Acta Crystallographica Section F
**Structural Biology and Crystallization Communications**
ISSN 1744-3091

# Crystallization and preliminary crystallographic studies of *Campylobacter jejuni* ChuZ, a member of a novel haem oxygenase family

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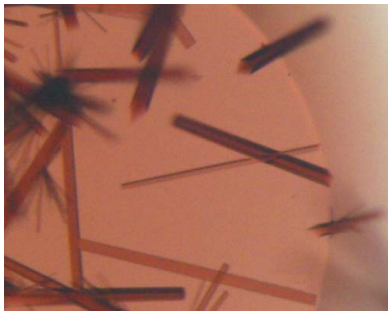
Received 27 May 2011
Accepted 1 July 2011





The haem oxygenase ChuZ from *Campylobacter jejuni*, a major enteric pathogen in humans, is part of the iron-acquisition mechanism that is involved in bacterial survival and persistence in hosts. The ChuZ–haemin complex has been purified and crystallized and diffraction data have been collected to 2.4 Å resolution. The ChuZ–haemin complex crystals belonged to space group $C222_1$, with unit-cell parameters $a$ = 106.474, $b$ = 106.698, $c$ = 52.464 Å, $\alpha = \beta = \gamma = 90^\circ$. The asymmetric unit contained one ChuZ monomer, with a Matthews coefficient of 2.58 Å$^3$ Da$^{-1}$.

## 1. Introduction

Iron is required by most living organisms. Iron acquisition is essential for the survival, persistence and pathogenicity of pathogenic bacteria invading animal hosts because free iron is maintained at very low levels by the hosts, partly in order to restrict microbial growth (Abraham *et al.*, 1996). Bacteria have developed several mechanisms to overcome iron shortages and to utilize iron in different forms (Wandersman & Delepelaire, 2004).

Because of the abundance of haem, usually in the form of haemoproteins, pathogenic bacteria in animal hosts prefer haem as the main source of iron (Otto *et al.*, 1992). They have developed sophisticated systems to capture haems, transfer them across bacterial cells and degrade them in order to retrieve free iron (Wilks & Burkhard, 2007). Haemoxygenases (HOs) play important roles in this process by catalyzing the oxidative cleavage of the porphyrin rings of haem. There is increasing evidence that HOs play a major role in iron availability in bacteria (Frankenberg-Dinkel, 2004).

HOs are monooxygenases that use haem both as a substrate and as a prosthetic group during cleavage. They utilize $O_2$ molecules as the oxidant and NADPH-cytochrome P450 reductase as the physiological electron donor in mammalian cells. In *in vitro* experiments, ascorbic acid can be used as the electron donor for HOs. All mammalian and most of the bacterial HOs studied to date share some sequence and structural similarities. They are all-$\alpha$-helical monomeric molecules. Recently, two new HOs, ChuZ from *Campylobacter jejuni* NCTC 11168 (Ridley *et al.*, 2006) and HugZ from *Helicobacter pylori* (Guo *et al.*, 2008), have been identified. These two proteins share 56% sequence identity with each other but do not have any sequence similarity to canonical HOs. Instead, they are weakly homologous to a family of FMN-binding proteins with a split-barrel fold. Genotyping experiments indicated that ChuZ is highly conserved in 32 clinical isolates and mutation of this protein leads to an inability to grow in an environment with haemin or haemoglobin as a sole source of iron. Haem degradation by *C. jejuni* requires ChuZ (Ridley *et al.*, 2006). Recent structural studies on *H. pylori* HugZ revealed that HugZ and *C. jejuni* ChuZ may form a new family of haem-binding proteins with split-barrel folds (Hu *et al.*, 2011). To further understand the structure and function of this new family of proteins, we expressed recombinant *C. jejuni* ChuZ protein in *Escherichia coli*, crystallized the protein and performed preliminary crystallographic studies. The results reported here laid a solid foundation for future studies of the high-resolution crystal structure of ChuZ.

## 2. Materials and methods

### 2.1. Cloning, expression and purification

The *chuZ* gene was amplified from the genome of *C. jejuni* and cloned into an expression vector derived from the pET22b plasmid (Novagen) and placed between *Nde*I and *Xho*I restriction sites. The insert was sequenced and found to be in complete agreement with the expected sequence. Because ChuZ has a strong innate affinity towards nickel–nitriloacetic acid resin, this affinity was utilized for its purification and no affinity tag was engineered into the construct.

The pET22b-*chuZ* plasmid was transformed into *E. coli* BL21 (DE3) competent cells (Invitrogen). The transformed cells were grown at 310 K in 1 l LB medium containing 100 µg ml$^{-1}$ ampicillin. Protein expression was induced by addition of isopropyl $\beta$-D-1-thio-galactopyranoside to a final concentration of 10 µ*M* when the optical density at 600 nm reached 0.6. The cells were grown for a further 12 h at 295 K and were then harvested by centrifugation at 4000 rev min$^{-1}$ for 30 min. The harvested cells were washed and resuspended in a solution consisting of 50 m*M* phosphate buffer pH 8.0, 300 m*M* NaCl and 10 m*M* imidazole and lysed by sonication at 273 K. The lysate was centrifuged at 16 000 rev min$^{-1}$ for 30 min (Sigma 3K30, 12150 rotor) and the supernatant was filtered (1 µm) to remove cell debris. The cell lysate was then loaded onto a nickel–nitriloacetic acid resin column (Novagen) and washed with 50 m*M* phosphate buffer pH 8.0 containing 300 m*M* NaCl and 20 m*M* imidazole. The ChuZ protein was eluted with the same solution containing 100 m*M* imidazole and was concentrated to 1 ml using a Millipore concentrator (10 kDa molecular-weight cutoff). The concentrated protein solution was loaded onto a HiTrap Desalting column (GE Healthcare) previously equilibrated with 50 m*M* Tris pH 8.0 buffer containing 150 m*M* NaCl. The protein was further purified on a HiLoad 16/60 Superdex 200 preparative-grade gel-filtration column (GE Healthcare) equilibrated with 20 m*M* Tris pH 8.0 and 100 m*M* NaCl. The protein fractions were pooled and stored at 277 K and the concentration was determined *via* the absorbance at 280 nm, assuming a molar extinction coefficient of 16 055 $M^{-1}$ cm$^{-1}$.

### 2.2. Reconstitution of ChuZ with haemin

The ChuZ–haemin complex was prepared as described previously (Wilks & Schmitt, 1998). Haemin solution (5 m*M* in DMSO) was slowly added to a solution of purified ChuZ in 20 m*M* Tris pH 8.0 and 100 m*M* NaCl to a final 2:1 molar ratio of haemin:ChuZ. Sodium azide dissolved in water at 0.5 *M* concentration was then added to the haemin–ChuZ mixture to a final concentration of 5 m*M*. The solution was incubated at 277 K overnight with shaking. After centrifugation, the supernatant was concentrated to 1 ml and loaded onto a HiLoad 16/60 Superdex 200 prep-grade gel-filtration column (GE Healthcare) equilibrated with 20 m*M* Tris pH 8.0 buffer containing 5 m*M* sodium azide. The majority of the ChuZ–haemin complex eluted as dimers based on the retention time. The fractions containing pure ChuZ–haemin complex were pooled and concentrated by ultrafiltration for crystallization.

### 2.3. Crystallization of the ChuZ–haemin complex

The crystallization conditions were determined by the microbatch technique using 72-well crystallization plates and several crystallization kits from Hampton Research (Index, SaltRx, PEG/Ion Screen, Crystal Screen, PEG/Tacsimate and PEGRx). Each drop was formed by mixing equal volumes (1 µl) of protein and reservoir solutions and was covered with 10 µl paraffin oil and left at 293 K. While ChuZ crystals were observed in several different conditions, those from PEGRx 1 condition No. 8 [0.1 *M* MES monohydrate pH 6.0, 22%(*v*/*v*) PEG 400] were found to have the best quality and this condition was used as the starting point for optimization. After more than 500 trials to optimize the crystallization conditions by varying the pH and the types and concentrations of PEGs, salts, buffers and organic compounds, needle-like crystals of diffraction quality were obtained from 0.1 *M* MES, 24%(*v*/*v*) PEG 400 and 0.1 *M* imidazole pH 6.5. Optimal protein concentration was screened independently and a final concentration of approximately 170 mg ml$^{-1}$ as determined by absorbance at 280 nm was used to obtain the crystals used for data collection.

### 2.4. Data collection and processing

All diffraction data sets were collected on beamline 17U of Shanghai Synchrotron Radiation Facility (SSRF; Shanghai, People's Republic of China) using a MAR 225 CCD detector. For cryoprotection, crystals were soaked in reservoir solution containing glycerol [concentration increased to 10%(*v*/*v*) stepwise] for 1 min before being mounted on nylon CryoLoops and flash-cooled in a cold nitrogen stream at 95 K. A data set to 2.4 Å resolution was collected at a wavelength of 0.9794 Å with a crystal-to-detector distance of 180 mm and 1° oscillation per frame. The exposure time for each frame was 1 s and the total oscillation range was 270°. The *HKL*-2000 package (Otwinowski & Minor, 1997) was used to index and integrate the collected frames.

## 3. Results

During the initial crystallization condition screening experiments, needle-like crystals were observed after 2 d and grew to dimensions of about 0.1 × 0.1 × 0.7 mm in a few days (Fig. 1). As a result of optimization, crystals suitable for high-resolution diffraction data collection were obtained *via* the microbatch method by mixing 1 µl ChuZ–haemin solution at a concentration of 170 mg ml$^{-1}$ with 1 µl reservoir solution consisting of 0.1 *M* MES, 24%(*v*/*v*) PEG 400 and 0.1 *M* imidazole pH 6.5. High protein concentration proved to be essential to obtain diffraction-quality ChuZ–haemin crystals: crystals obtained from identical conditions but with a lower protein concentration (85 mg ml$^{-1}$) diffracted to 3.5 Å resolution. We also found that ChuZ crystals obtained using the microbatch method were generally of better quality than those obtained using the hanging-drop method.

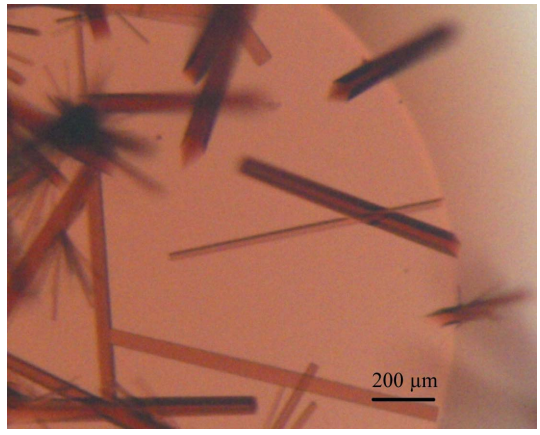



**Figure 1**
Crystals of the ChuZ–haemin complex.

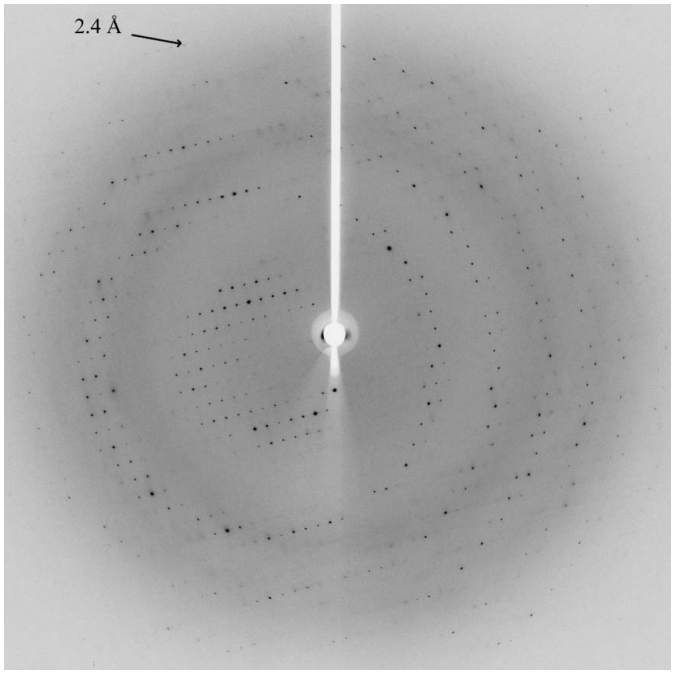



**Figure 2**
A representative diffraction pattern from a ChuZ–haemin complex crystal.

Data-collection statistics for the native crystal are given in Table 1. While we intended to collect more frames to improve the multiplicity and completeness, we had to stop data collection at 270° when the crystal quality deteriorated considerably owing to radiation damage. The crystal diffracted to a resolution of 2.4 Å (Fig. 2) and belonged to space group $C222_1$, with unit-cell parameters $a = 106.474$, $b = 106.698$, $c = 52.464$ Å, $\alpha = \beta = \gamma = 90^\circ$. Because the $a$ and $b$ unit-cell parameters were very similar to each other, we explored the possibility that the crystal might belong to a tetragonal space group. However, data integration in tetragonal space groups always resulted in very high $R_{\rm merge}$ values, indicating that the ChuZ crystals belonged to an orthorhombic space group. Calculations with the program *POINTLESS* (Evans, 2006) also showed $C222_1$ to be the correct space group. The Matthews coefficient was calculated to be 2.53 Å$^3$ Da$^{-1}$, with a solvent content of 51.4%, assuming one ChuZ–haemin monomer with a molecular weight of 29 319 Da in the asymmetric unit.

Structure determination by the molecular-replacement method is under way using the program *Phaser* (McCoy *et al.*, 2007) with the structure of *H. pylori* HugZ (Hu *et al.*, 2011) as the search model. Structural analysis of ChuZ and comparisons with HugZ will provide us with insight into the structure–function relationships of this new HO family from pathogenic bacteria.

**Table 1**
Data-collection statistics for a ChuZ–haemin complex crystal.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Space group | $C222_1$ |
| Unit-cell parameters | |
| $a$ (Å) | 106.474 |
| $b$ (Å) | 106.698 |
| $c$ (Å) | 52.464 |
| $\alpha = \beta = \gamma$ (°) | 90 |
| Wavelength (Å) | 0.9794 |
| Resolution (Å) | 50–2.4 (2.44–2.40) |
| No. of unique reflections | 11199 |
| Completeness (%) | 93.8 (64.1) |
| Multiplicity | 7.8 (4.1) |
| Mean $I/\sigma(I)$ | 46.0 (4.0) |
| $R_{\rm merge}$† (%) | 7.2 (39.3) |

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the $i$th intensity measurement of reflection $hkl$ and $\langle I(hkl)\rangle$ is its average.

We thank Z. Li and C. Li of the Department of Neurology, The Second Hospital of Hebei Medical University for providing the genome of *C. jejuni* NCTC 11168. We thank the staff at beamline 17U of Shanghai Synchrotron Radiation Facility for their help with data collection. This project was supported by the National 973 Program (2011CB911103, 2011CB910304 and 2009CB522606) and National Natural Science Foundation of China grant 81072493.

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