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Crystallization and preliminary X-ray diffraction studies of the complete modular endolysin from Cp-1, a phage infecting *Streptococcus pneumoniae*

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Endolysin from the phage Cp-1 (Cpl-1) cleaves the glycosidic β1,4-bonds between the *N*-acetylmuramic acid and the *N*-acetylglucosamine of the pneumococcal cell wall. Cpl-1 has been crystallized using the hanging-drop vapour-diffusion method at 291 K. Diffraction-quality orthorhombic crystals of the native protein were obtained only after addition of the detergent *n*-decyl-β-D-maltoside. Crystals belong to space group $C222_1$, with unit-cell parameters $a = 77.949$, $b = 95.782$, $c = 129.282$ Å. Diffraction data to a resolution of 2.1 Å were collected at a synchrotron facility.

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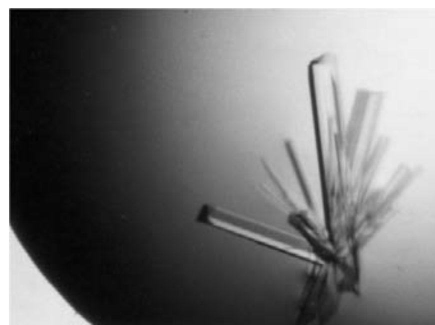
1. Introduction

The pneumococcal bacteriophage Cp-1 (*Podoviridae* family) contains a lytic *cpl1* gene which encodes the Cpl-1 lysozyme (339 amino acids, 39 249 Da; García *et al.*, 1987). The Cpl-1 lysozyme binds to the bacterial envelope and cleaves the glycosidic *N*-acetylmuramic acid-(β1,4)-*N*-acetylglucosamine bond of the glycan chains, breaking down the peptidoglycan backbone for progeny liberation. Lysozymes are usually classified into four types based on amino-acid sequence similarity: hen egg-white lysozyme (HEWL), bacteriophage T4 lysozyme (T4L), goose egg-white lysozyme (GEWL) and *Chalaropsis* lysozyme. Cpl-1 belongs to the latter group (ch-type). Despite their low sequence identity, the first three types share several structural features (Monzingo *et al.*, 1996). On the other hand, the first three-dimensional structure of a ch-type lysozyme shows a new muramidase fold (Rau *et al.*, 2001). In addition to the catalytic domain and in contrast to other well structurally characterized lysozymes, Cpl-1 requires a cell-wall-binding module to display complete functionality. This module enhances the substrate-binding efficiency by specific recognition of the choline residues present in pneumococcal cell-wall teichoic acids. The same recognition mechanism is shared by the host enzymes (LytC lysozyme, LytA amidase and LytB glucosaminidase) involved in lysis and daughter-cell separation after pneumococcal division as well as by cell-wall lytic amidases from other phages infecting pneumococci (López *et al.*, 1997). Structural and biochemical studies have shown that these proteins are formed by two different modules (Usobiaga *et al.*, 1996; Varea *et al.*, 2000). One of them is involved in cell-wall attachment by choline recognition and the other is responsible for the lytic activity. Until now, only the crystal structure of a shortened choline-binding domain of the major pneumococcal autolysin (LytA amidase) has been reported (Fernández-Tornero *et al.*, 2001). The X-ray crystallographic structure of Cpl-1 would therefore be the first complete structure of a lytic enzyme capable of hydrolyzing the pneumococcal cell wall. Therefore, the Cpl-1 structure will provide new and relevant valuable information about the modular organization of Cpl-1 lysozyme, the interactions established between the catalytic and the cell-wall-binding modules and their influence on protein function and stability. Oral administration of cell-wall hydrolases encoded by pneumococcal phages controls the streptococcal carrier state and kills infectious streptococci present in the nasopharynx of infected mice (Loeffler *et al.*, 2001). Therefore, the knowledge of the structure of these enzymes will be helpful in the establishment of a novel 'enzybiotic' treatment against streptococcal-mediated diseases.

2. Cpl-1 lysozyme production and purification

Cpl-1 lysozyme was purified from the cell extracts of *Escherichia coli* DH1 (pCIP100) by affinity chromatography on DEAE cellulose according to the method of Sanz *et al.* (1988). Lysozyme activity was assayed according to the procedure of Mosser & Tomaszs (1970). The homogeneity of the protein was confirmed by analytical ultracentrifugation and by SDS–PAGE. Fractions containing Cpl-1 lysozyme were pooled together and extensively dialyzed against 20 m*M* Tris–HCl buffer pH 8.0. The enzyme was then concentrated at 277 K with a

10 kDa cutoff Amicon protein concentrator (YM-10) to approximately 13 mg ml^{-1}. The final protein concentration was determined spectrophotometrically using a molar absorption coefficient of 117 350 M^{-1} cm^{-1} at 280 nm.



(*a*)

(*b*)

Figure 1
Effect of detergent on Cpl-1 crystals. (*a*) Crystals grown at 291 K in 1.6 *M* sodium formate, 0.1 *M* sodium citrate pH 6.1. (*b*) Crystals obtained in the same conditions of precipitant and buffer, but with the addition of 1.8 m*M* *n*-decyl-β-D-maltoside.

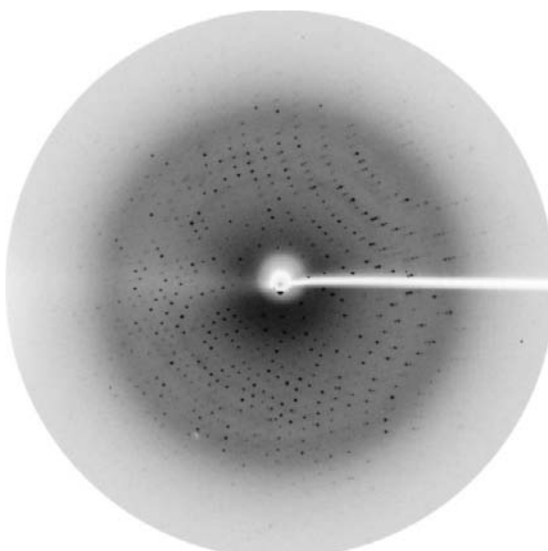


Figure 2
An X-ray diffraction pattern from Cpl-1 crystals (oscillation range 1.0°).

3. Crystallization

Prior to crystallization, the protein was diluted to 8 mg ml^{-1}. The preliminary crystallization conditions were established using the sparse-matrix sampling technique (Jancarik & Kim, 1991) with the hanging-drop vapour-diffusion method using Crystal Screens I and II (Hampton Research). 1 µl volumes of protein and well solution were used and each hanging-drop was equilibrated against 500 µl of well solution. Sea-urchin-like crystals were obtained from 2.0 *M* sodium formate and 0.1 *M* sodium acetate trihydrate buffer at pH 4.6. Further screening of this condition resulted in bar-shaped crystals at 1.6 *M* sodium formate plus 0.1 *M* sodium citrate buffer at pH 6.1 (Fig. 1*a*). Unfortunately, the diffraction pattern from these crystals was of very poor quality. Of 24 different detergents tested as additives in this condition (Detergent Screen 1, Hampton Research), only *n*-decyl-β-D-maltoside produced a new crystal form. Depending on detergent concentration, octahedral crystals with slightly convex faces coexisted with the previous bar-shaped ones (Fig. 1*b*). Optimization of these conditions to 1.7 *M* sodium formate, 0.1 *M* sodium citrate buffer pH 6.0 and 1.8 m*M* *n*-decyl-β-D-maltoside made it possible to obtain good-quality crystals suitable for diffraction, the maximum size being 0.5 × 0.3 × 0.3 mm. The drop volume of the best crystals was 10 µl, formed by 4 µl of protein solution, 1 µl of the detergent and 5 µl of well solution. Interestingly, the pH value for crystallization was the same as that reported for maximal enzymatic activity in phosphate buffer (García *et al.*, 1999). Crystals usually grew in 15–30 d at 291 K.

4. X-ray diffraction experiments

Cpl-1 crystals were soaked for 3 min in several cryoprotectant solutions in order to find suitable conditions for flash-freezing. The best results were obtained after soaking crystals in a solution consisting of 4.0 *M* sodium formate and 0.1 *M* sodium citrate pH 6.0. The ability of sodium formate to act as a cryosalt has recently been reported (Rubinson *et al.*, 2000). Interestingly, glycerol, commonly used as a cryoprotector, resulted in crystal degradation. Single crystals were mounted in fibre loops and frozen at 100 K in a nitrogen stream. Cpl-1 crystals were initially screened on a MAR Research (Germany) IP area detector with an Enraf–Nonius FR591 rotating-anode generator operating at 40 kV and 90 mA (Fig. 2).

Table 1
Data-collection statistics for Cpl-1 crystals.

Values in parentheses correspond to the highest resolution shell.

Crystal data	
Space group	$C222_1$
Unit-cell parameters	
a (Å)	77.949
b (Å)	95.782
c (Å)	129.282
Data collection	
Temperature (K)	100
Wavelength (Å)	1.004
Resolution (Å)	38.5–2.1 (2.2–2.1)
Unique data	28430
Redundancy	4.7 (3.9)
Completeness (%)	99.3 (95.9)
$I/\sigma(I)$	6.8 (2.0)
R_{sym}†	0.07 (0.34)

† $R_{sym} = \sum|I - I_{av}|/\sum I$, where the summation is over symmetry-equivalent reflections.

To improve the resolution, synchrotron radiation was used. X-ray diffraction was measured with a CCD detector using the BM14 beamline at the ESRF Grenoble synchrotron-radiation source. All data were processed and scaled using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). Crystals grown in 1.7 *M* sodium formate, 0.1 *M* sodium citrate buffer pH 6.0 and 1.8 m*M* *n*-decyl-β-D-maltoside were orthorhombic, with unit-cell parameters a = 77.949, b = 95.782, c = 129.282 Å, $\alpha = \beta = \gamma = 90°$. Based on the molecular weight and unit-cell volume, a Matthews coefficient of 3.0 Å^3 Da^{-1} (Matthews, 1968) suggests a monomer in the asymmetric unit and a solvent content of 60%. Data-collection statistics are summarized in Table 1.

Heavy-atom derivatives were prepared to perform MAD/MIR experiments at ESRF. Crystals of Cpl-1 soaked for 26 h in a well solution containing 10 m*M* phenylmercuryl acetate provided a promising anomalous signal which might give useful phasing information.

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