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Properties of a class C β-lactamase from *Serratia marcescens*

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A β-lactamase produced by a penicillin-resistant strain of *Serratia marcescens* was isolated and purified. The $k_{cat.}$ value for benzylpenicillin was about 5% of that observed for the best cephalosporin substrates. However, the low K_m of the penam resulted in a high catalytic efficiency ($k_{cat.}/K_m$) and the classification of the enzyme as a cephalosporinase might not be completely justified. It also exhibited a low but measurable activity against cefotaxime, cefuroxime, cefoxitin and moxalactam. Substrate-induced inactivation was observed both with a very good (cephalothin) or a very bad (moxalactam) substrate. The active site was labelled by β-iodopenicillanate. Trypsin digestion produced a 19-residue active-site peptide whose sequence clearly allowed the classification of the enzyme as a class C β-lactamase.

INTRODUCTION

The emergence of penicillin-resistant strains of *Serratia marcesens* has become a distinct clinical problem. Many strains synthesize an inducible β-lactamase that exhibits a high activity against cephalosporins (Sawai *et al.*, 1968). Tajima *et al.* (1981) have purified two β-lactamases from a clinical isolate. One was similar to the class A penicillinases (Ambler, 1980). The second clearly exhibited high cephalosporinase and poor penicillinase activities. In the present paper we describe the isolation and properties of a cephalosporinase produced by another clinically isolated strain of *Serratia marcescens* and the labelling and sequencing of a peptide containing the active-site serine residue. These data clearly establish the *Serratia* enzyme as a class C β-lactamase showing a high degree of homology to other class C enzymes.

MATERIALS AND METHODS

Organism

Serratia marcescens strain SC8247 was a clinical isolate characterized by a high degree of resistance to β-lactam antibiotics due to the inducible production of a β-lactamase. It was a gift from Dr. Spaulding, Temple University Medical School, Philadelphia, PA, U.S.A. The strain was conserved at 4 °C on 1.5% nutrient agar (Difco 0001) slants.

Portions (25 ml) of an overnight culture in 1% yeast extract (Difco 0127–01) were used to inoculate 500 ml samples of the same medium in 1-litre flasks. After 3 h on a rotatory shaker at 37 °C, cefoxitin was added at a final concentration of 25 μM. The cells were harvested 3 h after the induction.

Materials

Tosylphenylalanylchloromethane-('TPCK')-treated trypsin was from Millipore Corp. (Bedford, MA, U.S.A.).

Cephalothin, cephazolin, cephaloridine and moxalactam were from Eli Lilly and Co. (Minneapolis, MN, U.S.A.), cefuroxime was from Glaxo Group Research (Greenford, Middx., U.K.), cefotaxime was from Hoechst–Roussel (Romainville, France), benzylpenicillin was from Rhône–Poulenc (Paris, France) and cefoxitin was from Merck, Sharp and Dohme (Rahway, NJ, U.S.A.). All the antibiotics were kindly given by the respective companies.

Nitrocefin was purchased from Becton–Dickinson (Cockeysville, MD, U.S.A.), and β-iodopenicillanate, ^{3}H-labelled in the β-methyl group (2.4 mCi/mmol), was the sample synthesized by De Meester *et al.* (1985).

Determination of protein and assay of enzyme activity

Protein concentrations were determined by direct reading of the absorbance at 280 nm, by staining with Coomassie Blue (Bradford, 1976) or by measuring the concentration of free amino groups amenable to dinitrophenylation after complete acid hydrolysis

The enzyme activity was assayed with 100 μM-cephaloridin ($\Delta\epsilon_{260} = -12000\ M^{-1}\cdot cm^{-1}$), cefotaxime ($\Delta\epsilon_{260} = -6000\ M^{-1}\cdot cm^{-1}$) or nitrocefin ($\Delta\epsilon_{482} = 15000\ M^{-1}\cdot cm^{-1}$) as substrates. Incubations were performed in 50 mM-sodium phosphate buffer, pH 7.0. When necessary, enzyme samples were diluted in the same buffer containing 0.1 mg of bovine serum albumin/ml. One unit hydrolyses 1 μmol of substrate/min at 30 °C.

Purification of the enzyme

Negligible enzyme activity was found in the culture supernatant. The enzyme was freed from the cells by sonication after resuspending the cell pellet from a 5-litre culture in 300 ml of 200 mM-glycine/NaOH buffer, pH 9.0. The suspension was divided in 30 ml portions, which were sonicated for 2 × 3 min. The cell debris was separated by centrifugation (at 48000 *g* for 40 min). The pellet was then submitted to a second, identical, cycle of sonication–centrifugation. The combined supernatants were completely clarified by filtration through 0.2 μm-pore-size Millipore MF membranes.

At that stage it was found that the enzyme strongly and rapidly adsorbed on glass. All purification steps and

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dilutions were therefore performed with the use of plastic tubes and containers. Surprisingly, this adsorption phenomenon did not seem to interfere with the enzyme assay (see below).

The effect of pH on the stability of the enzyme was studied after 100-fold dilution of the sonication supernatant in the following buffers, the concentration of the buffering component being 50 mM in all cases: sodium acetate/acetic acid (pH 4 and 5), sodium cacodylate/cacodylic acid (pH 6), sodium phosphate (pH 7.0), Tris/HCl (pH 8 and 9), glycine/NaOH (pH 9.2 and 10) and potassium borate/NaOH (pH 9.8). Determination of the activity of samples after incubation at 20 °C for 0, 24, 48 and 150 h indicated that the enzyme was only stable at pH 9 and 10. At a lower pH most of the activity was lost after 24 h. The enzyme was first purified by $(NH_4)_2SO_4$ fractionation and chromatography on CM-cellulose and Sephadex G-75. However, a slight modification of the affinity-chromatography method described by Cartwright & Waley (1984) was found to be more simple and efficient.

Taking the stability range of the enzyme into account, the buffers were modified as follows. Adsorption on the type-L (hydrophilic arm) column was performed in 200 mM-glycine/NaOH buffer, pH 9.0, containing 0.5 M-NaCl. The enzyme was eluted with 0.5 M-potassium borate buffer, pH 9.0, containing 0.5 M-NaCl. The fractions absorbing at 280 nm were pooled, concentrated by ultrafiltration (Amicon UM10 membranes) and dialysed against a 0.1% $K_2B_4O_7$ solution adjusted to pH 9.0 and containing 10% (v/v) ethylene glycol. The samples used for kinetic studies were dialysed against 200 mM-glycine/NaOH buffer, pH 9.0.

Isoelectric focusing

Isoelectric focusing was performed in an LKB Multiphor apparatus with prepared PAG plates in the pH range 3.5–9.5. β-Lactamase activity was identified by the use of nitrocefin.

Determination of M_r

The M_r of the enzyme was determined by SDS/polyacrylamide-gel electrophoresis by the procedure of Laemmli & Favre (1969). The standard mixture (Bio-Rad Laboratories, Richmond, CA, U.S.A.) contained phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor and lysozyme.

Amino acid analysis and sequence

Samples were hydrolysed for 24, 48 and 72 h in azeotropic HCl at 116 °C. Analyses were performed on a Dionex D-300 automatic analyser. *N*-Terminal residues were determined by dansylation (5-dimethylaminonaphthalene-1-sulphonylation) (Bruton & Hartley, 1970; Joris *et al.*, 1983). The amino acid sequence of the active-site peptide was determined by using the Applied Biosystems 47 A gas-phase Sequenator (Joris *et al.*, 1985) with off-line analyses of phenylthiohydantoin derivatives on a Waters h.p.l.c. installation comprising the Intelink system and a 440 fixed-wavelength detector set at 254 and 313 nm. Methanolic HCl was used as the converting agent in the sequence. The separations were carried out on a 4.6 mm × 250 mm cyanopropyl column (IBM, Danbury, CT, U.S.A.) with a gradient program comprising tetrahydrofuran in the buffer component as suggested by Touchstone (A.B.I. User's Bulletin no. 3).

Peptide purification

The Sephadex G-25 (fine grade) column (115 cm × 1 cm) was equilibrated in 50 mM-NH_4HCO_3. Two reverse-phase columns were used on the Pharmacia fast protein liquid chromatography system. (i) A Pro-RPC HR 5/10 column was equilibrated with 10 mM-NH_4HCO_3 in water; after injection of the sample, the percentage of acetonitrile in the buffer was linearly increased from 0 to 60% (v/v) over a period of 40 min (flow rate 0.5 ml/min). (ii) A Pep-RPC HR5/5 column was equilibrated with 0.1% (v/v) trifluoroacetic acid in water; after injection of the sample, the percentage of acetonitrile in the buffer was similarly increased from 0 to 60% (v/v). In both cases the absorbance at 214 nm was monitored continuously and the absorbance of each fraction (0.3 ml) was subsequently determined at 325 nm. The radioactivity of 5 μl samples was also measured.

Spectra were obtained and spectrophotometric measurements made with a Beckman DU8 spectrophotometer. Kinetic parameters were determined by continuously monitoring the variation of absorbance at 482 nm (nitrocefin), 260 nm (other cephalosporins) or 235 nm (benzylpenicillin) and transmitting the readings to an Apple II microcomputer via an RS232C interface. The integrated form of the Henri–Michaelis equation was used to compute K_m and V as described by De Meester *et al.* (1986). In all cases enzyme and substrate dilutions were made in buffers containing 0.2 mg of bovine serum albumin/ml.

RESULTS

Purification of the enzyme

Starting with 5 litres of culture, the combined supernatants, after two cycles of sonication–centrifugation, typically contained about 80 cefotaxime units. The yield of the affinity chromatography was about 80%. On the basis of an absorbance of 1.3 at 280 nm for a 0.1% solution (see below), a purified preparation contained 24 mg of protein for 64 cefotaxime units. Gel electrophoresis in the presence of SDS, performed on a 30 μg sample, revealed the presence of only one band, whose mobility corresponded to an M_r of about 37000.

The protein concentration in a solution exhibiting an absorbance of 1.0 at 280 nm was estimated at 0.73 and 0.90 mg/ml respectively by staining with Coomassie Blue (Bradford, 1976) and by dinitrophenylation after acid hydrolysis. An intermediate $A_{280}^{1\%,\,1\,cm}$ value of 13.0, in agreement with spectral and radioactivity measurements performed after complete inactivation by β-iodo[^{3}H]-penicillanate (De Meester *et al.*, 1986), was finally accepted for the pure enzyme.

Isoelectric pH

The activity migrated to the end of the gel and focused at a pH of about 9.5. This value should, however, be considered with caution, since that part of the pH gradient was found to be less reliable.

Amino acid composition

As shown by Tables 3 and 4, ionic strength and pH had compares it with that of the enzyme purified by Tajima *et al.* (1981).

Kinetic parameters

In a preliminary investigation, the substrates, at a

Table 1. Amino acid compositions of *Serratia marcescens* β-lactamase and of the active-site peptide

The composition of the enzyme in terms of residues/molecule is calculated on the basis of an M_r of 37700. The values in parentheses are those reported by Tajima *et al.* (1981) for a similar enzyme from another strain of *Serratia marcescens*. Abbreviation: N.D., not determined.

	Amino acid composition				
	β-Lactamase			Active-site peptide	
Residue	(nmol/mg of protein)	(residues/ molecule)		(nmol/ sample)	(residues/ molecule)
Lys	486	18	(18)	1.52	2
His	153	6	(4)	—	—
Arg	389	14	(12)	—	—
Trp*	N.D.	5	(4)	N.D.	N.D.
Asx	882	32	(35)	—	—
Thr	532	19	(18)	1.94	3
Ser	551	20	(17)	1.19	2
Glx	104	37	(34)	2.85	4
Pro	710	26	(25)	0.76	1
Gly	627	23	(28)	1.42	2
Ala	1210	44	(39)	—	—
Cys	N.D.	N.D.	(0)	—	—
Val	596	21	(19)	0.55	1
Met	313	11	(12)	—	—
Ile	455	16	(15)	0.62	1
Leu	911	33	(28)	1.59	2
Tyr	362	13	(20)	—	—
Phe	215	8	(10)	0.54	1
Total		346			19

* Determined on the basis of the absorbance at 280 nm (Cantor & Schimmel, 1980) with $A^{1\%}_{280}{}^{1\ cm} = 13.0$.

200 μM concentration (500 μM for benzylpenicillin), were incubated with the enzyme in 50 mM-Hepes/NaOH buffer, pH 8.0, containing 0.2 M-NaCl and 0.2 mg of bovine serum albumin/ml. The kinetic parameters K_m and V were deduced from the analysis of the complete time courses of substrate disappearance. When the reaction rate was increased by increasing the concentration of enzyme, it was found that for cephalothin and moxalactam both K_m and $V/[E]_0$ decreased with increasing enzyme concentrations. The $V/K_m \cdot [E]_0$ ratio, however, remained unchanged. These results indicated substrate-induced inactivation, a phenomenon that has often been observed with β-lactamases (Citri & Zyk, 1982). The initial rate (first 40 s) measured with 25 μM-, 50 μM- and 100 μM-moxalactam did not vary significantly. With cephalothin, when the reaction reached 90% of completion in less than 60 s, K_m and $V/[E]_0$ were independent of the amount of enzyme, which indicated that the substrate-induced inactivation had negligible effect. It was also found that such a rapid procedure allowed an accurate determination of A_∞. Since this was very important for a valid determination of the kinetic parameters (Cornish-Bowden, 1975), experiments involving the good substrates (cephaloridine, cefazoline and nitrocefin) were also performed under conditions where hydrolysis was completed in 1–2 min. Table 2 shows the kinetic constants determined for nine substrates. For cephalothin and moxalactam the values were those obtained for the 'native' form of the enzyme, before inactivation became significant.

Influence of ionic strength and pH

As shown by Tables 3 and 4, ionic strength and pH had little influence on k_{cat}. The value of K_m, however, increased with increasing NaCl concentration and was minimal between pH 8 and 9.

Table 2. Kinetic parameters of *Serratia marcescens* β-lactamase at pH 8.2

The values were obtained in 50 mM-Hepes buffer, pH 8.2, containing 0.2 M-NaCl and 0.2 mg of bovine serum albumin/ml and are given as means ± S.D. for at least four measurements. For cephalothin and moxalactam the data refer to the native form of the enzyme. With nitrocefin the same results were obtained in both quartz and plastic cuvettes.

Substrate	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Cephaloridine	1100 ± 50	275 ± 25	4.0×10^6
Cephazoline	1300 ± 100	540 ± 45	2.4×10^6
Nitrocefin	1240 ± 60	40 ± 2	31×10^6
Cefotaxime	1.7 ± 0.2	12 ± 3	0.14×10^6
Cefuroxime	0.8 ± 0.15	0.6 ± 0.06*	1.3×10^6
Cefoxitin	0.014 ± 0.002	0.3 ± 0.03*	47000
Benzylpenicillin	75 ± 3	1.7 ± 0.2*	44×10^6
Cephalothin	1100 ± 50	67 ± 4	16×10^6
Moxalactam	0.12 ± 0.01	60 ± 7*	2000

* Determined as K_i values by using the tested compound as a competitive substrate versus 50 μM-nitrocefin. Initial rates for the hydrolysis of nitrocefin were measured over a 30 s period, with the exception of cefoxitin, for which accumulation of the acyl-enzyme was slow and the hydrolysis of nitrocefin was measured at the steady state. From the pre-steady-state data, a value of 38000 ± 4000 $M^{-1} \cdot s^{-1}$ was computed for the ratio k_2/K, which agrees well with the value of 47000 $M^{-1} \cdot s^{-1}$ obtained for $k_{cat.}/K_m$.

Table 3. Influence of ionic strength on the kinetic parameters of *Serratia marcescens* β-lactamase for cephaloridine

All experiments were performed in 50 mM-Hepes buffer, pH 8.2, containing 0.2 mg of bovine serum albumin/ml.

[NaCl] (M)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
0	950±100	200±20	4.7×10^6
0.1	1050±20	240±3	4.4×10^6
0.2	1100±50	275±25	4×10^6
0.4	1150±100	303±35	3.8×10^6
0.8	1120±100	314±35	3.6×10^6

Table 4. Influence of pH on the kinetic parameters of *Serratia marcescens* β-lactamase for cephaloridine

All buffers contained 0.2 M-NaCl and 0.2 mg of bovine serum albumin/ml and were 50 mM in the buffering component (pH 5.1–6, sodium cacodylate; pH 7.0, sodium phosphate; pH 8.2–8.8, Hepes/NaOH; pH.9.7, glycine/NaOH). The conductivity of all buffers was adjusted to 2.0±0.1 mS.

pH	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
5.1	1110±140	610±50	1.8×10^6
6.0	1080±70	400±50	2.6×10^6
7.0	960±70	355±40	2.7×10^6
8.2	1100±50	275±25	4×10^6
8.8	970±10	240±5	4×10^6
9.7	610±50	280±25	2.2×10^6

Rate of substrate-induced inactivation

The absorbance at 260 nm of a 200 μM-cephalothin solution was recorded for 20 min in the presence of a concentration of enzyme (approx. 2 ng/ml) such that less than 20% of the substrate was hydrolysed after 20 min. Under those conditions the decrease in the reaction rate was due only to substrate-induced inactivation. The buffers were those described on Table 4. The values of the inactivation rate constants computed as described by De Meester *et al.* (1986) were $0.74 \times 10^{-3} \pm 0.09 \times 10^{-3}$, $4.5 \times 10^{-3} \pm 0.5 \times 10^{-3}$ and $2.9 \times 10^{-3} \pm 0.2 \times 10^{-3}$ s^{-1} at pH 5.1, 8.2 and 8.8 respectively. The corresponding constant for moxalactam was measured only at pH 8.2. A value of $1.8 \times 10^{-3} \pm 0.2 \times 10^{-3}$ s^{-1} was found, which was not influenced by variation of moxalactam concentration between 25 and 100 μM.

Labelling and isolation of an active-site peptide

The enzyme (10 ml of a 15 μM solution) was treated with 30 μM-β-iodo[^{3}H]penicillanate. After 60 min no residual activity could be detected and the absorbance at 325 nm remained constant. The solution was dialysed against water to eliminate the excess of β-iodopenicillanate and freeze-dried. The dry residue was dissolved in 600 μl of 8 M-urea and incubated at 37 °C for 60 min. Trypsin (80 μg in 680 μl of 50 mM-NH_4HCO_3) was added and the digestion was performed at 37 °C for 60 min. The reaction was stopped by addition of 80 μg of soya-bean trypsin inhibitor. The sample was first filtered through the Sephadex G-25 column. Two fractions absorbing at 325 nm were separated. The first one was excluded from the gel and probably consisted of undigested enzyme. A u.v.-absorption spectrum of the second was recorded. Maxima were observed at 315 and 280 nm and the A_{315}/A_{280} ratio was 1.0:1, indicating a clear enrichment of the chromophore obtained after re-arrangement of the β-iodopenicillanate. The fractions were freeze-dried, redissolved in 10 mM-NH_4HCO_3 and submitted (three runs) to chromatography on the Pro-RPC column. One major peak absorbed both at 214 and 315 nm and was also radioactive. Dansylation and hydrolysis performed on 0.3 nmol indicated the presence of two *N*-terminal groups, alanine and glutamic acid/glutamine, in similar quantities. The peptide was then further purified by chromatography on the Pep-RPC column. Dansylation of a portion of the fraction corresponding to the sole radioactive peak indicated only one *N*-terminal residue, glutamic acid/glutamine. No maximum near 280 nm was observed in the u.v.-absorption spectrum and the A_{315}/A_{280} ratio was 3:1, probably indicating the absence

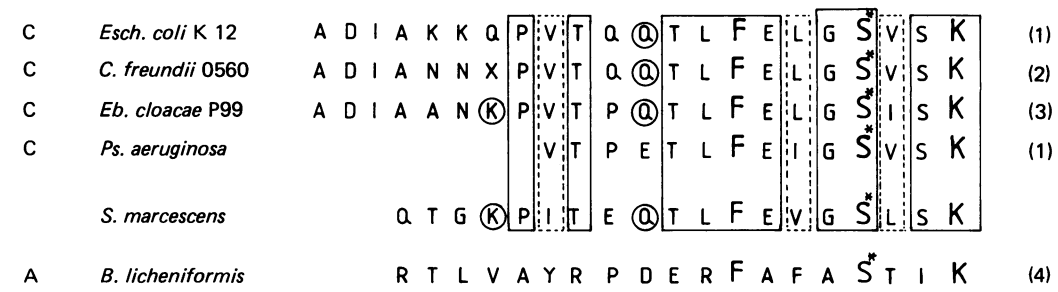

Fig. 1. Sequence of the active-site peptide of *Serratia marcescens* β-lactamase and comparison with corresponding peptide regions of other β-lactamases

Automatic Edman degradation of the *S. marcescens* peptide was carried out for 21 cycles, but no sequence information was obtained after residue 19. The repetitive yield calculated between Gln-9 and Gln-1 was 87.7%. The initial yield for Gln-1 was 726 pmol accompanied by 256 pmol of Glu. The amount of Lys-19 was 52 pmol. The boxes surround the residues that are conserved in all class C enzymes (continuous lines) or that exhibit very similar side chains (broken lines). The encircled residues are common to the *S. marcescens* enzyme and some, but not all, other class C enzymes. The residues common to both classes A and C are represented in **bold** type. References: (1) Knott-Hunziker *et al.* (1982); (2) Lindberg & Normark (1986); (3) Joris *et al.* (1984); (4) Ambler (1980).

of tyrosine and tryptophan residues from the peptide. This was confirmed by the amino acid analysis (Table 1) and by the sequence (Fig. 1), which was determined with 2.5 nmol of the peptide. Fig. 1 shows an alignment of the determined sequence with the previously determined sequences of class C β-lactamases and of a representative of class A. By analogy with the class C sequences, it was clear that serine-16 of the peptide corresponded to the active-site serine residue.

DISCUSSION

The general properties of the *Serratia marcescens* β-lactamase described in the present paper are very similar to those of the cephalosporinase studied by Tajima *et al.* (1981). We found a somewhat lower content of tyrosine residues (see Table 1) and minor differences in the kinetic parameters for some substrates (compare our Table 2 with Table 3 in Tajima *et al.*, 1981). The determination of the sequence of the peptide obtained by trypsin digestion of the enzyme after labelling of the active site with β-iodopenicillanate clearly established the enzyme as a member of class C. Fig. 1 shows a high degree of homology around the active site of all class C β-lactamases. It seems that these enzymes represent a rather homogeneous group. This might be related to the fact that up to now all the studied class C enzymes are synthesized by Enterobacteriaceae (*Enterobacter*, *Citrobacter*, *Escherichia* and *Serratia*) and another Gram-negative rod (*Pseudomonas*).

Cephaloridine, cefazolin, nitrocefin and cephalothin were very good substrates ($k_{cat.} > 1000\ s^{-1}$). Cefotaxime, cefuroxime, cefoxitin and moxalactam were very slowly hydrolysed. Since the K_m values for the poor substrates were rather low, it seemed likely that the acyl-enzymes formed with these compounds were relatively stable. With benzylpenicillin a rather low $k_{cat.}$ was found (6–7% of those for cephaloridine and cefazoline). However, K_m was also very low and, in consequence, the catalytic efficiency, $k_{cat.}/K_m$, was similar to that for the best substrates ($44 \times 10^6\ M^{-1} \cdot s^{-1}$). This was reminiscent of the behaviour of the *Pseudomonas aeruginosa* chromosomal β-lactamase, another class C enzyme, when the same substrate was used (Bicknell *et al.*, 1983). The pH-dependence of the kinetic parameters, however, sharply differed from that reported for the *Pseudomonas* enzyme: we found very little variation of $k_{cat.}$ between pH 5 and 8.8, but $k_{cat.}/K_m$ was maximum at about pH 8.5.

Substrate-induced inactivation was observed with cephalothin and moxalactam. This phenomenon, first studied by Citri *et al.* (1976), seems rather general with β-lactamases. It is best documented with class A enzymes (Citri & Zyk, 1982; Carrey *et al.*, 1984), but similar results were obtained with class C enzymes (De Meester *et al.*, 1986). In the present case, substrate-induced inactivation was observed with a very good (cephalothin) and a very bad (moxalactam) substrate, whose side chains were very different. It thus seems quite difficult to try to predict whether a substrate will behave as a hysteretic inactivator or not on the basis of its kinetic parameters or of its structural characteristics. The molecular origin of that phenomenon remains rather mysterious: a given substrate can exhibit a normal Michaelian behaviour with some enzymes, and induce progressive and reversible inactivation of others. One might assume substrate-induced inactivation to depend mainly on kinetic factors: if all acylated β-lactamases were prone to unfolding, acyl-enzyme accumulation would consistently result in inactivation. The very low K_m values which were observed with some substrates of the *Serratia* β-lactamase were probably indicative of rather high concentrations of acyl-enzyme at the steady state. This was clearly not a sufficient condition for inducing a hysteretic behaviour of the enzyme/substrate system.

The results obtained by Faraci & Pratt (1985) indicated that the PC1 β-lactamase of *Staphylococcus aureus* catalysed the hydrolysis of cephalosporins with good 3′-leaving groups (e.g. cephaloridine and cephalothin) via a branched pathway in which the acyl-enzyme could undergo either direct hydrolysis or transformation into a relatively inert intermediate by elimination of the 3′-substituent. This inert intermediate was the same for cephaloridine and cephalothin and was hydrolysed with a half-life of 10 min. In the present work we found no indication of the accumulation of such an intermediate with cephaloridine. In fact, the value of $k_{cat.}$ for that substrate was about 10^6-fold higher with the *Serratia* enzyme than with the *Staphylococcus* enzyme. The substrate-induced inactivation observed with cephalothin might be due to the accumulation of a slowly hydrolysed intermediate. However, the situation was again very different from that observed with the staphylococcal enzyme. In that case no direct hydrolysis of the acyl-enzyme was detected, and the product was only formed by the slow hydrolysis of the nearly inert intermediate. With the *Serratia* enzyme the acyl-enzyme was rapidly hydrolysed ($\geqslant 1100\ s^{-1}$) and the residual activity at the steady state was far from negligible (about 30% at pH 8.2). The differences between the two enzymes are thus so important that it seems difficult, at this stage, to discuss the behaviour of the class C *Serratia* β-lactamase on the basis of the model proposed by Faraci & Pratt (1985).

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