Expression, purification and preliminary crystallographic analysis of a haem-utilizing protein, HutX, from *Vibrio cholerae*

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Vibrio cholerae, the causative agent of cholera, has developed a variety of mechanisms to obtain the limited-availability iron from human hosts. One important method for iron acquisition is through haem-uptake systems. Although the transport of haem has been widely studied, the fate of haem once it enters the cytoplasm remains an open question. Here, preliminary X-ray crystallographic analysis was performed on HutX, a member of the conserved haem-utilization operon from *V. cholerae* strain N16961. The crystals of HutX were found to belong to the orthorhombic space group $C22_21$, with unit-cell parameters $a = 50.1$, $b = 169.0$, $c = 81.8$ Å. There are two protein molecules in the asymmetric unit, with a corresponding Matthews coefficient V_M of 2.06 Å³ Da⁻¹ and a solvent content of 40.28%.

1. Introduction

Iron acquisition is indispensable for *Vibrio cholerae*, and iron plays a crucial role in the survival and pathogenicity of this bacterium (Wyckoff *et al.*, 2007). Nevertheless, iron availability is limited in most aerobic environments as well as in the human host. *V. cholerae* has evolved multiple iron-uptake systems, and one effective mechanism is the transport and utilization of haem (Stoebner & Payne, 1988). Several proteins have been identified to be involved in the *V. cholerae* haem iron-utilization system, including HutA, HutR and HasR, which are haem receptors in the outer membrane (Henderson & Payne, 1993, 1994; Mey & Payne, 2001), the TonB1 system, which transduces energy from the inner membrane to haem receptors (Occhino *et al.*, 1998), HutBCD, which is a periplasmic binding protein-dependent ABC transport system thought to transport haem across the inner membrane (Occhino *et al.*, 1998), and HutWXZ, which are putative haem-utilization proteins in the cytoplasm (Wyckoff *et al.*, 2004).

The HutX protein consists of 167 amino acids. A BLAST search against the National Center for Biotechnology Information (NCBI) database suggested that HutX belongs to the ChuX_HutX superfamily, and it is thought to act as a haem-carrier protein. HutX shares 37% sequence identity with ChuX from the pathogenic *Escherichia coli* O157:H7. Previous research showed that the structure of ChuX consists of two biological dimers in the asymmetric unit. Each dimer contains two putative haem-binding sites (Suits *et al.*, 2009). A number of site-directed mutants were generated to probe the haem-binding sites in the ChuX protein. However, owing to the lack of a protein–haem complex structure, the mechanism of haem utilization in these proteins remains unclear.

Table 1

Macromolecule-production information.

The restriction sites in the primers and the additional N-terminal residues in the sequence are underlined.

Source organism	<i>V. cholerae</i> O1 biovar El Tor strain N16961
DNA source	Genome
Forward primer	GAAATTCATATGTCATTACAACAGCAAGTC
Reverse primer	GAAATTCGAGTCAGTGTGCTGCTGCATTGC
Cloning vector	pET-15b(+)
Expression vector	pET-15b(+)
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MGSSHHHHHSSGLVPRGSHKHVHMSLQQQVA-QLLEQQPTLLPAAMAEQLNVTEFDIVHALP-EEMVAVVDGSHAQTILESLPEWGPVTTIMT-IAGSIFEVKAPFPKGVARGYYNLMGRDGE-LHGHLKLENISHVALVSKPFMGRESHYFGF-FTAQGENAFKIYLRDEKRELIPEQVARFK-AMQQQH

Table 2

Crystallization.

Method	Vapour diffusion
Plate type	Hanging drop
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	10–15
Buffer composition of protein solution	10 mM Tris–HCl pH 8.0, 100 mM NaCl
Composition of reservoir solution	0.2 M sodium acetate, 0.1 M Tris–HCl pH 8.5, 28% (w/v) PEG 4000
Volume and ratio of drop	4 µl, 1:1
Volume of reservoir (µl)	500

The crystals of HutX obtained in this study were of sufficient quality to collect high-resolution data sets, and preliminary X-ray crystallographic analysis was performed on the data sets. Attempts to obtain a HutX–haem complex are still ongoing.

2. Materials and methods

2.1. Macromolecule production

The gene encoding the HutX fragment (residues 3–165) was PCR-amplified from the *V. cholerae* O1 biovar El Tor strain N16961 genome with forward (5'-GAAATTCATATGTCATTACAACAGCAAGTC-3') and reverse (5'-GAAATTCCTCGAGTCAGTGTGCTGCTGCATTGC-3') primers (restriction sites are underlined; Table 1). The PCR fragments were digested with the NdeI and XhoI restriction enzymes prior to insertion into the pET-15b(+) vector (Novagen). The construct was then transformed into *E. coli* BL21 (DE3) cells. The cells were cultured in Luria–Bertani (LB) medium supplemented with 100 mg ml⁻¹ ampicillin at 310 K to an optimal OD₆₀₀ of 0.8. Expression of HutX was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.12 mM and the induced cells were cultured for an additional 12 h at 288 K.

The cells were harvested by centrifugation at 6000g for 15 min and resuspended in lysis buffer (25 mM Tris–HCl pH 8.0, 200 mM NaCl). After sonication, the cell lysate was centrifuged for 50 min at 28 500g. The supernatant was loaded

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL17U, SSRF
Wavelength (Å)	0.97870
Temperature (K)	100
Detector	MAR Mosaic 225 CCD
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	1.3
Space group	C222 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	50.1, 169.0, 81.8
α , β , γ (°)	90.0, 90.0, 90.0
Mosaicity (°)	0.646
Resolution range (Å)	50.00–2.00 (2.07–2.00)
Total No. of reflections	145751
No. of unique reflections	23449
Completeness (%)	97.6 (87.4)
Multiplicity	6.2 (4.6)
$\langle I/\sigma(I) \rangle$	21.32(3.62)
$R_{\text{merge}}^{\dagger}$ (%)	8.4 (32.3)
Overall <i>B</i> factor from Wilson plot (Å ²)	40.97

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean intensity of multiply recorded reflections.

onto an Ni-chelating Sepharose (GE Healthcare) affinity column pre-equilibrated with lysis buffer. The affinity column was extensively washed with wash buffer (25 mM Tris–HCl pH 8.0, 200 mM NaCl, 20 mM imidazole) to remove contaminants. The HutX protein was eluted with elution buffer (25 mM Tris–HCl pH 8.0, 100 mM NaCl, 500 mM imidazole). An ion-exchange column (Source 15Q, GE Healthcare) and a size-exclusion chromatography column (Superdex 200, GE Healthcare) were used for further purification. The yield of the purified protein was ~35 mg from 4 l of bacterial culture. The protein purity after nickel-affinity chromatography was analyzed by SDS–PAGE (Fig. 1). Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

For crystallization trials, the HutX protein samples were concentrated to 10–15 mg ml⁻¹. Initial crystallization experi-

Figure 1
SDS–PAGE analysis of the HutX protein purified by nickel-affinity chromatography. Lane *M*, protein marker (labelled in kDa); lane *C*, cell lysate; lane *S*, supernatant; lane *F*, flowthrough fraction; lane *W*, wash fraction; lane *E*, elution fraction.

ments were performed manually by the sitting-drop vapour-diffusion method at 289 K. A total of 576 different conditions from commercially available kits (Index, Crystal Screen, Crystal Screen 2, PEGRx 1, PEGRx 2, SaltRx 1, SaltRx 2 and Natrix from Hampton Research and Wizard I, Wizard II and Wizard III from Emerald Bio) were used for screening. 1.2 μ l protein sample was mixed with 1.2 μ l reservoir solution and equilibrated against 100 μ l reservoir solution. About two weeks later, crystals were observed in Crystal Screen conditions Nos. 22 and 39. Crystals from condition No. 22 [0.2 M sodium acetate, 0.1 M Tris–HCl pH 8.5, 30% (w/v) PEG 4000] were selected for further optimization using the hanging-drop procedure, in which 2 μ l protein sample was mixed with 2 μ l reservoir solution and equilibrated against 500 μ l reservoir solution. The concentration of PEG 4000 was changed from 20 to 40%, and diffraction-quality crystals (Fig. 2*a*) were grown using a reservoir solution consisting of 0.2 M sodium acetate,

0.1 M Tris–HCl pH 8.5, 28% (w/v) PEG 4000. The crystals from a single drop were washed several times in reservoir solution and dissolved in water for analysis by SDS–PAGE (Fig. 2*b*). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

For data collection, the optimized crystals were flash-cooled in liquid nitrogen with 15–20% (v/v) glycerol as a cryoprotectant. X-ray diffraction data were collected at 100 K on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF; Shanghai, People's Republic of China) equipped with an MAR Mosaic 225 CCD detector. The data were integrated and scaled with the *HKL-2000* program suite (Otwinowski & Minor, 1997). The diffraction data statistics are given in Table 3.

Figure 2
(*a*) Crystals of the HutX protein from *V. cholerae*. (*b*) SDS–PAGE analysis of the HutX protein from washed and dissolved crystals. Lane *M* contains protein marker (labelled in kDa). (*c*) An X-ray diffraction image of a $C222_1$ crystal of HutX. The resolution in Å is indicated by the purple circles. (*d*) Self-rotation function peaks ($\chi = 180^\circ$) calculated by *MOLREP*.

Figure 3
(a) Absorbance spectra of HutX with haem. (b) Gel-filtration analysis of recombinant HutX on Superdex 200 10/300 GL.

3. Results and discussion

Recombinant HutX protein (residues 3–165) with an N-terminal His tag was purified by nickel-affinity, ion-exchange and size-exclusion chromatography, and exhibited good behaviour during purification. SDS-PAGE analysis confirmed the purity of HutX. The molecular weight of the HutX protein was determined to be approximately 20 kDa

(Fig. 1), which is consistent with the calculated value of 21 025 Da. UV-absorption spectral analyses were performed to explore the haem-binding ability of HutX (Fig. 3a). The Soret peak for the HutX–haem complex was at 406 nm, which was significantly different from those of haem and the HutX protein, indicating that HutX binds to haem.

Crystals were obtained at 289 K by the hanging-drop vapour-diffusion technique. The crystals grew to maximal dimensions within two weeks (Fig. 2a). A diffraction data set for HutX was collected to a resolution of 2.0 Å (Fig. 2c, Table 3) at 100 K. The HutX crystal belonged to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 50.1$, $b = 169.0$, $c = 81.8$ Å. The Matthews coefficient V_M (Matthews, 1968) was calculated to be $2.06 \text{ Å}^3 \text{ Da}^{-1}$ and the corresponding solvent content was estimated to be 40.28%, suggesting that there are two protein molecules in the asymmetric unit, which was consistent with the self-rotation function peaks (Fig. 2d) calculated by *MOLREP* (Vagin & Teplyakov, 2010). The dimeric nature of HutX in solution was confirmed by gel-filtration analysis (Fig. 3b).

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