

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

Begoña Monterroso,<sup>a,b</sup> Armando Albert,<sup>a</sup> Martín Martínez-Ripoll,<sup>a</sup> Pedro García,<sup>c</sup> José Luis García,<sup>c</sup> Margarita Menéndez<sup>b</sup> and Juan A. Hermoso<sup>a\*</sup>

<sup>a</sup>Grupo de Cristalografía Macromolecular y Biología Estructural, Instituto Rocasolano, CSIC, Serrano 119, 28006 Madrid, Spain,

<sup>b</sup>Departamento de Química-Física de Macromoléculas Biológicas, Instituto Rocasolano, CSIC, Serrano 119, 28006 Madrid, Spain, and <sup>c</sup>Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain

Correspondence e-mail: xjuan@iqfr.csic.es

Endolysin from the phage Cp-1 (Cpl-1) cleaves the glycosidic  $\beta$ 1,4-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- $\beta$ -D-maltoside. Crystals belong to space group *C*222<sub>1</sub>, 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.

Received 17 April 2002

Accepted 1 July 2002

## 1. Introduction

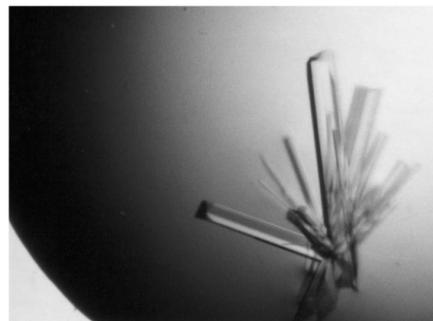
The pneumococcal bacteriophage Cp-1 (*Podoviridae* family) contains a lytic *cplI* gene which encodes the Cpl-1 lysozyme (339 amino acids, 39 249 Da; García *et al.*, 1987). The Cpl-1 lysozyme binds to the bacterial envelope and cleaves the glycosidic *N*-acetylmuramic acid- ( $\beta$ 1,4)-*N*-acetylglucosamine 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 'enzyme' treatment against streptococcal-mediated diseases.

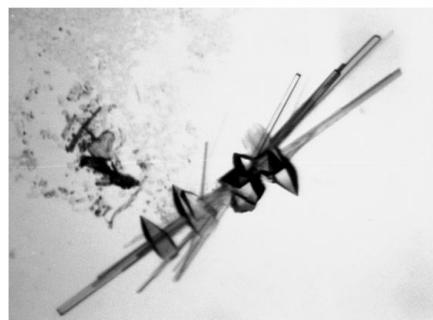
## 2. Cpl-1 lysozyme production and purification

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 & Tomasz (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 mM 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  $13 \text{ mg ml}^{-1}$ . The final protein concentration was determined spectrophotometrically using a molar absorption coefficient of  $117\,350 \text{ M}^{-1} \text{ cm}^{-1}$  at 280 nm.



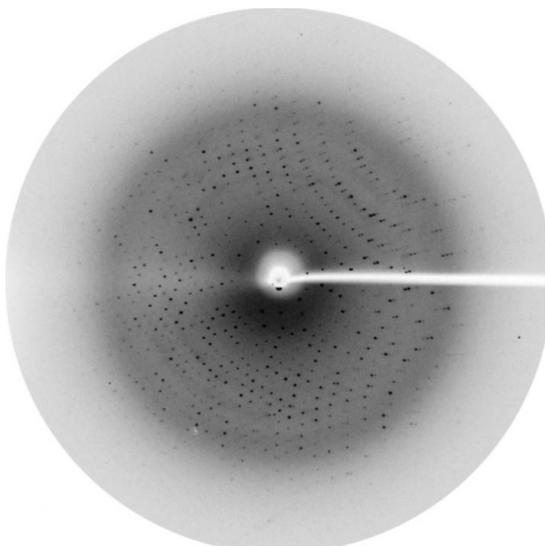
(a)



(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 mM *n*-decyl- $\beta$ -D-maltoside.



**Figure 2**

An X-ray diffraction pattern from Cpl-1 crystals (oscillation range  $1.0^\circ$ ).

### 3. Crystallization

Prior to crystallization, the protein was diluted to  $8 \text{ mg ml}^{-1}$ . 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  $\mu\text{l}$  volumes of protein and well solution were used and each hanging-drop was equilibrated against 500  $\mu\text{l}$  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. 1a). 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- $\beta$ -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. 1b). Optimization of these conditions to 1.7 M sodium formate, 0.1 M sodium citrate buffer pH 6.0 and 1.8 mM *n*-decyl- $\beta$ -D-maltoside made it possible to obtain good-quality crystals suitable for diffraction, the maximum size being  $0.5 \times 0.3 \times 0.3 \text{ mm}$ . The drop volume of the best crystals was 10  $\mu\text{l}$ , formed by 4  $\mu\text{l}$  of protein solution, 1  $\mu\text{l}$  of the detergent and 5  $\mu\text{l}$  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 initi-

**Table 1**

Data-collection statistics for Cpl-1 crystals.

Values in parentheses correspond to the highest resolution shell.

Crystal data	
Space group	C222 <sub>1</sub>
Unit-cell parameters	
<i>a</i> (Å)	77.949
<i>b</i> (Å)	95.782
<i>c</i> (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>I</i> / $\sigma$ ( <i>I</i> )	6.8 (2.0)
<i>R</i> <sub>sym</sub> <sup>†</sup>	0.07 (0.34)

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

ally 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).

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 *CCP4* 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 mM *n*-decyl- $\beta$ -D-maltoside were orthorhombic, with unit-cell parameters  $a = 77.949$ ,  $b = 95.782$ ,  $c = 129.282 \text{ Å}$ ,  $\alpha = \beta = \gamma = 90^\circ$ . Based on the molecular weight and unit-cell volume, a Matthews coefficient of  $3.0 \text{ Å}^3 \text{ Da}^{-1}$  (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 mM phenylmercuryl acetate provided a promising anomalous signal which might give useful phasing information.

We gratefully acknowledge the BM14 Spanish beamline at ESRF for access to synchrotron radiation and for helpful assistance in data collection. This work was supported by grants from the Spanish Ministerio de Ciencia y Tecnología (BIO2000-0747, BIO2000-1307 and BCM2000-1002) Comunidad Autónoma de Madrid (Programa de Grupos Estratégicos).

## References

- Collaborative Computational Project, Number 4 (1994). *Acta Cryst. D* **50**, 760–763.
- Fernández-Tornero, C., López, R., García, E., Giménez-Gallego, G. & Romero, A. (2001). *Nature Struct. Biol.* **8**, 1020–1024.
- García, J. L., García, E., Arrarás, A., García, P., Ronda, C. & López, R. (1987). *J. Virol.* **61**, 2573–2580.
- García, P., González, M. P., García, E., García, J. L. & López, R. (1999). *Mol. Microbiol.* **33**, 128–138.
- Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
- Leslie, A. G. W. (1992). *Crystallographic Computing 5: From Chemistry to Biology*, edited by D. Moras, A. D. Podjarny & J. C. Thierry, pp. 39–50. Oxford University Press.
- Loeffler, J. M., Nelson, D. & Fischetti, V. A. (2001). *Science*, **294**, 2170–2172.
- López, R., García, E., García, P. & García, J. L. (1997). *Microb. Drug Resist.* **3**, 199–211.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Monzingo, A. F., Marcotte, E. M., Hart, P. J. & Robertus, J. D. (1996). *Nature Struct. Biol.* **3**, 133–140.
- Mosser, J. L. & Tomasz, A. (1970). *J. Biol. Chem.* **245**, 287–298.
- Rau, A., Hogg, T., Marquardt, R. & Hilgenfeld, R. (2001). *J. Biol. Chem.* **276**, 31994–31999.
- Rubinson, K. A., Ladner, J. E., Tordova, M. & Gilliland, G. L. (2000). *Acta Cryst. D* **56**, 996–1001.
- Sanz, J. M., López, R. & García, J. L. (1988). *FEBS Lett.* **232**, 308–312.
- Usobiaga, P., Medrano, F. J., Gasset, M., García, J. L., Saiz, J. L., Rivas, G., Laynez, J. & Menéndez, M. (1996). *J. Biol. Chem.* **271**, 6832–6838.
- Varea, J., Saiz, J. L., López-Zumel, C., Monterroso, B., Medrano, F. J., Arrondo, J. L. R., Iloro, I., Laynez, J. L., García, J. L. & Menéndez, M. (2000). *J. Biol. Chem.* **275**, 26842–26855.