A class-A β -lactamase from *Pseudomonas stutzeri* that is highly active against monobactams and cefotaxime

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A β -lactamase produced by *Pseudomonas stutzeri* was purified to protein homogeneity, and its physicochemical and catalytic properties were determined. Its profile was unusual since, in addition to penicillins, the enzyme hydrolysed second- and third-generation ' β -lactamase-stable' cephalosporins and monobactams with similar efficiencies. On the basis of the

characteristics of the interaction with β -iodopenicillanic acid, the enzyme could be classified as a class-A β -lactamase. However, when compared with most class-A β -lactamases, it exhibited significantly lower k_{cat}/K_m values for the compounds usually considered to be the best substrates of these enzymes.

INTRODUCTION

The selective pressure exerted by the extensive utilization of β -lactamase antibiotics has resulted in the appearance of numerous resistant strains. The major resistance factor is the production of β -lactamases. On the basis of their catalytic mechanisms and primary structures, these enzymes are at present divided into four classes (Ambler, 1980; Ambler et al., 1991; Joris et al., 1991). Class-B enzymes are metalloproteins which contain an essential Zn^{2+} ion. Enzymes in the other three classes, A, C and D, are active-site serine enzymes which can be distinguished by their sequences and, to some degree, by their specificity profiles.

Class-A enzymes can be chromosome- or plasmid-encoded (Bush, 1989; Boissinot and Levesque, 1990; Perilli et al., 1991). In the latter case, the transferable properties of the plasmids have been responsible for their spreading to many pathogenic species and genera (Zschech and Murray, 1991), making them important contributors to chemotherapeutic failures. In addition, a few single-point mutations in these plasmid-encoded β -lactamases can significantly alter their activity spectra, leading to the emergence of the 'extended-spectrum β -lactamases' (Barthelemy et al., 1988; Sougakoff et al., 1988) which exhibit a dangerously increased efficiency against so-called ' β -lactamase-stable' cephalosporins, such as cefotaxime and ceftazidime.

In contrast, the same phenomenon has not been reported for the chromosome-encoded class-A or class-C β -lactamases. In consequence, we have performed an extensive survey of β -lactamase production in various Italian hospitals (Abruzzo-Lazio area) oriented to the detection of chromosome-encoded enzymes with extended specificity profiles.

A *Pseudomonas stutzeri* strain was found that exhibited an unusual resistance pattern to β -lactam antibiotics (Amicosante et al., 1991), mainly to penicillins and third-generation cephalosporins. It produced two types of β -lactamase. One had an M_r value of 39000 and an isoelectric point of 8.2 and its properties were typical of class-C β -lactamases. The second enzyme, with an M_r value of 29000 and an isoelectric point of 5.2–5.4, appeared to be very active against ceftazidime and cefotaxime, indicating that it could be considered as an extended-spectrum β -lactamase, which, exceptionally, included monobactams among its good substrates.

MATERIALS AND METHODS

Chemicals and antibiotics

Polybuffer 74, Mono P HR5/20, Sephadex G-75 and ampholine PAGE-plates (3.5–9.5) were from Pharmacia (Uppsala, Sweden). 6 β -Iodopenicillanate was a gift from Pfizer Central Research (Sandwich, Kent, U.K.). Nitrocefin was supplied by Glaxo Research Group (Greenford, Middx., U.K.). Benzylpenicillin, ampicillin, oxacillin, cephalothin and cephaloridin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cefotaxime and ceftazidime were kindly given by Hoechst Italia (Milano, Italy) and Glaxo Italia (Verona, Italy) respectively. Sulbactam was from Pfizer (Rome, Italy), carumonam was from Hoffman-La Roche (Basel, Switzerland) and aztreonam and tigemonam were from Squibb.

β -Lactamase activity and protein determination

β -Lactamase activity was measured at 30 °C in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.05 mg/ml BSA using cefotaxime as substrate. One unit hydrolyses 1 μ mol/min at 30 °C at a saturating substrate concentration. Protein concentrations were determined as described by Bradford (1976).

Bacterial strains and growth conditions

A *Pseudomonas stutzeri* strain was isolated from the blood of an immunocompromised patient. Characterization was achieved with the help of the API 20NE from BioMérieux. Cultures were grown overnight at 37 °C in brain/heart infusion.

β -Lactamase production and purification

The bacterial pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.0. Cells were disrupted by sonication. The clarified supernatant was loaded on to a Sephadex G-75 gel-permeation column. The active fractions were collected, pooled and loaded on a phenylboronic acid–Sephacrose column (PBA–Sephacrose) as reported by Cartwright and Waley (1984). The enzyme was eluted with 0.5 M NaCl in 0.5 M borate buffer, pH 7.0, dialysed and finally purified to homogeneity by fast chromatofocusing on a Mono P HR5/20 column over the pH range 6.5–4.0. The pH

Abbreviation used: PBA, phenylboronic acid.

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of the active fractions was immediately adjusted to 7.0 by the addition of phosphate buffer. The last step allowed the separation of three active fractions. The first did not bind to the column and contained a β -lactamase of high isoelectric point (8.2), the properties of which (M_r 39000 and transient inactivation by cefotaxime) indicated that it belonged to class C. The two other fractions were eluted at pH 5.4 and 5.2 and were further studied.

M_r determinations were performed by electrophoresis in 15% (w/v) polyacrylamide gels in the presence of SDS as described by Laemmli (1970). Isoelectric point values were measured using ampholine PAGE-plates (3.5–9.5) in a Multiphor II apparatus cooled to 8 °C.

Determination of kinetic parameters

Kinetic parameters were determined by recording the complete time courses of substrate hydrolysis. The wavelengths and absorption coefficient variations have been previously reported (Amicosante et al., 1988). Very low K_m values were determined in competition experiments using 100 μ M nitrocefin as substrate. All kinetic data were obtained at 30 °C in 0.1 M sodium phosphate buffer, pH 7.0, containing 50 μ g/ml BSA. Values represent means of five different experiments performed at various substrate and enzyme concentrations. The data were collected using microcomputer-linked Uvikon 860 or Perkin-Elmer Lambda 2 spectrophotometers and analysed as described by De Meester et al. (1987).

RESULTS

Purification, apparent M_r and pI determination

Table 1 shows the different purification steps. The final preparation was about 95% pure as judged by SDS/PAGE. The overall purification yield was 9%. The last step allowed the detection of two different β -lactamase forms exhibiting isoelectric points of 5.2 and 5.4 (Figure 1). The two isoforms appeared to have the same activity (see below) and the same apparent M_r , 29000 (not shown).

Interaction with non- β -lactam compounds

The presence of EDTA (final concentration 10 mM) did not significantly decrease the β -lactamase activity (10% loss after 1 h of incubation). This result showed that bivalent cations were not involved in the folding or the catalytic pathway. At a concentration of 10 μ M, *p*-chloromercuribenzoate completely inactivated the enzyme (0.27 μ M) after 90 min of incubation at 30 °C.

Figure 1 Elution profile from the Mono P column

A sample containing 2 mg of protein was loaded on the Mono P HR5/20 column equilibrated with 25 mM BisTris, pH 6.5, and eluted with Polybuffer 74 (dilution 1:10, pH 4). Fractions (1 ml) were collected, their pH was measured and subsequently adjusted to 7.0 with 50 mM sodium phosphate buffer, pH 7.5, containing 5% ethylene glycol. Peaks A and B contained β -lactamase activity and corresponded to pI values of 5.4 and 5.2 respectively. Peak C contained the putative class-C β -lactamase. ----, pH.

Kinetic parameters for β -lactam compounds

The experimental values are given in Table 2. The *Ps. stutzeri* enzyme exhibited a broad activity spectrum against β -lactam compounds. The k_{cat} and k_{cat}/K_m values for penicillins and cephalosporins were very similar. Surprisingly, some k_{cat} values were even higher for cephalosporins than for penicillins. Cefotaxime and cefuroxime, usually considered as β -lactamase-stable molecules, were hydrolysed with higher k_{cat} values than

Table 1 Purification of the β -lactamases produced by *Ps. stutzeri*

The starting material was obtained from 8 litres of culture. Cefotaxime was used as substrate. The activity values are $\pm 5\%$.

Step	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification factor	Yield (%)
Crude extract	390	2	780	1	100
Sephadex G-75 chromatography	32	14	448	7	57
PBA-Sephacrose 4B chromatography	1.6	130	208	65	26
Chromatofocusing					
Form pI 5.4	0.15	410	61	205	8
Form pI 5.2	0.03	350	10	175	1.2

Table 2 Substrate profile of the *Ps. stutzeri* β -lactamase

The parameters were usually determined for the pI 5.4 form. When determined, those for the pI 5.2 form are given in parentheses. Results are means $\pm$ S.D. for three to five different experiments. N.D., not determined.

Substrates	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m (μ M $^{-1}$ · s^{-1})
Nitrocefin	35 $\pm$ 5 (45 $\pm$ 5)	11 $\pm$ 2 (10 $\pm$ 1)	0.32 $\pm$ 0.04 (0.22 $\pm$ 0.05)
Cephalothin	20 $\pm$ 4	6 $\pm$ 1	0.32 $\pm$ 0.03
Cefotaxime	209 $\pm$ 20 (230 $\pm$ 40)	21 $\pm$ 3 (16 $\pm$ 4)	0.1 $\pm$ 0.02 (0.07 $\pm$ 0.01)
Cefuroxime	144 $\pm$ 30	26 $\pm$ 4	0.18 $\pm$ 0.02
Ceftazidime	160 $\pm$ 33	6 $\pm$ 1	0.03 $\pm$ 0.003
Cephalexin	60 $\pm$ 11	7 $\pm$ 1	0.12 $\pm$ 0.02
Penicillin G	16 $\pm$ 2* (23 $\pm$ 3)	5 $\pm$ 0.3† (12 $\pm$ 2)	0.32 $\pm$ 0.015 (0.52 $\pm$ 0.07)
Ampicillin	105 $\pm$ 10	15 $\pm$ 1	0.14 $\pm$ 0.01
Carbenicillin	6.6 $\pm$ 0.9*	0.33 $\pm$ 0.03†	0.05 $\pm$ 0.004
Oxacillin	0.4 $\pm$ 0.03*	0.12 $\pm$ 0.01‡	0.3 $\pm$ 0.03
Cloxacillin	0.94 $\pm$ 0.08*	0.15 $\pm$ 0.02‡	0.016 $\pm$ 0.002
Clavulanic acid	0.32 $\pm$ 0.02*	N.D.	N.D.
Imipenem	1.1 $\pm$ 0.2*	0.007 $\pm$ 0.001†	0.007 $\pm$ 0.005
Sulbactam	3.0 $\pm$ 0.4*	0.25 $\pm$ 0.03†	0.08 $\pm$ 0.008

* K_m values were determined as K_i values using nitrocefin as reporter substrate.

† k_{cat} values were determined by direct hydrolysis at V_{max} .

‡ The k_{cat} and K_m values were calculated after the reaction had reached the steady state.

Table 3 Kinetic parameters for the interaction between monobactams and the *Ps. stutzeri* β -lactamase

The values for benzylpenicillin are also given for comparative purposes. k_{cat} values were determined by monitoring the hydrolysis of the antibiotics at $[S] \geq K_m$. Results are means $\pm$ S.D. for three to five different experiments.

Substrates	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m (μ M $^{-1}$ · s^{-1})
Benzylpenicillin	35 $\pm$ 5	11 $\pm$ 2	0.32 $\pm$ 0.04
Aztreonam	10 $\pm$ 2*	3 $\pm$ 0.3	0.33 $\pm$ 0.015
Tigemonam	7 $\pm$ 0.7*	0.8 $\pm$ 0.1	0.11 $\pm$ 0.014
Carumonam	170 $\pm$ 30*	6 $\pm$ 1	0.035 $\pm$ 0.003

* K_m values were determined as K_i values using nitrocefin as reporter substrate.

penicillins such as ampicillin. Sulbactam was efficiently hydrolysed with a k_{cat} of 0.25 s^{-1} , and no concomitant inactivation of the enzyme was observed over a 30 min period with 250 μ M sulbactam. Imipenem and cefoxitin (not shown) were rather resistant to enzyme hydrolysis (k_{cat} 5 $\times 10^{-3}$ –7 $\times 10^{-3}$ s^{-1}). For cefoxitin, the slow formation of a transient acylenzyme enabled the evaluation of the acylation rate ($k_2/K = 10^3$ M $^{-1}$ · s^{-1}). The presence of a burst was detected during the hydrolysis of oxacillin and cloxacillin. The reported k_{cat} values were then determined for the second phase of antibiotic hydrolysis after the burst was completed. The K_m values were obtained using nitrocefin as a reporter substrate.

The kinetic parameters for most substrates were measured with the β -lactamase that had pI value of 5.4 since it was the most abundant form. Within the limits of experimental error, the second form (pI 5.2) had a similar activity pattern, as shown by the values reported in Table 2. The β -lactamase displayed good

activity towards monobactams such as aztreonam, tigemonam and carumonam (Table 3).

Interaction with β -iodopenicillanic acid

The enzyme was rapidly inactivated by β -iodopenicillanic acid. No significant turnover could be detected, and the formation of the inactivated species was concomitant with the appearance of a maximum at 325 nm in the difference spectrum (acylenzyme minus enzyme). The pseudo-first-order rate constant for inactivation was measured at inactivator concentrations ranging from 0.3 to 8 μ M and a hyperbolic dependence was found. From these data, the individual k_2 (0.068 $\pm$ 0.002 s^{-1}) and K (1.39 $\pm$ 0.16 μ M) values could be computed with the help of the Enzfitter program (Leatherbarrow, 1987).

DISCUSSION

Ps. stutzeri is ubiquitous in soil and water, but also in various clinical specimens (Palleroni et al., 1970). It can be associated with clinical difficulties such as septicaemia, urinary-tract or post-operative and traumatic infections.

A *Ps. stutzeri* strain presenting a high level of resistance to third-generation cephalosporins was found to produce two non-inducible chromosome-encoded β -lactamases (M_r 39000 and 29000). The first enzyme had an isoelectric point of 8.2. The activity against cloxacillin, clavulanic acid and cefotaxime confirmed that this enzyme was a class-C β -lactamase. Two forms of the second β -lactamase were found with different pI values of 5.4 and 5.2 but similar catalytic properties. Ragged N-termini could explain the presence of the two isoforms. This problem, often encountered with β -lactamases, has been discussed in depth before (Franceschini et al., 1991; Matagne et al., 1991). The fact that it remained active in the presence of EDTA and the rapid inactivation by β -iodopenicillanate indicated that the enzyme was probably a class-A enzyme, although the low K value observed with the latter inactivator was similar to that observed with a class-D enzyme (P. Ledent and J.-M. Frère, unpublished work). However, the substrate profile and the chromosomal encoding of the β -lactamase were not in favour of its inclusion in class D. The enzyme was also sensitive to low concentrations of *p*-chloromercuribenzoate. This phenomenon could be due to the presence of a cysteine residue near the active-site area as observed for some other class-A enzymes (Amicosante et al., 1988, 1990).

The β -lactamase from *Ps. stutzeri* showed a really broad activity spectrum with similar activities against penicillins and cephalosporins, which again underlined the fact that the identification of class-A enzymes as penicillinases can be misleading. The third-generation cephalosporins, cefotaxime and ceftazidime, were rapidly hydrolysed. The low K_m and k_{cat} values observed for oxacillin, cloxacillin and sulbactam explained their behaviour as good inhibitors when in competition with better substrates. It was interesting to note that no progressive inactivation was observed at $[S] \geq K_m$ over a period of 30 min, in contrast with the behaviour of these compounds towards class-C enzymes. With cloxacillin and oxacillin, biphasic kinetics were obtained, as with several other class-A enzymes. Finally, imipenem and cefoxitin were slowly hydrolysed but the k_{cat} values were not negligible.

When compared with other class-A enzymes, the *Ps. stutzeri* β -lactamase showed rather low enzyme efficiencies and k_{cat} values for compounds usually considered as good class-A substrates (ampicillin, benzylpenicillin). Surprisingly, this β -lactamase had exceptionally high efficiencies against monobactams such as aztreonam, carumonam and tigemonam. Indeed, the k_{cat}/K_m

values for aztreonam and benzylpenicillin were similar. Moreover, carumonam, which is usually a very poor β -lactamase substrate, still exhibited a k_{cat}/K_m value of $35000 \text{ M}^{-1} \cdot \text{s}^{-1}$, orders of magnitude higher than those observed with most other class-A enzymes (A. Matagne and J.-M. Frère, unpublished work; Bush et al., 1982). These results underline the previously mentioned heterogeneity of the catalytic properties of class-A β -lactamases (Matagne et al., 1990). No striking similarity could be detected when comparing the specificity profile of the *Ps. stutzeri* β -lactamase with those of other class-A enzymes produced by *Pseudomonas* (CARB enzymes; Labia et al., 1981).

The properties of the β -lactamase are somewhat reminiscent of several mutants of the TEM enzymes, which also exhibited decreased and relatively high activities against benzylpenicillin and aztreonam respectively (Sowek et al., 1991). One might thus expect some sequence similarity between the TEM variants and the *Ps. stutzeri* enzyme. Conversely, similar substrate profiles might result from less related structures representing an example of converging evolution subsequent to the divergence of class-A enzymes from a common ancestor. The cloning and sequencing, which are at present under way, should shed more light on this interesting alternative.

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