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Crystallization and preliminary X-ray diffraction analysis of the Cmr2–Cmr3 subcomplex in the CRISPR–Cas RNA-silencing effector complex

Clustered, regularly interspaced, short palindromic repeat (CRISPR) loci, found in prokaryotes, are transcribed to produce CRISPR RNAs (crRNAs). The Cmr proteins (Cmr1–6) and crRNA form a ribonucleoprotein complex that degrades target RNAs derived from invading genetic elements. Cmr2dHD, a Cmr2 variant lacking the N-terminal putative HD nuclease domain, and Cmr3 were co-expressed in *Escherichia coli* cells and co-purified as a complex. The Cmr2dHD–Cmr3 complex was co-crystallized with 3'-AMP by the vapour-diffusion method. The crystals diffracted to 2.6 Å resolution using synchrotron radiation at the Photon Factory. The crystals belonged to the orthorhombic space group *I*222, with unit-cell parameters $a = 103.9$, $b = 136.7$, $c = 192.0$ Å. The asymmetric unit of the crystals is expected to contain one Cmr2dHD–Cmr3 complex with a Matthews coefficient of $3.0 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 59%.

1. Introduction

Many prokaryotes employ a defence system against foreign genetic elements, such as plasmids and phages, which consists of clustered, regularly interspaced, short palindromic repeat (CRISPR) loci and CRISPR-associated (Cas) proteins (Marraffini & Sontheimer, 2008; van der Oost *et al.*, 2009; Deveau *et al.*, 2010; Karginov & Hannon, 2010; Terns & Terns, 2011). CRISPR loci are composed of conserved repeated sequences separated by variable spacer sequences which are identical to parts of the foreign DNAs. The primary transcripts from CRISPR loci are processed into short single-stranded RNA fragments (crRNAs) by the dedicated Cas proteins. The crRNAs and Cas proteins form effector complexes to cleave the invading genetic elements, in which the crRNAs act as guides for targeting mediated by base complementarity (Hale *et al.*, 2009; Garneau *et al.*, 2010; Jore *et al.*, 2011; Semenova *et al.*, 2011; Wiedenheft, Lander *et al.*, 2011; Jinek *et al.*, 2012).

There are two types of effector complexes in the CRISPR–Cas defence system: one targets DNA and the other targets RNA. In *Escherichia coli*, one crRNA and five protein subunits (CasA–E) form an effector complex known as Cascade (CRISPR-associated complex for antiviral defence; Brouns *et al.*, 2008). The Cascade complex recognizes invading DNAs by using crRNA and subsequently induces the cleavage of invading DNAs by the Cas3 nuclease/helicase. Structural analyses of the Cascade complex and its individual protein subunits provided detailed insights into the mechanism by which the invading DNAs are cleaved (Jore *et al.*, 2011; Wiedenheft, Lander *et al.*, 2011). DNA-targeting effector complexes also exist in many prokaryotes, such as *Bacillus halodurans*, *Pseudomonas aeruginosa*, *Streptococcus thermophilus* and *Staphylococcus epidermidis* (Marraffini & Sontheimer, 2008; Garneau *et al.*, 2010; Wiedenheft, Duijn *et al.*, 2011; Nam *et al.*, 2012).

RNA-targeting effector complexes have been found in *Pyrococcus furiosus* and *Sulfolobus solfataricus* (Hale *et al.*, 2009; Zhang *et al.*, 2012). The effector complex from *P. furiosus* consists of six Cmr proteins (Cmr1–6) and a 39- or 45-nucleotide (nt) crRNA (Hale *et al.*, 2009, 2012). The crRNAs from *P. furiosus* contain an invariant 8 nt tag at the 5' end, which is essential for the CRISPR-mediated RNA interference (Hale *et al.*, 2009, 2012). The sequence at the 3' end of the crRNA (31 or 37 nt, not including the 8 nt tag) is derived from the

Table 1

Data-collection statistics.

Values in parentheses are for the last shell.

Space group	<i>I</i> 222
Unit-cell parameters (Å)	<i>a</i> = 109.9, <i>b</i> = 136.7, <i>c</i> = 192.0
Wavelength (Å)	0.98
Resolution (Å)	50–2.60 (2.64–2.60)
Measured reflections	373906
Unique reflections	42206
<i>R</i> _{merge} [†]	0.063 (0.333)
<i>I</i> / <i>σ</i> (<i>I</i>)	50.5 (5.0)
Completeness (%)	99.7 (99.5)
Multiplicity	8.9 (7.0)

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity over symmetry-equivalent measurements.

spacer sequence and functions as a guide for targeting the complementary RNA (Hale *et al.*, 2009, 2012). The *P. furiosus* Cmr effector complex specifically cleaves the target RNA at a fixed distance (14 nt) from the 3' end of the crRNA (Hale *et al.*, 2009, 2012).

In contrast to the Cascade complex, the roles of the individual Cmr proteins in the Cmr effector complex have not been elucidated owing to a paucity of structural information. Recently, the crystal structure of a *P. furiosus* Cmr2 derivative (Cmr2dHD), which lacks its N-terminal HD nuclease domain, revealed that this protein contains two adenylyl cyclase-like domains that bind ADP and divalent metal ions at their domain boundary (Cocozaki *et al.*, 2012). However, the amino-acid residues responsible for ADP- and metal-ion-binding are not involved in the crRNA-guided RNA-cleavage activity of the effector complex. Therefore, the role of Cmr2 in the complex has remained elusive. A biochemical study of the Cmr effector complex in *S. solfataricus* revealed that Cmr2 associates with Cmr3 (Zhang *et al.*, 2012), but the precise interaction mechanism and the functional role of the Cmr2–Cmr3 complex in the activity have not been determined. We now report the crystallization and preliminary X-ray diffraction analysis of the complex of *P. furiosus* Cmr2dHD and Cmr3 bound with 3'-AMP, to clarify the crRNA-guided RNA-cleavage reaction by the Cmr effector complex.

2. Materials and methods

2.1. Protein preparation

The PCR-amplified genes encoding Cmr2dHD (residues 216–871) and Cmr3 were prepared as an operon construct, which was cloned into the pET28b expression vector. The recombinant Cmr2dHD, with an N-terminal His tag, and Cmr3 were co-expressed at 293 K in *E. coli* strain BL21-CodonPlus (DE3)-RIL. The harvested cells were resuspended in buffer *A* (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM imidazole) supplemented with 1 mM PMSF and 1 mM benzamidine and disrupted by sonication. After centrifugation, the clarified lysate was incubated at 338 K for 20 min. The heat-treated lysate was centrifuged and the supernatant was applied onto a Ni–NTA (Qiagen) column previously equilibrated with buffer *A*. The Cmr2dHD–Cmr3 complex was eluted from the Ni–NTA column with buffer *B* (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 250 mM imidazole). The eluate from the Ni–NTA column was dialysed against buffer *C* (20 mM Tris–HCl pH 7.0, 150 mM NaCl, 1 mM DTT) and was then loaded onto a HiTrap Heparin column (GE Healthcare) equilibrated with buffer *C*. A linear gradient was developed from 150 to 1000 mM NaCl in buffer *C*. The fractions rich in the Cmr2dHD–Cmr3 complex were combined and dialysed against buffer *D* (20 mM Tris–HCl pH 9.0, 100 mM NaCl, 1 mM DTT). The solution was then

applied onto a RESOURCE Q column (GE Healthcare), which had previously been equilibrated with buffer *D*, and the elution was performed with a linear gradient of 100–1000 mM NaCl in buffer *D*. The fractions enriched in the Cmr2dHD–Cmr3 complex were combined and dialysed against buffer *E* (20 mM Tris–HCl pH 7.5, 200 mM NaCl, 1 mM DTT). The purified complex was concentrated to 11 mg ml^{−1}, using Amicon Ultra-15 Centrifugal Filters (30 000 Da molecular-weight cutoff; Millipore).

2.2. Crystallization and X-ray data collection

Crystallization screening was performed by the sitting-drop vapour-diffusion method at 293 K. Sitting drops were prepared by mixing 1 µl reservoir solution with 1 µl protein solution (5 mg ml^{−1}) containing 2 mM ADP and 2 mM MgCl₂ and were equilibrated against 100 µl of reservoir solution. The crystals grew in reservoir consisting of 15% 2-propanol, 20 mM MgCl₂, 50 mM MES pH 6.0. The crystallization conditions were then optimized in the presence of 2 mM 3'-AMP, instead of ADP, by the hanging-drop vapour-diffusion method and crystals suitable for X-ray diffraction analysis were obtained under conditions consisting of 12.5–15% 2-propanol, 18 mM MgCl₂, 45 mM MES pH 6.0, 20 mM ammonium acetate, 10 mM sodium acetate, 3% PEG 4000.

The crystals were cryoprotected in the optimized crystallization conditions containing 25% glycerol and were flash-cooled in a nitrogen stream at 95 K. X-ray diffraction data were collected on beamline BL-17A of the Photon Factory, Ibaraki, Japan, using an ADSC Q270 CCD detector. The raw data were processed and scaled with *HKL-2000* (Otwinowski & Minor, 1997). The processing statistics are summarized in Table 1.

3. Results and discussion

The interaction between Cmr2 and Cmr3 has recently been suggested by Zhang *et al.* (2012). To elucidate the roles of the Cmr proteins in the RNA-silencing mechanism, we attempted to determine the crystal structure of the Cmr2–Cmr3 complex. Our first approach to prepare the individual *P. furiosus* Cmr2 and Cmr3 proteins was unsuccessful because only Cmr3 was produced as an inclusion body. To solve this problem, the genes encoding *P. furiosus* Cmr2dHD (residues 216–

Figure 1
The crystal of the 3'-AMP-bound Cmr2dHD–Cmr3 complex.

Figure 2
The diffraction patterns of the 3'-AMP-bound Cmr2dHD–Cmr3 complex.

871), a Cmr2 protein lacking the putative HD nuclease domain, and full-length Cmr3 (residues 1–322) were co-expressed in *E. coli* cells by using an operon construct, with a single His tag at the N-terminus of Cmr2dHD. The deletion of the putative HD nuclease domain in *P. furiosus* Cmr2 reportedly had no effect on the RNA-cleavage activity by the Cmr effector complex (Cocozaki *et al.*, 2012). Eventually, the co-expression of these proteins allowed us to obtain the soluble Cmr3 protein in complex with Cmr2dHD. The interaction between Cmr2dHD and Cmr3 is strong, and the proteins were purified as a complex through several chromatography steps. Analytic gel-filtration chromatography on a Superdex 200 HR 10/300 column revealed that the Cmr2dHD–Cmr3 complex migrated as a single peak corresponding to a molecular weight of 118 kDa (data not shown). Based on the molecular masses of Cmr2dHD (76 kDa) and Cmr3 (36 kDa), this result indicated that the Cmr2dHD–Cmr3 complex forms a 1:1 heterodimer.

The purified Cmr2dHD–Cmr3 complex was used for crystallization screening by the sitting-drop vapour-diffusion method at 293 K. We obtained crystals of the Cmr2dHD–Cmr3 complex in the presence of 2 mM ADP under conditions consisting of 15% 2-propanol, 20 mM MgCl₂, 50 mM MES pH 6.0. The crystallization conditions were then optimized in the presence of 3'-AMP by the hanging-drop vapour-diffusion method. As a result, crystals suitable for X-ray diffraction grew using a reservoir consisting of 12.5–15% 2-propanol, 18 mM MgCl₂, 45 mM MES pH 6.0, 20 mM ammonium acetate, 10 mM sodium acetate, 3% PEG 4000 and reached maximum dimensions of 200 × 300 × 300 µm within a week (Fig. 1). Electrophoretic analysis by SDS–PAGE demonstrated that the crystals contained both Cmr2dHD and Cmr3 (data not shown). The 3'-AMP-containing crystals diffracted to 2.6 Å resolution (Fig. 2) and belonged to the orthorhombic space group *I*222, with unit-cell parameters *a* = 103.9, *b* = 136.7, *c* = 192.0 Å. On the basis of the molecular masses of recombinant Cmr2dHD (76 kDa) and Cmr3 (36 kDa), the asymmetric unit of the crystal is expected to contain one Cmr2dHD–Cmr3

complex, with a Matthews coefficient of 3.0 Å³ Da^{−1} and a solvent content of 59% (Matthews, 1968). Very recently, the crystal structure of the Cmr2dHD–Cmr3 complex from *P. furiosus* was determined at 2.85 Å resolution (Shao *et al.*, 2013). The crystals were grown in a reservoir containing 21–25% PEG 1500 at 303 K. Although their crystallization conditions are quite different from those reported here, both crystals belonged to the same space group with similar unit-cell parameters. However, the diffraction quality of our crystals was slightly higher (2.6 Å resolution) than that reported by Shao *et al.* (2013). Therefore, we expect that our analysis may provide more precise structural information. Moreover, in contrast to their crystals, which were complexed with ATP (Shao *et al.*, 2013), we crystallized the Cmr2dHD–Cmr3 in complex with 3'-AMP and thus our crystals could reveal new details about the nucleotide-binding site. Attempts to solve the 3'-AMP-bound Cmr2dHD–Cmr3 complex structures by the molecular-replacement method are in progress.

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