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Crystallization and preliminary X-ray analysis of the outer membrane pyoverdine receptor FpvA from *Pseudomonas aeruginosa*

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FpvA, the pyoverdine outer-membrane receptor from *Pseudomonas aeruginosa*, is involved in iron uptake when bacteria grow under iron limitation. Crystals of the *in vivo* pyoverdine-loaded FpvA were obtained under several crystallization conditions using different detergents. A native data set was collected at 3.6 Å resolution and a three-wavelength MAD data set was collected at 3.6 Å resolution using crystals of selenomethionine-substituted protein. The crystals grew under similar conditions and both belong to space group *C*2, but have different unit-cell parameters.

1. Introduction

Iron is one of the most abundant elements in the Earth's crust and is utilized by most living organisms (Posey & Gherardini, 2000). Iron is used in various cellular pathways involving, for example, oxidoreduction reactions, in which iron can lose or gain one electron. Under aerobic conditions its availability is very low as it forms ferric oxyhydroxide, which is insoluble in water. In cells it is stored as an inorganic complex by ferritin, bacterioferritin, transferrin or lactoferrin and its concentration in fluids is very low ($\sim 10^{-18}$ *M*; Posey & Gherardini, 2000). Receptor proteins in the outer membrane of Gram-negative bacteria are involved in the uptake of vitamin B_{12} and iron-loaded siderophores. Siderophores are low-molecular-weight iron-carrier molecules produced in the extracellular medium by microorganisms. They solubilize iron and deliver it to iron-deficient cells. The siderophores display a high structural diversity (Neilands, 1995) and are transported, when loaded with iron, across the outer membrane *via* highly specific outer membrane receptors. The energy required for transport is provided by the proton motive force, probably through interactions between the N-terminal part of the receptor and the energy-transducing TonB–ExbB–ExbD complex of the cytoplasmic membrane (Moeck & Coulton, 1998). In the last few years, the structures of the ferrichrome transporter FhuA (Ferguson *et al.*, 1998), the ferric enterobactin transporter FepA (Buchanan *et al.*, 1999) and the ferric citrate transporter FecA (Ferguson *et al.*, 2002), all from *Escherichia coli*, have been solved using the MAD method.

Pseudomonas aeruginosa is an opportunistic pathogen of a broad range of host organisms (He *et al.*, 2004). The *fpvA* gene product of *P. aeruginosa* is an outer membrane protein (FpvA; MW = 86 469 Da) involved in iron transport *via* pyoverdine (Pvd), the major siderophore produced by this bacterium (Poole *et al.*, 1993). Like the *E. coli* ferric citrate FecA receptor, FpvA belongs to a subfamily of siderophore outer-membrane receptors that are able to regulate *via* their N-terminal end the expression level of the receptor itself and of other proteins involved in iron uptake (Braun *et al.*, 2003). Moreover, these two receptors are able to bind the corresponding apo and ferric siderophore (Schalk *et al.*, 2002; Yue *et al.*, 2003).

Owing to the importance of *P. aeruginosa* in human infections and its growing resistance to antibiotics, we undertook crystallographic study of the so-called '*in vivo*' FpvA–pyoverdine complex (FpvA-Pvd; Folschweiller *et al.*, 2002). Here, we describe the crystallization and preliminary data analysis of *in vivo* FpvA-Pvd and the corresponding selenomethionine-labelled protein [(SeMet)-FpvA-Pvd].

2. Materials and methods

2.1. Expression and purification

FpvA-Pvd was purified from the FpvA-overproducing and Pvd-producing K691-(pPVR2) *P. aeruginosa* strain (Schalk *et al.*, 1999) grown in succinate medium (Demange *et al.*, 1990) in the presence of 150 µg ml^{-1} carbenicillin. (SeMet)-FpvA-Pvd was produced in the PAO503/pPVR2 *P. aeruginosa* strain auxotrophic for methionine. The cells were grown in succinate medium supplemented with 150 µg ml^{-1} L-selenomethionine (Acros) in the presence of 150 µg ml^{-1} carbenicillin. (SeMet)-FpvA-Pvd was purified using the same protocol as for FpvA-Pvd. A selenomethionine incorporation of greater than 95% was determined by MALDI mass spectroscopy (unpublished results).

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Table 1
Crystallographic data statistics for native FpvA-Pvd.

Values in parentheses are for data in the highest resolution shell.

Space group	*C*2
Wavelength (Å)	0.85505
Resolution (Å)	20.0–3.6 (3.73–3.60)
Total observations	147095
Unique reflections	80653
Completeness (%)	90.3 (87.2)
$I/\sigma(I)$	4.4
R_{sym} (%)	24.5 (52.3)

Table 2
Crystallographic MAD data statistics and crystallographic data statistics after merging of the Friedel mates at the Se peak (last column).

Values in parentheses are for data in the highest resolution shell.

Wavelength (Å)	0.979413	0.979632	0.977801	0.979413
Resolution (Å)	20.0–3.6 (3.7–3.6)	20.0–3.6 (3.7–3.6)	20.0–3.6 (3.7–3.6)	20.0–3.6 (3.7–3.6)
Total observations	143225	143783	126298	143238
Unique reflections	81262	81580	80495	41896
Completeness (%)	96.6 (81.9)	96.6 (81.5)	94.9 (70.0)	98.4 (96.0)
$I/\sigma(I)$	6.24 (0.81)	6.55 (0.69)	5.88 (1.21)	8.46 (1.14)
R_{sym} (%)	10.6 (45.4)	10.5 (55.3)	10.1 (32.1)	12.9 (37.5)

2.2. Crystallization

Crystallization experiments were carried out at 295 K with the Screen Lite sparse matrix from Hampton Research in Linbro Plates using the sitting-drop vapour-diffusion method. 1 µl protein solution was mixed with an equal volume of reservoir solution and equilibrated by vapour diffusion with the same precipitant. Several crystallization conditions were found using different detergents. The detergent exchanges were performed by gel-filtration chromatography or by ultrafiltration at 1770*g* using a 30 kDa molecular-weight cutoff (Amicon, Millipore).

2.3. Data collection and processing

X-ray diffraction experiments on native protein were performed at 100 K using crystals mounted in cryoloops and flash-frozen in liquid ethane. A data set was collected on beamline X06 at SLS using a MAR CCD detector. The three-wavelength MAD data set was collected on beamline BM30 at ESRF using a MAR CCD detector from a single crystal cryocooled in liquid nitrogen. The native data set was processed and scaled using *DENZO* and *SCALEPACK* (Otwinoski & Minor, 1997). The MAD data set was processed and scaled using *XDS* (Kabsch, 1993).

3. Results and discussion

Two procedures can be used to prepare FpvA-Pvd (Schalk *et al.*, 1999). The complex can either be produced *in vivo* by *P. aeruginosa* and subsequently purified or it can be prepared *in vitro* starting with a purified empty receptor incubated with Pvd. Time-resolved spectroscopy studies on FpvA-Pvd have shown a different proteic environment depending on how the complex was formed (Folschweiller *et al.*, 2002). For the crystallization studies described here, the first procedure was used, leading to the *in vivo* complex.

FpvA-Pvd and (SeMet)-FpvA-Pvd crystals were grown under similar conditions. 1–4 µl protein (5 mg ml^{-1}) in 0.75% C_8E_5 (Bachem) was mixed with an equal volume of crystallization solution composed of 13–16% PEG 4K, 20–25% ethylene glycol as cryoprotectant in 0.1 *M* sodium citrate buffer pH 5.6. Crystals appeared rapidly and reached their maximal size after a few days. They were long thin rods reaching up to 500 µm in length and approximately 100 µm in width (Fig. 1).

Native data were processed at 3.6 Å owing to the decay of the crystal in the X-ray radiation (Table 1). The crystal belongs to space group *C*2, with unit-cell parameters a = 144.9, b = 234.6, c = 236.9 Å, β = 102.3°. According to the Matthews coefficient calculation (Matthews, 1968), the asymmetric unit should contain six molecules with a corresponding V_M of 3.8 Å^3 Da^{-1} and a solvent content of 67.5%. Analysis of the self-rotation function calculated using *GLRF* (Tong & Rossmann, 1990) shows a non-crystallographic threefold axis in the κ = 120° section (peak height 516.03) perpendicular to the b axis and a non-crystallographic twofold axis (peak height 479.78) resulting from the orientation of the non-crystallographic threefold axis, suggesting the presence of two trimers in the asymmetric unit. Attemtps to solve the phase problem by molecular replacement using the atomic coordinates of the three iron-siderophore receptors solved by the MAD method were unsuccessful.

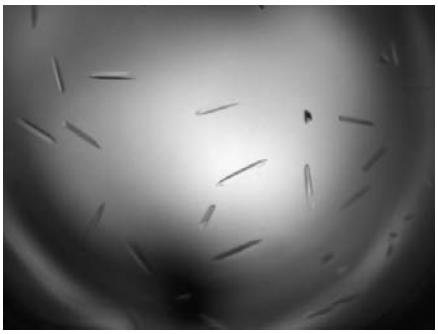

Figure 1
(SeMet)-FpvA-Pvd crystals. The crystals reached typical dimensions of 0.5 × 0.1 × 0.1 mm.

In order to solve the phase problem, the selenomethionine-labelled receptor was overexpressed and a three-wavelength MAD data set was collected at 100 K at the inflection point, peak and high-energy remote of the Se *K* edge on beamline BM30 at ESRF. The statistics are summarized in Table 2. The (SeMet)-FpvA-Pvd crystals belong to space group *C*2, with unit-cell parameters a = 137.5, b = 231.3, c = 121.7 Å, β = 104.6°. Analysis of the self-rotation function shows also the same non-crystallographic symmetry as observed for the FpvA-Pvd crystals. On the basis of the solvent content (65.8%; V_M = 3.6 Å^3 Da^{-1}; Matthews, 1968) and analysis of the self-rotation function, three molecules were expected in the asymmetric unit. Trials to find 3 × 11 Se atoms were then carried out using *CNS_SOLVE* (Brünger *et al.*, 1998) and *SOLVE* (Terwilliger & Berendzen, 1999), but no solution was found for the phase problem. The peak data were also merged (Table 2) and molecular-replacement calculations using *AMoRe* (Navaza, 1994) were performed in the 10–5 Å resolution range with the atomic coordinates of FhuA (Ferguson *et al.*, 1998) as a template. Several solutions were found for the cross-rotation function, three of which led to a good solution after the translation and the fitting steps. The correlation coefficient and R factor were 35 and 51%, respectively. Crystal-packing analysis confirmed this solution, also showing the non-crystallographic threefold and twofold axes. Model rebuilding is now under way. The failure to solve the phase problem using the native data set was probably more related to the large number of FpvA-Pvd molecules in the asymmetric unit than to the low sequence identity between the primary structures of the siderophore receptors.

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