PENICILLINASE ACTIVE SITES: LABELLING OF SERINE-44 IN β-LACTAMASE I BY 6β-BROMOPENICILLANIC ACID

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1. Introduction

β-Lactamases inactivate penicillins and cephalosporins by catalysing the hydrolysis of the β-lactamase ring in these clinically-valuable antibiotics. The resistance of pathogenic bacteria is frequently due to their production of β-lactamases. Yet we know little in chemical terms about catalysis by these enzymes. Until now, affinity labelling has not been carried to a successful conclusion with any β-lactamase. β-Lactamase I from *Bacillus cereus* has been used here; it is readily prepared [1], the sequence has been studied [2], it is homologous with the enzymes from pathogens whose structure is known [3], and it is being examined crystallographically [4]. The reagent used here for affinity labelling, 6-β-bromopenicillanic acid, inactivates β-lactamase I in a rapid, stoicheiometric reaction [5], and the rate of inactivation is decreased by the presence of substrate [6]. We now report that the site labelled is serine-44. This result invites us to regard certain β-lactamases as serine enzymes.

2. Materials and methods

β-Lactamase I was prepared and assayed as in [1]. Trypsin, aminopeptidase M and carboxypeptidase A were those previously used [7]. The 6-α-bromopenicillanic acid, ^{3}H-labelled in its 2-β-methyl group, spec. radioact. 22.9 μCi/μmol, was the sample in [5]. Peptides were fractionated by paper chromatography and paper electrophoresis as in [7,8], except that the solvents contained 0.2% (v/v) thiodiglycol as antioxidant [9]. Radioactive peptides were detected by fluorography [10]. Amino acid analyses were carried out on a LKB Biochrom 4101 analyser. Radioactivity was determined with 'Unisolve 1' scintillation fluid (Koch-Light Labs) in a Nuclear-Chicago Unilux IIA scintillation counter.

3. Results

3.1. *Isolation of labelled peptide*

6-α-Bromo[^{3}H]penicillanic acid was partially converted into the 6-β-isomer, which is the epimer that inactivates β-lactamase I, before labelling the enzyme [5,6] (table 1). Preliminary experiments showed that with relatively large amounts of trypsin the undenatured labelled enzyme could be digested (without prolonged incubations) to give essentially one radioactive peptide (as detailed in table 1). The composition and N-terminus showed that this corresponded to peptide T7 [2], comprising residues 40–47:

Phe–Ala–Phe–Ala–Ser–Thr–Tyr–Lys

3.2. *Further degradation of tryptic peptide by peptidases*

Digestion of the labelled tryptic peptide (2 nmol) with carboxypeptidase A (10 μg) for 16 h at 27°C gave lysine and tyrosine, identified as their bis-dansyl (dimethylaminonaphthalenesulphonyl) derivatives [11]. This suggested that the label was not bound to either of these residues. Positive identification of the site labelled was obtained by digestion of the labelled

Table 1
Composition of labelled tryptic peptide

Amino acid	Residues/mol	
	Found	Calc.
Thr	1.1	1
Ser	1.2	1
Ala	2.1	2
Tyr	0.8	1
Phe[a]	1.8	2
Lys	1.0	1
^3H	1.2	

[a] N-Terminal amino acid identified as dansyl derivative [11]

Potassium 6-α-bromo[^{3}H]penicillanate (32 mg) in 5 ml of 20 mM sodium pyrophosphate (pH 9.2) was kept for 70 h at 27°C, and the solution then added to β-lactamase I (64 mg) in 7 ml 0.5 M NaCl, 1 mM EDTA, maintained at pH 7 in a pH-stat at 30°C. The enzyme, which was inactivated within 1 min, was isolated by gel-filtration [5]; yield 56 mg, spec. radioact. 24 μCi/μmol. Labelled enzyme (46 mg, 24 × 10^6 cpm) in 5 ml 1% (w/v) ammonium bicarbonate was treated with trypsin (10 mg) in 1 ml 1 mN HCl for 2.5 h at 37°C. The mixture was fractionated by paper chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.); the radioactive band (R_F 0.68) was further fractionated by paper electrophoresis at pH 1.8 (20% v/v in acetic acid, 2% v/v in formic acid) for 68 min at 60 V/cm. The radioactive band had mobility 0.58 relative to methyl green. The above values are molar ratios; the last column gives the values calculated for the tryptic peptide T7 [2]

tryptic peptide (35 nmol) in 0.4 ml 0.4% ammonium bicarbonate with 1.7 mg aminopeptidase M preparation for 2 h at 37°C. The products were fractionated by paper electrophoresis at pH 1.8 (42 min at 80 V/cm): the radioactive product had travelled 12 cm, whereas serine had travelled 24 cm. After hydrolysis of the radioactive product, the sole amino acid was serine (8 nmol, 30% yield, after allowance has been made for samples withdrawn). A paper blank, treated similarly, gave < 0.5 nmol serine.

These results showed that the labelled residue in the tryptic peptide is serine and hence, unless there is a migration of the label, for which there is no evidence, the residue in the enzyme that is labelled is serine-44. The parent tryptic peptide T7 [2] was basic; the peptide obtained in the present experiments was neutral (zero net charge on paper electrophoresis, at pH 6.5) when the tryptic digest was submitted directly to electrophoresis. After fractionation by paper chromatography and paper electrophoresis, however, the labelled peptide was basic. The structure of the moiety derived from the 6β-bromopenicillanic acid suggests, initially, a 2,3-dihydro-1,4-thiazine (see section 3.3); the nature of the subsequent change that leads to the tryptic peptide being basic is currently under investigation.

3.3. *Ultraviolet absorption of the labelled enzyme*

The ultraviolet absorption of the labelled enzyme shows a new maximum at 326 nm; this is lost after treatment with alkali, conditions under which the label is shed [5]. Thus the A_{326} is derived from the moiety introduced by reaction with 6β-bromopenicillanate. The absorption shifts (reversibly) to 314 nm when the protein is unfolded in 3 M guanidinium chloride. These observations are consistent with the suggestion, for the structure of the moiety derived from the 6β-bromopenicillanic acid, of a 2,3-dihydro-1,4-thiazine [12].

4. Discussion

The serine residue that is labelled in the experiments reported here is conserved in the amino acid sequences of all the other β-lactamases that are known [3]. This serine residue thus becomes an excellent candidate for an active site component, although further work is necessary to decide whether an acyl enzyme is an intermediate during hydrolysis of substrates.

We now consider whether the present findings may be generalised. 6β-Bromopenicillanic acid effectively inactivates β-lactamases from *Staphylococcus aureus*, and *Escherichia coli* (strain W3310), *Bacillus licheniformis*, and *Pseudomonas aeruginosa* as well as β-lactamase I from *B. cereus*, but the Zn^{2+}-requiring β-lactamase II from the latter organism is not inactivated [5,6] and the candidates for active-site groups considered for β-lactamase II (i.e., the Zn^{2+}-ligands) do not include serine [13].

The other enzymes that interact with penicillins (and related β-lactams) are those that catalyse peptide cross-linking in peptidoglycin synthesis during growth of bacterial cell walls [14]; here, too, serine features [15,16]. Whether there are further resemblances

remains to be found out. Similarly, if further work confirms that β-lactamases are serine enzymes, the possibility exists that there will be some features in common with other serine enzymes, such as proteinases, peptidases, esterases or phosphatases.

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