Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Structural studies of the transpeptidase domain of PBP1a from *Streptococcus pneumoniae*

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Received 22 January 2003
Accepted 26 March 2003

The synthesis of the bacterial cell wall requires enzymes which are localized both in the cytoplasm and in the periplasm. Penicillin-binding proteins (PBPs) catalyze the last, crucial steps in peptidoglycan biosynthesis and several of them are essential for bacterial survival. High-molecular-mass PBPs can be bifunctional (class A) or monofunctional (class B) and to date no structural information on any class A PBP is available. To initiate the determination of the three-dimensional structure of a class A PBP, crystals of the transpeptidase domain of PBP1a from *Streptococcus pneumoniae* were prepared by limited proteolysis of the full-length molecule and purification by anion-exchange chromatography and gel filtration. The samples crystallize in space group $C222_1$, contain one molecule per asymmetric unit and diffract X-rays to 2.7 Å. Selenomethionine-labelled crystals have been prepared and structure solution is under way.

1. Introduction

Streptococcus pneumoniae is the causative agent of a variety of upper respiratory tract infections as well as acute otitis media, bacteraemia and meningitis. Although streptococcal infections have been treated successfully with β-lactam antibiotics for over six decades, 25% of all invasive *S. pneumoniae* strains are presently resistant to penicillin (Pallares *et al.*, 2000). β-Lactams target bacteria by acylating the active-site serine of the transpeptidase domains of penicillin-binding proteins (PBPs), which catalyze key steps in the synthesis of the bacterial peptidoglycan. During the synthesis of this highly resistant cross-linked mesh, some PBPs catalyze glycosyltransfer or the interconnection of glycan strains (polymers of *N*-glucosamine and *N*-acetylmuramic acid residues), as well as cross-linking or transpeptidation of stem peptides which are attached to the glycan chains (Höltje, 1998).

The basis of β-lactam action reflects the fact that the transpeptidation substrate, the D-alanyl-D-alanine C-terminus of peptidoglycan stem peptides, has close structural analogy to the β-lactam ring. β-Lactams thus act as pseudosubstrates and acylate the PBP transpeptidation active sites, which deacylate very slowly and are incapable of cross-linking the peptidoglycan.

S. pneumoniae has five high-molecular-mass PBPs [1a, 1b, 2a, 2b and 2x, the first three of which contain both glycosyltransfer (GT) and transpeptidation (TP) domains] and one low-molecular-mass molecule (PBP3, which is believed to regulate peptidoglycan cross-linking levels). PBPs 1a, 1b and 2a belong to class A, while PBPs 2x and 2b are monofunctional class B molecules (Goffin & Ghuysen, 2002). Interestingly, clinical isolates of resistant pneumococci display PBP1a-, PBP2x- and PBP2b-encoding genes with mutations throughout the entire sequence (Hakenbeck *et al.*, 1999; Asahi *et al.*, 1999). In hospital environments, the specific resistance of streptococcal species to β-lactams such as cefotaxime and ceftriaxone is mediated by mutations within PBP2x and PBP1a (Munoz *et al.*, 1992). To date, no structural information is available on a class A PBP from any organism. Here, we report the expression, purification, crystallization and initial X-ray diffraction analyses of the transpeptidase (TP) domain of PBP1a from *S. pneumoniae*.

2. Experimental methods

2.1. Purification and crystallization of the PBP1a TP domain

PBP1a (719 residues) is composed of a short cytoplasmic region, a transmembrane domain and a periplasmic unit containing two domains, the glycosyltransfer (GT) and transpeptidation (TP) domains, which can be separated by limited trypsinization. In order to purify the TP domain of PBP1a, it was necessary to express the full-length protein as a GST fusion (without the cytoplasmic and transmembrane regions, henceforth called PBP1a*) and subsequently perform limited proteolysis. Attempts to clone and express the crystallizable TP domain in the absence of the

N-terminal GT domain led to aggregated protein.

E. coli MC1061 (pJAH143) cells were grown at 310 K in LB medium supplemented with 100 µg ml^{-1} ampicillin until the OD_{600nm} reached 1.0, after which the shaker temperature was reduced to 291 K and the cells were induced with 1 m*M* IPTG. Cultures were allowed to grow overnight with vigorous shaking. Subsequently, cells were harvested by centrifugation and sonicated in 25 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA, 0.2 *M* NaCl and 1% CHAPS. The lysate was subsequently centrifuged at 30 000*g* for 30 min and loaded onto a glutathione Sepharose column (Pharmacia) previously equilibrated and washed in the aforementioned buffer, with the exception of CHAPS. Full length GST-PBP1a* was obtained by eluting fractions in 10 m*M* reduced glutathione prepared in the same buffer. Protein-containing samples were dialyzed overnight against 25 m*M* Tris pH 8.0, 1 m*M* EDTA and 0.1 *M* NaCl.

The TP1a domain was prepared by trypsinizing GST-PBP1a* using trypsin:PBP1a* ratios of 1:20–100(*w*:*w*) for 1 h at room temperature. The samples were subsequently purified over a MonoQ column (Pharmacia) in 25 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA with a linear gradient from 0 to 0.5 *M* NaCl. Two closely eluting peaks were identified; both peaks produced TP1a crystals with the same morphology (Fig. 1). In order to improve crystal quality, each peak was purified using a Superose 200 column in 25 m*M* Tris–HCl pH 8.0 and 1 m*M* EDTA.

Initial crystallization tests were performed by employing commercial screens (Hampton), which yielded small needle-like crystals in 100 m*M* MES pH 6.5, 25% PEG MME 550 and 10 m*M* $ZnSO_4$ at 288 K. Both forms of the TP1a domain were subsequently crystallized by vapour diffusion using 100 m*M* MES pH 6.5–7.0, 10% PEG 1000 and 5 m*M* $ZnS0_4$. Crystals grown at pH 6.5 took several weeks to appear, while those grown at pH 7.0 appeared after a few days, although at any pH crystals took several months to grow to the final size used for data collection (50 × 30 × 30 µm). Crystals tended to grow faster if 7–9 m*M* $MgCl_2$, $CoCl_2$ or $MnCl_2$ was added to the crystallization drop, but the use of these additives was discontinued owing to low crystal quality. In order to reduce nucleation, 200 µl of silicone oil was overlaid on the mother-liquor bed prior to closure of the cover slip. Although the latter step slowed the rate of crystal growth, crystals grown under these conditions tended to be fewer and of better quality than those grown otherwise (Chayen, 1997).

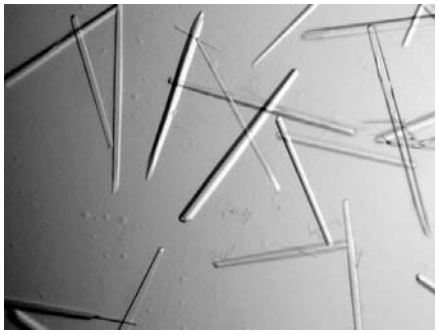

Figure 1
C-centred orthorhombic crystals of the transpeptidase domain of PBP1a from *S. pneumoniae*.

2.2. Cryoprotection and data collection

Cryoprotection was performed by initially introducing 10 µl of 0.1 *M* MES pH 6.5, 11% PEG 1000, 5 m*M* $ZnSO_4$ and 5% ethylene glycol into the crystallization drop. After a 5 min equilibration period, crystals were transferred into a larger volume of this solution. The amount of ethylene glycol in the cryoprotection buffer was slowly increased to 20% over a total period of 24 h, after which crystals were flash-cooled by rapid introduction into liquid nitrogen. Data were collected on the ID13 beamline equipped with a microdiffractometer (European Synchrotron Radiation Facility, Grenoble, France) at 0.96 Å with 1° oscillations.

For antibiotic soaking experiments, TP1a crystals were cryoprotected in the above-mentioned manner and were incubated immediately prior to the flash-cooling steps in cryosolution containing 300 µ*M* of either penicillin G, nitrocefin or cefotaxime for 3 h. Only crystals soaked in cefotaxime continued to diffract after soaking. Data from a cefotaxime-soaked crystal was collected on beamline ID14EH4 (ESRF Grenoble) at 0.976 Å with 1° oscillations. Integration, scaling and merging of data were performed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Crystals belong to space group $C222_1$, with unit-cell parameters $a = 112.6$, $b = 183.9$, $c = 54.9$ Å, and have one molecule per asymmetric unit. Data-collection statistics for both native and antibiotic soaked crystals are given in Table 1.

Table 1
Statistics of X-ray diffraction data for the TP1a domain from *S. pneumoniae*.

Values in parentheses are for the highest resolution shell.

	Apo form	Cefotaxime soak
Resolution limit (Å)	25–2.8	25–2.7
No. reflections measured	128804	115864
No. unique reflections	13743	16346
Completeness (%)	98.4 (99.8)	97.1 (86.6)
$I/\sigma(I)$	13.2 (1.7)	14.7 (2.9)
R_{sym} (%)	8.3 (37.1)	7.4 (35.0)

3. Results and discussion

Class A PBPs are essential for bacterial survival. In *E. coli*, disruption of both PBPs 1a and 1b is lethal, while compensation of one for the other is possible (Youssif *et al.*, 1985); a similar requirement has been observed for streptococcal molecules (Kell *et al.*, 1993). The lack of structural information regarding any class A PBP reflects their tendency to aggregate as well as their instability and the possible inherent flexibility between GT and TP domains.

In the past, expression and purification of PBP1a* proved to be challenging owing to the tendency of the protein to aggregate. In order to partially solve this problem, we modified the protocol developed by Di Guilmi *et al.* (1998) by incorporating 1% CHAPS into the lysis buffer. After purification, the yield of crystallization-quality TP1a domain is approximately 0.5 mg per litre of bacterial culture.

Following trypsinization of PBP1a, the TP domain that is generated is a 390-residue protein (Ser264–Arg653); depending on the amount of trypsin and the length of time employed, a secondary internal cleavage site following Arg545 was also observed, generating crystals of poor quality. In order to circumvent this problem, an Arg545Gln mutant was generated. The mutant protein was expressed and purified using the same protocol as the wild-type molecule, although the yield was slightly inferior. Trypsinization of Arg545Gln TP1a yielded a unique band containing the complete transpeptidase domain.

For both the wild-type and Arg545Gln forms, both TP1a peaks purified on MonoQ eluted as monomers on gel filtration and crystallized under similar conditions and in the same space group. Diffraction to 2.7 Å was observed using a synchrotron X-ray source and a CCD detector. Crystals displayed considerable fragility towards the X-ray beam, as well as non-isomorphism. Although transpeptidase domains contain catalytic motifs reminiscent of class A β-lactamases [S*XX*K, S*X*N, K(T/S)G] and are expected to be structurally related to such molecules, amino-acid similarities between the TP domain of PBP1a and other peptidoglycan-metabolizing enzymes of

known structure, such as PBP2x from *S. pneumoniae* (Parès *et al.*, 1996), PBP2a from *Staphylococcus aureus* (Lim & Strynadka, 2002) (both monofunctional class B molecules) or PBP5 from *E. coli* (Davies *et al.*, 2001), are approximately 15%, making the employment of molecular replacement for structure solution a non-viable option. The search for suitable heavy-atom derivatives has revealed a strong tendency to non-isomorphism, with the binding of a variety of heavy-atom salts being capable of modifying the unit-cell parameters by up to 10 Å. Selenomethionine-labelled protein has been prepared and crystallized and crystal optimization is under way. The solution of the three-dimensional structure of the apo and antibiotic complexed form of the transpeptidase domain of streptococcal PBP1a will not only provide valuable insight into the mechanism of drug resistance in pathogens, but will also be the first structural information regarding a class A PBP from any species.

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