ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1989, p. 2160–2163
0066-4804/89/122160-04$02.00/0

Vol. 33, No. 12

Structural Features Related to Hydrolytic Activity against Ceftazidime of Plasmid-Mediated SHV-Type CAZ-5 β-Lactamase

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Tryptic peptides of the novel ceftazidimase CAZ-5 were sequenced by manual Edman degradation and aligned according to strong homology (more than 98%) with SHV-1 and SHV-2 β-lactamase sequences. CAZ-5 differed from SHV-1 by five amino acid substitutions. Unusually high activity of CAZ-5 towards ceftazidime was imputed to substitution of a Lys for a Glu at position 214 of the mature protein.

Broad-spectrum cephalosporins such as cefotaxime and related compounds, which are very effective against gram-negative bacteria, have been extensively used in hospital practice. However, infections caused by members of the family *Enterobacteriaceae* resistant to newer cephalosporins were soon observed (23, 24). In part, the resistance was related to overproduction of chromosomally mediated cephalosporinase involving a stable derepression of enzyme synthesis (17, 20). Otherwise, transferable resistance to broad-spectrum cephalosporins, first detected in the Federal Republic of Germany (15, 16), was responsible, especially in France, for outbreaks of nosocomial infections (6, 14, 26). Whatever the main bacterial phenotype (cefotaxime resistance, ceftazidime resistance, or multiresistance), it was associated with the synthesis of new plasmid-mediated β-lactamases (5, 7, 8, 10, 12, 21, 25).

Recently, such as enzyme, CAZ-5, also called SHV-4 (22), was isolated (Hôpital Begin, St. Mandé, France) from *Klebsiella pneumoniae* 210-2, a strain resistant to broad-spectrum cephalosporins. The kinetic constants of CAZ-5 did not notably differ from those of the cefotaxime-hydrolyzing β-lactamase SHV-2, with the exception of the hydrolysis rates for ceftazidime and aztreonam, which were markedly increased (Table 1). In DNA hybridization analysis under conditions of high stringency with a probe bearing the SHV-2 β-lactamase structural gene, CAZ-5 was found to belong to the SHV β-lactamase family (M. Barthélémy, J. Péduzzi, A. Chenon, J. C. Nicolas, A. Thabaut, K. Tiwari, A. Morand, and R. Labia, 28th Intersci. Conf. Antimicrob. Agents Chemother., paper no. 480, 1988).

Previously, we showed that cefotaxime-hydrolyzing activity of SHV-2 β-lactamase was the result of a single substitution within the amino acid sequence of the SHV-1 enzyme (2). To elucidate the mutational events that altered the SHV sequence and conferred unusual activity towards ceftazidime, we determined the primary structure of CAZ-5 β-lactamase.

The resistance gene was transferred to *Escherichia coli* K-12 C600 resistant to sodium azide. The β-lactamase was purified by a previously described protocol (4) from an *E. coli* mutant strain that overproduces CAZ-5 enzyme. Purified protein gave one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, M_r value of 28,000 ± 500, and on isoelectric focusing analysis, pI of 7.8. The amino acid composition of CAZ-5 β-lactamase did not differ markedly from that of the SHV-1 protein (3) and was in good agreement with that deduced from the amino acid sequence analysis. Eight first N-terminal sequence analyses each yielded one residue with the sequence Ser-Pro-Gln-Pro-Leu-Glu-Gln-Ile- identical to those of SHV-1 and SHV-2.

The S-carboxymethylated protein was digested by trypsin (Sigma Chemical Co.), and the resulting peptide mixture was resolved by high-performance liquid chromatography on a Waters μBondapak C_{18} column (10 μm; 4 by 300 mm) (Fig. 1). Elution was performed with 0.1% trifluoroacetic acid in water as solvent A and acetonitrile as solvent B. A three-step linear gradient of solvent B was used (0 min, 5%; 35 min, 30%; 45 min, 45%; 60 min, 80%) with a flow rate of 1 ml/min. Apart from peak 16, which exhibited a higher hydrophobicity, the elution pattern of the tryptic peptides of CAZ-5 was identical to that of SHV-2 (2). Peaks 11 to 14, 3, and 5 to 22 were further chromatographed at pH 6.0 on the same column, with a linear gradient from 0.25 mM ammonium acetate buffer to 0.5 mM ammonium acetate buffer–acetonitrile (2:3, vol/vol). Peptide T-19, which eluted with

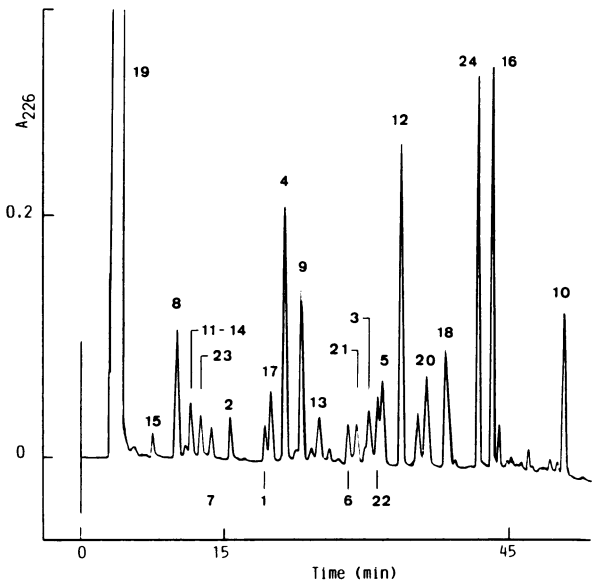

FIG. 1. Separation of peptides (15 nmol) after digestion of CAZ-5 β-lactamase with trypsin. Peptides were numbered in the order in which they occur in the sequence, starting from the N terminus. Peptide 20 was the fragment spanning residues 198 and 218.

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TABLE 1. Kinetic constants of CAZ-5 and SHV-2 β-lactamases[a]

Antimicrobial agent	CAZ-5		SHV-2	
	V_{max}[b]	K_m (μM)	V_{max}[b]	K_m (μM)
Penicillin G	100	3.5	100	3.5
Cefotaxime	115	25	70	41
Ceftazidime	52	60	6.5	24
Aztreonam	5	0.5	1	10

[a] Data are taken from reference 18.
[b] V_{max} are relative to that of benzylpenicillin (set at 100).

the buffer peak, was separated by an additional isocratic chromatography at pH 2.0. The amino acid compositions of the 24 tryptic peptides were determined, and all the peptides, except T-10 and T-20, were fully sequenced (Fig. 2) by the double-coupling method of Chang et al. (9) with slight modifications (3). To elucidate the unsequenced part of the 42-residue-long peptide T-10, the latter was subdigested by endoproteinase Asp-N (Boehringer GmbH, Mannheim, Federal Republic of Germany). Five peptides were separated by reverse-phase high-performance liquid chromatography at pH 2.0. The amino acid sequences of two of them, T-10–D-2 and T-10–D-5, which provide substantial data for structural elucidation of T-10, are shown in Fig. 2. The residue at position 126 was not unambiguously identified, and phenylalanine was deduced from amino acid composition analysis of peptide T-10–D-5. Three other peptides resulted from subfractionation of peptide T-10. In addition to the N-terminal fragment (positions 87 to 89), two peptides were the result of an atypical cleavage at the N-terminal peptide bond of glutamic acid at position 95, which split 50% of peptide T-10–D-2.

Primary structure determination of the region spanning residues 198 and 218 (peptide T-20) was complicated, since trypsin incompletely digested the β-lactamase at lysine-209 and arginine-215, whereas the peptide bond at lysine-214 was not affected. Tryptic cleavage yielded T-18 (10%), T-19 (8%), an unrecovered peptide corresponding to positions 216 to 218, and the parent peptide (36%). So, peptide T-20 was subdigested by endoproteinase Asp-N, and fragments T-20–D-1 and T-20–D-2 were recovered from reverse-phase high-performance liquid chromatography at pH 2.0 and sequenced. Thus, the region 198 to 218 was fully determined.

CAZ-5 enzyme was demonstrated to be very similar to SHV-2 β-lactamase in kinetic constants, amino acid composition, DNA hybridization analyses, and tryptic peptide mapping. Therefore, tryptic peptides, which shared more than 98% homology with those of other SHV β-lactamases, were aligned (Fig. 2). Comparison of the amino acid sequence of CAZ-5 β-lactamase with those of SHV-1 (3) and

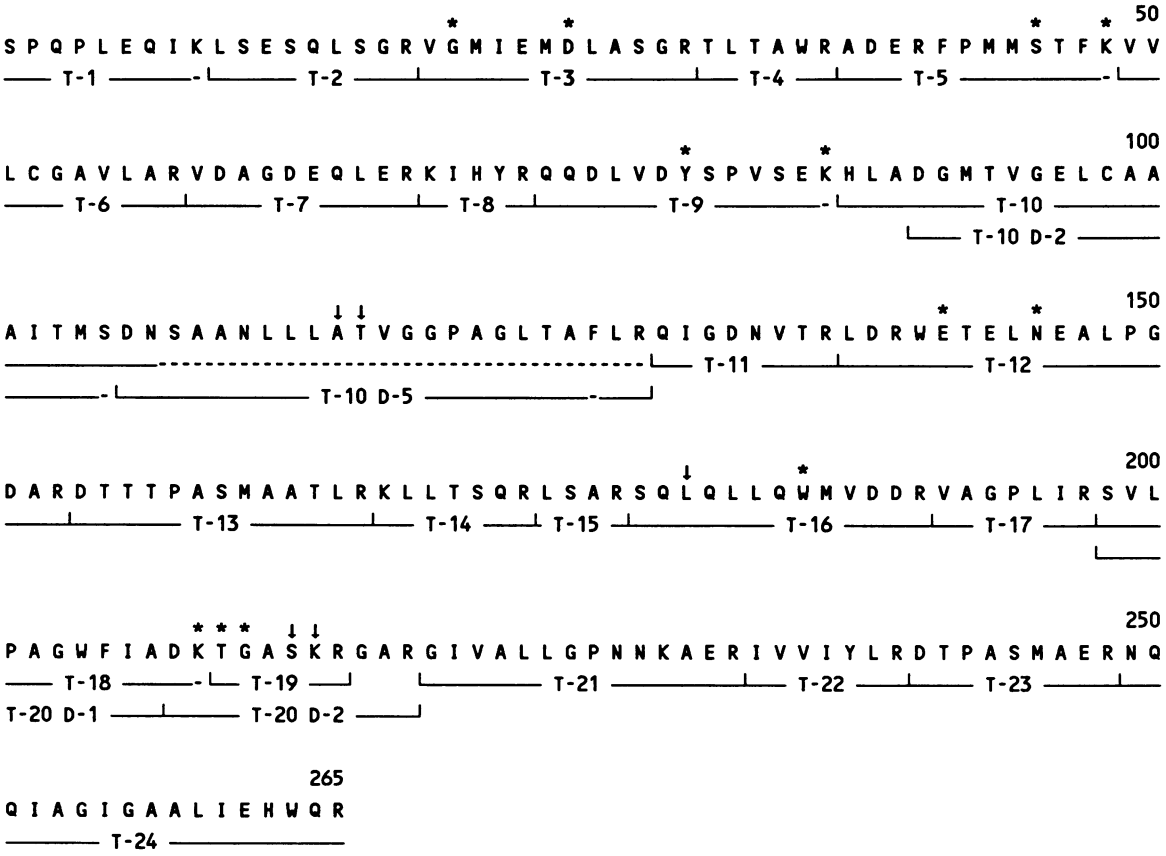

FIG. 2. Putative amino acid sequence of CAZ-5 β-lactamase. T indicates peptide derived from trypsin cleavage. T-10 D and T-20 D designate peptides obtained from subfractionation of T-10 and T-20 with endoproteinase Asp-N. Peptides were aligned by comparison with the sequence of the parent SHV enzymes (2, 3). Residues identified by manual Edman degradation are underlined by solid lines. Broken lines indicate residues checked only by amino acid compositions. Arrows indicate positions which were not retained in the SHV-1 amino acid sequence. Asterisks indicate amino acid residues conserved within the family of active-site-serine penicillin-recognizing enzymes as defined in reference 13.

TABLE 2. Amino acid substitutions between primary structures of SHV-1, SHV-2, and CAZ-5 β-lactamases

Residues	Amino acid at position[a]:				
	115 (140)	116 (141)	180 (205)	213 (238)	214 (240)
SHV-1	Thr	Ala	Arg	Gly	Glu
SHV-2	Thr	Ala	Arg	Ser	Glu
CAZ-5	Ala	Thr	Leu	Ser	Lys

[a] Numbers in parentheses indicate corresponding positions in the numbering system of Ambler (1). Residues at different positions are taken from the amino acid sequences of SHV-1 (3), SHV-2 (2), and CAZ-5 (this paper).

SHV-2 (2) revealed, respectively, five and four amino acid substitutions (Table 2).

Compared with SHV-1 β-lactamase, SHV-2 and CAZ-5 had a substitution of serine for glycine at position 213. This serine residue is believed to be responsible for the activity of SHV-2 β-lactamase towards broad-spectrum cephalosporins (2). This hypothesis implied that residue 213 was close enough to the catalytic cavity so that the hydroxyl group of serine could interact with the oxime group of the oxyimino cephalosporin. Thus, the enzyme-substrate complex would be stabilized with a new hydrogen bond. The crystal structure of SHV-type β-lactamases is not known. Nevertheless, SHV-type enzymes are class A β-lactamases, which have amino acid sequences and secondary structures in common (1, 13). Amino acid alignment of several active-site-serine enzymes, including class A β-lactamases, revealed seven conserved boxes holding identical or homologous residues which should be involved in the catalytic process (13). The serine residue at position 213 was very close to one of these boxes (Fig. 2). Moreover, the crystal structure of β-lactamase from *Staphylococcus aureus* PC1, a class A β-lactamase, has been reported (11). From the postulated three-dimensional structure of the substrate-enzyme complex several residues were indicated as interacting with the substrate. Most of these residues, including the residue corresponding to position 213, took part in or were located near one of the seven retained domains. Therefore, the hypothesis that serine-213 might participate in the enzyme-oxyimino cephalosporin complex is quite strong.

Within the CAZ-5 amino acid sequence, position 214 held a lysine residue which certainly was in a suitable position to interact with the substrate, as was assumed previously (19). Ceftazidime is distinguishable from other broad-spectrum cephalosporins by the presence of a carboxylic function in the alkyloxyimino group. Such a function is also present in the side chain of the monobactam aztreonam. It was then credible that formation of a salt bond with lysine-214 might alter the catalytic process of ceftazidime and aztreonam hydrolyses.

In opposition to SHV-2, CAZ-5 had three extra positions that held different residues within the SHV-1 sequence (Table 2). These three residues, alanine-115, threonine-116, and leucine-180, were not located near one of the critical domains (Fig. 2) and were hardly likely to interact directly with the catalytic cavity. Elsewhere, an alanine residue at position 115 and a threonine residue at position 116 have been observed in the amino acid sequence, deduced from plasmid DNA analysis, of a cefotaxime-hydrolyzing β-lactamase from *Salmonella typhimurium* which was indistinguishable from SHV-2 β-lactamase by isoelectric focusing and kinetic constant determinations (A. Chenon and J. C. Nicolas, personal communication). The remaining sequence of the protein was identical to that of SHV-2 previously determined (2).

We conclude that, in the SHV β-lactamase family, substitution of a serine for a glycine residue at position 213 (position 238 in Ambler's numbering) of the amino acid sequence would be associated with hydrolytic activity towards broad-spectrum cephalosporins consequent to formation of a new stabilizing hydrogen bond. An extra substitution of a lysine for a glutamic acid residue at position 214 (position 240 in Ambler's notation), allowing formation of a salt bond with a ceftazidime or aztreonam substrate, would be responsible for the high levels of resistance to these antibiotics.

We thank A. Thabaut for the gift of *K. pneumoniae* 210-2.

This work was supported in part by a grant from the Institut National de la Santé et de la Recherche Médicale (CRE 872007).

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