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The active site of the P99 β-lactamase from *Enterobacter cloacae*

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Labelling the β-lactamase of *Enterobacter cloacae* P99 with a poor substrate or a mechanism-based inactivator points to an active-site serine residue in a sequence closely resembling that of the *ampC* β-lactamase. These results establish the P99 enzyme as a class-C β-lactamase, and the concurrence of the two approaches helps to confirm the reliability of determining active-site sequences with the aid of mechanism-based inactivators.

β-Lactamases are clinically important enzymes notable for their efficiency and diversity. That this diversity may not be extreme as it appears is suggested by the division of β-lactamases into three classes (Ambler, 1980; Jaurin & Grundström, 1981). It is interesting that although members of both classes A and C are 'serine enzymes' (Knott-Hunziker *et al.*, 1979, 1980, 1982*a*,*b*; Cohen & Pratt, 1980; Fisher *et al.*, 1980, 1981) their structures differ so much that they are regarded as evolutionarily distinct (Jaurin & Grundström, 1981). Many Gram-negative bacteria produce chromosomally encoded β-lactamases, and the recent increase in infections due to *Enterobacter cloacae* (Neu, 1983) has focused attention on the β-lactamase (called P99) that certain strains produce abundantly, and which has been crystallized (Charlier *et al.*, 1983). The active-site residues of β-lactamases have been identified with β-lactams behaving either as mechanism-based inhibitors or substrates that turn over slowly. In the work reported here, both methods have been used.

Materials and methods

Enterobacter cloacae, strain P99, was grown, and the β-lactamase purified, as described by Ross (1975) and Cartwright & Waley (1984). Chymotrypsin and tosylphenylalanylchloromethane ('TPCK')-treated trypsin were from Millipore (Freehold, NJ, U.S.A.). 6β-Iodopenicillanate was kindly given by Dr. Kemp (Pfizer Research, Sandwich, Kent, U.K.) and [^{3}H]cloxacillin (sp. radioactivity 4.1 μCi/μmol) was the sample prepared previously (Knott-Hunziker *et al.*, 1982*a*).

Automatic sequencing was performed with an Applied Biosystem gas-phase sequenator for the peptides labelled with inhibitor (Hewick *et al.*, 1981), or was done as described by Campbell *et al.* (1981) for the peptides labelled with substrate. Manual sequencing was carried out by the dansyl–Edman procedure (Bruton & Hartley, 1970).

Abbreviations used: dansyl, 5-dimethylaminonapthalene-1-sulphonyl; h.p.l.c., high-pressure liquid chromatography; SP-Sephadex, sulphopropyl-Sephadex.

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Results

Preparation of labelled enzyme

The enzyme (4.5mg, 120nmol) in 0.5ml of 50mM-phosphate, pH7, was labelled with inhibitor by treatment with 6β-iodopenicillanate (1.5mol/mol of enzyme). The appearance of a characteristic u.v. absorbance at 325nm (ε 12000 $M^{-1} \cdot cm^{-1}$) was observed. This maximum shifted to 315nm on denaturation with urea. Labelling with cloxacillin was possible because the $k_{cat.}$ for the hydrolysis of this substrate was very low (about 0.01 min^{-1} at pH7 and 30°C). The enzyme (0.5mM) was incubated with [^{3}H]cloxacillin in 50mM-sodium phosphate (pH7)/0.5M-NaCl, for 1min at 0°C; the reaction was stopped by adding 1 vol. of acetic acid. Gel filtration was then carried out on Sephadex G-25 in 30% (v/v) acetic acid to

isolate labelled enzyme. The extent of labelling was 0.8–0.9mol of ^3H/mol of enzyme.

Isolation of peptides labelled with inhibitor

The solution of enzyme labelled with inhibitor was made 2M with respect to urea, 0.5mg of trypsin was added and, after 60min at 37°C, a second portion of 0.5mg of trypsin was added and incubation continued for 60min. The digest was then fractionated on a column (140cm × 1cm) of Sephadex G-25. Fractions eluted with water, having absorbance at both 305 and 254nm (determined with fixed-wavelength detectors), were pooled (average K_d 0.38, where K_d is the distribution coefficient of the peptide). H.p.l.c. gave one major peak detected by A_{305} (Fig. 1*a*). The corresponding fractions (eluted between 42 and 45min) were further purified by a second run under similar conditions, yielding peptide T1, which was 22 residues long and had *N*-terminal alanine (Table 1). The sequence, determined on 2nmol with the gas-phase sequenator, is given in Fig. 2; the penultimate, and antepenultimate residues (Ile, Ser) were not identified with certainty. Hence 8nmol of T1 were digested with chymotrypsin and the peptide with absorbance at 305nm (T1C1) purified by h.p.l.c. The *N*-terminal residue was glutamic acid (Table 1), and the sequence, determined manually, showed that T1C1 comprised residues 16–20 (Fig. 2), and that the serine residue labelled by inhibitor was serine-19.

Isolation of peptides labelled with substrate

The labelled enzyme (20mg) was digested with 0.4mg of trypsin in 1% NH_4HCO_3/2M-urea for 2h

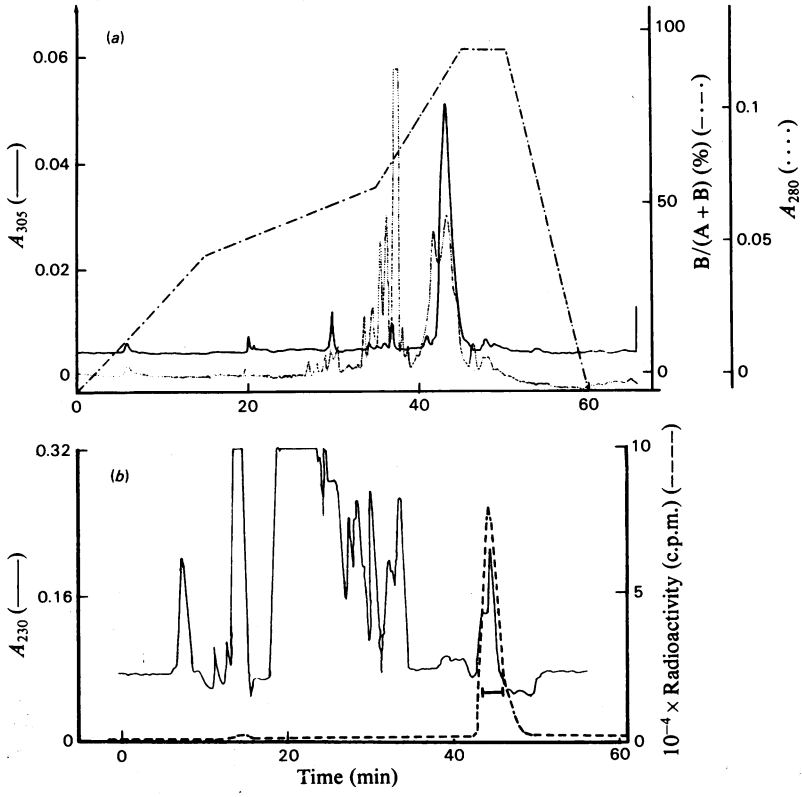

Fig. 1. *Fractionation of tryptic peptides by h.p.l.c.*

(*a*) Of the material purified on Sephadex G-25, 50% was dissolved in 0.4ml of buffer A and applied to a reverse-phase Nucleosil-7 C_{18} Macherey–Nagel column (10mm × 250mm). Buffer A was 50mM-NH_4HCO_3 in water and buffer B a 2:3 (v/v) mixture of buffer A and acetonitrile. The shape of the gradient [expressed as 100 × B/(A + B)] is shown on the Figure and the flow rate was 2ml·min^{-1}. Labelled peptides were detected by their A_{305}. (*b*) Peptide T2, labelled with [^3H]cloxacillin and previously fractionated on Sephadex G-25 (K_d = 0.6) and SP-Sephadex, was applied to a reverse-phase Lichrosorb RP (5μm) column (4.6mm × 250mm). Buffer A was 20mM-triethylamine acetate, pH4.5, in water and buffer B a 2:1:2 (by vol.) mixture of acetonitrile, propanol and buffer A. The gradient was linear over 60min and the flow rate 1ml·min^{-1}. Labelled peptides were detected by their radioactivity.

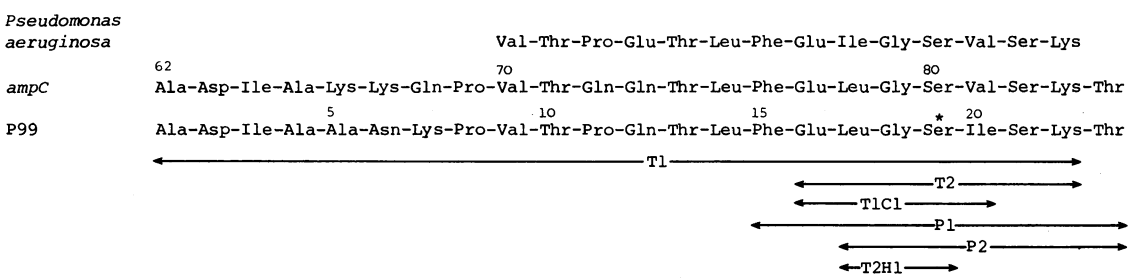

Fig. 2. *Active-site sequences of class-C β-lactamases*

The sequence that is deduced for the 23-residue fragment of the P99 β-lactamase is shown; the asterisk marks the serine residue that is labelled by substrate and inhibitor. Peptides obtained by the action of trypsin and pepsin are denoted by 'T' and 'P', respectively; further digestion of tryptic peptides T1 and T2 with chymotrypsin or thermolysin gave peptides T1C1 and T2H1 respectively. The sequence 1–19 of T1 was determined with the gas-phase sequenator. All the other peptides were sequenced except the last one. Above the continuous sequence are shown the sequences of *ampC* β-lactamase (middle line) (Jaurin & Grundström, 1981) and *Pseudomonas aeruginosa* β-lactamase (top line) (Knott-Hunziker *et al.*, 1982*a*). The numbering of the *ampC* β-lactamase is derived from the complete sequence of the gene, including the signal peptide.

Table 1. *Composition of labelled peptides*

The Table gives residues/molecule from amino acid analysis; the values in parentheses refer to residues not found by the Edman sequence analysis. The integral values in columns (3), (5), (7), (11) and (13) are from the sequence analysis, and in column (9) are calculated for residues 17–19. The electrophoretic mobility (*m*) at pH6.5 was calculated relative to Asp = −1 (Offord, 1977). Columns (1) and (6) refer to peptide labelled with 6β-iodopenicillanate, and columns (2), (4), (8), (10) and (12) refer to radioactive peptides labelled with [^{3}H]cloxacillin. *N*-Terminal residues were identified by the dansyl procedure (Bruton & Hartley, 1970). T, P, T1C1 and T2H1 are explained in the legend to Fig. 2.

		Composition (residues/molecule)											
	Peptide ...	T1			T2		T1C1		T2H1		P1		P2	
Amino acid	Column ...	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
Asp		2.1	1.9	2	(0.4)		(0.3)				1.0	1	1.0	1
Thr		2.0	1.5	2							2.2	2	2.0	2
Ser		2.0	1.6	2	1.4	2	1.3	1	0.9	1	0.9	1		
Glu		2.0	2.1	2	0.4	1	1.2	1	(0.4)		1.2	1	1.2	1
Pro		1.9	1.9	2										
Gly		1.4	1.7	1	1.5	1	1.0	1	1.1	1	1.2	1	1.2	1
Ala		2.8	3.1	3	(1.3)									
Val		1.2	1.3	1										
Ile		1.8	1.9	2	1.0	1	0.5	1			0.9	1	1.0	1
Leu		2.0	2.0	2	1.7	1	1.1	1	1.0	1	1.0	1	1.1	1
Phe		0.9	0.9	1							1.0	1		
Lys		1.8	2.0	2	0.9	1					0.8	1	0.8	1
^{3}H* ...			0.9		0.8				0.9		1.0		1.0	
m ...			−0.3		−0.27				−0.44		−0.31		−0.15	
N-Terminus ...		Ala	Ala		Glu		Glu		Leu		Phe		Leu	
No. of residues ...			22		7		5		3		9		7	

* Mol of labelled substrate bound/mol of peptide.

at 37°C and the digest fractionated on a column (150cm × 0.9cm) of Sephadex G-25 (superfine grade) in 0.1M-acetic acid at 4°C. The radioactive fraction (K_d 0.34) contained peptide T1, and a second radioactive fraction (K_d 0.6) contained peptide T2 (Table 1); further tryptic hydrolysis converted peptide T1 into peptide T2. The labelled enzyme was also digested with 1% pepsin in 1mM-HCl/3M-guanidinium chloride for 60min at 37°C. The tryptic or peptic digests, after fractionation on

Sephadex G-25, were further fractionated on SP-Sephadex 50 and by h.p.l.c.; the peptic digest yielded two peptides, P1 and P2 (Table 1). The h.p.l.c. fractionation of the Sephadex fractions containing peptide T2 is shown on Fig. 1(*b*).

The amino acid sequence of peptide T2 showed that it comprised residues 16–22 (Fig. 2). The sequences of peptides P1 and P2 showed that they comprised residues 15–23 and 17–23 respectively. Finally, peptide T2 was digested with thermolysin and the digest fractionated by h.p.l.c. The radioactive tripeptide (T2H1) (Table 1) contained the labelled serine in the sequence Leu-Gly-Ser and comprised residues 17–19 (Fig. 2). Since serine is the only amino acid in this tripeptide with a reactive side chain, it is serine-19 that is labelled by substrate.

Discussion

The results on the P99 β-lactamase in Fig. 2 establish that the same serine residue is labelled by 6β-iodopenicillanate, a 'branched-pathway' β-lactamase inactivator, and by cloxacillin, an 'inhibitory substrate' (Cartwright & Waley, 1983). The sequence of the 18 residues before, and the four residues after, serine-19 is firmly based on the structures of six peptides. The sequence of peptides containing the active-site serine residues of the β-lactamases of *Pseudomonas aeruginosa* and *Escherichia coli* K12 (*amp*C gene) has been previously established (Knott-Hunziker *et al.*, 1982*a*). The corresponding sequence obtained in the present work is closely similar to that of the *ampC* β-lactamase: 18 out of 23 residues are identical. Similarly, 11 out of 14 residues are the same in the P99 and *Pseudomonas aeruginosa* β-lactamases. In fact, among the 14 residues corresponding to positions 70–83 in the *ampC* β-lactamase, ten are identical in these three β-lactamases (Fig. 2). Our results clearly establish the *E. cloacae* P99 β-lactamase as a member of class C, and the serine residue labelled as the counterpart of serine-80 in the *ampC* β-lactamase (the position of peptide T1 in the sequence still requires determination).

It was observed with various penicillin-sensitive enzymes that the homology was much more pronounced in the immediate surroundings of the penicillin-binding serine residue (Frère & Joris, 1984). Although these homologies were not as strong as those observed in the present study, one may wonder whether, in the case of the class C β-lactamases, the homology extends further away from the active serine residue. This problem requires further investigation.

The isolation of an acyl-enzyme from cloxacillin and the P99 β-lactamase suggests that this covalent intermediate is important in catalysis. Kinetic studies are necessary to decide whether this intermediate is on the main reaction pathway.

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