# β-LACTAMASE ACTION: ISOLATION OF AN ACTIVE-SITE SERINE PEPTIDE FROM THE *PSEUDOMONAS* ENZYME AND A PENICILLIN

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Received 23 September 1980

## 1. Introduction

β-Lactamases (penicillinases) comprise an efficient and diverse group of enzymes that are often responsible for the resistance of pathogens to β-lactam antibiotics. An inducible, cell-bound chromosomal β-lactamase from *Pseudomonas aeruginosa* NCTC 8203 was first described by Sabath et al. [1] and was later obtained in partially purified form [2]. A mutant of *Ps. aeruginosa* 18S was found to produce a similar β-lactamase constitutively [3], and this enzyme has been purified to apparent homogeneity [4]. Those β-lactamases that have been studied structurally fall into two classes, A and B [5]. The *Pseudomonas* enzyme however, with its relatively high $M_r$ [2,4], has been put forward as a candidate for a third structural class [5]. We suggested in [6] that class A β-lactamases might be serine enzymes, serine being the amino acid residue labelled by a specific inactivator. This hypothesis is now broadened: we identify a penicilloyl-serine at the active site of the *Pseudomonas* enzyme, by the isolation of a labile acyl-enzyme.

## 2. Materials and methods

The *Pseudomonas* β-lactamase used was a highly purified preparation of the cell-bound enzyme produced by mutant 1822 S/H from strain 18S. [$^{14}$C]-Cloxacillin, 6-(5-[5-$^{14}$C]methyl-3-*o*-chlorophenylisoxazole-4-carboxyamido)penicillanic acid, spec. radioact. 28 μCi/mmol, was synthesised from ethyl [3-$^{14}$C]acetoacetate [7]. [$^{3}$H]Cloxacillin was prepared from the acid chloride of 5-methyl-3-*o*-chlorophenylisoxazole-4-carboxylic acid [7] and 6-aminopenicillanic acid labelled with $^{3}$H in its 2β-methyl group [8] and isolated as the crystalline sodium salt, spec. radioact. 4.1 μCi/μmol. β-Lactamase activity was measured in the pH-stat., or by the spectrophotometric method [9]. The penamaldate assay, and other methods were as in [4,10,11].

## 3. Results

### 3.1. *Isolation of the acyl-enzyme*

Preliminary experiments on the rate of hydrolysis of cloxacillin by the lactamase suggested a $k_{cat}$ of 1–10 min$^{-1}$, a low enough value to permit the ready isolation of an intermediate. Thus the enzyme was incubated briefly with radioactive cloxacillin and then separated from excess substrate by gel filtration

Table 1
Labelling of the β-lactamase by cloxacillin (mol/mol)

| Eluant for gel filtration | Basis for measurement of labelling | Extent of labelling |
|---|---|---|
| Sodium dodecyl sulphate (20 mM) | Radioactivity, $^{14}$C | 0.8 |
| | Penamaldate, $HgCl_2$ titre | 1.2 |
| | Penamaldate, $A_{280}$ | 1.1 |
| Acetic acid 30% (w/v) | Radioactivity, $^{3}$H | 1.04 ± 0.03 |

β-Lactamase (0.4–0.7 mM) and [$^{14}$C]- or [$^{3}$H]cloxacillin (10–40 molar proportions) in 0.2 M sodium phosphate pH 7, 0.5 M NaCl were allowed to react for 1 min at 0°C or 40 s at 20°C, and the solution then diluted with an equal volume of either 40 mM sodium dodecylsulphate or glacial acetic acid. Gel filtration was carried out on Sephadex G-25 superfine. The last entry in the table gives the mean ± SEM from 19 expt. The high $M_r$ peak was absent in a control experiment in which the acetic acid was added before the cloxacillin

under denaturing conditions. Acylation was stoichiometric, whether judged by incorporation of radioactivity from cloxacillin (labelled in either the side chain or the nucleus) or by the penamaldate assay for penicilloyl groups (table 1). Experiments in which the radioactive substrate was diluted with non-radioactive substrate for 10–60 s before the labelling was terminated by denaturation gave a half-life for the acyl-enzyme of ~9 s at 30°C, pH 7. Hence the acyl-enzyme is reactive enough to be an intermediate on the main pathway, although further work on the kinetics is necessary to test this hypothesis.

Table 2
Composition of labelled peptides (residues/mol)

| Amino acid | Tryptic peptide | | Thermolysin peptide |
|---|---|---|---|
| | Expt. (a) | Expt. (b) | |
| Asp | 0.3 | 0.6 | |
| Thr | 2.2 | 1.6 | |
| Ser | 2.2 | 1.7 | 1.0 |
| Glu | 2.4 | 1.9 | |
| Pro | 0.8 | 0.8 | |
| Gly | 1.5 | 1.2 | 1.1 |
| Ala | 0.2 | 0.5 | |
| Val | 1.7 | 2.0 | |
| Ile | 0.8 | 1.0 | 0.9 |
| Leu | 0.7 | 1.8 | (0.4) |
| Phe | 0.7 | 1.0 | |
| Lys | 0.9 | 0.8 | |
| $^3H$ | | 0.9 | 0.8 |
| *m* | −0.18 | −0.34 | −0.39 |

The labelled enzyme (8 mg), suspended in 1.5 ml 1% (w/v) ammonium bicarbonate, was treated with 3.5 mg trypsin in 0.25 ml of mN HCl for 2.3 h at 37°C. Expt. (a): the mixture was fractionated by paper electrophoresis at pH 6.5 (145 min at 56 V/cm), the radioactive band eluted, treated with 5% (v/v) triethylamine for 90 min at 37°C to split off the penicilloyl group [12], and electrophoresis at pH 6.5 then done again (80 V/cm for 35 min). The peptide was no longer radioactive and the anionic mobility had decreased from −0.34 to −0.18. Expt. (b): the tryptic digest was fractionated with 0.5% formic acid on Sephadex G-25 superfine (140 cm long × 0.9 cm diam.) at 4°C, and the radioactive peak further fractionated by HPLC on a Lichrosorb RP-8 (10 μm) column (Owens Polyscience, Macclesfield) with a linear gradient from 50 mM triethylamine formate (pH 3.5), 5% (v/v) propan-1-ol to the same buffer in 60% (v/v) propan-1-ol. The radioactive thermolysin peptide was prepared by digesting the tryptic peptide (60 nmol) with 0.2 mg thermolysin (Calbiochem-Behring) in 1% (w/v) ammonium bicarbonate, 2 mM $CaCl_2$, for 50 min at 37°C and then fractionating the digest by HPLC as above. The table gives molar ratios, values of <0.2 mol/mol being omitted. The last row gives the electrophoretic mobility (*m*) at pH 6.5 [13]

### 3.2. *Isolation of labelled peptides*

The labelled protein, after freeze-drying from aqueous acetic acid and digestion with trypsin, gave only one radioactive peptide. The peptide, which was isolated by several methods (table 2), had N-terminal valine and probably contained 14 residues. Further digestion with thermolysin gave a smaller radioactive peptide, in which only one amino acid had a potentially reactive sidechain. The dansyl-Edman procedure [14,15] (after treatment with triethylamine to remove the penicilloyl group) gave the sequence:

Ile–Gly–Ser.

## 4. Discussion

The isolation of an acyl-enzyme, and the identification of serine as the amino acid acylated, constitute the first clues to the mechanism of action of the *Pseudomonas* enzyme. Previous views about the role of serine in β-lactamases were based on the inactivators (table 3). The recent well-based kinetic evidence for an acyl-enzyme intermediate does not distinguish between serine and threonine, and utilized a very poor substrate [16], whereas the value of $k_{cat}/K_m$ for cloxacillin and the *Pseudomonas* enzyme (of the order of $10^6$ $M^{-1} . s^{-1}$) is quite high: by this criterion, cloxacillin is a good substrate. We may now conclude that at least two classes of β-lactamases are serine enzymes.

Table 3
Active-site sequences in β-lactamases and D-alanine carboxypeptidases

| Residue labelled | Sequence | Enzyme |
|---|---|---|
| Ser | Phe–Ala–Ser–Thr | β-Lactamase I [6] |
| Ser | Ile–Gly–Ser | *Ps.* β-lactamase |
| Ser or Thr | Met–Met–Ser–Thr | *E. coli* β-lactamase [16] |
| Ser or Thr | Tyr–Ala–Ser–Thr | *Staph. aureus* β-lactamase [17] |
| Ser | Ile–Ala–Ser–Met | *B. subtilis* carboxypeptidase [12] |
| Ser | Val–Gly–Ser | *Streptomyces* R61 carboxypeptidase [18] |

The sequences immediately around the active-site serine in β-lactamases and D-alanine carboxypeptidases are set out in table 3. As far as can be judged from such a limited comparison, the sequence most similar to the *Pseudomonas* β-lactamase is the *Streptomyces* R61 carboxypeptidase, but further work is needed to show whether there is extensive homology between these enzymes.

**Acknowledgements**

The support of the Medical Research Council is gratefully acknowledged. We thank Mr N. Gascoyne for carrying out the amino acid analyses, Glaxo Research Laboratories and Beecham Research Laboratories for gifts, and Professor Sir Edward Abraham for advice and encouragement; S. G. W. is a member of the Oxford Enzyme Group.

## References

[1] Sabath, L. D., Jago, M. and Abraham, E. P. (1965) Biochem. J. 96, 739–752.
[2] McPhail, M. and Furth, A. J. (1973) Biochem. Soc. Trans. 1, 1260–1263.
[3] Flett, F., Curtis, N. A. C. and Richmond, M. H. (1976) J. Bacteriol. 127, 1585–1586.
[4] Berks, M., Redhead, K. and Abraham, E. P. unpublished.
[5] Ambler, R. P. (1980) Phil. Trans. Roy. Soc. Lond. B 289, 321–331.
[6] Knott-Hunziker, V., Waley, S. G., Orlek, B. S. and Sammes, P. G. (1979) FEBS Lett. 99, 59–61.
[7] Doyle, F. P., Hanson, J. C., Long, A. W. W., Nayler, J. H. C. and Stove, E. R. (1963) J. Chem. Soc. 5838–5845.
[8] Usher, J. J., Loder, B. and Abraham, E. P. (1975) Biochem. J. 151, 729–739.
[9] Waley, S. G. (1974) Biochem. J. 139, 789–790.
[10] Kiener, P. A., Knott-Hunziker, V., Petursson, S. and Waley, S. G. (1980) Eur. J. Biochem. in press.
[11] Corran, P. H. and Waley, S. G. (1974) Biochem. J. 139, 1–10.
[12] Waxman, D. J. and Strominger, J. L. (1980) J. Biol. Chem. 255, 3964–3976.
[13] Offord, R. E. (1977) Methods Enzymol. 47, 51–69.
[14] Gray, W. R. (1972) Methods Enzymol. 35, 333–344.
[15] Bruton, C. J. and Hartley, B. S. (1970) J. Mol. Biol. 52, 165–178.
[16] Fisher, J., Belasco, J. G., Khosla, S. and Knowles, J. R. (1980) Biochemistry 19, 2895–2901.
[17] Cartwright, S. J. and Coulson, A. F. W. (1980) Phil. Trans. Roy. Soc. Lond. B 289, 370–372.
[18] Frere, J. M., Duez, C., Ghuysen, J. M. and Vandekerkhove, J. (1976) FEBS Lett. 70, 257–260.