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Purification, Crystallisation and Preliminary X-ray Analysis of Penicillin Binding Protein 4 from *Escherichia coli*, a Protein Related to Class A β -Lactamases

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Crystals of the penicillin binding protein 4 (PBP4) from *Escherichia coli* have been obtained at 37°C from liquid to liquid diffusion experiments in capillaries. PBP4 was dissolved in a 1.0 M ammonium sulphate solution, buffered at pH 7.2, to a concentration of 5 mg/ml, and was layered on top of a 1.6 to 2.2 M ammonium sulphate solution. Crystals appeared within four to six weeks. They belong to space group *C222* with cell dimensions $a = 68.5$ Å, $b = 100.5$ Å and $c = 137.0$ Å, and diffract to at least 2.8 Å resolution. There is one molecule with a molecule mass of 49,568 Da in the asymmetric unit.

Keywords: crystallisation; penicillin; binding protein; β -lactamase; murein; peptidoglycan.

The cell wall provides a bacterium with mechanical stability. It is an unusual biopolymer that surrounds the whole bacterial cell as a single macro-molecule. The polymer is composed of long glycan strands cross-linked by short peptide bridges, thus forming a network-like structure, murein or peptidoglycan. The short peptides show a unique combination of L and D amino acids. Penicillin and other β -lactam antibiotics irreversibly inhibit a number of proteins involved in murein synthesis, hydrolysis and repair (for reviews see Waxman & Strominger (1984); Frère & Joris (1985); Ghuysen (1991)). These so-called penicillin binding proteins (PBPs) have been shown to bind penicillin covalently at a conserved active site serine (Keck *et al.*, 1985). In *Escherichia coli*, seven penicillin binding proteins have been characterised (for review see Matsushashi, 1994). These penicillin binding proteins are generally divided into two classes: the higher molecular mass PBPs (PBP1a, PBP1b, PBP2 and PBP3) and the lower molecular mass PBPs (PBP4, PBP5 and PBP6). The higher molecular mass PBPs are bifunctional enzymes: they are generally DD-transpeptidases, but in addition they have transglycosylase activity. They are essential for murein metabolism during bacterial growth and division, and they are the lethal targets

of the β -lactam antibiotics (Spratt & Pardee, 1975; Suzuki *et al.*, 1978). The lower molecular mass PBPs are, in general, monofunctional DD-carboxypeptidases, and they do not seem to be essential for the growth and division of *E. coli*. Except for PBP4, which was described as a soluble protein (Mottl & Keck, 1991), all PBPs are membrane-bound proteins. PBP4 is the only PBP which exhibits DD-endopeptidase activity in addition to DD-carboxypeptidase activity (Korat *et al.*, 1991). The enzyme can cleave the D-alanyl- γ -meso-2,6-diaminopimelyl (D-Ala- γ -A₂pm) peptide bond in cross-links of the peptidoglycan. The enzyme is thought to be involved in the recycling and maturation of murein, and it has been suggested that PBP4 is the endopeptidase normally involved in breaking cross-links to allow the insertion of new glycan strands into the murein sacculus (Burman & Park, 1984). However, the endopeptidase activity that may degrade the murein sacculus is a constant threat to the integrity of the cell wall, and there is indeed some evidence that PBP4 plays a role in the autolysis of the cell (Kitano *et al.*, 1980).

PBP4 shows a peculiar behaviour towards inhibitors: it is the only PBP for which boronic acids are competitive inhibitors (Then *et al.*, 1990). Boronic acids are specific inhibitors of another class of penicillin-interacting proteins, the β -lactamases (Crompton *et al.*, 1988; Martin *et al.*, 1994). The enzyme is also very sensitive to benzyl-penicillin

Abbreviations used: PBP, penicillin binding protein; D-Ala- γ -A₂pm, D-alanyl- γ -meso-2,6-diaminopimelyl; PEG, polyethylene glycol.

and ampicillin, and penems are bound with high specificity (Spratt, 1977; Matsuhashi *et al.*, 1982; Hashizume *et al.*, 1984). This all seems to indicate that PBP4 has an active site structure divergent from other PBPs of *E. coli*.

PBP4 has a molecular mass of 49.5 kDa and consists of 457 amino acid residues. Analysis of the amino acid sequence shows that PBP4 possesses the three fingerprints, SXXK, SXN and KTG (Mottl *et al.*, 1991), that have been found in all penicillin binding proteins and most β -lactamases. SXXK and KTG are absolutely conserved in all penicillin interacting proteins (with the exception of class B β -lactamases, which are metalloproteins), whereas the conservation of SXN appears to be less strict. On the basis of a sequence comparison with other PBPs and β -lactamases, and the spacing of the three fingerprints, it was suggested that PBP4 and class A β -lactamases share a common evolutionary ancestor, but that PBP4 has an additional domain of 188 amino acids inserted between the SXXK and SXN elements (Mottl *et al.*, 1991).

To substantiate the evolutionary relationship between PBP4 and class A β -lactamase, we carried out multiple sequence alignments according to Higgins & Sharp (1988) and hydrophobic cluster

analysis (Lemesle-Varloot *et al.*, 1990). The three fingerprints served as reference points for the alignment. The final alignment of the sequences of PBP4 (Mottl *et al.*, 1991) and the class A β -lactamases from *Bacillus licheniformis*, *E. coli* (TEM1) and *Staphylococcus aureus* (PC1) (Neugebauer *et al.*, 1981; Sutcliffe, 1978; Ambler, 1975) shows 21% identical residues; 51% of the residues are similar (see Figure 1). From this alignment it was found that all residues involved in catalysis (Ser70, Lys73, Ser130, Asp131, Asn132, Lys234, Thr235 and Gly134) are conserved except Glu166. Biochemical and structural analysis of mutants revealed that Glu166 plays a critical role in the de-acylation step that is responsible for the hydrolysis of the β -lactam antibiotics in β -lactamases (Knox *et al.*, 1993). In PBP4, where residue 166 is a Gln, the β -lactam antibiotics are covalently bound to the active site Ser70 and the de-acylation of the covalently bound intermediate occurs only very slowly.

A comparison of the three-dimensional structures of β -lactamases (Dideberg *et al.*, 1987; Sutton *et al.*, 1987; Moews *et al.*, 1990; Oefner *et al.*, 1990; Herzberg, 1991; Jelsch *et al.*, 1992; Strynadka *et al.*, 1992) and the DD-carboxypeptidase of *Streptomyces* R61 (Kelly *et al.*, 1986; Samraoui *et al.*, 1986) shows

Figure 1. Multiple sequence alignment of the mature PBP4 sequence and the three class A β -lactamases for which the X-ray structure has been determined. The sequence of the additional domain in PBP4 is omitted. The sequence numbering is according to Ambler *et al.* (1991) with allowance for the deletion of 188 amino acids that constitute the extra domain in PBP4. Identical and homologous residues are depicted in bold; homologous residues are classified according to the definition in the SIMPLIFY program of the GCG Sequence Analysis Package (Devereux *et al.*, 1984). The secondary structure prediction has been made on the basis of the alignment with the *Bacillus licheniformis* β -lactamase. Abbreviations: Bliche (*Bacillus licheniformis* β -lactamase), Saureus (*Staphylococcus aureus* PC1 β -lactamase), Ecoli (*Escherichia coli* R-TEM β -lactamase). The insertion point of the extra domain is indicated with ■; the fingerprints characteristic of β -lactamase are indicated with •.

pronounced structural homology. Above all, the scaffolding of the active site is almost identical. The active site serine in the SXXK motif is always located at a short single turn of the 3_{10} helix that precedes α -helix H2, the KTG element forms part of a β -sheet lining the active site, and the SXN box is part of a loop which protrudes into the catalytic centre. On the basis of the clear sequence similarity, we assume that the 3D-structure of PBP4 resembles that of class A β -lactamases with the addition of an extra domain. A residue-by-residue threading (Bowie *et al.*, 1991) of the sequence of PBP4 on the structure of the β -lactamase from *B. licheniformis* has shown that the PBP4 sequence is compatible with a β -lactamase fold.

The extra domain of 188 amino acids does not have any significant relationship with other sequences in the Protein Identification Resource database. Assuming a similar fold of the core of PBP4 and β -lactamase, the insertion of 188 amino acids is located on the surface of β -lactamase in a loop starting at residue 89. This loop immediately follows the helix that bears the active-site serine. Deletion of residues 116 to 238 does not affect the penicillin binding properties of the enzyme, although it seems to have some effect on the de-acylation properties of the penicillin-protein complex. It has been suggested that the additional domain serves a regulatory function with regard to the autolytic DD-endopeptidase activity of PBP4 (Mottl *et al.*, 1992).

Although crystals have been reported for the lower molecular mass PBP5 from *E. coli* K12 (Ferreira *et al.*, 1988) and recently of a higher molecular mass PBP2x from *Streptococcus pneumoniae* (Charlier *et al.*, 1993), no 3D structure of these PBPs is available. Since PBP4 does not have a membrane anchor and large amounts of this protein can be obtained (Korat *et al.*, 1991), it seems to be very suitable for crystallisation purposes.

Since the development of bacterial resistance to the common antibiotics like penicillins and other β -lactams is threatening the treatment of many infectious diseases, new antibiotics are needed (Travis, 1994). The three-dimensional structure of a penicillin binding protein may yield detailed information on the target enzymes of this widely used class of antibiotics. PBP4 could serve as a starting point for the design of novel antibiotics. Furthermore, the structure of PBP4 would reveal the unique organisation of this protein and could give an indication of the function of the additional domain of 188 amino acids. The structure could also be used to study the endopeptidase activity.

The purification of PBP4 was slightly modified compared to earlier reports (Mottl & Keck, 1991). A 55 l quantity of Luria broth supplemented with 0.5% (w/v) glucose was inoculated with 500 ml fresh pre-culture of *E. coli* MC1061 carrying the PBP4 expression plasmid pPROF2AB (Korat *et al.*, 1991) in a 65 litre mobile pilot plant fermentor (New Brunswick). The pre-cultures were grown in the presence of 50 μ g/ml kanamycin. The bacteria were aerated (20–30 l/min) and agitated at 55 revs/min at a temperature of 30°C until the A_{600} of the solution

reached 0.44. Then the temperature was switched to 42°C and the stirring was stopped. After 75 minutes, the A_{600} levelled off at 0.78 and the cells were harvested in a cooled continuous centrifuge. The cell pellet was resuspended in 250 ml 50 mM Tris-HCl buffer (pH 8). Lysis was started by adding 120 μ g/ml HEW lysozyme and 10 mM $MgSO_4$. After stirring for 15 minutes on ice, lysis was completed by means of a French Press (>10,000 psi); 5 μ l/250 ml Benzonase (Merck) was added and the suspension was ultra-sonicated at 400 W for 2 minutes. After adding 3 litres of cold 50 mM Tris-HCl buffer (pH 8.0) with 5 mM $MgSO_4$, the homogenate was cleared by centrifugation at 25,000 g for two hours at 4°C. The supernatant was stored at –20°C in portions of 400 ml. The supernatant (400 ml) was treated with a covalent affinity column, Affigel 10 (Biorad) to which ampicillin was coupled. Coupling of ampicillin in aqueous solution at alkaline pH leads to partial hydrolysis of the antibiotic and the affinity resin. Coupling in methanol, however, leads to a highly substituted gel with excellent binding properties (up to 10 mg PBP4/ml gel). A 25 ml quantity of Affigel 10 was washed with methanol, and 300 mg ampicillin in 100 ml methanol was added. After the overnight coupling reaction the non-bound ampicillin was removed by further washing with methanol. Subsequently, the gel was washed with 50 mM Tris-HCl buffer (pH 8.0) with 5 mM $MgSO_4$.

The crude protein supernatant was added to the gel suspension and the mixture was shaken for two hours at 4°C. The gel was first washed with large volumes of Tris-buffer (50 mM Tris-HCl (pH 8.0), 5 mM $MgSO_4$) on a sintered glass funnel. Next the gel was washed with: (1) 1 M NaCl in Tris buffer; and (2) 40% (v/v) ethylene glycol in Tris buffer, and finally again with Tris buffer without NaCl or ethylene-glycol. To liberate the penicillin binding proteins, the gel was washed with a very cold 50 mM glycine NaOH buffer (pH 10.5) with 5 mM $MgSO_4$, and then transferred in 25 ml of this buffer to a water bath at 37°C. The resin was treated five times with 25 ml of elution buffer. The elution was complete after 48 hours. Active-site labelling with [^{14}C]benzylpenicillin has shown that after this procedure the activity of the protein is still intact (Mottl & Keck, 1991).

The eluates containing PBP4 were pooled and filtered using a 0.22 μ m Gs filter. The last step in the purification scheme was an ion-exchange chromatography step with a TSK DEAE 650-s (100 $\times$ 10 mm) column on an FPLC system (Pharmacia). PBP4 was eluted with a NaCl gradient (250 to 350 mM NaCl in glycine NaOH buffer (pH 10.5), 5 mM $MgSO_4$). The PBP4-containing fractions were pooled and dialysed against 50 mM Tris-HCl buffer (pH 8.0) with 5 mM $MgSO_4$ and 0.5 M NaCl. Usually 1 gram of dry *E. coli* cells yield 2.75 mg of PBP4.

Attempts to crystallise PBP4 were started with the sparse matrix approach (Jancarik & Kim, 1991), and although crystalline material was obtained with several PEGs (polyethylene glycol 300, 4,000 and

10,000) as precipitant, the conditions could not be optimised and only very intergrown crystals were obtained. A brute force approach using the hanging drop method (McPherson, 1982) also only yielded very intergrown crystals and spherulites with either ammonium sulphate or PEG as a precipitant. Switching to liquid-liquid diffusion in capillaries, however, gave usable crystals. The optimal conditions for obtaining crystals are: 5 μ l of a 5 mg/ml PBP4 solution in 10 mM cacodylate (dimethyl arsenic acid) buffer or BES (*N,N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid) buffer (pH 7.4) with 1 M ammonium sulphate, 100 mM NaCl, 5 mM MgSO₄ and 3 mM EDTA, which are layered on top of 5 μ l of a precipitant solution that contains 1.8 to 2.2 M ammonium sulphate, 100 mM cacodylate buffer (pH 7.4), 100 mM EDTA and 10% of PEG400. The experiments are done at 37°C. Crystals appeared after two weeks and grew to a size of 0.1 mm $\times$ 0.05 mm $\times$ 0.5 mm in 1 to 2 months. Although the crystals usually grow as a bunch emanating from one single point, pieces can be dislodged from them, which can be mounted as single crystals.

Rotation images of 1° were collected from several crystals on a MAR image plate at the X11 beam-line at the EMBL outstation, Hamburg, using auto-indexing software (Kabsch, 1993), and from a careful analysis of the intensity distribution of the reflections, the space-group was determined to be C222 with cell dimensions $a = 68.5$ Å, $b = 100.5$ Å and $c = 137.0$ Å. Assuming that there is one molecule in the asymmetric unit with a molecular mass of 49,568 Da, the volume per unit mass, $V_M = 2.38$ Å³/Da. This value is well within the range of 1.6 to 3.6 typically found in protein crystals (Matthews, 1977). The percentage solvent in the crystals would then be 49%.

A partial dataset to 3.2 Å from a single crystal has been collected at station 9.5 at the Daresbury synchrotron facilities. The crystals appeared to be rather sensitive to X-ray radiation, and only data for 35° rotation could be collected. Further data collection is in progress. We hope to elucidate the PBP4 structure using molecular replacement with β -lactamase as a starting model or multiple isomorphous replacement, if the molecular replacement fails.

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