The putative siderophore-interacting protein from *Vibrio anguillarum*: protein production, analysis, crystallization and X-ray crystallographic studies

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Received 26 January 2018

Accepted 29 March 2018

Edited by R. L. Stanfield, The Scripps Research Institute, USA

Keywords: *Vibrio anguillarum*; siderophore-interacting proteins; iron release; siderophores.

Supporting information: this article has supporting information at journals.iucr.org/f

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Siderophore-interacting proteins (SIPs) play an important role in iron acquisition in many bacteria. SIPs release iron from the internalized ferric siderophore complex by reducing ferric iron to ferrous iron, but how the iron is reduced is not well understood. Here, a *sip* gene was identified in the genome of *Vibrio anguillarum* 775. To further understand the catalytic mechanism of the protein, the SIP was overexpressed in *Escherichia coli* Rosetta (DE3) cells, purified and crystallized for X-ray diffraction analysis. The crystal diffracted to 1.113 Å resolution and belonged to space group $P2_1$, with unit-cell parameters $a = 64.63$, $b = 58.47$, $c = 70.65$ Å, $\beta = 114.19^\circ$.

1. Introduction

Iron plays a key role in many cellular processes such as cell respiration and the tricarboxylic acid cycle (Crosa *et al.*, 2004). When iron is deficient in their surroundings, bacteria secrete siderophores, small-molecular compounds that chelate ferric iron with high affinity and specificity, and then transport the ferric siderophore into the cells. Although the ferrisiderophore complex is absorbed by the cells, only free iron that has been released from the complex can be further used. This iron-release procedure usually involves hydrolytic cleavage of the siderophore by esterases (Brickman & McIntosh, 1992) or the reduction of the ferric iron to ferrous iron, which is much less strongly chelated by the siderophore (Miethke *et al.*, 2011). Many bacteria employ key enzymes called siderophore-interacting proteins (SIPs) to accomplish the reduction of iron.

The *sip* gene was originally identified in *Vibrio cholerae*. It encodes ViuB, a 271-amino-acid cytoplasmic protein that is involved in the utilization of ferric vibriobactin (Butterton & Calderwood, 1994). Homologues of ViuB were subsequently widely found in other bacteria and these SIPs are involved in bacterial growth and virulence (Ryndak *et al.*, 2010; Tu *et al.*, 2014). SIPs are FAD-containing proteins that catalyze NAD(P)H-dependent iron reduction (Bamford *et al.*, 2008; Miethke *et al.*, 2011; Li *et al.*, 2015). Nevertheless, how SIPs release the iron from the ferric siderophore complex by reduction is unclear. Based on the length of their C-terminal sequences, SIPs can be divided into two groups: members of group I contain a short C-terminal sequence and are represented by YqiH from *Escherichia coli* (Miethke *et al.*, 2011), while members of group II contain an extra C-terminal sequence and are represented by FscN from *Thermobifida fusca* (Li *et al.*, 2015). Interestingly, YqiH can reduce ferric iron from a broad spectrum of siderophores (Miethke *et al.*,

Table 1
Macromolecule-production information.

Source organism	<i>V. anguillarum</i> 775
DNA source	Genomic DNA
Forward primer†	AAACCATGGTGAAAAAGAAACAGAACCTAA ACG
Reverse primer†	AAACTCGAGTTATGACTGAGTTTGTGCATCC
Cloning vector	pETM-11
Expression vector	pETM-11
Expression host	<i>E. coli</i> Rosetta (DE3)
Complete amino-acid sequence of the construct produced‡	GAMVKKETEPKRSFYPLTVADSSQISPSLQR ITLQGDSIGHFTLENEGDIKLLFSEDGG TDLRLTPDQRQIMRTYIIRSFATNNRI EIDFVRHEAEDKQCGFAVRWAMHTAIGDT ISIAQPGKAQGLNPNKWFLLAADMTAIP ALAAQLKRLPRDAKGYAVIEIEHVDDKQA LQAPENIEISWVVKDSSTNLATSVIEKNW LGENGSVWCACEFDTMRALREYFRNEKEI AKDYIYISSYWKRGVSEDGHKQLKQTDQ TQS

† The NcoI and XhoI restriction sites are underlined. ‡ The vector residues are underlined.

2011), while FscN shows specificity towards fuscachelin (Li *et al.*, 2015). Whether the substrate specificity is related to the extra C-terminal region is unknown.

V. anguillarum 775, a pathogenic bacterium that causes vibriosis in the aquaculture industry worldwide (Frans *et al.*, 2011), contains a putative *sip* gene (Li & Ma, 2017). The encoded SIP is similar to FscN from *T. fusca* in having a long C-terminal region; however, it probably has a different ferric siderophore preference because *V. anguillarum* 775 produces anguibactin, a siderophore that is distinct from fuscachelin, to acquire iron (Lemos *et al.*, 2010). To better understand the structural basis for the substrate preference of the SIP proteins, we subjected *V. anguillarum* 775 SIP (vaSIP) to a crystallographic study. Here, we report the purification, characterization and crystallization of recombinant vaSIP. The crystal diffracted to 1.113 Å resolution, which is the highest resolution observed so far for a member of the SIP family.

2. Materials and methods

2.1. Protein production and analysis

The *sip* gene fragment was amplified by polymerase chain reaction (PCR) from *V. anguillarum* 775 genomic DNA using the primers shown in Table 1. The PCR product was ligated into pETM-11 vector (EMBL) between NcoI and XhoI restriction sites. After transformation into *E. coli* Rosetta (DE3) cells, the recombinant protein with an N-terminal 6×His-tag fusion was overexpressed by an auto-induction method (Studier, 2005). Firstly, the cells were grown in 10 ml seed medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 2 mM MgSO₄, 0.2× trace elements, 8% (w/v) glucose, 25 mM Na₂HPO₄, 25 mM KH₂PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄] supplemented with 0.05 mg ml⁻¹ kanamycin and 0.034 mg ml⁻¹ chloramphenicol at 37°C and 150 rev min⁻¹. After 8 h, the expression strain was incubated in auto-induction medium [seed medium supplemented with 0.05% (w/v) glucose, 0.5% (w/v) glycerol and 0.2% (w/v) α-lactose] to induce protein expression for 24 h at 20°C and 180 rev min⁻¹.

The cells were harvested by centrifugation and then resuspended in buffer A (150 mM NaCl, 50 mM Tris–HCl pH 8.0). The resuspended cells were lysed by ultrasonic disruption and the lysate was centrifuged at 20 000g for 60 min at 4°C. The supernatant was applied onto a Ni–NTA column (GE Healthcare) that had been pre-equilibrated with buffer A. After washing with buffer A supplemented with 20 mM imidazole, the protein was eluted with buffer A supplemented with 200 mM imidazole. The eluted protein was exchanged into buffer A using a PD-10 column (GE Healthcare) and was then cleaved at 4°C by *Tobacco etch virus* protease (TEV) overnight. The TEV-digested protein was again subjected to a Ni–NTA column (GE Healthcare) to remove the uncleaved protein and TEV. The tag-free protein was finally purified using a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated with buffer B [150 mM NaCl, 10 mM HEPES pH 7.5, 1 mM dithiothreitol (DTT)]. The purified protein was pooled and concentrated for characterization and crystallization. The purity of the recombinant vaSIP was analyzed by SDS–PAGE and the oligomerization was determined using a Superdex 200 Increase column (GE Healthcare) equilibrated with buffer B. The column was calibrated with ferritin (440 kDa), catalase (250 kDa), bovine serum albumin (67 kDa), ovalbumin (44 kDa) and myoglobin (16.7 kDa). The absorption spectrum (250–600 nm) of the protein was measured using a UV-1801 spectrophotometer (Rayleigh, People's Republic of China).

2.2. Crystallization

Crystallization experiments were carried out by the sitting-drop vapour-diffusion method at 20°C. Crystallization conditions were screened using commercial kits (Crystal Screen, Crystal Screen 2 and Index from Hampton Research). Drops consisting of 0.7 µl protein solution and 0.7 µl reservoir solution were equilibrated against 70 µl reservoir solution in 96-well plates (Dragonfly, People's Republic of China).

Figure 1
Crystals of vaSIP. The crystal block used for X-ray diffraction data collection is circled.

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	48-well plates
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	60
Buffer composition of protein solution	10 mM HEPES, 150 mM NaCl pH 7.5, 1 mM DTT
Composition of reservoir solution	0.1 M KBr, 30% (w/v) mPEG 2000
Volume and ratio of drop	2.8 µl (1:1 mixture of protein and reservoir solution)
Volume of reservoir (µl)	100

Optimization of precipitant concentrations, pH values, protein concentrations and drop volumes of the initial screening conditions was performed in 48-well plates (XTalQuest, People's Republic of China) at 20°C. To verify the crystallized protein, the crystals collected from the optimized condition were washed three times in the reservoir solution. These

crystals were then dissolved in 5 ml buffer *B* (150 mM NaCl, 10 mM HEPES pH 7.5, 1 mM DTT) and concentrated to about 50 µl with 10 kDa cutoff concentrators (Vivaspin 6, Sartorius) for SDS-PAGE analysis. This dilution and re-concentration approach was performed to attempt to remove the remaining mPEG 2000 from the crystals, as PEG can influence protein mobility in SDS-PAGE (Odom *et al.*, 1997). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

A block from a single crystal of vaSIP (Fig. 1) was selected for X-ray diffraction analysis. Before flash-cooling in liquid nitrogen, the crystal was briefly soaked in a cryoprotectant consisting of 0.1 M KBr, 35% (w/v) mPEG 2000. Diffraction data were collected at 100 K on beamline BL19U1 of the National Facility for Protein Science Shanghai (NFPS) at Shanghai Synchrotron Radiation Facility (SSRF), People's Republic of China. 360 images were obtained using a 1° oscillation width. The data were processed using *autoPROC* (Kabsch, 2010; Vonrhein *et al.*, 2011). The statistics of data collection and processing are provided in Table 3. A native Patterson function (50–1.2 Å resolution) was calculated to detect translational noncrystallographic symmetry (NCS) using *FFT* (Winn *et al.*, 2011), while a self-rotation function (10–2 Å resolution) was calculated to detect rotational NCS using *POLARRFN* (Winn *et al.*, 2011).

3. Results and discussion

The vaSIP protein was successfully purified using Ni-NTA and Superdex 200 columns. The purified protein was analyzed by 12% SDS-PAGE, showing a single band with a molecular weight of about 30 kDa (Fig. 2*a*). This is consistent with the theoretical molecular weight of vaSIP (30 014 Da) calculated

Figure 2

Protein analysis of vaSIP. (*a*) Analysis of vaSIP on a 12% SDS-PAGE gel. Lane *M*, molecular-weight markers (labelled in kDa); lane 1, purified vaSIP; lane 2, dissolved crystals of vaSIP. (*b*) Molecular-weight estimation of vaSIP based on size-exclusion chromatography (Superdex 200 Increase). The vaSIP elutes with a retention volume of 15.74 ml. Molecular-weight standards (ferritin, 440 kDa; catalase, 250 kDa; bovine serum albumin, 67 kDa; ovalbumin, 44 kDa; myoglobin, 16.7 kDa) were used to calculate the calibration curve. [The *x* axis is the elution volume and the *y* axis is the logarithm of the molecular weight. The relationship between *x* and *y* is $y = -0.206x + 7.7433$.] The elution volume of vaSIP corresponds to an apparent molecular weight of 31.7 kDa. (*c*) The UV-visible absorption spectrum of vaSIP. Two absorbance peaks at 388 and 469 nm are indicated by arrows.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL19U1, NFPS, SSRF
Wavelength (Å)	0.97852
Temperature (K)	100
Detector	PILATUS 6M
Crystal-to-detector distance (mm)	198.99
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	0.1
Space group	$P2_1$
a, b, c (Å)	64.63, 58.47, 70.65
α, β, γ (°)	90.00, 114.19, 90.00
Mosaicity (°)	0.175
Resolution range (Å)	64.449–1.113 (1.116–1.113)
Total No. of reflections	1092888 (4224)
No. of unique reflections	166723 (883)
Completeness (%)	88.8 (43.3)
Multiplicity	6.6 (4.8)
$\langle I/\sigma(I) \rangle$	17.7 (2.1)
R_{meas}	0.061 (0.814)
$CC_{1/2}$	0.999 (0.669)
Overall B factor from Wilson plot (Å ²)	8.605

by *ProtParam* (<http://web.expasy.org/protparam>). To determine the oligomerization state of vaSIP, the purified protein was subjected to Superdex 200 Increase size-exclusion chromatography. According to the plot of $\log(\text{molecular weight})$ versus elution volume using known molecular-weight standards, the elution volume of vaSIP corresponds to an apparent molecular weight of about 31.7 kDa, indicating that vaSIP exists as a monomer in solution (Fig. 2*b*). In the UV–visible spectrum, the light-yellow protein showed absorption peaks at 388 and 469 nm, indicating that vaSIP is probably combined with FAD (Fig. 2*c*).

vaSIP at different concentrations was used to screen for initial crystallization conditions. Six crystallization conditions (Nos. 6 and 28 of Crystal Screen, and Nos. 4, 55, 64 and 96 of Index) were identified at a protein concentration of 60 mg ml⁻¹. Subsequently, condition No. 96 of Index was subjected to optimization. The best crystals were grown in a drop consisting of 1.4 µl protein solution (60 mg ml⁻¹) and 1.4 µl reservoir solution [0.1 M KBr, 30% (w/v) mPEG 2000] equilibrated against 100 µl reservoir solution. Plate-shaped and rod-shaped crystals of vaSIP appeared in the same drop within 1 d (Fig. 1). These crystals showed a yellowish colour, similar to the crystals of YqjH from *E. coli* (Bamford *et al.*, 2008) and *Shewanella frigidimarina* SIP (Trindade *et al.*, 2016), indicating the presence of the FAD cofactor. SDS–PAGE analysis of the dissolved crystals showed a single protein band of about 30 kDa, indicating that the full-length protein had crystallized (Fig. 2*a*). However, these single crystals tended to form clusters. We had to carefully break the clusters to obtain single-crystal blocks for the diffraction experiment.

One block from a rod-shaped single crystal (Fig. 1) was briefly incubated in a cryoprotectant solution consisting of 0.1 M KBr, 35% (w/v) mPEG 2000 before being plunged into liquid nitrogen. Diffraction data were collected using a PILATUS 6M detector on beamline BL19U1 of the NFPS at SSRF, People's Republic of China. The crystal diffracted to ultrahigh resolution (Fig. 3), so we moved the detector to the shortest distance allowed by the instrument settings to collect data. The diffraction data were processed with *autoPROC* using default parameters. The data statistics showed that the diffraction data is of high quality and could extend to better than 1.113 Å resolution, with an $\langle I/\sigma(I) \rangle$ value of greater than 2 in the outermost shell (Table 3). Unfortunately, we only

Figure 3

X-ray diffraction image of a vaSIP crystal with resolution rings shown.

collected incomplete data in the outermost shell, with a completeness of about 44%. Collecting data at a shorter wavelength could be helpful to achieve a higher resolution as well as better completeness of the data in the outermost shell. The crystal belonged to space group $P2_1$, with unit-cell parameters $a = 64.63$, $b = 58.47$, $c = 70.65$ Å, $\beta = 114.19^\circ$. The Matthews coefficient calculation suggested the presence of two monomers in the asymmetric unit, with a V_M of 2.05 Å³ Da⁻¹ and a corresponding solvent content of 39.97% (Matthews, 1968; Kantardjieff & Rupp, 2003). The native Patterson map showed a significant off-origin peak at (0.5, 0, 0.5) (Supplementary Fig. S1), indicating that the two monomers in the asymmetric unit are related by pseudo-translational symmetry. Therefore, the unit cell appears to be B -centred and pseudo- 2_1 axes that are parallel to the b direction would be expected. However, the self-rotation function did not show any significant NCS peaks. It seems that the signal from the pseudo- 2_1 NCS is masked by the 2_1 crystallographic symmetry. Interestingly, similar NCS symmetry was observed in the crystal of *S. frigidimarina* SIP, which also belongs to space group $P2_1$ but has different unit-cell parameters (Trindade *et al.*, 2016).

We searched the Protein Data Bank using the *BlastP* server and identified two homologous proteins to vaSIP. One is from *T. fusca* (PDB entry 4yhb; 2.2 Å resolution; Li *et al.*, 2015) and the other is from *S. putrefaciens* (PDB entry 2gpj; 1.89 Å resolution; Joint Center for Structural Genomics, unpublished work). These two proteins show sufficient sequence identity to vaSIP (39% for PDB entry 2gpj from *Shewanella putrefaciens* and 30% for PDB entry 4yhb from *T. fusca*) and their structures can be utilized as search models to solve the structure of vaSIP using molecular replacement. The vaSIP crystal diffracted to 1.113 Å resolution, which is the highest resolution to be observed for a member of the SIP family. The crystal structure of vaSIP would facilitate detailed understanding of the structural basis for substrate preference in the SIP family.

Acknowledgements

We thank the staff of the BL19U1 beamline of the National Facility for Protein Science Shanghai (NFPS) at Shanghai

Synchrotron Radiation Facility for assistance during data collection.

Funding information

This work was supported by awards from the 1000 Talents Program of China, the 100 Talents Program of the Chinese Academy of Sciences and the AoShan Talents Program of Qingdao National Laboratory for Marine Science and Technology (Grant No. 2015ASTP) to QM.

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