Crystal structure of the putative cytoplasmic protein STM0279 (Hcp2) from *Salmonella typhimurium*

Qing-Peng Lin,^a Zeng-Qiang Gao,^b Zhi Geng,^b Heng Zhang^{b*} and Yu-Hui Dong^{b*}

Received 17 April 2017

Accepted 15 July 2017

Edited by N. Sträter, University of Leipzig, Germany

Keywords: type VI secretion system; crystal structure; haemolysin co-regulated protein; hexameric rings; T6SS assembly.

PDB reference: Hcp2 from *S. typhimurium*, 5xeu

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

^aSchool of Life Sciences, University of Science and Technology of China, Hefei 230027, People's Republic of China, and ^bBeijing Synchrotron Radiation Facility, Institute of High Energy Physics, Chinese Academy of Sciences, People's Republic of China. *Correspondence e-mail: zhangheng@ihep.ac.cn, dongyh@ihep.ac.cn

STM0279 is a putative cytoplasmic protein from *Salmonella typhimurium* and was recently renamed haemolysin co-regulated protein 2 (Hcp2), with the neighbouring STM0276 being Hcp1. Both of them are encoded by the type VI secretion system (T6SS) of the *Salmonella* pathogenicity island 6 (SPI-6) locus and have high sequence identity. The Hcp proteins may function as a vital component of the T6SS nanotube and as a transporter and chaperone of diverse effectors from the bacterial T6SS. In this study, the crystal structure and the oligomeric state in solution of Hcp2 from *S. typhimurium* (StHcp2) were investigated. The crystal structure refined to 3.0 Å resolution showed that the protein is composed of a β -barrel domain with extended loops and can form hexameric rings as observed in known Hcp homologues. Mutation of the extended loop was found to partly destabilize the hexameric conformation into monomers or cause the production of inclusion bodies, suggesting it has an important role in hexameric ring formation.

1. Introduction

The type VI secretion system (T6SS) is a novel multi-protein needle-like apparatus which is distributed widely in Gram-negative bacteria (Cascales & Cambillau, 2012; Silverman *et al.*, 2012). It plays an important role in many processes in bacterial life cycles, such as interspecies competition, biofilm formation and virulence-related processes (Hood *et al.*, 2010; Russell *et al.*, 2011; Ho *et al.*, 2014; Jiang *et al.*, 2014; Vettiger & Basler, 2016). The haemolysin co-regulated protein (Hcp) secreted by all characterized T6SSs binds specifically to cognate effector molecules as a chaperone and receptor of substrates, as well as being postulated to form part of the T6SS secretion tube. The structures of several Hcp homologues have been reported, such as Hcp1 and Hcp3 from *Pseudomonas aeruginosa* (Douzi *et al.*, 2014; Federico *et al.*, 2015; Lim *et al.*, 2015; Jobichen *et al.*, 2010; Osipiuk *et al.*, 2011). Significantly, all of them are composed of a β -barrel domain forming hexameric ring oligomers with an inner diameter of ~ 40 Å. The internal pore can only accommodate small folded proteins (< 20 kDa) or unfolded proteins, such as Tse1–3 from *P. aeruginosa* and EvpC from *Edwardsiella tarda* (Zheng & Leung, 2007). Negative-staining electron microscopy and mutational studies revealed that *P. aeruginosa* Tse1–3 bind to the inner surface of the Hcp hexamer (Silverman *et al.*, 2013).

STM0276 and STM0279 are encoded by the type VI secretion system (T6SS) of the *Salmonella* pathogenicity

© 2017 International Union of Crystallography

Table 1

Macromolecule-production information.

Source organism	<i>S. typhimurium</i>
DNA source	Genomic DNA
Forward primer	CGCGGATCCATGTCTTATGACATTTTCTG
Reverse primer	CCGCTCGAGTTAAATTTCTTTGTTGGCC
Cloning vector	pET-28a
Expression vector	pET-28a
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MGSSHHHHSSGENLYFEHMASMTGGQQMGRGM-SYDIFLKIDGIDGESMDKHKNEIEVLSWRWN-IHQESTMHAGSGLSGKVSVTNLSFEHYIDRA-SPNLFKYCSSGKHIPQAILVMRKAGGNPLEYL-KYTFTDLIIAMVSPSGSQGGEIASRESIELSF-STVKQEYVVQNQQGGSGGTITAGYDFKANKEI

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	48-well plate
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 150 mM NaCl
Composition of reservoir solution	2.0 M ammonium sulfate, 0.1 M ammonium acetate pH 5.6
Volume and ratio of drop	1 µl, 1:1
Volume of reservoir (µl)	1

island 6 (SPI-6) locus and share high sequence identity. They belong to the Hcp family and function as vital components of the T6SS nanotube, and were renamed *StHcp1* and *StHcp2*, respectively. Moreover, *StHcp1*, rather than *StHcp2*, is required to kill *Klebsiella oxytoca* *in vitro* by specific interaction with the T6SS peptidoglycan (PG) amidase effector Tae4 (Sana *et al.*, 2016). In this study, we present the crystal structure and oligomerization study in solution of *StHcp2*.

2. Materials and methods

2.1. Macromolecule production

The gene encoding full-length *StHcp2* was amplified from the *S. typhimurium* genomic DNA, and was cloned into pET-28a-plus (introducing an N-terminal TEV cleavage site, constructed by our laboratory). The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) cells for expression. Protein expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside at 289 K. The supernatant was loaded onto a 2 ml Ni-NTA resin column (GE Healthcare) and eluted with buffer *B* [25 mM Tris-HCl pH 8.0, 50 mM NaCl, 5%(v/v) glycerol] containing 250 mM imidazole. The proteins were further purified by ion-exchange chromatography and subsequent gel-filtration chromatography. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

StHcp2 was concentrated to ~10 mg ml⁻¹ using Millipore Amicon Ultra 10 kDa centrifugal filters. Crystallization screening was performed with kits from Hampton Research and Qiagen using the sitting-drop vapour-diffusion method at

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL19U1, SSRF
Wavelength (Å)	0.9792
Temperature (K)	100
Detector	PILATUS 6M
Crystal-to-detector distance (mm)	400
Rotation range per image (°)	1.0
Total rotation range (°)	360
Exposure time per image (s)	0.5
Space group	C2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	102.645, 147.786, 85.687
α , β , γ (°)	90.00, 123.74, 90.00
Mosaicity (°)	1.2
Resolution range (Å)	50.0–3.00
Total No. of reflections	58666
No. of unique reflections	21754
Completeness (%)	99.1 (99.5)
Multiplicity	2.7
$\langle I/\sigma(I) \rangle$	16.2 (1.5)
$R_{\text{p.i.m.}}$	0.054 (0.395)
R_{meas}	0.090 (0.664)
Overall <i>B</i> factor from Wilson plot (Å ²)	81.1

Table 4

Structure refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	32.8–3.00
Completeness (%)	98.6
σ Cutoff	1.34
No. of reflections, working set	19915
No. of reflections, test set	1055
Final R_{cryst} (%)	23.9
Final R_{free} (%)	28.0
No. of non-H atoms	5934
R.m.s. deviations	
Bonds (Å)	0.009
Angles (°)	1.220
Average <i>B</i> factor (Å ²)	104
Ramachandran plot	
Favoured regions (%)	88.9
Additionally allowed (%)	10.7
Outliers (%)	0.4

293 K. The initial crystal was grown by mixing 1 µl protein solution with 1 µl reservoir solution consisting of 2.0 M ammonium sulfate, 0.1 M ammonium acetate pH 4.6. The optimal crystallization conditions were refined to 2.2 M ammonium sulfate, 0.1 M ammonium acetate pH 5.6. 20% glycerol added to the crystallization condition was used as a cryoprotective solution to flash-cool the crystals in liquid nitrogen. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Before data collection, crystals were soaked for 5 s in a cryoprotectant consisting of 20%(v/v) glycerol in the crystal mother liquor and were then flash-cooled in liquid nitrogen. The temperature was held at 100 K in a cold nitrogen-gas stream during data collection. X-ray diffraction data were collected using a PILATUS 6M detector on beamline BL19U1 at the Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, People's Republic of China. All data sets were

processed with *HKL-2000* (Otwinowski & Minor, 1997). Data-collection and processing statistics are summarized in Table 3.

2.4. Structure solution and refinement

The initial phases were calculated using *Phaser* (McCoy *et al.*, 2007) with *Burkholderia pseudomallei* Hcp1 (PDB entry 3wx6; Lim *et al.*, 2015) as the search model. The phases from *Phaser* and the structure factors from *HKL-2000* were combined in *ARP/wARP* (Perrakis *et al.*, 1999). Some biases were reduced manually in *Coot* (Emsley & Cowtan, 2004). The program *phenix.refine* (Afonine *et al.*, 2012) was used to refine the structure and to append water molecules. *MolProbity* (Chen *et al.*, 2010) was used to validate the structure.

Refinement statistics are summarized in Table 4. *PyMOL* (<http://www.pymol.org>) was used to prepare structural figures.

3. Results

3.1. Overall structure of *S. typhimurium* Hcp2

The structure of *StHcp2* was solved by molecular replacement using the structure of Hcp1 (PDB entry 3wx6) from *B. pseudomallei* in space group *C2* as a search model, and was refined to final *R* and *R*_{free} factors of 0.24 and 0.28, respectively, at 3.0 Å resolution (Table 1). The asymmetric unit contains six *StHcp2* molecules with overall dimensions of

Figure 1

Overall structure of *StHcp2*. (a) Cartoon representation showing the *StHcp2* structure in rainbow (from the N-terminus in blue to the C-terminus in red). The missing residues are roughly depicted using dotted lines. (b) The *StHcp2* hexameric ring: a cartoon representation of one hexamer generated by the sixfold crystallographic symmetry (top view). The α-helix of each monomer is placed almost parallel to the crystallographic axis. The diameter of the internal ring is 40 Å, while the external diameter is 80 Å. (c) Stacking of two representative *StHcp2* hexameric rings (shown as light and dark grey surfaces) which form a tube-like architecture. (d) Structure-based sequence alignment of representative Hcp family members, including those from *Salmonella typhimurium* (*St_Hcp2*), *Pseudomonas aeruginosa* (*Pa_Hcp1*), *Edwardsiella tarda* (*Et_Hcp1*), *Acinetobacter baumannii* (*Ab_Hcp1*), *Escherichia coli* (*Ec_Hcp1*) and *Burkholderia pseudomallei* (*Bp_Hcp1*), performed using *ClustalX* (v1.81) and *ESPrpt* 3.0. Conserved residues are boxed in blue; identical and conserved residues are highlighted on a red background and as red letters, respectively.

$\sim 103 \times 148 \times 86$ Å comprising 158 residues (Tyr3–Glu160). The residues Gln36–Gly49 inclusive, as well as Gly90–Pro93, Glu118–Ile119 and Gln139–Gly144, were not observed in the electron-density map and were not included in the current model.

The *StHcp2* structure revealed a tight β -barrel domain (12 Å diameter) formed by two β -sheets that are comprised of four and five strands each (Fig. 1*a*). The barrel is flanked by an 11-residue α -helix (Ala65–Ser75) and an extended loop formed by Arg31–Leu56 (some residues are missing; discussed below). Similar loops that were defined in several Hcp homologues can protrude more than 20 Å away from the β -barrel core. The hexameric rings could be generated by sixfold symmetry (Fig. 1*b*). This stacking of hexameric rings can form a tube-like assembly (Fig. 1*c*), with an outer diameter of 80 Å and an inner diameter of 40 Å. A similar hexameric ring stacking which forms a nanotube has been reported in Hcp homologues (Douzi *et al.*, 2014; Federico *et al.*, 2015; Lim *et al.*, 2015; Jobichen *et al.*, 2010; Osipiuk *et al.*, 2011).

A DALI search (http://ekhidna.biocenter.helsinki.fi/dali_server) for globally similar proteins was performed within the Protein Data Bank (PDB). Significant structural similarity was found between *StHcp2* and Hcp homologues from other species, such as *Yersinia pestis* Hcp (PDB entry 3v4h; r.m.s.d. of 1.0 Å for 125 C α atoms; Center for Structural Genomics of Infectious Diseases, unpublished work), *P. aeruginosa* Hcp1

(PDB entry 1y12; r.m.s.d. of 1.1 Å for 131 C α atoms; Mougous *et al.*, 2006), *E. tarda* EvpC (PDB entry 3eaa; r.m.s.d. of 1.2 Å for 131 C α atoms; Jobichen *et al.*, 2010) and enteroaggregative *E. coli* Hcp1 (PDB entry 4hkh; r.m.s.d. of 1.5 Å for 124 C α atoms; Douzi *et al.*, 2014).

3.2. Oligomerization

The oligomerization state of wild-type *StHcp2* in solution was studied by analytical ultracentrifugation, using the purified protein at ~ 1.0 mg ml $^{-1}$. The results showed two peaks corresponding to the apparent molecular weights of tetrameric (86.2 kDa; $\sim 25\%$) and hexameric (143.0 kDa; $\sim 75\%$) *StHcp2*, while a dodecamer was not observed (Fig. 2*a*). Therefore, the model of ring interface interactions for this structure is likely to be the result of the crystal packing in this particular condition (Fig. 1*c*). In solution, EvpC (a homologue of *StHcp2*) from *E. tarda* exists as a dimer at low concentration and as a hexamer at higher concentration (Jobichen *et al.*, 2010). In addition, the Hcp from *A. baumannii* exists exclusively as a hexamer (Federico *et al.*, 2015), while that from *B. pseudomallei* exists predominantly as a hexamer (with a small amount of dodecamer; Lim *et al.*, 2015). It should be noted that the variable oligomerization state in solution may not reflect the intact status of Hcp *in vivo*, and the hexamer is

Figure 2

Mutation studies on the extended loop of *StHcp2* reveal its important role in stabilizing the overall structure of the hexameric rings. (a) Analytical ultracentrifugation analysis of the oligomerization state of wild-type *StHcp2* and the E37A mutant in solution, showing the partial disassembly of the hexameric ring into monomers (20.4 kDa) for the E37A mutant. (b) Structural superimposition of *StHcp2* (magenta) with *P. aeruginosa* Hcp1 (*PaHcp1*; PDB entry 1y12; green) and *B. pseudomallei* Hcp1 (*BpHcp1*; PDB entry 3wx6; yellow). The neighbouring subunit of the *StHcp2* ring is shown as a surface in wheat. The extended loop (Arg31–Leu56) in *StHcp2* can be defined based on the high similarity between the Hcp structures, although this loop conformation is variable in different species. The missing residues (Gln36–Gly49) are roughly depicted using dotted lines. (c) Expression of the Q36A and E37A mutants and the truncation Δ (E38–G49) in *E. coli*.

predominant in all species that are associated with nanotube formation.

3.3. The extended loop plays an essential role in stabilizing the overall structure of hexameric rings

The extended loop has been found to act as a key contact point between the interacting monomers of adjacent hexameric rings (Lim *et al.*, 2015). In our *StHcp2* structure, this loop corresponding to residues Arg31–Leu56 can be defined based on the sequence alignment of Hcp homologues with remarkable similarity (Figs. 1c and 2b), although the residues Glu36–Gly49 are missing in the flexible loop.

In order to evaluate the role of the extended loop in *StHcp2*, the Q36A and E37A mutants and the truncation Δ (E38–G49) were created to study their effect on the oligomerization state of wild-type *StHcp2* in solution. Unexpectedly, both the Q36A mutant and the Δ (E38–G49) truncation exist as inclusion bodies when overexpressed in *E. coli* and little recombinant protein could be obtained in the supernatant (Fig. 2c), indicating that these mutations may significantly affect the overall fold. Analytical ultracentrifugation showed that an additional peak corresponding to an apparent molecular weight of a monomer (20.4 kDa; $\sim$ 4.0%) appeared for the E37A mutant (Fig. 2a), as well as two peaks corresponding to tetramers (76.2 kDa; $\sim$ 26.5%) and hexamers (131.1 kDa; $\sim$ 69.5%) similar to those in wild-type *StHcp2*. This monomeric status has not previously been reported for known Hcp homologues.

4. Discussion

Our mutation studies on the extended protruding loop revealed that this loop plays an important role in stabilizing the overall conformation of the *StHcp2* monomer as well as the hexameric ring. Structural analysis showed that this loop is very close to the interface of the two subunits (Fig. 2b) and participates in the multiple contacts of the hexameric ring in the structures of Hcp homologues (Douzi *et al.*, 2014; Federico *et al.*, 2015; Lim *et al.*, 2015; Jobichen *et al.*, 2010; Osipiuk *et al.*, 2011). Here, we find that mutation of Glu37 to alanine (E37A) in the loop may partly destabilize the hexameric conformation into monomers. Meanwhile, the mutation of Gln36 to alanine (Q36A) or truncation of residues 38–49 [Δ (E38–G49)] may destroy the natural conformation and cause the formation of inclusion bodies. A recent study showed that the extended loop in *B. pseudomallei* Hcp1 (PDB entry 3wx6) shows a significant shift compared with other Hcp homologues, and mediates multiple contacts in the hexameric ring interface (Lim *et al.*, 2015). Q46A and E47A mutations in this loop caused Hcp to be unable to stack as dodecamers and are likely to disrupt the formation of the tube-like assembly (Lim *et al.*, 2015). These key residues in the extended loop will restrict the Hcp hexameric ring and may further collapse the tube assembly. Meanwhile, the conservation in these extended loops is quite low (apart from the highly conserved Gly45; Fig. 1d), and their conformations in known Hcp structures are

variable, which may cause their different roles in stabilizing the Hcp nanotube.

In conclusion, this structure–function study on *StHcp2* uncovers the critical role of the extended loop, which plays an essential part in stabilizing the overall conformation of the hexamer subunits. This will help us to further understand the role of *StHcp2* in the *S. typhimurium* T6SS assembly.

Acknowledgements

We thank the staff of beamline stations BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF) and 3W1A at Beijing Synchrotron Radiation Facility (BSRF) for providing technical support and for many fruitful discussions. We thank Xiaoxia Yu from the Institute of Biophysics, CAS for technical help with analytical ultracentrifugation analysis.

Funding information

Funding for this research was provided by: Strategic Priority Research Program of CAS (award No. XDB08030103); National Natural Science Foundation of China (award Nos. 31670059, 31570744).

References

- Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H. & Adams, P. D. (2012). *Acta Cryst.* **D68**, 352–367.
- Cascales, E. & Cambillau, C. (2012). *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **367**, 1102–1111.
- Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). *Acta Cryst.* **D66**, 12–21.
- Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* **D60**, 2126–2132.
- Douzi, B. S. S., Spinelli, S., Blangy, S., Roussel, A., Durand, E., Brunet, Y. R., Cascales, E. & Cambillau, C. (2014). *PLoS One*, **9**, e86918.
- Federico, M., Ruiz, E. S., Spínola-Amilibia, M., Torreira, E., Culebras, E. & Romero, A. (2015). *PLoS One*, **10**, e0129691.
- Ho, B. T., Dong, T. G. & Mekalanos, J. J. (2014). *Cell Host Microbe*, **15**, 9–21.
- Hood, R. D. *et al.* (2010). *Cell Host Microbe*, **7**, 25–37.
- Jiang, F., Waterfield, N. R., Yang, J., Yang, G. & Jin, Q. (2014). *Cell Host Microbe*, **15**, 600–610.
- Jobichen, C., Chakraborty, S., Li, M., Zheng, J., Joseph, L., Mok, Y.-K., Leung, K. Y. & Sivaraman, J. (2010). *PLoS One*, **5**, e12910.
- Lim, Y. T., Jobichen, C., Wong, J., Limmathurotsakul, D., Li, S., Chen, Y., Raida, M., Srinivasan, N., MacAry, P. A., Sivaraman, J. & Gan, Y.-H. (2015). *Sci. Rep.* **5**, 8235.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordóñez, C. L., Lory, S., Walz, T., Joachimiak, A. & Mekalanos, J. J. (2006). *Science*, **312**, 1526–1530.
- Osipiuk, J., Xu, X., Cui, H., Savchenko, A., Edwards, A. & Joachimiak, A. (2011). *J. Struct. Funct. Genomics*, **12**, 21–26.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Perrakis, A., Morris, R. & Lamzin, V. S. (1999). *Nature Struct. Biol.* **6**, 458–463.
- Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W. & Mougous, J. D. (2011). *Nature (London)*, **475**, 343–347.

- Sana, T. G., Flaughnatti, N., Lugo, K. A., Lam, L. H., Jacobson, A., Baylot, V., Durand, E., Journet, L., Cascales, E. & Monack, D. M. (2016). *Proc. Natl Acad. Sci. USA*, **113**, E5044–E5051.
- Silverman, J. M., Agnello, D. M., Zheng, H., Andrews, B. T., Li, M., Catalano, C. E., Gonen, T. & Mougous, J. D. (2013). *Mol. Cell*, **51**, 584–593.
- Silverman, J. M., Brunet, Y. R., Cascales, E. & Mougous, J. D. (2012). *Annu. Rev. Microbiol.* **66**, 453–472.
- Vettiger, A. & Basler, M. (2016). *Cell*, **167**, 99–110.
- Zheng, J. & Leung, K. Y. (2007). *Mol. Microbiol.* **66**, 1192–1206.