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Crystallization of a Genetically Engineered Water-soluble Primary Penicillin Target Enzyme

The High Molecular Mass PBP2x of *Streptococcus pneumoniae*

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A genetically engineered water-soluble derivative of PBP2x of *Streptococcus pneumoniae* has been produced, purified and crystallized in a form suitable for X-ray diffraction analysis. The best crystals have been grown at 15°C, from solutions containing 8% polyethylene glycol 10,000 at pH values ranging from 3·9 to 6·0. These crystals diffract to a resolution of 3·5 Å and have a space group $P6_122$ (or enantiomorph) with unit cell dimensions of $a = b = 162{\cdot}2$ Å, $c = 171{\cdot}8$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$. The molecular mass and cell dimensions suggest that there is one molecule of enzyme per asymmetric unit. The breakdown of a chromogenic cephalosporin derivative diffused into a crystal reveals clearly that the enzyme is active in the crystalline state.

Keywords: crystallization; membrane protein; penicillin binding protein; *Streptococcus pneumoniae*

The deleterious effects of penicillin on growing bacterial cells involve inhibition of essential membrane-bound enzymes functioning in murein biosynthesis. These so-called penicillin-binding proteins (PBPs‡) interact with beta-lactam antibiotics by formation of a covalent penicilloyl- (cephalosporoyl-) complex *via* an active site serine residue. The acyl-enzyme complex is enzymatically inactive; upon release of the beta-lactam moiety by hydrolysis, active enzyme is restored. According to sequence homologies with PBPs of *Escherichia coli*, the only organism where the *in vitro* function of these enzymes has been studied in detail, PBPs are divided into high molecular mass (hmm) PBPs catalyzing essential transpeptidation reactions, and low molecular mass (lmm) PBPs acting as D,D-carboxypeptidases, which appear to be dispensable for cell growth (for a review, see Ghuysen, 1991). In addition to the penicillin binding domain hmm PBPs contain a long N-terminal domain that performs a penicillin-intensive transglycosylation reaction, and usually a C-terminal extension of unknown properties.

Hmm and lmm PBPs form, together with class A, C and D beta-lactamases, the protein family of penicillin-interacting serine enzymes. Despite their different biological functions they share structural features, such as two conserved amino acid sequences located in their penicillin-sensitive catalytic domain: the SXXK-box with the active site serine, and the triad KT(S)G.

The three-dimensional structures of several beta-lactamases have been solved by X-ray crystallographic analysis (Dideberg *et al.*, 1987; Herzberg &

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‡Abbreviations used: PBPs, penicillin-binding proteins; hmm, high molecular mass; lmm, low molecular mass; IPTG, Isopropyl-β-D-thiogalactopyranoside.

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Moult, 1987; Sutton *et al.*, 1987; Oefner *et al.*, 1990; Moews *et al.*, 1990; Jelsch *et al.*, 1992; Strynadka *et al.*, 1992) and were shown to be remarkably similar in polypeptide folding compared to the small soluble carboxypeptidase/transpeptidase of *Streptomyces* R61 (Kelly *et al.*, 1986; Samraoni *et al.*, 1986). The architecture of these proteins serves as a model for the penicillin-binding domain of hmm PBPs. At the present time, only one lmm PBP has been crystallized: the water-soluble derivative of PBP5 from *E. coli* K12 (Ferreira *et al.*, 1988) yielded crystals suitable for X-ray analysis. However, lack of isomorphism between native and heavy atom derivative crystals has always prevented the establishment of the structure of this protein. For hmm PBP, no satisfactory crystals have been obtained so far and the structural arrangement of their N-terminal domains and their C-terminal extensions remains completely unknown.

Structural information of hmm PBPs is of special interest since they represent the important primary targets for beta-lactam antibiotics. In several bacterial species like *Streptomyces pneumoniae*, intrinsic penicillin resistance is achieved by modification of essential PBPs into variants with decreased affinity for the antibiotic. The first PBP that is affected in resistant pneumococci appears to be PBP2x (Laible & Hakenbeck, 1987; Laible *et al.*, 1991). This protein represents a promising model enzyme for studying the structural prerequisites for the interaction of a hmm PBP with beta-lactams in detail. A number of point mutations resulting in succesive decrease in penicillin affinity have been characterized in a number of cefotaxime resistant mutants (Laible & Hakenbeck, 1991), indicating protein sites critical for interaction with the antibiotic. A soluble, active derivative (PBP2x*) has been constructed recently by deleting the hydrophobic membrane anchoring domain by molecular genetic techniques, and high overexpression in an *E. coli* system facilitated the purification of sufficient amounts of active enzyme suitable for crystallization trials (Laible *et al.*, 1992).

For the crystallization studies described here, the following modifications of the published purification procedure were introduced. To avoid the observed unstable constitutive expression from the recombinant plasmid pCG 31-26 in *E. coli* DH5, in which the PBP2x gene is cloned behind the *lacZα'* promoter, the plasmid was transformed into the *lacIq* repressor-carrying *E. coli* strain JM101. Overexpression was induced by addition of 0·1 mM IPTG, for two hours at an $A_{610\,nm}$ value of 0·6. Approximately 50 g of cells were used for isolating large amounts of PBP2x*. Cells were resuspended in 200 ml 50 ml Tris·HCl buffer pH 8·0, 5 mM $MgCl_2$, 0·02% PMSF and DNase, and passed twice through a French pressure cell press (1·45 Pa). Supernatant containing the soluble PBP2x*, obtained after centrifugation for one hour at 100,000 *g*, was dialyzed against 10 mM Tris·HCl, pH 8, 0·02% sodium azide (buffer A) and fractionated over a 400 ml EMD-TMAE-fractogel column (Merck, Germany) equilibrated with the same buffer at a flow rate of 5 ml/min. After washing with 5 l buffer A, PBP2x* was eluted with a linear gradient using buffer A + 0·3 M NaCl. The fractions containing the protein (around 200 mM NaCl) were pooled and dialyzed twice against buffer A followed by dialysis against 25 mM Bis-Tris buffer pH 6·3, 0·02% sodium azide (buffer C). Final purification was achieved by chromatofocusing on a PBP47 column (50 × 1 cm) (Pharmacia, Sweden). A total of 5 ml polybuffer 47, diluted 20-fold was applied to the column equilibrated in buffer C before PBP2x* was loaded. For elution, polybuffer 47 was applied until the elution buffer reached a pH of 4·0. Samples containing PBP2x* (around pH 5·0) were pooled, dialyzed against buffer A containing 1 mM EDTA and concentrated by ultrafiltration to a protein concentration of >10 mg/ml as determined by Bradford (1976). Usually 1 g cells yielded 2 mg purified PBP2x.

PBP2x* was monitored on immunoblots using a specific rabbit antiserum directed against PBP2x*. Enzymatic activity of PBP2x* was tested in a penicillin-binding assay and by active site titration with [^{14}C] Penicillin G (>98% penicillin bound: Mottl & Keck, 1991) and by measuring the hydrolysis of the thioester substrate S2e (Adam *et al.*, 1991).

For crystallization, hanging-drop vapor diffusion methods were carried out systematically. Several crystal forms were obtained under various conditions. The largest crystals (>0·3 mm) have three types of morphology. Bipyramidal crystals (form A) were grown in 10 μl droplets of an equi-volume mixture of 25 mg protein/ml solution and a reservoir solution containing 8% PEG 10,000 in 100 mM acetate buffer, pH 4, at 15°C. These crystals diffracted up to 3·5 Å and are suitable for X-ray analysis. We obtained prismatic crystals (form B) under similar conditions but at a pH between 5 and 6. Finally, well-shaped isotrope crystals (form C) appeared after two weeks under the same conditions but they diffracted only up to 7 Å.

For X-ray analysis, crystals were mounted in glass capillaries containing some mother liquor. Diffraction patterns were recorded on the FAST diffractometer system (Enraf–Nonius) using the MADNES software (Messerschmidt & Pflugrath, 1987). The reflections of the best diffracting crystals (form A and B) could be indexed on a hexagonal lattice corresponding to unit cell parameters $a = 162{\cdot}2$ Å, $c = 171{\cdot}8$ Å. The space group was determined by inspecting the intensity distribution. The data showed systematic absences along $00l$ for $l \neq 6n$, which allowed confirmation that the space group is $P6_122$ (International Tables no. 178) or $P6_522$ (no. 179). Large (0·5 mm × 0·5 mm × 0·6 mm) single crystals were used to collect native data using the FAST system. A total of 35,317 observations were reduced to 7804 unique reflections with a merging R-factor of 7·7% on intensities.

Crystal density was measured with a Ficoll

density gradient to be 1·145 g/ml. Given the volume of the asymmetric unit and using a value $\bar{v} = 0{\cdot}737$ for the partial specific volume of the unsolvated protein we confirm the presence of one molecule in the asymmetric unit.

For the poorly diffracting crystals no attempt was made to measure a full data set, however, using only strong reflections we found a tetragonal lattice with unit cell dimensions $a = 171{\cdot}5$ Å, $c = 133{\cdot}3$ Å.

An activity assay confirmed that a functional enzyme has been crystallized. Two crystals were firstly soaked in a stabilizing solution (15% PEG 10,000, 100 mM acetate, pH 4). One crystal was transferred to the same solution containing 10 mM Cefuroxime for two hours, then washed three times with the stabilizing solution. Finally, both crystals were moved in a new drop of the same stabilizing solution containing 1 mM Nitrocefin. This chromogenic cephalosporin derivative changes its color from yellow to red upon hydrolysis of the endocyclic amide bond. In a few minutes, the native crystal became red while the other stayed transparent demonstrating hydrolyzing activity in the first case, and a complete inhibition of PBP2x* by Cefuroxime in the second case. After half an hour, the untreated crystal turned completely red. A slight release of the Cefuroxime was only observed after 24 hours.

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