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Expression, crystallization and preliminary crystallographic data analysis of PigI, a putative L-prolyl-AMP ligase from the prodigiosin synthetic pathway in *Serratia*

Prodigiosin, a member of the prodiginines, is a tripyrrole red pigment synthesized by *Serratia* and some other microbes. A bifurcated biosynthesis pathway of prodigiosin has been proposed in *Serratia* in which MBC (4-methoxy-2,2'-bipyrrrole-5-carbaldehyde) and MAP (2-methyl-3-*N*-amyl-pyrrole) are synthesized separately and then condensed by PigC to form prodigiosin. The first step for the synthesis of MBC is the activation of L-proline by PigI, but its catalytic mechanism has remained elusive. To elucidate its mechanism, recombinant PigI was purified and crystallized. Crystals obtained by the sitting-drop method belonged to space group *P*1 and diffracted to 2.0 Å resolution, with unit-cell parameters $a = 51.2$, $b = 62.8$, $c = 91.3$ Å, $\alpha = 105.1$, $\beta = 90.1$, $\gamma = 92.2^\circ$. Matthews coefficient analysis suggested two molecules in the asymmetric unit, with a V_M of $2.6 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 52.69%.

1. Introduction

Serratia are Gram-negative bacteria that are taxonomically defined members of the Enterobacteriaceae (Giri *et al.*, 2004) which have been isolated from soil, water, insects and clinic patients (Grimont & Grimont, 2005). Normally environmental isolates of *Serratia* produce the tripyrrole pigment prodigiosin (Pig; 2-methyl-3-pentyl-6-methoxyprodigiosin; Harris *et al.*, 2004), a molecule that is a typical secondary metabolite, only appearing in the later stages of bacterial growth (Williams *et al.*, 1971; Williams, 1973; Williamson *et al.*, 2006), whereas in contrast to the environmental isolates, most clinical isolates with no pigments must rely on other biochemical markers for identification (Hejazi & Falkner, 1997; von Graevenitz, 1980).

Prodigiosin is a red hydrophobic pigment associated with the membrane fraction of *Serratia* cells. Even though the significance of prodigiosin in the host cell remains elusive, its nonphysiological functions have been described extensively and include antifungal, antibacterial, antiprotozoal/antimalarial, immunosuppressive and anticancer activities (Campàs *et al.*, 2003; D'Alessio *et al.*, 2000; Montaner *et al.*, 2000; Mortellaro *et al.*, 1999; Pérez-Tomás *et al.*, 2003; Zhou *et al.*, 2010).

The biosynthetic pathway for prodigiosin has been proposed to be a bifurcated pathway in which 2-methyl-3-amylpyrrole (MAP) and 4-methoxy-2,2'-bipyrrrole-5-carboxyaldehyde (MBC) are synthesized separately and then enzymatically condensed into 2-methyl-3-amyl-6-methoxyprodigiosene or prodigiosin by PigC (Williamson *et al.*, 2005; Harris *et al.*, 2004) (Fig. 1). Most of the genes in the biosynthetic gene cluster participating in the synthesis of prodiginines have been identified (Harris *et al.*, 2004; Williamson *et al.*, 2005). Fig. 1 shows the model of the biosynthetic pathway of prodigiosin in *Serratia*. PigA, PigG, PigH, PigI and PigJ have been demonstrated *in vitro* to comprise the assembly-line portion of the pathway that synthesizes 4-hydroxy-2,20-bipyrrrole-5-methanol (HBM; Garneau-Tsodikova *et al.*, 2006).

The MBC synthetic pathway is initiated by the conversion of L-proline to L-prolyl-AMP; the L-prolyl-AMP is then covalently tethered to the phosphopantetheinylated form of PigG by PigI. The L-prolyl-S-PigG is then desaturated to pyrrolyl-2-carboxyl-S-PigG by PigA, a 42 kDa flavoprotein desaturase (Garneau-Tsodikova *et al.*, 2006). Several crystallographic studies on the gene products in the prodigiosin biosynthetic gene cluster have been attempted, but none

Figure 1
The biosynthetic pathway of prodigiosin. The figure is reproduced from Williamson *et al.* (2005).

of them concerns the initiation of the MBC synthetic pathway (Whicher *et al.*, 2011; Liu *et al.*, 2012; Cho *et al.*, 2008). To elucidate the mechanism of the first step of MBC biosynthesis, we cloned and expressed the PigI gene from the previously isolated *Serratia* sp. FS14 (Zheng *et al.*, 2011) in *Escherichia coli*; the protein was then purified and crystallized.

2. Materials and methods

2.1. Gene cloning, protein expression and purification

The predicted open reading frame coding for PigI was amplified by PCR from a sample of *Serratia* sp. FS14 (Zheng *et al.*, 2011) with two primers covering the whole coding sequence. The oligonucleotides (restriction sites are shown in bold) 5'-ATAT**CATATG**-GCAACCTTCATTTACCG containing an *Nde*I restriction-enzyme site and 5'-ACT**CTCGAG**TCGCGCATTACCTCGGAC containing an *Xho*I site were used as the forward and reverse primers, respectively. The amplified PCR product was digested with *Nde*I and *Xho*I, and then subcloned into pET-24b (Novagen) in order to give a hexahistidine tag in the C-terminal part of the expressed protein. The construct was verified by restriction-enzyme digestion and DNA sequencing, and the encoded protein sequence encoded 491 residues and shared 99% identity with the previously reported sequence of

PigI from *S. marcescens* WW4 [GenBank AGE16900.1; the three different residues are S113(N), K269(R) and R430(K), where the letters in parentheses refer to strain WW4]. The PigI-encoding plasmid was transformed into *E. coli* C43 (DE3) cells for expression (Miroux & Walker, 1996). The cells were grown at 310 K in LB medium supplemented with kanamycin (30 µg ml⁻¹). Protein over-production was induced at an OD₆₀₀ of 1.2 with 0.5 mM IPTG and the cells were grown for a further 3 h at 303 K.

After harvesting by centrifugation, the cell pellet was resuspended in binding buffer consisting of 50 mM Tris-HCl pH 8.8, 300 mM NaCl, 5 mM imidazole, 10% glycerol and supplemented with 1 mM PMSF just prior to sonication. Sonication was carried out on ice with a 200 W output for a total time of 30 min with 1 s pulse-on and 3 s pulse-off. The cell lysate was clarified by centrifugation for 30 min at 15 000 rev min⁻¹ (Hitachi R20A2 rotor) and the target protein was purified by immobilized metal-affinity chromatography (IMAC) on Ni-NTA Superflow (Qiagen). The protocol used was as follows. The supernatant was loaded onto an Ni-NTA column previously equilibrated with binding buffer. After washing the column twice with six bed volumes of binding buffer containing first 5 and then 20 mM imidazole, the target protein was then eluted with elution buffer (50 mM Tris-HCl pH 8.8, 300 mM NaCl, 10% glycerol and 50 mM imidazole) (Fig. 2). Elution fractions were pooled and dialysed against 200 volumes of a solution consisting of 20 mM imidazole pH

Table 1

Data collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9792
Temperature (K)	100
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1.0
Total rotation range (°)	180
Space group	<i>P</i> 1
Unit-cell parameters (Å, °)	<i>a</i> = 51.2, <i>b</i> = 62.8, <i>c</i> = 91.3, <i>α</i> = 105.1, <i>β</i> = 90.1, <i>γ</i> = 92.2
Resolution range (Å)	20–2.0 (2.1–2.0)
Observed reflections	136034 (16449)
Unique reflections	68955 (8442)
Multiplicity	1.97 (1.95)
<i>R</i> _{merge} † (%)	10.9 (43.7)
Completeness (%)	91.9 (82.5)
<i>I</i> / <i>σ</i> (<i>I</i>)	8.16 (2.46)
<i>B</i> factor (Å ²)	27.25
Mosaicity (°)	0.243

† $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the observed intensity of the *i*th measurement and the mean intensity of the reflection with indices *hkl*, respectively.

6.5, 200 mM NaCl, 10% glycerol for 14 h. The protein sample was then centrifuged for 30 min at 15 000 rev min^{−1} and the supernatant was used for the methylation process using the protocol described by Walter *et al.* (2006).

After methylation, the protein sample was then centrifuged to remove precipitate. The supernatant containing methylated PigI was then concentrated and loaded onto a Superdex 200 (Pharmacia Biotech) column which was pre-equilibrated with the same buffer as used for dialysis and the column was then eluted with 1.2 bed volumes of the same buffer. The elution pattern showed two protein peaks, which were analysed by SDS–PAGE and the first small peak was found to be highly oligomeric protein (Fig. 3). The fraction corresponding to the second peak was pooled, concentrated and stored at 277 K. The protein purification was performed at 277 K.

Figure 2

Coomassie-stained SDS–PAGE analysis of PigI purified by IMAC. The contents of the lanes are as follows: lane 1, marker; lane 2, whole cell lysate; lane 3, supernatant of centrifugation after sonication; lane 4, flowthrough after the supernatant was loaded onto the Ni–NTA column; lanes 5 and 6, protein samples washed with binding buffer containing 5 and 20 mM imidazole, respectively; lanes 7, 8 and 9, the target protein eluted with buffer containing 50 mM imidazole. Molecular weights (kDa) are marked on the left and pigI protein is at 53 kDa.

2.2. Crystallization

Before the crystallization, L-proline was mixed with the protein at a final concentration of 1 mM. Initial screening was performed at 295 K by the sitting-drop vapour-diffusion method in 48-well plates. Crystallization trials were set up using Hampton Research screening kits with protein concentrations of 5 and 10 mg ml^{−1}. The crystallization drop was composed of 1 µl protein solution and 1 µl reservoir solution. The initial condition was further optimized and the best diffracting crystals were obtained with a reservoir solution consisting of 0.2 M ammonium sulfate, 0.1 M sodium cacodylate trihydrate pH 6.5, 28–34% (w/v) polyethylene glycol (PEG) 8000. Crystals grew to full size within several weeks (Fig. 4).

2.3. Data collection

Prior to mounting, the crystals were briefly soaked for several seconds in a cryoprotectant solution consisting of 0.2 M ammonium sulfate, 0.1 M sodium cacodylate trihydrate pH 6.5, 32% (w/v) PEG

Figure 3

Purification of pigI by gel-filtration chromatography. The chromatogram shows two main peaks; SDS–PAGE (12%) analysis of the proteins contained within these fractions shows that both peaks are PigI. The contents of the lanes are as follows: lane M, marker (labelled in kDa); lanes 1, 2 and 3, the protein sample from the bottom, middle and summit of the first small peak, respectively; lanes 4–8, protein samples from the bottom, middle and summit of the second large peak.

Figure 4

Crystal of PigI. The crystal shown is about 100 × 50 × 15 µm in size. The scale bar is 50 µm in length.

Figure 5
Diffraction image of a PigI crystal.

8000, 15%(v/v) glycerol and were then picked up in nylon loops and flash-cooled in liquid-nitrogen. Complete X-ray diffraction data sets were collected on beamline BL17U1 of the Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, People's Republic of China using an ADSC Q315r detector. The crystal-to-detector distance was set to 300 mm and the exposure time per frame to 1.2 s for data collection. The crystal was rotated through a total of 180° with an oscillation range of 1.0° per frame. The raw data were processed and scaled using the *XDS* package (Kabsch, 2010*a,b*). The data-collection and processing statistics are summarized in Table 1.

3. Results and discussion

Recombinant PigI with a His₆ tag at the C-terminus was successfully expressed in *E. coli* C43 (DE3) cells and was in a completely soluble form with a yield of about 20 mg purified protein per litre of culture. After purification by IMAC on Ni-NTA Superflow, a strong band corresponding to the expected molecular weight of PigI was observed on SDS-PAGE. Initially, despite extensive crystallization screening, no crystals were obtained for native PigI. Previous studies showed that alkylating the ϵ -amino group of lysines alters the hydrophobicity, solubility and pI of the protein, which may promote crystallization by altering sample-sample, sample-solvent and crystal-packing interactions (Rayment, 1997; Kim *et al.*, 2008; Walter *et al.*, 2006). Based on this theory, we analysed the protein sequence of PigI and found that the lysine composition of PigI is quite high (11 lysines); we therefore methylated the PigI protein and re-performed the crystallization screening with methylated PigI and methylated PigI with L-proline. In this round of screening, thin plate-shaped crystals were obtained at 295 K from the condition 0.2 M ammonium sulfate, 0.1 M sodium cacodylate trihydrate pH 6.5, 30%(w/v) PEG 8000 for the methylated PigI with L-proline. After optimization, high-quality diffraction data were collected to 2.0 Å resolution using synchrotron X-rays (Fig. 5). The crystals belonged to space group *P*1, with unit-

cell parameters $a = 51.2$, $b = 62.8$, $c = 91.3$ Å, $\alpha = 105.1$, $\beta = 90.1$, $\gamma = 92.2^\circ$ (Table 1).

Structure determination of PigI will be attempted by molecular replacement (MR) using the crystal structures of D-alanyl carrier protein ligase (DltA) as a template [PDB entries 3e7x, 3e7w (Yonus *et al.*, 2008), 3fcc, 3fce (Osman *et al.*, 2009) and 3dhv (Du *et al.*, 2008); 30% amino-acid sequence identity].

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