Biochimica et Biophysica Acta, 377 (1975) 431—443

BBA 67416

PURIFICATION AND PROPERTIES OF A CONSTITUTIVE β-LACTAMASE FROM *PSEUDOMONAS AERUGINOSA* STRAIN DALGLEISH

ANNA J. FURTH*

Sir William Dunn School of Pathology, University of Oxford, Oxford (U.K.)

(Received September 2nd, 1974)

Summary

1. The β-lactamase (penicillin amido-β-lactamhydrolase EC 3.5.2.6) appeared to be periplasmic rather than truly intracellular, since it was released by freeze-thawing without gross morphological changes in the cell.

2. The partially purified enzyme had p*I* between 5.0 and 5.5, mol. wt 32 000 and a broad pH vs activity profile with a maximum at pH 8.

3. The cephalosporins tested were hydrolysed less rapidly than most of the penicillins, and the K_m values for penicillins were lower than for cephalosporins. However cloxacillin was hydrolysed very slowly although it was strongly bound.

4. The substrate-induced inactivation common to many β-lactamases was particularly marked with cephaloridine and cloxacillin. The cloxacillin-induced inactivation was shown to be reversible.

Introduction

The β-lactamase enzymes (EC 3.5.2.6.) hydrolyse the β-lactam ring in penicillins and cephalosporins, and are a major factor in the bacterial resistance to these antibiotics. Infections with *Pseudomonas aeruginosa*, which is particularly resistant to antibiotic therapy, were successfully treated by carbenicillin [1], but the emergence of carbenicillin-resistant strains of this organism was soon reported [2]. Several of these strains were able to destroy carbenicillin, presumably by β-lactamase activity, and *Ps. aeruginosa* strain Dalgleish, which was clearly shown to produce a β-lactamase [3], is one such example. The β-lactamase concerned was characterised by its rapid hydrolysis of both carbenicillin and ampicillin, and was the subject of a preliminary investigation by Newsom et al. [4]. The present paper describes the isolation of milligram

* Present address: Biology Department, The Open University, Milton Keynes, U.K.

quantities of a more highly purified preparation of this enzyme, and a further study of its properties.

Methods

Materials

Benzylpenicillin was bought from Glaxo Laboratories, Greenford, U.K. Epibenzylpenicillin was a gift from Professor Vanderhaeghe of the Rega Institute, Leuven, Belgium, α-sulphobenzylpenicillin from Takeda Chemical Industries, Osaka, Japan and other penicillins from Beecham Research Laboratories, Brentford, U.K. Cephalosporin 87/312 [5], cephaloridine and cephalosporin C were gifts from Glaxo, and cephalothin and cephalexin from Eli Lilly and Co. Ltd, Basingstoke, U.K. Lysozyme, DNAase, ovalbumin, bovine serum albumin, and soybean trypsin inhibitor were bought from BDH Ltd, trypsin (DCC-treated) from Sigma, Carbowax PEG 20M from Union Carbide and yeast extract from Oxoid Ltd, London, U.K. Sodium tetrathionate was prepared by the method of Gilman et al. [6].

Organism

Ps. aeruginosa strain Dalgleish was a gift from Professor Richmond, Dept. of Bacteriology, University of Bristol. It was originally isolated from a patient with an oesophageal tumour [3].

Growth of cultures

The organism was grown in liquid CCY medium as prepared by Gladstone and van Heyningen [7], except that yeast diffusate was replaced by yeast extract (25%, w/v). The inoculum was grown in 5 × 100 ml 4-baffle shake flasks [8], each containing 100 ml of medium. A small drop of brain heart infusion agar on which the organism was maintained was introduced into each flask on the tip of a platinum loop. The flasks were shaken at 220 rev./min and 37°C for 8 h, and the contents used to inoculate 5 l of production culture. This was grown in a 7 l New Brunswick fermentation vessel, type FS 307. The culture was stirred at 740 rev./min with an air flow of 4 l per min at 37°C. Antifoam (silicon MS antifoam A from Hopkins and Williams) was added when necessary. After 4.5 h the absorbance was 26, when read on a Spekker absorptiometer with a neutral grey filter H 508. (For a similar organism *Ps. aeruginosa* N.C.T.C.8203, viable counts have shown that this corresponds to 10^{10} bacteria per ml.) The culture was harvested by centrifuging at 4000 × *g* for 45 min. Cells were stored frozen.

Sonication

Sonication was performed with an MSE 100 W sonicator in a 15-ml ice-cooled cup for 3 min. A 9-mm probe with amplitude setting at 4 μm was used.

Microscopy

Light microscopy was performed with a phase-contrast light microscope (Leitz orthoplan) at ×1250 magnification.

For electron microscopy the cells were fixed and embedded by the method described by Kellenberg et al. [9].

Freeze-thaw treatment of whole cells

Approx. 1 ml of packed cells which had been harvested at late log phase and stored frozen, were allowed to thaw and mixed with an equal volume of 0.2 M acetate buffer at pH 4.8, containing DNAase (10 μg/ml) and *p*-chloromercuribenzoate (10^{-3} M). (The mercuribenzoate was present to prevent proteolytic digestion of the β-lactamase, a phenomenon which is described under Results.) The sample was frozen and thawed a further three times, in an acetone/solid CO_2 bath and a 37° C water bath, respectively. A further 2 ml of buffer was added, and the preparation was stirred for 20 min at room temperature, and then centrifuged at 33 400 × *g* for 1 h. The supernatant was assayed for β-lactamase activity, and the pellet observed under the microscope.

Lysozyme/EDTA/trypsin treatment of whole cells

Freshly harvested cells were washed in 0.01 M Tris · HCl buffer at pH 8, and resuspended in 0.03 M Tris · HCl buffer at pH 8 containing 0.25 M sucrose. In this way approx. 15 ml of packed cells were resuspended in a total volume of 60 ml. Lysozyme/EDTA/trypsin were added to a final concentration of 0.01 mg/ml (lysozyme), 0.25 mg/ml (EDTA) and 0.5 mg/ml (trypsin). The cells were incubated at 25° C and samples removed after periods of 5 to 90 min. The cells were examined with a light microscope, while the bulk of the sample was centrifuged at 33 400 × *g* for 1 h. Any β-lactamase released was determined by assaying the supernatant.

Preparation of cell extract

200 ml of packed cells were worked up at a time, and the enzyme was extracted into 0.2 M acetate buffer, pH 4.8, containing 0.01 M sodium tetrathionate.

Frozen cells were allowed to thaw and then immediately mixed with an equal volume of extraction buffer in a 2-litre round-bottomed flask. DNAase (10 μg/ml) was added to reduce viscosity. The suspension was shell-frozen in acetone/solid CO_2 and then thawed in a 37° C water bath. After a total of three freeze-thawings, a further 200 ml of extraction buffer were added, and the suspension stirred at 4° C for 1 h. Cell debris was removed by centrifuging at 33 400 × *g* for 60 min.

The supernatant was dialysed exhaustively against 0.02 M acetate buffer, pH 4.8. A precipitate which formed during this period was removed by further centrifugation at 33 400 × *g* for 60 min.

Ion-exchange chromatography

DE-32 cellulose was obtained from Whatman Biochemicals Ltd; DE-50 powder which had been obtained from the same firm in 1961 is said to be equivalent to their present DE-1. Both resins were precycled and equilibrated with starting buffer according to the maker's instructions.

Chromatography was performed in a perspex column 9 cm × 22 cm closed at each end by a rubber bung. The resin bed was supported by a nylon net held in place by the lower bung. A flow rate of 336 ml/h (53 $ml \cdot cm^{-1} \cdot h^{-1}$) was achieved by a head of 100 cm. Fractions were collected at 2.5-min intervals.

Gel filtration on Sephadex G-75 or G-100

This was performed in 0.1 M acetate buffer, pH 5.7, containing 1 M NaCl, in a column 2.5 cm × 52 cm at a flow rate of 17 ml/h. A sample of 2.5 ml (4.5 mg of protein per ml) was made 5% in sucrose and layered over the resin surface under a head of buffer. 100 fractions were collected at 10-min intervals.

Gel filtration on Sephadex G-25

Separation of enzyme and cloxacillin was performed in 0.5 M sodium phosphate buffer at pH 7, in a column 0.9 cm × 5.7 cm of Sephadex G-25. Sample (0.3 ml) was applied to the exposed resin surface and washed under with 0.1 ml of buffer. Elution was at 66 ml/h using a gravity head of 12 cm, and collecting fractions at 0.5-min intervals. The void volume (2.7 ml) was known from a calibration run with dextran blue, and therefore Fractions 4 and 5 were retained as the protein-containing eluate.

Molecular weight

Molecular weight was determined by the method of Andrews [10] on Sephadex G-100 in 0.1 M acetate buffer, pH 5.7, containing 1 M NaCl. The same column, sample application and flow rate was used as above. Bovine serum albumin (67 000), ovalbumin (45 000), soybean trypsin inhibitor (21 500) and lysozyme (14 000) were chosen as standards.

Polyacrylamide gel electrophoresis

At pH 9.5 this was performed in 0.094 M Tris buffer containing 0.12% tetramethylene diamine (v/v); glycine was added to adjust the pH [11]. For runs at pH 5, the buffer was 0.35 M β-alanine containing 0.48% tetramethylene diamine (v/v); acetic acid was added to adjust the pH. Gels were 5% (w/v) in acrylamide, and were poured into tubes 0.5 cm × 10 cm and run at a constant current of 5 mA/gel for 50 min [11]. Protein was detected by staining in coomassie brilliant blue [11]. β-Lactamase activity was detected after eluting the enzyme from 5-mm gel slices, by incubating in 0.025 M citrate/phosphate buffer at pH 6.3 for 1 h at 37°C; 100-μl aliquots were then assayed against cephalosporin 87/312.

Isoelectric focusing

Isoelectric focusing was performed in an LKB Multiphor, according to the makers' instructions. The water-cooled apparatus was maintained at 10°C, and Ampholines of p*I* range 3.5—9.5 in 5% (w/v) acrylamide gel were used. A voltage of 50 V/cm was maintained for 4.5 h (current approx. 5 mA), following a prefocusing run of 2 h. Protein and β-lactamase activity were detected as for polyacrylamide gel electrophoresis.

Enzyme assays

1. Routine assays were performed with benzylpenicillin as substrate, by the pH-stat alkalimetric titration method. The apparatus consisted of a pH meter type 26, titrator type 11, autoburette type ABU 13 with 0.25-ml burette assembly, and titrigraph type SBR 2c, all from Radiometer, Copenhagen. Assays were performed at 30°C and pH 7, by addition of 40 mM NaOH to 2 ml of substrate (1 mg/ml of benzylpenicillin).

Units were expressed as μmol hydrolysed/min per ml of enzyme, and it was assumed that one equivalent of acid was liberated per mol of penicillin hydrolysed, and 2 equivalents per mol of cephalosporin [12]. This method was also used to determine relative V values with a variety of cephalosporin and penicillin substrates.

2. K_m values were determined from assays by the microiodometric method of Novick [13].

3. Activity with cephalosporin 87/312 was determined colorimetrically at 30° C in 0.05 M triethanolamine · HCl buffer at pH 7 by the method of O'Callaghan et al. [5].

Protein concentration

Protein concentration was determined by a modified Biuret method [14], with bovine serum albumin as standard. Column eluates were monitored automatically by measurement of the absorbance at 280 nm, using an LKB Uvicord II detector and recorder type 6520. Absolute values were obtained by comparing the absorbance of peak fractions with that obtained in a Unicam SP800 spectrophotometer.

Chloride concentration

Cl^- concentration was determined from conductivity measurements, using a Radiometer conductivity meter type CDM 2c. A standard curve was prepared by measuring conductivities of solutions containing known amounts of NaCl dissolved in 0.025 M triethanolamine · HCl buffer.

Nucleic acid

Nucleic acid was determined from the ratio of absorbances at 260 and 280 nm [15].

Results

Cellular location

No activity could be detected in the medium on harvesting. The cell-bound enzyme was fully released by freeze-thawing, a treatment which left the cells largely intact, and suggested that the enzyme was periplasmic rather than truly intracellular. In preliminary experiments treatment was carried out on a concentrated cell suspension prepared from 1 ml of packed cells. Details are given in Methods, under "Freeze-thaw treatment of whole cells". There was 96% release of the enzyme, as compared to the total that could be liberated by sonication, and the cells were still mobile and rod-shaped when viewed under the light microscope. This treatment apparently initiated further changes, as could be seen when the freeze-thawed cells were diluted into isotonic sucrose at pH 5, and incubated at 25° C. After 4 h they began to change shape, and after 17 h all the cells were spherical rather than rod-shaped. They were also osmotically sensitive, and lysed when transferred to 0.1 M sucrose at pH 5. An electron micrograph of the cells before transfer showed vesicles budding off from the wall, and in some cells there was considerable cytoplasmic damage.

Treatment of freshly harvested cells with lysozyme/EDTA or with

lysozyme/EDTA/trypsin (see Methods) released only 6.5% of the enzyme, and the cells remained rod-shaped even after several hours incubation.

Preparation of cell extract

Since the purification procedure could be considerably simplified by starting from a periplasmic rather than a whole cell extract, the enzyme was released by freeze-thawing. 200 ml of packed cells were worked up at a time, as described under Methods.

Preliminary experiments suggested that the periplasmic extract contained a native protease capable of partially digesting the β-lactamase to a less active form. This proteolysis was investigated more fully on the partially purified enzyme, where it was found to be inhibited by sulphydryl reagents (see below). For large scale preparations as here, sodium tetrathionate was added with the extraction buffer. This reagent converts protein thiols to sulphenyl thiosulphate groups [16], and was found to give sufficient protection from proteolysis when added to extraction buffer only. It could be omitted from buffers used in later steps of the purification.

After freeze thawing, the cells were removed by centrifugation at 33 000 × *g* and the supernatant containing the enzyme was dialysed exhaustively against 0.02 M acetate buffer at pH 4.8. A bulky white precipitate which formed during dialysis was removed by further centrifugation.

Purification

The procedure is summarised in Table I. There were difficulties in purifying the enzyme by either gel filtration or ion-exchange chromatography which appeared to be caused by non-ionic interaction between enzyme and resin backbone. This was particularly noticeable where the enzyme was firmly adsorbed to ion-exchange resin under starting buffer conditions. It proved difficult to get sharp desorption, even when eluting under conditions where the enzyme would not have been adsorbed initially. Pretreatment of the cell extract by acetone or $(NH_4)_2SO_4$ did not improve the chromatographic behaviour of the enzyme.

The high recoveries seen in Step 2 of Table I were achieved by avoiding conditions where the enzyme was adsorbed. For this step the starting material was the periplasmic extract which had been dialysed against 0.02 M acetate

TABLE I

PURIFICATION OF β-LACTAMASE FROM *PS. AERUGINOSA* STRAIN DALGLEISH

Cell extract was prepared from 200 ml of packed frozen cells. In Step 4, only 20% of the eluate from the previous step was worked up at a time, and therefore the activity resulting from Step 4 has been recalculated to allow comparison with the rest of the preparation. Enzyme activity was determined as μmol of benzylpenicillin hydrolysed/min; mg of protein was determined by the Biuret method.

Procedure	Total activity (units × 10^{-3})	Yield (percent)	Specific activity (units/mg)
1. Cell extract	6.4	100	2.9
2. DE-1 eluate	6.4	100	5.8
3. DE-32 eluate	4.1	64	96
4. G-100 eluate	3.9	61	264

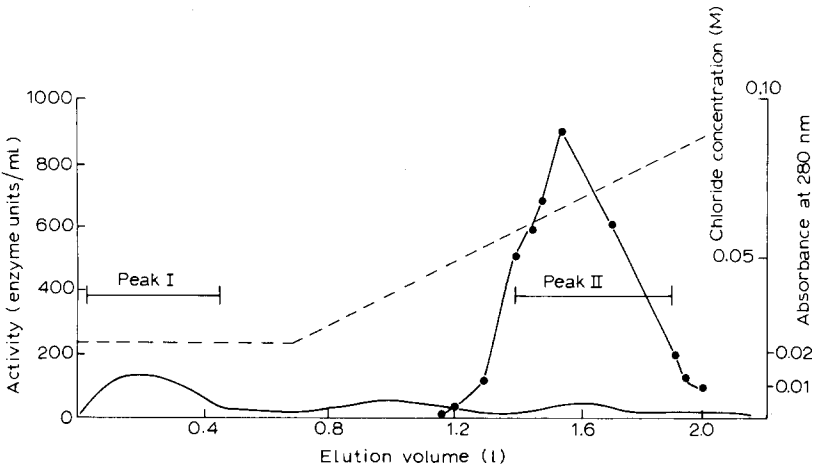

Fig. 1. Gradient elution of β-lactamase on DE-32 cellulose. Enzyme activity against benzylpenicillin (●———●) was determined by titrimetric assay, protein concentration (———) by absorbance at 280 nm, and Cl^- concentration (------) from conductivity measurements. Bars represent the fractions pooled. The enzyme solution (520 mg of protein in 1.2 litres of 0.025 M triethanolamine · HCl buffer at pH 6.7) was applied to a 9 cm × 22 cm column. Details of ion-exchange chromatography are given in Methods. The salt gradient was produced from 1 litre of starting buffer and 1 litre of starting buffer containing 0.1 M NaCl.

buffer at pH 4.8. It contained approx. 5 g of protein in 620 ml, and was passed rapidly through the column of DE-1 cellulose equilibrated in the same buffer. There was complete adsorption of nucleic acid and 100% recovery of enzyme activity in the unadsorbed protein fraction.

This unadsorbed fraction, which contained 520 mg of total protein, was dialysed against 0.025 M triethanolamine · HCl buffer at pH 6.7 before gradient chromatography on DE-32 cellulose (Step 3). The elution diagram is shown in Fig. 1. Approx. 430 mg of unadsorbed material were removed by starting buffer elution (Peak I), leaving the enzyme firmly bound to the resin. Application of a salt gradient gave a rather broad enzyme-active peak containing 60 mg of protein in 550 ml, and representing 64% of the applied activity. There was a 6.5-fold increase in specific activity over that of the starting material.

The bulk of the enzyme sample was stored at this stage as a precipitate in $(NH_4)_2SO_4$. Concentration followed by precipitation was achieved by placing the sample in a dialysis sac in solid $(NH_4)_2SO_4$. The buffering by the 0.025 M triethanolamine · HCl present was sufficient to maintain the pH below 7, and there was no loss of activity over several months.

In Step 3, a 10-mg portion of the DE-32 eluate was dialysed against 0.1 M acetate buffer (pH 5.7) containing 1 M NaCl, concentrated on Carbowax and applied to a column of Sephadex G-100 as described under Methods. The elution diagram is shown in Fig. 2. There was 95% recovery of activity and a 4-fold increase in specific activity. Approx. one-half of the inactive material was eluted in the void volume as Peak I, and the enzyme emerged sharply in an elution volume of 160 ml. (In contrast to this behaviour, chromatography on Sephadex G-75 gave a diffuse, flat peak of activity. The non-ionic interaction between resin and enzyme mentioned earlier may be a factor in this otherwise

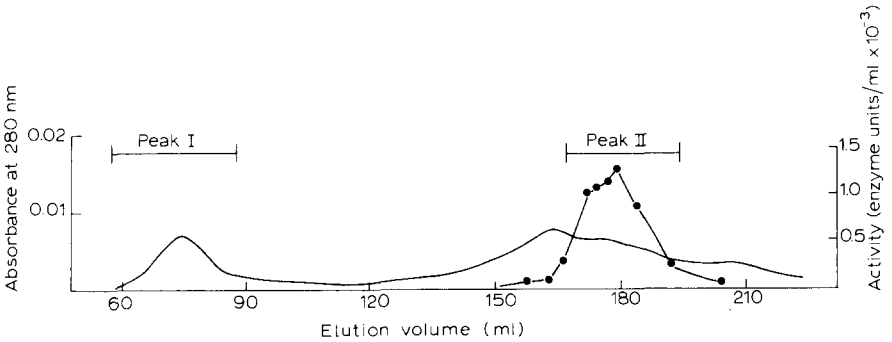

Fig. 2. Gel filtration of β-lactamase on Sephadex G-100. Enzyme activity against benzylpenicillin (●———●) was determined by titrimetric assay and protein concentration (———) by the absorbance at 280 nm. Details of gel filtration are given under Methods. Bars represent the fractions pooled.

unexplained behaviour. Similar anomalies have been reported on changing from Bio-Gel A 5M to the more highly cross-linked A 0.5M [17].)

The enzyme-active fractions from Sephadex G-100 were pooled, concentrated and stored by treatment with solid $(NH_4)_2SO_4$ as before.

Homogeneity

The above procedure resulted in a 100-fold purification from the periplasmic extract. Polyacrylamide gel electrophoresis at pH 9.5 showed one major band and four minor bands. The major band, which had a mobility one-third that of bromophenol blue, was the only enzyme-active component.

This partially purified preparation was used in all further studies on the enzyme.

Molecular properties of the enzyme

1. Proteolytic digestion. The periplasmic extract appeared to contain a native protease capable of partially digesting the β-lactamase to a less active form. There was over 50% loss of β-lactamase activity when the partially purified preparation was allowed to stand at pH 8.4 for 4 days (in 0.05 M triethanolamine · HCl buffer at 4°C). This inactivation was accompanied by a characteristic change in polyacrylamide gel electrophoresis pattern. Superimposed on the original pattern of one major and four minor bands were four very diffuse, fast-moving and intensely-staining components. In the presence of 10^{-3} M *p*-chloromercuribenzoate, but not of 10^{-3} M EDTA, no such new bands were seen. This suggests that sulphydryl reagents but not EDTA can inhibit the proteolytic digestion.

Trypsin caused complete inactivation of the partially purified β-lactamase during 1.5 h at 25°C and pH 6. The two proteins, each at 1 mg/ml concentration, were incubated together in 0.05 M imidazole buffer containing 0.25 M $CaCl_2$. The digestion products were apparently too small to be detected on polyacrylamide gel electrophoresis.

2. Molecular weight. This was determined from chromatography on Sephadex G-100 at pH 5.7 to be 32 000. Details of marker proteins are given in the experimental section.

3. Isoelectric point. The partially purified enzyme gave three bands on isoelectric focusing, corresponding to p*I* 5.0, 5.1 and 5.5. The two most acidic bands were the most intense. A low p*I* value is consistent with the ion-exchange behaviour of the enzyme.

Enzymic properties of the enzyme

1. Specific activity. The partially purified enzyme had a specific activity towards benzylpenicillin of 264 μmol hydrolysed/min per mg at 30° C. Taking a molecular weight of 32 000 this would be equivalent to a "molecular activity" of 8.5 μmol/min per μmol were the enzyme homogeneous. This is towards the lower end of the range of molecular activities given by Citri [18] for a series of purified β-lactamases.

2. Effect of pH on enzyme activity. With benzylpenicillin as substrate a broad, flat pH vs activity curve was obtained, with a maximum at pH 8 (see Fig. 3).

3. Substrate specificity. As shown in Table II, the enzyme tended to hydrolyse cephalosporins much less rapidly than penicillins, although both cephaloridine and cephalosporin 87/312 were exceptions to this. The enzyme also had a rather lower affinity for cephalosporins, as seen by the K_m values. The three penicillins oxacillin, cloxacillin and dicloxacillin were hydrolysed only very slowly, although the K_i for cloxacillin showed that it at least was strongly bound. Epibenzylpenicillin, in which the stereochemical configuration at the C-6 carbon is inverted, underwent no detectable hydrolysis.

4. Effect of non-lactam compounds on enzyme activity. There was no inhibition of benzylpenicillin hydrolysis in the presence of 10^{-3} M EDTA or 10^{-3} M *p*-chloromercuribenzoate.

Substrate-induced inactivation

Non-linear progress curves were observed during hydrolysis of several cephalosporins and penicillins, particularly cloxacillin and cephaloridine. With 10^{-3} M cloxacillin the enzyme activity was reduced to 45% of its v_o value after 10 min and to 26% after 15 min. This was not due to substrate depletion, since addition of fresh enzyme to an assay mixture containing fully inactivated enzyme gave an immediate rise in the hydrolysis rate, back to the original v_o

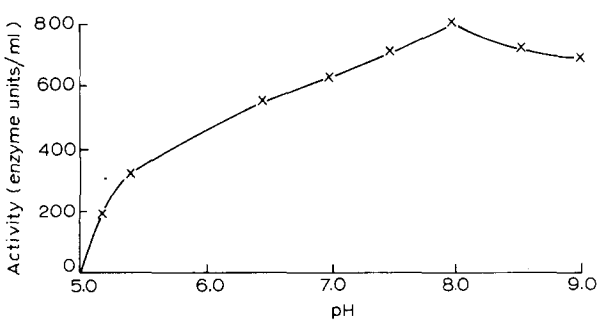

Fig. 3. Effect of pH on activity of β-lactamase towards benzylpenicillin. To avoid possible interference by specific buffer ions, the substrate was dissolved in water and activity was determined by titrating to a range of end-points between pH 5 and pH 9.

TABLE II

SUBSTRATE SPECIFICITY OF β-LACTAMASE FROM *PS. AERUGINOSA* STRAIN DALGLEISH

Cephalosporin 87/312 was assayed by spectrophotometric assay. For other substrates, V values were determined by titrimetric assay at a substrate concentration of 1 mg/ml, and K_m values were determined from assays by the iodometric method. The Eadie-Hofstee plot [41] was used for calculations.

Substrate	Relative V	K_m (M)
Benzylpenicillin	100	$1 \cdot 10^{-6}$
Carbenicillin	150	$2 \cdot 10^{-6}$
α-Sulphobenzylpenicillin	56	—
Ampicillin	88	—
Methicillin	16	—
Phenoxymethylpenicillin	80	$3 \cdot 10^{-6}$
Phenoxyethylpenicillin	46	—
Phenoxypropylpenicillin	39	—
Oxacillin	8.3	—
Cloxacillin	0.4	$5 \cdot 10^{-5}$
Dicloxacillin	0.3	—
Penicillin N	45	—
6-Epibenzylpenicillin	< 2	—
Cephalosporin C	2	—
Cephalexin	8	$4 \cdot 10^{-4}$
Cephaloridine	40	—
Cephalothin	4	—
Cephalosporin 87/312	31	$3 \cdot 10^{-4}$

value. When the concentration of substrate relative to enzyme was further increased by using 10^{-2} M cloxacillin, the inactivation was even more rapid, and the hydrolysis rate fell to an undetectable level after only 14 min. Since the K_m value is 10^{-5} M the active site should be fully saturated at concentrations well below 10^{-3} M. Probably reaction also takes place at areas other than the active site, at the higher concentrations of cloxacillin.

Binding of benzylpenicillin at the active site appeared to stabilise the enzyme against cloxacillin-induced inactivation. This substrate had a K_m of 10^{-6} M and was hydrolysed much faster than cloxacillin. With 10^{-3} M benzylpenicillin, progress curves were linear for at least 30 min under the standard assay conditions. Addition of 10^{-3} M cloxacillin to such a reaction mixture caused no detectable inhibition. This is in sharp contrast to the inhibition of cephaloridine hydrolysis noted by Newsom et al. [4]. The explanation probably lies in the relative binding affinities for the different substrates. The K_m for cephaloridine could not be precisely determined because of the non-linear progress curves mentioned earlier, but the enzyme was by no means saturated even at $5 \cdot 10^{-5}$ M cephaloridine. This suggests that both cloxacillin and cephaloridine are bound much less tightly then benzylpenicillin.

Benzylpenicillin was able not only to protect the enzyme from inactivation by cloxacillin, but also to cause some degree of reactivation when added to enzyme pretreated with cloxacillin. An enzyme sample was incubated with 10^{-2} M cloxacillin for 3 min at room temperature. The activity towards 10^{-3} M benzylpenicillin was then determined by titrimetric assay under the standard conditions. The volume of enzyme/cloxacillin solution taken for assay was such that the final concentration of cloxacillin was $1.25 \cdot 10^{-3}$ M, i.e. still sufficient

to cause considerable inactivation on its own. The initial activity towards benzylpenicillin was only 25% of that before cloxacillin treatment, but the progress curve was markedly concave upwards, and after 4 min the activity had returned to 30% of its original value.

Complete reactivation of this same sample could be more effectively achieved by rapid chromatography of the enzyme/cloxacillin mixture. Chromatography was performed on Sephadex G-25 in 0.5 M sodium phosphate buffer at pH 7, as detailed under Methods. The protein-containing eluate was then incubated at 37°C for 20 min, as in the method of Sagai and Saito [19]. When reassayed against benzylpenicillin the enzyme was found to have fully recovered its original activity.

Discussion

Release of the β-lactamase from *Ps. aeruginosa* strain Dalgleish apparently took place very rapidly after freeze-thaw damage, and long before any change in cell shape could be seen. This suggests that the enzyme is located near the surface where, as Richmond and Sykes [20] have pointed out, it would be of great advantage when considering antibiotic resistance in the bacterial cell as a whole. There are examples of both periplasmic and cytoplasmic β-lactamases amongst Gram-negative bacteria, but the generalisation [21] that periplasmic enzymes are chromosomally mediated has now been discounted [22]. There is no evidence that the β-lactamase from *Ps. aeruginosa* Dalgleish is R-factor mediated, yet the observations made here clearly show that it is periplasmic.

Freeze-thawing is a comparatively simple method for cell opening and has the advantage of liberating only the periplasmic rather than whole cell contents. This treatment was also sufficient to initiate spheroplast formation without the usual addition of hydrolytic enzymes. The exact nature of freeze-thaw damage to bacterial cell walls is not clear, but it is possible that here it causes susceptible regions to become accessible to an autolysin in the cell wall. It has been reported that an autolysin from *Ps. aeruginosa* is released into the medium from ageing cells [23], and Cheng et al. [24] have suggested that lysozyme added externally to strain ATCC 9027 gains access to susceptible peptidoglycan areas only after damage to the "molecular sieve layer".

Protoplast formation in non-halophilic strains of *Ps. aeruginosa* has very seldom been reported [24,25], and it is by now well established the cell walls of pseudomonads differ significantly from those of other Gram-negative bacteria such as *Escherichia coli*, and that lysozyme/EDTA treatment on its own is insufficient for spheroplast formation. The present work has shown that there are apparently also differences between strains of *Ps. aeruginosa*. Whereas the cells from strain L24 [25] gave spheroplasts after only 10 min treatment in lysozyme/EDTA/trypsin, those from strain Dalgleish studied here remained rod-shaped even after several hours. (pH was 8, not 5, as in freeze-thawing.)

The protease responsible for partial digestion of the β-lactamase was not further investigated, but it would appear to be distinct from the *p*-chloromercuribenzoate-insensitive, EDTA-sensitive proteases isolated from other strains of *Ps. aeruginosa* [26,27].

Ps. aeruginosa produces a number of different β-lactamases, one of which

is inducible. This enzyme has been extensively studied [28—31], and can be induced in a wide variety of *Ps. aeruginosa* strains, including the Dalgleish strain used here [32]. It is primarily a cephalosporinase, and quite distinct from the constitutive enzyme studied here. The substrate specificity of the constitutive β-lactamase from *Ps. aeruginosa* strain Dalgleish was first reported by Newsom et al. [4] and has been extended in the present study to confirm that most of the penicillins tested are hydrolysed faster than the cephalosporins. It has now also been shown that the enzyme tends to have a greater affinity for penicillins than for cephalosporins. The high activity towards cephaloridine is an exception, which is also found with some other β-lactamases [33]. Somewhat surprisingly, the Gram-positive *Staphylococcus aureus* [34—36] produces a β-lactamase similar in some respects to the Dalgleish enzyme. Both enzyme hydrolyse cephalosporin C much more slowly than penicillins, except for cloxacillin and methicillin which are poor substrates for both. Carbenicillin is also hydrolysed rapidly by the *S. aureus* enzyme, but compared with benzylpenicillin there is a marked rise in K_m to 10^{-3} M, said to be associated with the introduction of polar groups into the side-chain [36]. No such effect is seen with the Dalgleish enzyme, and there are obviously significant differences at the active sites. This difference is emphasised by both the shape and optima of the pH vs activity curves; the Dalgleish enzyme shows a broad peak at pH 8, and the *S. aureus* enzyme a sharp peak at pH 6.8.

Non-linear progress curves have frequently been reported for β-lactamases, following the first observation by Crompton et al. [34]. The question of substrate-induced conformational changes has been discussed in reviews by Citri [18,37] and by Abraham [38]. It has been proposed that enzyme inactivation is initiated by a substrate-induced conformational change, which may then be followed by irreversible covalent changes. A conformational change should be reversible, but in the few cases where this has been reported, conditions for reactivation seem to be very critical. Dyke [39] demonstrated reactivation of *S. aureus* β-lactamase if the cloxacillin-induced inactivation was not allowed to proceed beyond 15%. Zyk and Citri [40] in a preliminary report imply that divalent cations may be important. Sagai and Saito [19] give details of a procedure for 100% reactivation, following 86% dicloxacillin-induced inactivation of *S. aureus* enzyme. By applying their procedure to the *Ps. aeruginosa* Dalgleish enzyme it was possible to demonstrate full recovery of activity after 75% inactivation by cloxacillin. Therefore, with both these enzymes substantial inactivation can be reversed. This confirms that the initial substrate-induced change which produces inactivation is a reversible one. Although there are many reports of substrate-induced inactivation of β-lactamases, the reversibility has seldom been demonstrated. This may be partly because subsequent irreversible changes take place rather rapidly, and partly because substrate binding induces a substantial conformational change verging on partial denaturation. Refolding from this state may require precisely defined conditions in order to take place at an observable rate.

Acknowledgements

I am very grateful to Professor E.P. Abraham for his help and encourage-

ment. I would like to thank Mr T.J. Beesley for kindly performing the fermentor runs, the M.R.C. for financial support, Dr N.G. Heatley for kindly performing the isoelectric focusing, and Dr D. Kay for kindly performing the electron microscopy.

References

1 Acred, P., Brown, D.M., Knudsen, E.T., Rolinson, G.N. and Sutherland, R. (1967) Nature 215, 25—30
2 Lowbury, E.J.L., Lilly, H.A., Kidson, A., Ayliffe, G.A.J. and Jones, R.J. (1969) Lancet 2, 448—452
3 Newsom, S.W.B. (1969) Lancet 2, 1141
4 Newsom, S.W.B., Sykes, R.B. and Richmond, M.H. (1970) J. Bacteriol. 101, 1079—1080
5 O'Callaghan, C.H., Morris, A., Kirby, S.M. and Shingler, A.H. (1972) Antimicrobiol. Agents Chemother. 1, 283—288
6 Gilman, A., Philips, F.S., Koelle, E.S., Allen, R.P. and St.John, E. (1946) Am. J. Physiol. 147, 115—126
7 Gladstone, G.P. and van Heyningen, W.E. (1957) Br. J. Exp. Pathol. 38, 123—237
8 Smith, B., Warren, S.G., Newton, G.G.F. and Abraham, E.P. (1967) Biochem. J. 103, 877—890
9 Kellenberger, E., Ryter, A. and Seychaud, J. (1958) J. Biophys. Biochem. Cytol. 4, 671—678
10 Andrews, P. (1964) Biochem. J. 91, 222—233
11 Smith, I. (1968) in Chromatographic and Electrophoretic Techniques (Smith, I., ed.), Vol. 2, Edn 2, pp. 365—418, Heinemann, London
12 Sabath, L.D., Jago, M. and Abraham, E.P. (1965) Biochem. J. 96, 739—752
13 Novick, R.P. (1962) Biochem. J. 83, 236—240
14 Goa, J. (1953) Scand. J. Clin. Lab. Invest. 5, 218—222
15 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384—421
16 Pihl, A. and Lange, R. (1962) J. Biol. Chem. 237, 1356—1362
17 Sargent, M.G. and Lampen, J.O. (1970) Arch. Biochem. Biophys. 136, 167—177
18 Citri, N. (1971) in The Enzymes (Boyer, P., ed.), Vol. 4, edn 3, pp. 23—46, Academic Press, New York
19 Sagai, H. and Saito, T. (1973) J. Antibiot. 26, 315—319
20 Richmond, M.H. and Sykes, R.B. (1973) Adv. Microbiol. Physiol. 9, 31—88
21 Neu, H.C. (1968) Biochem. Biophys. Res. Commun. 32, 258—263
22 Curtis, N.A.C., Richmond, M.H. and Stanisich, R.V. (1973) J. Gen. Microbiol. 79, 163—166
23 Collins, F.M. (1964) J. Gen. Microbiol. 34, 379—388
24 Cheng, K.-J., Ingram, J.M. and Costerton, J.W. (1970) Can. J. Microbiol., 16, 1319—1324
25 Weiss, R.L. and Fraser, D. (1973) J. Bacteriol. 113, 963—968
26 Morihara, K. (1964) J. Bacteriol 88, 745—757
27 Matsubara, H. and Feder, J. (1971) in The Enzymes (Boyer, P., ed.), Vol. 3, edn 3, pp. 721—795, Academic Press, New York
28 Sabath, L.D., Jago, M. and Abraham, E.P. (1965) Biochem. J. 96, 739—752
29 Garber, N. and Friedman, J. (1970) J. Gen. Microbiol. 64, 343—352
30 Zyk, N., Kalkstein, A. and Citri, N. (1972) Isr. J. Med. Sci. 8, 1906—1911
31 McPhail, M. and Furth, A.J. (1973) Biochem. Trans. Soc. 1, 1260—1263
32 Sykes, R.B. and Richmond, M.H. (1971) Lancet 2, 342—344
33 Datta, N. and Richmond, M.H. (1965) Biochem. J. 98, 204—209
34 Crompton, B., Jago, M., Crawford, K., Newton, G.G.F. and Abraham, E.P. (1962) Biochem. J. 83, 52—63
35 Richmond, M.H. (1965) Biochem. J. 94, 584—593
36 Hou, J.P. and Poole, J.W. (1973) J. Pharmacol. Sci. 62, 783—788
37 Citri, N. (1973) Adv. Enzymol. 37, 397—648
38 Abraham, E.P. (1974) in Biosynthesis and Enzymatic Hydrolysis of Penicillins and Cephalosporins, University of Tokyo Press, p. 69
39 Dyke, K.G.H. (1967) Biochem. J. 103, 641—646
40 Zyk, N. and Citri, N. (1973) Ninth Int. Congr. Biochem., Stockholm, abstracts p. 122
41 Eadie, G.S. (1942) J. Biol. Chem. 146, 85