The production and molecular properties of the zinc β -lactamase of *Pseudomonas maltophilia* IID 1275

Roy BICKNELL,* Ezard L. EMANUEL,* Jean GAGNON† and Stephen G. WALEY*

*Sir William Dunn School of Pathology and †Department of Biochemistry University of Oxford,
South Parks Road, Oxford OX1 3RE, U.K.

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The production and purification of a tetrameric zinc β -lactamase from *Pseudomonas maltophilia* IID 1275 were greatly improved. Three charge variants were isolated by chromatofocusing. The subunits each contain two atomic proportions of zinc and (in two of the variants) one residue of cysteine. The thiol group is not required for activity, nor does it appear to bind to the metal. Replacement of zinc by cobalt, cadmium or nickel takes place at a measurable rate, and gives enzymes that are less active than the zinc enzyme. The properties of this enzyme differ from those of the other known zinc β -lactamase, β -lactamase II from *Bacillus cereus*. The amino acid sequence of the *N*-terminal 32 residues was determined; there is no similarity to the *N*-terminal sequences of other β -lactamases.

In the 16 years following the recognition that β -lactamase II from *Bacillus cereus* 569 was a zinc metalloenzyme (Sabath & Abraham, 1966), many β -lactamases have been isolated and characterized, but none that required metal ions for activity. β -Lactamase II has remained the sole member of structural class B (Ambler, 1980). Thus the isolation of a zinc-dependent β -lactamase from *Pseudomonas maltophilia* (Saino *et al.*, 1982), a pathogen, was noteworthy, and raised the question of whether this enzyme was related to β -lactamase II. The present paper describes improved procedures for obtaining β -lactamase L1 from cells grown in bulk (15 litres); the enzyme was obtained with a 4-fold higher specific activity than hitherto. Kinetic studies confirmed the central role of the tightly bound Zn^{2+} ion. Classification by amino acid sequence is the preferred method for β -lactamases, and so structural studies were initiated. The *N*-terminal region was studied. The independent origin of class A and class C β -lactamases, enzymes with serine at the active site, has been suggested (Jaurin & Grundström, 1981; Knott-Hunziker *et al.*, 1982). The work reported below makes it likely that there will also turn out to be more than one structural class of zinc β -lactamase.

Materials and methods

Materials

Pseudomonas maltophilia strain IID 1275 was kindly supplied by Professor Y. Saino (Gunma

University, Gunma, Japan), benzylpenicillin and cephalosporin C by Glaxo Laboratories (Greenford, Middx. U.K.), clavulanic acid by Beecham Research Laboratories (Betchworth, Surrey, U.K.), 6 β -iodopenicillanic acid by Leo Products (Copenhagen, Denmark) and the hydroxybenzylpenicillanic acid sulphone (Foulds *et al.*, 1985) by Professor P. G. Sammes (University of Leeds, Leeds, U.K.). Nitrocefin was from Becton Dickinson (Oxford, U.K.). Materials for chromatofocusing were from Pharmacia (Milton Keynes, Bucks., U.K.). β -Lactamase II was as previously described (Bicknell *et al.*, 1983).

Methods

Protein was measured by the method of Bradford (1976), with bovine serum albumin as standard, and cysteine was determined with Ellman's reagent as described by Habeeb (1972). After ion-exchange chromatography, the determination of protein was on the basis $A_{280}^{1\%} = 10$, and that the subunit of M_r 31600 (see below), gave 1.0 SH group/subunit.

Electrophoresis was carried out on 10% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). Isoelectric focusing was performed on Ampholine PAG plates, pH 3.5–9.5 or pH 5.0–8.2, from LKB Produkter (Bromma, Sweden). β -Lactamase activity was detected with nitrocefin.

Amino acid analysis of hydrolysates of 0.3 mg of protein was carried out on an LKB Biochrom 4101 analyser after hydrolysis *in vacuo* for 24, 48 or 72 h

with 6M-HCl containing 1 p.p.m. of 2-mercaptoethanol. Tryptophan was estimated from the formula:

$$\text{No. of residues of tryptophan} = [(1.1 \times \epsilon) - (n_{\text{Tyr}} \times 1.3)]/5.5$$

where ϵ is the absorption coefficient in $\text{mm}^{-1} \cdot \text{cm}^{-1}$ and n_{Tyr} is the no. of residues of tyrosine. The *N*-terminal amino acid was determined on protein after oxidation with performic acid (Gray, 1972).

The amino acid sequence of the *N*-terminal 32 residues was determined on protein that had been reductively alkylated by 80mM-iodoacetic acid in the presence of 40mM-dithiothreitol in 6M-guanidinium chloride (Bibring & Baxandall, 1978). The sequence was determined on 60nmol of protein on a Beckman 890C sequenator as described by Campbell *et al.* (1981).

The unit of β -lactamase activity was the hydrolysis of 1 μmol of benzylpenicillin/min in the pH-stat at pH 7 at 30°C in the presence of 0.5M-NaCl and 0.1mM-ZnSO₄. The pH-dependence of k_{cat} was measured with 30mM-benzylpenicillin (zero-order kinetics were observed) with the precautions noted by Bicknell *et al.* (1983).

The attempted hydrolysis of DL-alanylglycine (50mM) or glycyl-DL-phenylalanine (15mM) in 0.5M-Mops/NaOH buffer, pH 7, containing 1mM-ZnCl₂ by either 25 μM - β -lactamase L1 or 100 μM - β -lactamase II was carried out at 37°C for 16h. The reaction mixtures were analysed by t.l.c. on cellulose plates with butan-1-ol/acetic/water (40:9:20, by vol.).

Isolation of β -lactamase L1. Growth of *Ps. maltophilia* IID 1275 was essentially as described by Saino *et al.* (1982), but the induction differed. After 30min growth in the 15-litre fermenter, benzylpenicillin was added to a final concentration of 1mg/ml. The same amount of benzylpenicillin was again added when the increase in A_{600} reached 2, in about 30min; typically, two-further additions were made during the 8h incubation. The cells, after being spun down, were extracted four times with 50mM-sodium phosphate buffer, pH 7, containing 1% (w/v) Triton X-100. The extract was freed of nucleic acids by precipitation with 0.4% protamine sulphate, made 0.5mM with respect to phenylmethanesulphonyl fluoride, and dialysed against 50mM-Tris/HCl buffer, pH 8, containing 0.1mM-ZnCl₂. Ion-exchange chromatography on QAE-(quaternary ammonium ethyl)-Sephadex at pH 8 was carried out essentially as described by Saino *et al.* (1982), who reported an isoelectric point of 6.9 for β -lactamase L1. Hence the next step was chromatography on CM-Sephadex at pH 6, in 50mM-Mes/NaOH buffer containing 1mM-ZnCl₂; elution was carried out with a linear gradient of 0–0.3M-NaCl in the Mes/ZnCl₂ buffer.

Chromatofocusing over the range pH 8 to pH 6 was carried out on Sephadex PBE 94 essentially as described by Pharmacia, except that 20ml of resin was used. There were three peaks containing enzyme, which were dealt with separately. After concentration by ultrafiltration, exclusion chromatography on a column (60cm long $\times$ 1cm diam.) of Sephacryl S200 was carried out to remove Ampholines; the buffer was 50mM-Tris/HCl, pH 8, containing 0.1M-NaCl and 0.1mM-ZnCl₂, which was also used to store the enzyme (at 4°C) after concentration. No activity was lost on storage for 4 months.

Dicobalt β -lactamase L1. β -Lactamase L1 (0.81mg) in 30ml of 10mM-Mes/NaOH buffer pH 6.5, was treated with 2mM-CoCl₂; assays showed that the enzymic activity had declined to a steady value after 30min. The pH was raised to 8 with 200mM-Mops/NaOH buffer containing 20 μM -CoCl₂, and the solution was concentrated to 0.3ml by ultrafiltration (YM10 membrane). The solution was twice diluted and concentrated again, and then dialysed; there were two atomic proportions of cobalt per subunit, but no (0.04 ± 0.05) zinc, by atomic absorption analysis.

Results and discussion

Production of β -lactamase L1 Ps. maltophilia

The yield of β -lactamase L1 was greatly influenced by the conditions of induction; without inducer, no β -lactamase was detected. Repeated addition of benzylpenicillin proved best; usually three additions were made during 8h (see the Materials and methods section). The yield of enzyme was increased 6-fold by increasing the concentration of benzylpenicillin added from 0.1mg/ml (as used by Saino *et al.*, 1982) to 1mg/ml. When the benzylpenicillin was added as the culture was entering the exponential phase of growth, growth was retarded for 4h. By this time, the benzylpenicillin had been hydrolysed and growth again became rapid. Meanwhile, the β -lactamase activity increased as a response to the inducer, but then fell. The fall in activity was probably due to proteolysis, which may also have been responsible for the variant ionic forms (see below). The situation is complex: the inducer is destroyed by the β -lactamase that it is inducing, and the β -lactamase is probably being destroyed by other enzymes. In practice it was necessary to measure growth and β -lactamase activity at 15min intervals throughout the fermentation so that inducer was added at the best times.

Isolation of β -lactamase L1

β -Lactamase L1, as is usual for β -lactamases from Gram-negative bacteria, was cell-bound,

presumably located in the periplasm (Richmond & Curtis, 1974). Incomplete release of the enzyme from cell debris after freeze-thawing was initially a problem, which was solved by extraction with 1% (w/v) Triton X-100. Indeed, the use of this detergent made freeze-thawing unnecessary. The detergent, which was shed during later steps, was not necessary to keep the enzyme in solution (contrast Connolly & Waley, 1983). The purification consisted of two ion-exchange steps followed by chromatofocusing on Sephadex PBE 94 (Table 1). The other β -lactamase produced, L2, was not adsorbed on QAE-Sephadex, and was isolated by affinity chromatography on a boronic acid gel (Cartwright & Waley, 1984). β -Lactamase L1 was unstable below pH 8 unless Zn^{2+} ions were added, and the ion-exchange chromatography at pH 6 was carried out in the presence of 1 mM- ZnCl_2 . The elution profile from chromatofocusing on Sephadex PBE 94 showed three bands (Fig. 1): each had the same specific activity, and each was EDTA-sensitive. They are referred to below as charge variants. After concentration of the fractions, Ampholines were removed by exclusion chromatography on Sephacryl S200. Each charge variant gave a single, enzymically active, band on isoelectric focusing.

Properties of charge variants

The charge variants shared most properties, including metal content (the non-integral value was probably due to loss of zinc from the enzyme on dialysis), but differed in thiol content (Table 2).

The charge variant with pI7.2 may arise by oxidation of the thiol group. The tetramers (see below) apparently (and unexpectedly) contained no or four thiol groups/molecule, and were not

Fig. 1. Fractionation of β -lactamase L1 by chromatofocusing
Chromatofocusing was carried out on a Sephadex PBE94 gel with a linear pH gradient from pH 8 to pH 6. The charge variants were eluted at pH 7.2, 7.1 and 6.5, and had specific activities of 490, 500 and 515 units/mg and comprised 18%, 45% and 37% of the total respectively.

Table 1. Purification of β -lactamase L1

Details of the steps are given in the Materials and methods section. The overall yield of β -lactamase L1 was 20 mg from 190 g wet wt. of cells.

Stage of purification	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Recovery (%)
Cell extract	105 000	0.9		(100)
Dialysed extract	83 400	0.9		80
After QAE-Sephadex	65 300	53	58	62
After CM-Sephadex	30 200	290	320	29
After chromatofocusing and exclusion chromatography	10 000	500	560	10

Table 2. Charge variants of β -lactamase L1

	Properties of charge variants		
	6.5	7.1	7.2
Eluted at pH	6.5	7.1	7.2
% of β -lactamase	37	45	18
Specific activity (units/mg of protein)	515	500	490
Zn(II) content (atoms/subunit)	1.7*	1.7*	1.7*
Thiol content (SH groups/subunit)	0.92	0.98	<0.06
Subunit M_r	31 600	31 600	31 600
M_r	†	123 000	†

* Estimated uncertainty of method ± 0.05 .
† Shown to be tetrameric, but M_r not accurately determined.

Fig. 4. Effect of metal replacement on β -lactamase L1 activity

(a) The hydrolysis of 30 mM-benzylpenicillin by 19.5 nM- β -lactamase L1 at 30°C at pH 6.5 in the presence of 0.5 M-NaCl and 0.1 mM-ZnCl₂ was measured in the pH-stat; at the time shown by the arrow, CoCl₂ (final concn. 2 mM) was added. (b) Variation of activity with time after preincubation of β -lactamase L1 (0.17 mg/ml) in 0.75 M-Mops buffer, pH 6.5, at 30°C with 2 mM-metal ions; the remaining activity was measured by assay with 3 mM-benzylpenicillin in the presence of 0.5 M-NaCl in the pH-stat at pH 8.

Replacement of zinc by other metals. Addition of CoCl₂ (final concentration 2 mM) to an assay in which 30 mM-benzylpenicillin was being hydrolysed in the presence of 0.1 mM-ZnCl₂ brought about a progressive decline of activity (Fig. 4a). The final rate was independent of the concentration of Co²⁺ over the range 20 μ M–20 mM. When other ions were tested (at 2 mM) like this, they fell into three categories: (a) no effect (even at 200 mM), Mg²⁺ and Ca²⁺; (b) decrease of rate to new zero-order rate, Co²⁺, Ni²⁺, Cd²⁺ and Mn²⁺; (c) complete loss in activity, Hg²⁺ and Cu²⁺ (Cu²⁺ was tested at 0.2 mM because it catalyses the hydrolysis of benzylpenicillin).

The replacement of zinc by other metals was also studied by incubating β -lactamase L1 with 2 mM-Ni²⁺ and with 2 mM-Co²⁺ at pH 6.5, with samples being periodically withdrawn for assay (Fig. 4b). The final rate was characteristic of the metal ion. The changes were fully reversible: addition of Zn²⁺ to 10 mM restored full activity.

Full activity could, however, not be restored by addition of metal ions to the apoenzyme; our preparations of the apoenzyme always lost activity, in contradistinction to those of Saino *et al.* (1982). This was not due to dissociation of the apoenzyme into monomers: exclusion chromatography (in the presence of EDTA) of β -lactamase L1 treated with EDTA gave results indistinguishable from those obtained with the tetrameric holoenzyme. There were two atoms of zinc/subunit; one has a catalytic role, and either or both may have a structural role.

The absorption spectrum of dicobalt β -lactamase L1 [found to contain two Co(II) atoms/subunit but no Zn(II)] (Fig. 5) resembled the spectra of the cobalt derivatives of carboxypeptidase A (Latt

Fig. 5. Absorption spectrum of dicobalt β -lactamase L1. The difference spectrum of dicobalt β -lactamase L1 (85 μ M) in 50 mM-Mops buffer, pH 8, containing 2 mM-CoCl₂ was scanned in a Cary spectrophotometer; the reference cell contained 2 mM-CoCl₂ in the same buffer.

& Vallee, 1971) and thermolysin (Holmquist & Vallee, 1974). The absorption coefficient (450 M⁻¹·cm⁻¹) was about 3 times as large, perhaps because there are two Co(II) atoms/subunit in β -lactamase L1. The absorption coefficient suggests that at least one cobalt atom is in a distorted tetrahedral or trigonal environment.

Substrates and inhibitors of β -lactamase L1. The presence of about 20% of minor component had no detectable effect on the kinetics; progress curves obeyed the integrated Michaelis–Menten equation with $K_m = 0.2$ mM for benzylpenicillin, as noted by Saino *et al.* (1982). The k_{cat} for the hydrolysis of penicillins is greater than that for the hydrolysis of

Table 4. Comparison of β -lactamase L1 and β -lactamase II

The kinetic parameter k_{cat} for benzylpenicillin was measured at 30°C, in the presence of 0.5M-NaCl in the pH-stat at pH 6 for β -lactamase II and at pH 8 for β -lactamase L1, and is expressed on the basis of one active site/subunit. For β -lactamase II, the metal ion concentration was 1 mM, but no metal ion was added to β -lactamase L1.

Property	β -Lactamase L1	β -Lactamase II
M_r by exclusion chromatography	123000	20000–30000
M_r by electrophoresis in sodium dodecyl sulphate	31600	26000*
Metal (Zn or Co) content	2/subunit	2/molecule
Thiol content	1/subunit†	1/molecule
Is thiol required for activity?	No	Yes
Is thiol a metal ligand?	No	Yes
$10^{-3} \times k_{\text{cat}}$ for zinc enzyme (min^{-1})	10	50
$10^{-3} \times k_{\text{cat}}$ for cobalt enzyme (min^{-1})	3.6	8
$10^{-3} \times k_{\text{cat}}$ for cadmium enzyme (min^{-1})	2.1	0.5
$10^{-3} \times k_{\text{cat}}$ for nickel enzyme (min^{-1})	5.4	<0.01
Biphasic kinetics with manganese enzyme*	No	Yes
Is k_{cat} for a penicillin generally much higher than that for a cephalosporin?	Yes	No
Inactivator (other than chelating agents)	None known	None known

* Bicknell (1984).

† For main charge variant; see Table 2.

cephalosporins (Saino *et al.*, 1982). The pH-dependence of k_{cat} for the hydrolysis of benzylpenicillin was broad and bell-shaped, characterized by a lower $\text{p}K_a$ of less than 5 (limit set by the stability of the enzyme) and a higher $\text{p}K_a$ of 10.1. β -Lactamase L1 was not inactivated by clavulanic acid, 6 β -iodopenicillanic acid, a hydroxybenzylpenicillanic acid sulphone (Knight & Waley, 1985), phenylboronic acid or methanesulphonyl fluoride. The only other β -lactamase known to be resistant to all these compounds is β -lactamase II from *B. cereus*.

Both β -lactamase L1 (Saino *et al.*, 1982) and β -lactamase II hydrolyse *N*-formimidoylthienamycin; the latter is particularly efficient (k_{cat} 12500 min^{-1} and K_m 1.6 mM, at 30°C at pH 6; Bicknell, 1984). *N*-Formimidoylthienamycin is unusual (among β -lactam antibiotics) in being hydrolysed by a renal dipeptidase (Kim & Campbell, 1982). Since the renal dipeptide (a zinc metalloenzyme) and the zinc β -lactamases share *N*-formimidoylthienamycin as a substrate, we wondered whether they also shared alanylglycine or glycylphenylalanine as a substrate. There were, however, no signs of hydrolysis of these dipeptides by β -lactamase L1 or β -lactamase II, after prolonged incubation; the enzymes had retained virtually all their activity. Peptides appear resistant to β -lactamases, although depsi-peptides are hydrolysed (Pratt & Govardhan, 1984).

Comparison of β -lactamase L1 and β -lactamase II

There are many differences between these two zinc β -lactamases (Table 4). Thus β -lactamase L1 is a tetramer, the thiol group is not required for activity, and Ni(II) can replace Zn(II). Their

specificities also differ. Finally, there is no resemblance between the *N*-terminal amino acid sequence of β -lactamase L1 and that of any other β -lactamase, even when the efficient method of sequence hydrophobicities (Sweet & Eisenberg, 1983) is used. Thus β -lactamase L1 may well belong to a new structural class of β -lactamases.

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