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Crystallization and preliminary X-ray diffraction analysis of the peptidylprolyl isomerase Par27 of *Bordetella pertussis*

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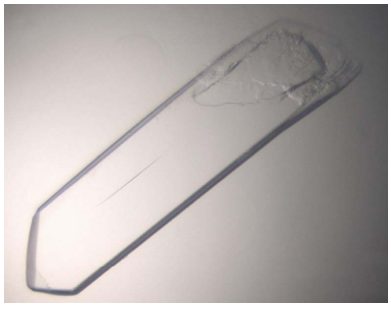
Proteins with both peptidylprolyl isomerase (PPIase) and chaperone activities play a crucial role in protein folding in the periplasm of Gram-negative bacteria. Few such proteins have been structurally characterized and to date only the crystal structure of SurA from *Escherichia coli* has been reported. Par27, the prototype of a new group of parvulins, has recently been identified. Par27 exhibits both chaperone and PPIase activities *in vitro* and is the first identified parvulin protein that forms dimers in solution. Par27 has been expressed in *E. coli*. The protein was purified using affinity and gel-filtration chromatographic techniques and crystallized in two different crystal forms. Form *A*, which belongs to space group *P*2 (unit-cell parameters $a = 42.2$, $b = 142.8$, $c = 56.0$ Å, $\beta = 95.1^\circ$), diffracts to 2.8 Å resolution, while form *B*, which belongs to space group *C*222 (unit-cell parameters $a = 54.6$, $b = 214.1$, $c = 57.8$ Å), diffracts to 2.2 Å resolution. Preliminary diffraction data analysis agreed with the presence of one monomer in the asymmetric unit of the orthorhombic crystal form and two in the monoclinic form.

1. Introduction

Proline isomerization occurs in numerous periplasmic proteins and is emerging as a potentially general mechanism for the control of protein folding and function. Proteins with peptidylprolyl isomerase (PPIase) activity have been classified into three distinct families: cyclophilins, FK506-binding proteins and parvulins (Gothel & Marahiel, 1999; Rahfeld *et al.*, 1994). Par27 is a novel periplasmic protein from the Gram-negative pathogen *Bordetella pertussis*, the whooping cough agent. This protein has recently been shown to be the prototype of a new group of parvulins (Hodak *et al.*, 2008). Par27 was initially identified in a search for accessory proteins implicated in the secretion of filamentous haemagglutinin (FHA), the major adhesin of *B. pertussis* (Clantin *et al.*, 2004, 2007; Hodak *et al.*, 2006). Par27 has been proposed to act as a general periplasmic chaperone in this bacterium (Hodak *et al.*, 2008).

The sequence analysis of Par27, a 241-residue protein, revealed similarities to the two parvulin domains of SurA, a periplasmic protein from *Escherichia coli*. SurA is known to possess two distinct activities: a chaperone activity involved in the biogenesis of several outer membrane proteins (OMPs) and a PPIase activity (Lazar *et al.*, 1998; Lazar & Kolter, 1996; Rouvière & Gross, 1996; Sklar *et al.*, 2007; Xu *et al.*, 2007; Stymest & Klappa, 2008). SurA contains two parvulin-like domains in tandem, only the second of which is catalytically active (Behrens *et al.*, 2001). The parvulin domains of SurA are flanked by an amino-terminal domain and a short carboxy-terminal tail that are absent in Par27, allowing conformational adaptations in these proteins that share similar catalytic domains.

The SurA crystal structure has been reported (Bitto & McKay, 2002), providing information on the parts of the protein that are responsible for these activities and to a certain extent on the structure



of the region that is conserved in Par27. SurA exhibits an asymmetric dumbbell shape in which the active PPIase domain forms a satellite module tethered to a larger core module that is implicated in the chaperone function and contains the N-terminal domain, the inactive PPIase domain and the C-terminal tail. This chaperone module facilitates the correct folding of OMPs in the absence of ATP or other energy sources (Behrens *et al.*, 2001). The mechanism by which protein folding is accomplished is poorly understood.

Interestingly, Par27 exhibits both chaperone and PPIase activities *in vitro* and is the first parvulin protein to be identified that forms dimers in solution (Hodak *et al.*, 2008). The central PPIase domain of Par27 is flanked by N- and C-terminal extensions that are found in a number of putative PPIases that are mostly present in β-proteobacteria (Hodak *et al.*, 2008). In order to progress in understanding the mechanism of protein folding in the periplasm of Gram-negative bacteria and to gain further insights into its structural determinants, we initiated a structural study of Par27 by X-ray crystallography. Here, we report the crystallization and preliminary X-ray characterization of this parvulin-like protein.

2. Experimental methods

2.1. Expression and purification of recombinant Par27

The construction of the Par27 expression plasmid, pHod1, has been described previously (Hodak *et al.*, 2008). This plasmid encodes the complete sequence of Par27 with the addition of the sequence MRGSHHHHHHGS at the N-terminal end. pHod1 was used to transform *E. coli* M15(pRep4) for expression. A single bacterial colony of M15(pRep4, pHod1) was used to inoculate an overnight culture of LB broth supplemented with ampicillin (100 µg ml^{-1}; A_{100}) and kanamycin (50 µg ml^{-1}; K_{50}) at 310 K. The culture was mixed with 25%(*w*/*v*) glycerol and stored at 193 K. A day before use, this stock solution of frozen bacteria was thawed, plated on a fresh agarose plate (LB + A_{100} + K_{50}) and incubated for 20 h at 310 K. Fresh bacterial colonies were used to inoculate an overnight preculture (LB + A_{100} + K_{50}) at 310 K. This preculture was used to inoculate 2 l fresh LB + A_{100} + K_{50} to an OD_{600} of 0.1; the culture was grown at 310 K on a rotating table (180 rev min^{-1}).

Protein expression was induced at an optical density (OD_{600}) of 0.6 with 0.1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) and the culture was grown for a further 3 h at 310 K. Cells were harvested by centrifugation (30 min at 6000*g* and 277 K) and bacterial pellets were stored at 253 K before further manipulation.

For protein purification, frozen cell pellets were thawed and resuspended in 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 5% glycerol, 50 µg DNase I and one antiprotease tablet (Complete EDTA-Free; Roche). Cells were disrupted by two consecutive runs in a French Press (6.9 MPa). Cells debris and insoluble material were removed from the crude extract by two centrifuge spins (30 min at 12 000*g* and 277 K). The supernatant was diluted in a final volume of 150 ml purification buffer (50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 5% glycerol, 5 m*M* imidazole) and filtered using a 0.22 µm filter.

A HiTrap chelating Sepharose column (5 ml; GE Healthcare) loaded with 100 m*M* $CoCl_2$ was used for purification of Par27 (Fig. 1*a*). The sample was loaded onto the column, which had previously been pre-equilibrated with purification buffer. Unbound material was washed off with 20 column volumes (CV) of purification buffer; the remaining contaminants were eliminated by successive washing steps of 10 CV each with increasing imidazole concentrations ranging from 10 to 100 m*M* with 10 m*M* increments. Par27 was eluted in purification buffer supplemented with 500 m*M* imidazole. The eluted fractions were analyzed by SDS–PAGE (15%, Coomassie Blue staining); fractions containing the target protein were pooled and concentrated. Approximately 15 ml eluted protein was concentrated to 3 ml in a Diaflow concentration device with a cutoff of 10 kDa (at 285 K). After spinning the concentrated protein using a microcentrifuge (15 min at 12 000*g* and 277 K), the supernatant was loaded onto a preparative gel-filtration column (Superdex75, Amersham Biosciences) equilibrated with protein crystallization buffer (10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl). Par27 was eluted in a single peak (Fig. 1*b*) and the 1 ml fractions were tested for homogeneity by SDS–PAGE (Fig. 1*c*). The fractions were pooled and concentrated to 100 mg ml^{-1} for crystallization screenings. The protein concentration was determined using the Bradford assay (Bradford, 1976; Protein Assay kit, Bio-Rad Laboratories) using BSA as a standard.

Selenomethionine (SeMet) incorporation was performed using standard procedures (Doublié, 1997) and the protein was purified using the same protocol as described above. Substitution of all eight methionines in recombinant Par27 by SeMet was demonstrated by MALDI–TOF spectrometry.

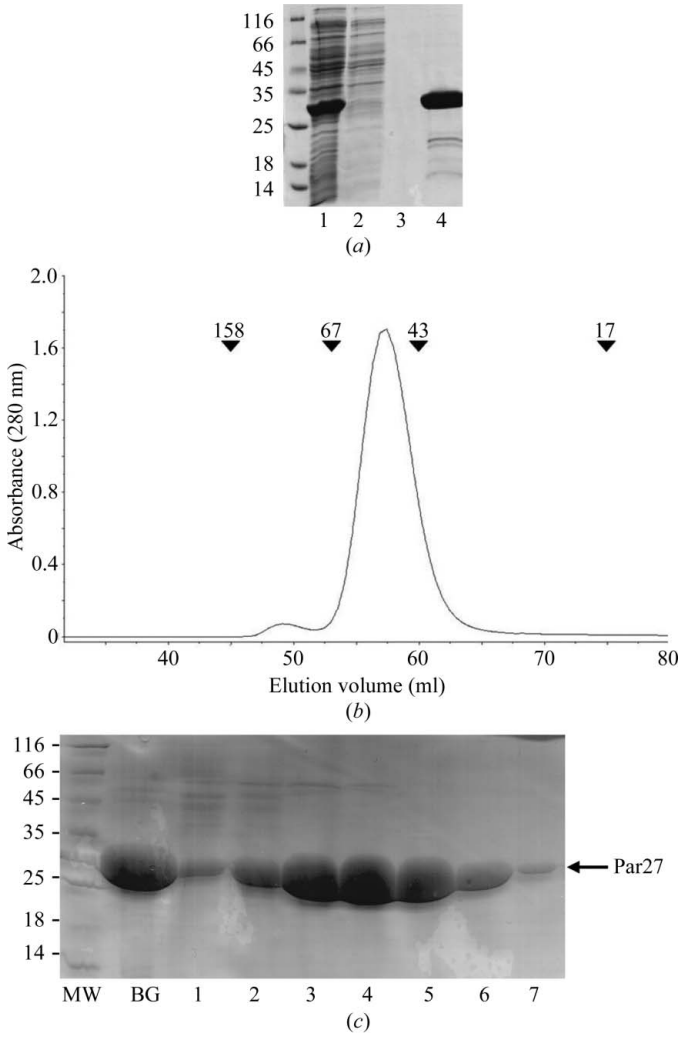
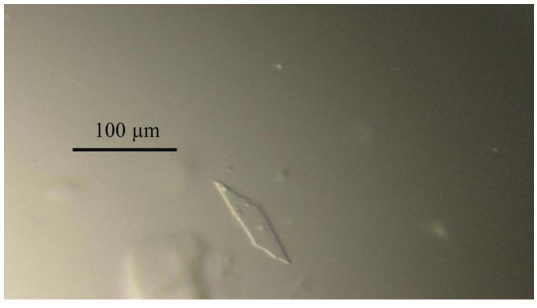


Figure 1
Purification of Par27. (*a*) Metal-chelate affinity chromatography. Lane 1, total extract after cell lysis; lane 2, flowthrough; lane 3, washing step; lane 4, elution with 500 m*M* imidazole. (*b*) Superdex75 16/60 preparative-grade gel filtration. The molecular weights of the protein standards are indicated with arrows at the top. (*c*) Coomassie Blue-stained SDS–PAGE of Par27 before gel filtration (BG) and Par27 fractions after gel filtration (1–7).

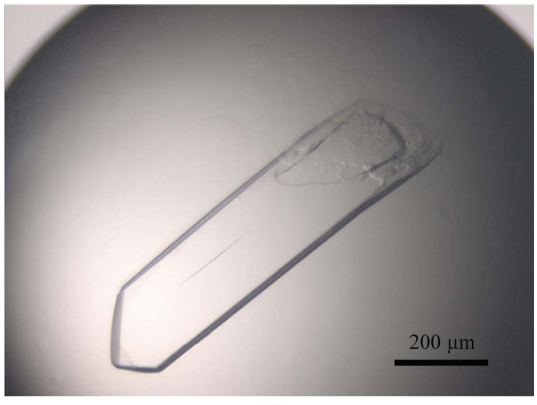
2.2. Crystallization

In all crystallization trials, recombinant Par27 was used at a concentration of 100 mg ml^{-1} in a buffer containing 10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl. The same protein concentration and buffer were used with the SeMet protein. Crystallization screening was carried out in 96-well plates (Greiner) in a sitting-drop vapour-diffusion setup using a CyBio-HTPC crystallization robot. In the screening, 0.5 µl protein solution was mixed with 0.5 µl precipitant solution and equilibrated against 50 µl precipitant solution. Initial crystallization screening using the pH Clear I Suite from Qiagen failed to give any positive results. Therefore, other commercially available screening kits were used, *i.e.* the PEGs Suite, MPD Suite, Classics Suite, Cryos Suite, Anions Suite and Cations Suite (all kits were purchased from Qiagen). Crystallization trials were carried out at 277 and 293 K. After 3 d, tiny crystals (30 µm) of form *A* appeared in condition A3 of the PEGs Suite screen, which contained 30%(*v*/*v*) PEG 400 in 0.1 *M* sodium acetate pH 4.6 at 293 K. The crystallization conditions were reproduced manually in larger drops containing 1 µl protein solution and 1 µl precipitant solution equilibrated against 0.5 ml precipitant solution using the hanging-drop vapour-diffusion technique. Larger crystals (∼100 µm) appeared after 7 d. At both temperatures tested (277 and 293 K) crystals grew in fine plates (Fig. 2*a*). Attempts to further improve the crystal quality by varying the precipitant concentration, pH or protein concentration or trying additive kits were not successful.

Despite all our attempts, crystal form *A* could not be obtained with the Se-labelled protein under similar crystallization conditions [30%(*v*/*v*) PEG 400 in 0.1 *M* sodium acetate pH 4.6 at 293 K]. Attempts to reproduce these crystals by varying the precipitant concentration and pH around these crystallization conditions also failed. An extensive crystallization screening was thus carried out manually with the Se-labelled protein. In the screening, 1 µl protein solution (100 mg ml^{-1} in a buffer containing 10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl) was mixed with 1 µl precipitant solution and then equilibrated against 0.5 ml precipitant solution. Commercially available screening kits were used, *i.e.* the pH Clear I Suite, the PEGs Suite, MPD Suite, Classics Suite, Cryos Suite, Anions Suite and Cations Suite. Crystallization trials were carried out at 277 and 293 K. No crystals were observed in the weeks following the screening setup. Finally, crystals (form *B*) appeared months later in one crystallization condition at 277 K in which the precipitant solution contained 40%(*v*/*v*) PEG 400 in 0.1 *M* citric acid pH 5.0.



(*a*)



(*b*)

Figure 2
(*a*) Crystal of Par27 in the primitive monoclinic space group *P*2 (form *A*). (*b*) Crystal of Par27 in space group *C*222 obtained using the selenomethionylated protein (form *B*).

Table 1
Data-collection statistics.

	Par27 form *A*	Par27 form *B*
Wavelength (Å)	0.9797	1.5418
Space group	*P*2	*C*222
Unit-cell parameters (Å, °)	*a* = 42.24, *b* = 142.79, *c* = 55.96, β = 95.11	*a* = 54.60, *b* = 214.13, *c* = 57.81
Resolution limits (Å)	10–2.8 (2.9–2.8)	15–2.22 (2.35–2.22)
$\langle I/\sigma(I)\rangle$	16.01 (4.54)	20.14 (4.97)
Observed reflections	62232	109882
Independent reflections	16191	15548
Redundancy	3.84 (3.87)	6.98 (6.46)
$R_{\rm merge}$† (%)	9.9 (38.5)	8.5 (29.9)
Completeness (%)	99.5 (99.9)	99.3 (97.0)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observed amplitude of reflection *hkl* and $\langle I_{hkl}\rangle$ is the mean amplitude for all observations *i* of reflection *hkl*.

2.3. Diffraction data collection and processing

Diffraction data were collected from crystal form *A* on the ID14-4 beamline at the ESRF (Grenoble, France). The Par27 crystals were flash-cooled in a nitrogen-gas stream at 100 K. Diffraction data were collected from a native crystal using X-rays of wavelength 0.979730 Å. Data were processed in the primitive monoclinic space group *P*2, with unit-cell parameters *a* = 42.24, *b* = 142.79, *c* = 55.96 Å, β = 95.108° and a completeness of 99.5% for the last resolution shell. The Matthews coefficient ($V_{\rm M}$; Matthews, 1968) was determined to be 2.98 Å^3 Da^{-1} with an estimated solvent content of 58.70%, assuming the presence of two molecules per asymmetric unit.

Diffraction data for the second crystal form obtained of Par27 using the selenomethionylated protein were tested on our in-house rotating-anode generator equipped with a MAR345 imaging-plate system. These crystals diffracted to at least 2.2 Å resolution. Data were processed in space group *C*222, with unit-cell parameters *a* = 54.5, *b* = 214.1, *c* = 57.8 Å. The Matthews coefficient ($V_{\rm M}$) was determined to be 2.97 Å^3 Da^{-1} with an estimated solvent content of 58.6%, here assuming the presence of one molecule per asymmetric unit. For both crystal forms *A* and *B* diffraction data were processed using the *XDS* package (Kabsch, 1993). The crystal parameters and data-collection statistics are summarized in Table 1.

3. Conclusion

In summary, Par27 from *B. pertussis* has been crystallized in two different crystal forms, one of which was obtained using selenomethionylated protein. This crystal form diffracts to 2.2 Å resolution in-house and is thus suitable for structure phasing and determination using the anomalous signal of the Se atoms. Data collection at the European Synchrotron Radiation Facility (ESRF, Grenoble) is planned for this crystal form. The comparison of this peptidylprolyl isomerase chaperone with other proteins sharing similar functions,

especially SurA, will shed further light on the molecular mechanisms of this important family of periplasmic proteins.

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