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Crystallization and preliminary X-ray crystallographic studies of VibE, a vibriobactin-specific 2,3-dihydroxybenzoate-AMP ligase from *Vibrio cholerae*

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Vibriobactin synthetases (VibABCDEFH) catalyze the biosynthesis of vibriobactin in the pathogenic bacterium *Vibrio cholerae*. VibE, a vibriobactin-specific 2,3-dihydroxybenzoate-AMP ligase, plays a critical role in the transfer of 2,3-dihydroxybenzoate to the aryl carrier protein domain of holo VibB. Here, the cloning, protein expression and purification, crystallization and preliminary X-ray crystallographic analysis of VibE from *V. cholerae* are reported. The VibE crystal diffracted to 2.3 Å resolution. The crystal belonged to space group $P2_1$, with unit-cell parameters $a = 56.471$, $b = 45.927$, $c = 77.014$ Å, $\beta = 95.895^\circ$. There is one protein molecule in the asymmetric unit, with a corresponding Matthews coefficient of 1.63 Å^3 Da^{-1} and solvent content of 24.41%.

1. Introduction

Iron is an essential element for most pathogenic bacteria and plays crucial roles in their growth, survival and pathogenicity (Marx, 2002; Braun, 2001). However, the concentration of free iron is extremely low in almost all aerobic environments and in mammalian hosts (Ratledge & Dover, 2000). To obtain enough iron, these pathogens have developed a variety of iron-acquisition systems; one prevalent mechanism is the biosynthesis and utilization of iron-sequestering compounds known as siderophores (Neilands, 1995; Crosa & Walsh, 2002). Siderophores are produced *in vivo* and are secreted into the bacterial living environment, where they can compete for iron with iron-binding proteins from the vertebrate host. The ferric siderophore complex is then transported into the cell with the assistance of corresponding TonB-dependent outer membrane receptors and ATP-binding cassette transporter system proteins (Klebba *et al.*, 1993; Postle, 1993; Higgins, 2001).

Like most pathogens, the organism *Vibrio cholerae* also has a complete requirement for iron (Wyckoff *et al.*, 2007) and produces a single catecholate-type siderophore vibriobactin consisting of three 2,3-dihydroxybenzoate (2,3-DHB) molecules, two L-threonine molecules and an uncommon norspermidine (NSPD) molecule that functions as the scaffold backbone of vibriobactin. During vibriobactin assembly, it has been shown that one 2,3-DHB molecule is directly attached to the norspermidine backbone, while the other two are joined to the backbone *via* L-threonine residues (Griffiths *et al.*, 1984).

The enzymes contributing to vibriobactin biosynthesis belong to the nonribosomal peptide synthetases (NRPSs; Marahiel *et al.*, 1997) and consist of several components, designated VibA, VibB, VibC, VibD, VibE, VibF and VibH (Fraser *et al.*, 2000). These seven proteins comprise the complete list of vibriobactin synthetases (Butterton *et al.*, 2000; Wyckoff *et al.*, 1997, 2001). VibA, VibB and VibC are required for the initial steps of 2,3-DHB synthesis from chorismate (Wyckoff *et al.*, 1997), while VibB, VibD, VibE, VibF and VibH are required for the late processes of vibriobactin assembly from 2,3-DHB, L-threonine and NSPD. Additionally, three ATP molecules are also involved in the late steps of vibriobactin production (Butterton *et al.*, 2000; Keating *et al.*, 2000*a*,*b*; Wyckoff *et al.*, 2001).

VibE, a vibriobactin-specific 2,3-DHB-AMP ligase, belongs to the large adenylate-forming enzyme superfamily (Keating *et al.*, 2000*a*). This group of adenylation enzymes are present either as an

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embedded component of a multi-domain NRPS module or as an independent protein (May *et al.*, 2002). The catalytic reaction of VibE is an ATP-dependent two-step process. The first reaction is the reversible activation of 2,3-DHB *via* the catalytic reaction between DHB and ATP, forming the intermediate DHB-AMP, which is known as the acyl adenylate, and releasing pyrophosphate. In the second step the activated substrate is specifically thioesterified to the phosphopantetheine (pPant) arm covalently attached to the aryl carrier protein (ArCP) domain of holo VibB (Weber & Marahiel, 2001; Stachelhaus *et al.*, 1996), producing DHB-S-VibB. The product DHB-S-VibB is then directly connected to the primary amine of the norspermidine backbone *via* the enzymatic activity of the amide synthetase VibH (Keating *et al.*, 2000*a*; Wyckoff *et al.*, 2001). Homologues of the free-standing adenylation enzyme VibE exist in a great number of siderophore-biosynthesis pathogens, such as the enterobactin synthetase EntE from *Escherichia coli* (Raymond *et al.*, 2003), the acinetobactin synthetase BasE from *Acinetobacter baumannii* (Drake *et al.*, 2010), the bacillibactin synthetase DhbE from *Bacillus subtilis* (May *et al.*, 2002) and so on.

VibE plays an essential role in the biosynthesis of vibriobactin and may serve as a potential target of antimicrobial drugs; however, its crystal structure is not yet available. In this paper, we report the overexpression, purification and crystallization of VibE from *V. cholerae*. The crystal of ligand-free VibE diffracted to 2.3 Å resolution and its preliminary X-ray analysis is presented here.

2. Materials and methods

2.1. Cloning, expression and purification

The *vibE* gene (GenBank accession No. AAG00564) was PCR-amplified from genomic DNA of *V. cholerae* using the forward primer 5′-GATGTC**CATATG**ACAACCGATTTTACCCCT-3′ and the reverse primer 5′-ACGTCT**CTCGAG**CGACATTAACGCTAAGC-C-3′ (restriction sites are shown in bold). The purified PCR product was digested with *Nde*I and *Xho*I and ligated into the pET-29b(+) vector (Novagen), producing the construct pET-29b-*vibE* with a C-terminal His tag (LEHHHHHH) to facilitate protein purification.

The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells for expression. The cells were grown in LB broth medium containing 50 µg ml^{-1} kanamycin at 310 K to an optimal OD_{600} of 0.9. Expression of VibE was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.15 m*M* and the induced cells were cultured for an additional 10 h at 288 K.

The cells were harvested by centrifugation at 6000*g* for 15 min. The pellet was resuspended in lysis buffer (20 m*M* Tris–HCl buffer pH 8.0, 200 m*M* NaCl) and lysed by sonication on an ice–water mixture. After sonication, the cell lysate was centrifuged for 45 min at 28 500*g*. The supernatant was loaded onto an Ni-chelating Sepharose (GE Healthcare) affinity column pre-equilibrated with lysis buffer. The affinity column was extensively washed with washing buffer (20 m*M* Tris–HCl buffer pH 8.0, 100 m*M* NaCl, 15 m*M* imidazole) to remove contaminants. VibE was eluted with elution buffer (20 m*M* Tris–HCl buffer pH 8.0, 100 m*M* NaCl, 250 m*M* imidazole). The VibE was then loaded onto an ion-exchange column (Source 15Q, GE Healthcare) and eluted using a 140 ml linear gradient of 0–0.4 *M* NaCl. Finally, the collected VibE fractions were purified using a size-exclusion chromatography column (Superdex 200, GE Healthcare) equilibrated with 10 m*M* Tris–HCl buffer pH 8.0 containing 100 m*M* NaCl. The target protein was pooled according to protein purity as analyzed by SDS–PAGE and the final protein concentration was 15 mg ml^{-1}. All purification processes were performed at 277 K.

Table 1
X-ray data-collection and processing statistics for the VibE crystal.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 56.471, b = 45.927, c = 77.014, β = 95.895
Resolution (Å)	50–2.30 (2.38–2.30)
Total No. of reflections	57070
Unique reflections	17163
Completeness (%)	95.3 (93.6)
Multiplicity	3.3 (2.9)
$\langle I/\sigma(I)\rangle$	17.5 (5.54)
$R_{\rm merge}$ (%)	6.4 (22.4)
Matthews coefficient (Å^3 Da^{-1})	1.63
Solvent content (%)	24.41

2.2. Crystallization

Initial crystallization screens for VibE were carried out at 293 K using the sitting-drop vapour-diffusion method by mixing 1.2 µl protein solution and 1.2 µl crystallization buffer. About two months later, crystals were obtained under the following condition: 0.2 *M* $LiNO_3$, 25% PEG 3350. The VibE crystal was optimized using the hanging-drop vapour-diffusion method. The crystal quality was improved when the concentration of PEG 3350 was reduced to 13%.

2.3. Data collection and processing

Diffraction data were collected from a single crystal on Shanghai Synchrotron Radiation facility (SSRF) beamline BL17ul. In order to prevent radiation damage, the crystals were soaked in cryoprotectant buffer consisting of reservoir solution supplemented with 15%(*v*/*v*) glycerol and were then flash-cooled in a 100 K nitrogen stream. A 2.3 Å resolution diffraction data set was collected and was processed using the *HKL*-2000 software suite (Otwinowski & Minor, 1997). The diffraction data statistics are given in Table 1.

3. Results and discussion

The *vibE* gene from *V. cholerae* was successfully cloned and expressed in *E. coli* BL21 (DE3). The recombinant VibE protein was purified *via* three steps: Ni column chromatography, ion-exchange chromatography and size-exclusion chromatography. SDS–PAGE analysis showed that the purity of VibE was >97% and the molecular weight of VibE was determined to be approximately 60 kDa, which is in good agreement with the calculated value of 60 123.57 Da.

Figure 1
Crystals of VibE protein from *V. cholerae*. The dimensions of the VibE crystals are approximately 0.15 × 0.05 × 0.02 mm.

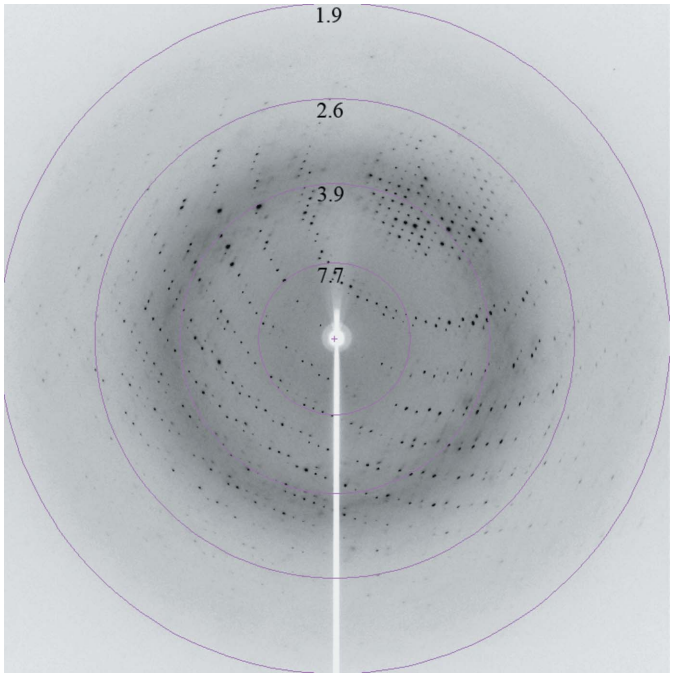

Figure 2
An X-ray diffraction image from a VibE crystal showing resolution circles (labelled in Å).

Optimized crystals were grown in a condition consisting of 0.2 *M* $LiNO_3$, 13% PEG 3350. An image of a VibE crystal is shown in Fig. 1. A diffraction data set was collected from a single crystal of this form and was processed to 2.3 Å resolution (Fig. 2, Table 1). The crystal belonged to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 56.471, b = 45.927, c = 77.014 Å, β = 95.895°. The Matthews coefficient (V_M; Matthews, 1968) was calculated to be 1.63 $Å^3$ Da^{-1} and the corresponding solvent content was estimated to be 24.41%, suggesting that there is only one protein molecule in the asymmetric unit.

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