A new method to study permeation of β-lactam antibiotics into reconstituted vesicles from the outer membrane of *Pseudomonas aeruginosa* NCTC 10662

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Received 26 July 1982

(P. aeruginosa) *Outer membrane* *F porin* *Lactam, β,permeation* *Vesicle resealing* *Lactamase, β*

1. INTRODUCTION

An impairment of antibiotic permeation through the outer membrane of Gram-negative bacteria is considered to be one of the main causes of bacterial resistance to β-lactam antibiotics [1]. It is particularly marked in *Pseudomonas aeruginosa*. Kinetic data on the permeation of β-lactams have been mathematically analysed in [2]. These authors took advantage of an inducible β-lactamase located within the periplasmic space of *Escherichia coli* and measured the degradation of β-lactams after permeation of the antibiotic through the outer membrane. Other workers have studied the kinetics of permeation of solutes using model membrane vesicles made either from outer membranes or from some of their constituents [3,4]. Methods for studying permeation have included the use of radiolabelled solutes [3] and light-scattering measurements based on the change in volume of the vesicles upon permeation of the solute [5]. Here, we describe a new, simple spectrophotometric method for studying β-lactam permeation into vesicles containing an enveloped β-lactamase. These vesicles were made from the outer membrane fraction of *P. aeruginosa* and from purified F porin. A new method for isolation of homogeneous F porin is also reported.

2. MATERIALS AND METHODS

2.1. *Preparation of membrane fractions*

Pseudomonas aeruginosa NCTC 10662 (wild-type) was grown in LB medium [6] supplemented with 0.4% glucose at 37°C under forced aeration. Bacteria were collected when the density of the culture reached 5×10^8 cells/ml. The procedures for cell disruption and isolation of a whole membrane fraction have been described [7]. Outer membranes were isolated from lysozyme-digested whole membranes as in [8].

2.2. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)*

Experiments were performed according to [9]. The starting concentration of acrylamide was 11%.

2.3. *Purification of the F porin*

Whole membranes were prepared as in [7]. They were resuspended in 10 mM Tris–HCl buffer (pH 7.3) containing 2% (w/v) SDS, 10% (v/v) glycerol and 0.7 M 2-mercaptoethanol [10] (3 mg membrane protein/ml). The suspension was incubated at 30°C for 30 min and then centrifuged at 30 000 × *g*, at 20°C for 60 min. The precipitate was collected and resuspended in $\frac{1}{10}$th the original volume of the same buffer supplemented with 0.5 M NaCl. Incubation of the membrane suspension and centrifugation were performed as before. The final supernatant was collected and subjected to preparative SDS–PAGE, carried out as above [9] using slab gels either 2 or 4 mm thick. At the end of the electrophoresis run, thin vertical strips were cut off from the gel, stained with Coomassie blue and destained to identify the position of F porin in the gel. Then a horizontal strip containing

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

the F porin was cut off. This strip was subjected to slab gel electrophoresis in a 4 phase system which consisted of the following layers from top to bottom:

(i) The strip of the first gel containing the F porin;
(ii) A 2 cm layer of the stacking gel described in [9];
(iii) A 3 cm layer of a gel containing 0.5% (w/v) agarose (electrophoretic grade) in 375 mM Tris–HCl buffer (pH 8.8) with 0.2% (w/v) SDS;
(iv) A longer layer of a supporting gel as the separation gel of [9].

The electrode buffer was as in [9] and before running the electrophoresis a small amount of bromophenol blue was diluted into the electrode buffer of the upper chamber to serve as a tracking dye. Electrophoresis was performed at room temperature and at 50 V. When the tracking dye had moved 2 cm in the agarose phase, electrophoresis was stopped and the agarose layer was removed and ground in a mortar with 0.2% SDS added. The resultant thick suspension was centrifuged at 180 000 × *g* for 3 h at 20°C and the supernatant collected. It was extensively dialyzed against water at 4°C for 5 days. Insolubilized F porin was collected by centrifugation at high speed (150 000 × *g*, for 3 h at 4°C) and resuspended in a small amount of water.

2.4. *Preparation of vesicles*

Vesicles were formed from isolated outer membranes following closely the technique in [8] which is itself a modification from [3]. The only modification that we introduced was to add *Bacillus cereus* β-lactamase at a final concentration 10-times higher (w/w) than that of the outer membrane protein. The same procedure was followed to prepare vesicles from purified F porin. About 0.8–1% of the added β-lactamase became entrapped within the vesicles.

2.5. *Other methods*

Lipopolysaccharide was extracted from membranes of *P. aeruginosa* NCTC 10662 by the phenol–water technique [11] and estimated according to [8].

Total lipids were extracted following the chloroform–methanol method in [12]. Total phospholipids were quantitated by measuring P_i after acid hydrolysis according to [13].

Protein concentrations were determined by the Folin phenol reagent method using bovine serum albumin as standard.

2.6. *Materials*

Nitrocefin was a generous gift from Glaxo Lab. (Greenford, Middlesex); cefamandole was obtained from Lilly Research Centre (Windlesham, Surrey), *Bacillus cereus* β-lactamase (EC 3.5.2.6) and triacylalycerol lipase (EC 3.1.1.3) were from Sigma Chemicals (St Louis MO). [^{3}H]Dextran was purchased from Amersham International (Amersham).

3. RESULTS AND DISCUSSION

F porin from our *P. aeruginosa* strain was purified to homogeneity as judged by its migration as a single band on electrophoresis. It was heat-sensitive, like the porin isolated from *P. aeruginosa* PA01 [14]. Our F porin is, at least partially, associated with peptidoglycan when extracted with high salt concentrations. The purified protein still retained a substantial amount of lipopolysaccharide that has not yet been accurately characterised and determined.

Hydrolysis of nitrocefin by the action of β-lactamase was followed spectrophotometrically by measuring the appearance of its degradation product(s) at 490 nm. The product(s) had $E_{1\,cm}^{275}$ of 15 000 [15]. Hydrolysis of cefamandole was followed at 275 nm; an $E_{1\,cm}^{275}$ of 6800 was calculated. Kinetic studies on β-lactamase-mediated hydrolysis of both β-lactams were carried out first using non-encapsulated β-lactamases. Analysis of the data revealed that enzymic hydrolysis followed typical Michaelis–Menten-type kinetics. Double reciprocal plots (not shown) yielded straight lines and a K_m of 20 μM for nitrocefin and 7 μM for cefamandole was obtained.

For permeation studies the same batch of freshly made vesicles was used, although data were found to be reproducible from batch to batch. We carried out the study using a broad range of β-lactam concentrations (0.5–100 μM). Practically maximal rates were achieved above 25 μM for cefamandole and 50 μM for nitrocefin.

Fig.1 shows a double reciprocal plot of nitro-

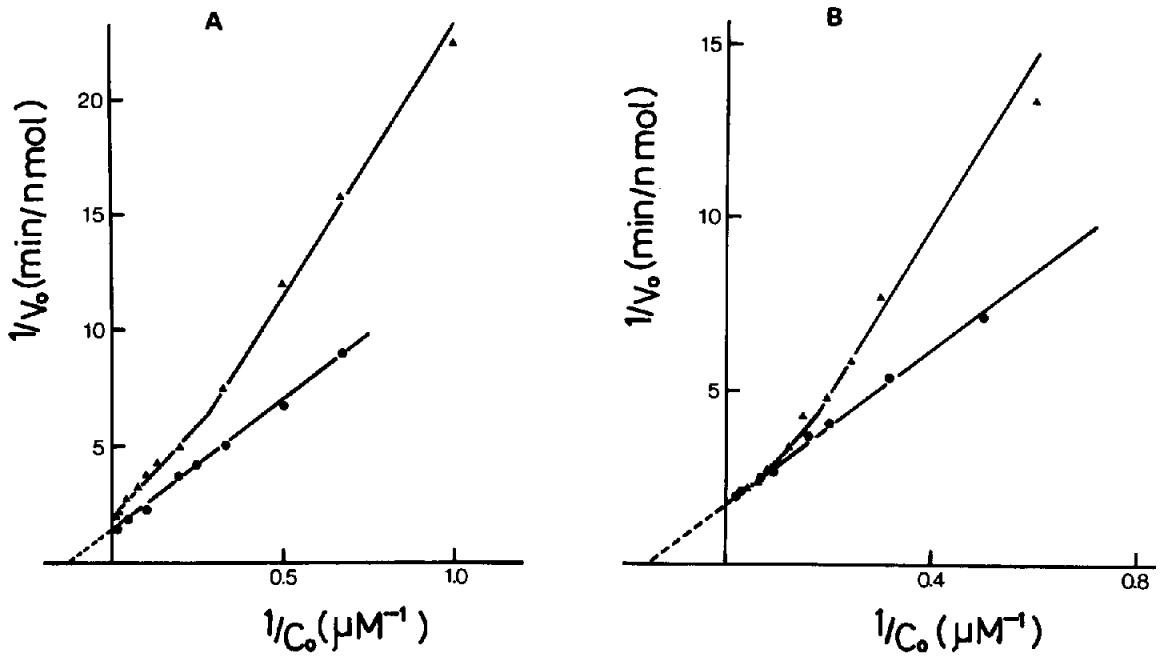

Fig.1. Double reciprocal plots of hydrolysis of: (A) nitrocefin; (B) cefamandole by *B.cereus* β-lactamase; (▲) β-lactamase encapsulated inside vesicles made from pure F porin; (●) β-lactamase released after disruption of the vesicles. Assays were performed by measuring the rate of β-lactam hydrolysis in a Perkin-Elmer 554 double beam spectrophotometer at 22°C. See text for details.

cefin and cefamandole hydrolysis caused by encapsulated β-lactamase inside F porin-loaded vesicles. This type of kinetics is characteristic of a process where the permeation of the enzyme substrate is, below certain concentrations, the rate limiting step of the overall process.

As discussed in [2,16], low concentrations of permeating substrate yielded lower rates than expected when free enzyme was used, while high concentrations of solute forced the permeation barrier so that maximum rates became equal for both the encapsulated and free enzyme. It is therefore necessary to use broad ranges of permeant concentration to study the effect of permeation and not to one single concentration of the substrate [16]. Michaelis–Menten-type kinetics was restored when β-lactamase was released from the vesicles. Attempts to break the vesicles by sonication or by treatment with moderate amounts of nonionic detergents such as Genapol and Triton X-100 (0.1%, 0.2%, w/v) resulted in inactivation of the β-lactamase. The encapsulated enzyme was best freed by digestion of the vesicles with lipase followed by a 30 s sonication pulse. These experiments were repeated with cefamandole, yielding similar results (fig.1B). From the slope of the straight portion of the lines (see fig.1) we were able to calculate relative $P \times A$-values (P, permeability coefficient; A, total area of permeation) of 0.040 and 0.033 cm^3/min for nitrocefin and cefamandole, respectively. They indicate that nitrocefin permeated into the F porin vesicles at a higher rate.

Similar studies were also carried out using vesicles made from whole outer membranes. The same qualitative patterns of kinetic behaviour were observed (not shown).

Among various control experiments we constructed vesicles from lipids and lipopolysaccharide, again containing β-lactamase inside. Negligible hydrolytic activity could be detected after addition of either nitrocefin or cefamandole. These observations confirm the interpretation that permeation of β-lactams involves movement through

the hydrophilic channels of the porin(s).

We believe that this model is highly suitable for measuring β-lactam permeation provided that:

(i) The β-lactams used are subsceptible to β-lactamase hydrolysis;

(ii) A good spectrophotometer preferably equipped with a device to correct for light-scattering is available, since the optical changes displayed by most β-lactams upon hydrolysis must be measured at short wavelengths.

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