 238 and 69 by an amino acid with a larger side chain might push the β3 strand out and away from the active Ser70. Extension of the space for the side chain at position 7 of the cephem nucleus may contribute to the extended-substrate specificities of both class A and C β-lactamases.

Another possibility is similar to that reported in the case of a mutation in the omega loop of class A TEM type β-lactamases. Arg164 of TEM-1 β-lactamases forms a strong ionic bond with Asp179, stabilizing the omega loop conformation. When Arg164 was replaced by His or Ser, perturbation of the omega loop was deduced by computer modeling and resulted in the substrate specificity being extended to oxyimino β-lactams. The increased proteolysis sensitivity (*21*) and the reduced expression levels (*22*) of the Arg164 mutant enzymes suggested that the spectrum extension to oxyimino β-lactams is attributable to an increase in the mobility of the omega loop. In the case of the class C β-lactamases reported in this study, the extended spectrum phenomenon was closely related to a decrease in the thermal stability of the enzyme activity. The mobile loop structure may enable the enzyme to form an unstable acyl intermediate.

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