Crystallization and preliminary X-ray diffraction analysis of the CRISPR–Cas RNA-silencing Cmr complex

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Clustered regularly interspaced short palindromic repeat (CRISPR)-derived RNA (crRNA) and CRISPR-associated (Cas) proteins constitute a prokaryotic adaptive immune system (CRISPR–Cas system) that targets and degrades invading genetic elements. The type III-B CRISPR–Cas Cmr complex, composed of the six Cas proteins (Cmr1–Cmr6) and a crRNA, captures and cleaves RNA complementary to the crRNA guide sequence. Here, a Cmr1-deficient functional Cmr (Cmr Δ 1) complex composed of *Pyrococcus furiosus* Cmr2–Cmr3, *Archaeoglobus fulgidus* Cmr4–Cmr5–Cmr6 and the 39-mer *P. furiosus* 7.01-crRNA was prepared. The Cmr Δ 1 complex was cocrystallized with single-stranded DNA (ssDNA) complementary to the crRNA guide by the vapour-diffusion method. The crystals diffracted to 2.1 Å resolution using synchrotron radiation at the Photon Factory. The crystals belonged to the triclinic space group *P*1, with unit-cell parameters $a = 75.5$, $b = 76.2$, $c = 139.2$ Å, $\alpha = 90.3$, $\beta = 104.8$, $\gamma = 118.6^\circ$. The asymmetric unit of the crystals is expected to contain one Cmr Δ 1–ssDNA complex, with a Matthews coefficient of 2.03 Å³ Da^{−1} and a solvent content of 39.5%.

1. Introduction

The CRISPR (clustered regularly interspaced short palindromic repeats)–Cas (CRISPR-associated) system, an adaptive immune system in prokaryotes, targets invading genetic elements, such as phages and plasmids, for degradation (van der Oost *et al.*, 2009; Karginov & Hannon, 2010; Marraffini & Sontheimer, 2010; Terns & Terns, 2011; Wiedenheft *et al.*, 2012). CRISPR loci are composed of conserved repeated sequences separated by variable spacer sequences derived from previously encountered invading genetic materials (Bolotin *et al.*, 2005; Mojica *et al.*, 2005; Pourcel *et al.*, 2005). After CRISPR loci are transcribed, the primary transcripts are processed within each repeat sequence to generate crRNAs (CRISPR RNAs) in a process catalyzed by the Cas6 endoribonuclease (Brouns *et al.*, 2008; Carte *et al.*, 2008; Hale *et al.*, 2008). Subsequently, the crRNA and Cas protein(s) form an interference complex to degrade the invading nucleic acid complementary to the spacer region of the crRNA (Brouns *et al.*, 2008; Hale *et al.*, 2009; Jinek *et al.*, 2012). Based on the signature Cas proteins Cas3, Cas9 and Cas10, the interference complexes are classified into the three major types I, II and III, respectively, which are further divided into several subtypes (Makarova *et al.*, 2011).

The subtype III-B interference complex (Cmr complex) consists of six Cmr proteins, Cmr1, Cmr2 (also known as Cas10), Cmr3, Cmr4, Cmr5 and Cmr6, as well as a crRNA, and

captures RNA complementary to the spacer region of the crRNA for degradation (Hale *et al.*, 2009; Staals *et al.*, 2013). The crRNAs isolated from the *Pyrococcus furiosus* Cmr complex have lengths of 39 or 45 nucleotides (nt), with an invariant 8 nt tag at the 5'-end which is derived from the repeat sequence and is essential for CRISPR-mediated RNA interference (Hale *et al.*, 2009, 2012). Variation in the lengths of the crRNAs, which differ in length by 6 nt, has also been reported in the crRNAs isolated from the *Thermus thermophilus* Cmr complex (Staals *et al.*, 2013). The Cmr complex degrades the target RNA at multiple positions with 6 nt intervals via a 5'-ruler mechanism (Staals *et al.*, 2013; Hale *et al.*, 2014). The individual crystal structures of Cmr1, Cmr2dHD (a Cmr2 derivative lacking the N-terminal HD nuclease domain), Cmr3 and Cmr5, as well as cryoelectron microscopy (cryo-EM) structures of the *P. furiosus* and *T. thermophilus* Cmr complexes, have been determined (Cocozaki *et al.*, 2012; Park *et al.*, 2013; Shao *et al.*, 2013; Osawa *et al.*, 2013b; Staals *et al.*, 2013; Spilman *et al.*, 2013; Sun *et al.*, 2014). The cryo-EM reconstructions of the Cmr complexes revealed the presence of a helical filament in the complex which is composed of several Cmr4 subunits (Spilman *et al.*, 2013; Staals *et al.*, 2013). However, the mechanisms of crRNA recognition and the periodic target cleavages from the 5'-end of the crRNA by the Cmr complex have remained elusive because of a lack of structural information for the fully assembled functional Cmr complex at atomic resolution. Intriguingly, the subtype III-A interference complex (Csm complex), which resembles the structure of the Cmr complex as demonstrated by cryo-EM analyses (Rouillon *et al.*, 2013; Staals *et al.*, 2014), degraded the target RNA in a similar manner to that of the Cmr complex (Staals *et al.*, 2014; Tamulaitis *et al.*, 2014). Therefore, structural determination of the Cmr complex will also pave the way towards elucidating the RNA-silencing mechanism by the subtype III-A Csm complex. We now report the crystallization and preliminary X-ray diffraction analysis of the functional Cmr complex in order to clarify the crRNA-guided RNA-cleavage reaction.

2. Materials and methods

2.1. Protein preparation

The Cmr2dHD–Cmr3 complex from *P. furiosus* (PfCmr2dHD–Cmr3) was produced and purified as described previously (Osawa *et al.*, 2013a,b). The operon gene encoding Cmr4–Cmr5–Cmr6 from *Archaeoglobus fulgidus* (AfCmr4–Cmr5–Cmr6) was PCR-amplified from genomic DNA and cloned into the NcoI–BamHI sites of pET-28b (Novagen). The AfCmr6 subunit in the AfCmr4–Cmr5–Cmr6 complex bears a C-terminal His₆ tag, which is derived from the PCR primer. Expression plasmids were transformed into *Escherichia coli* strain BL21-CodonPlus(DE3)-RIL and protein expression was induced with 0.1 mM IPTG when the culture reached an optical cell density (OD₆₀₀) of ~0.8, followed by shaking at 293 K for 18 h. The cells were harvested and lysed by sonication in 20 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM

imidazole, 1 mM PMSF. The cleared lysate was subjected to heat treatment at 338 K for 20 min. The heat-denatured proteins were removed by centrifugation and the supernatant was applied onto a Ni–NTA (Qiagen) column equilibrated with 20 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM imidazole. The AfCmr4–Cmr5–Cmr6 complex was eluted with the same buffer containing 250 mM imidazole. The eluate from the Ni–NTA column was dialyzed against 20 mM Tris–HCl pH 7.0, 200 mM NaCl. The dialyzed solution was loaded onto a HiTrap Heparin column (GE Healthcare) and the flowthrough fraction containing the complex was collected and dialyzed against 20 mM Tris–HCl pH 7.0, 400 mM NaCl. The dialysate was then loaded onto a RESOURCE Q column (GE Healthcare) and the flowthrough fraction containing the AfCmr4–Cmr5–Cmr6 complex was collected and dialyzed against 20 mM Tris–HCl pH 8.0, 200 mM NaCl, 1 mM DTT. The purified AfCmr4–Cmr5–Cmr6 complex was concentrated to approximately 10 mg ml^{−1} using Amicon Ultra-15 centrifugal filters (30K molecular-weight cutoff; Merck Millipore). The protein concentration of the AfCmr4–Cmr5–Cmr6 complex was determined using a molar extinction coefficient of 158 600 M^{−1} cm^{−1} at 280 nm.

2.2. Complex formation

The complex composed of PfCmr2dHD–Cmr3 and AfCmr4–Cmr5–Cmr6, which is referred to as chimeric Cmr23456 (*ChiCmr23456*), was formed by mixing the purified PfCmr2dHD–Cmr3 and AfCmr4–Cmr5–Cmr6 complexes in a 1.3:1 molar ratio. The mixture was incubated at 343 K for 10 min and on ice for 5 min. After centrifugation, the supernatant containing *ChiCmr23456* was purified by gel-filtration chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare) previously equilibrated with 20 mM Tris–HCl pH 8.0, 200 mM NaCl, 1 mM DTT. Fractions that were rich in *ChiCmr23456* were combined and concentrated to approximately 10 mg ml^{−1}. The protein concentration of *ChiCmr23456* was determined using a molar extinction coefficient of 306 660 M^{−1} cm^{−1} at 280 nm.

The Cmr1-deficient chimeric Cmr complex (*ChiCmrΔ1*) was reconstituted by mixing the purified *ChiCmr23456* complex and the 39-mer *P. furiosus* 7.01-crRNA in a molar ratio of 1.3:1 in the presence of 9.7 mM MgCl₂. The mixture was incubated at 343 K for 10 min and then on ice for 5 min. To this mixture, an equimolar amount of the 31-mer single-stranded DNA (ssDNA) complementary to the Pf7.01-crRNA guide was added. The final protein concentration of the mixture was adjusted to 5.1 mg ml^{−1} with buffer consisting of 20 mM Tris–HCl pH 8.0, 200 mM NaCl, 5 mM MgCl₂, 1 mM DTT. The solution was then incubated at 343 K for 10 min, followed by gradual cooling to 298 K to form the *ChiCmrΔ1*–ssDNA complex. After an incubation on ice for 5 min, the supernatant was used for crystallization. The nucleotide sequences of the Pf7.01-crRNA and the ssDNA used in the reconstitution experiment are AUUGAAAGUUGUAGUA-UGCGGUCCUUGCGGCUGAGAGCA and TGCTCTCA-GCCGCAAGGACCGCATACTACAA, respectively.

Table 1

Data-collection statistics.

Values in parentheses are for the last shell.

	Cryo-condition I	Cryo-condition II
Space group	<i>P</i> 1	<i>P</i> 1
Unit-cell parameters (Å, °)	<i>a</i> = 79.0, <i>b</i> = 79.6, <i>c</i> = 142.9, α = 90.6, β = 103.4, γ = 115.4	<i>a</i> = 75.5, <i>b</i> = 76.2, <i>c</i> = 139.2, α = 90.3, β = 104.8, γ = 118.6
Wavelength (Å)	0.98	0.98
Resolution (Å)	50–3.1 (3.28–3.10)	50–2.1 (2.22–2.10)
Measured reflections	214115	1125455
Unique reflections	54626	148845
$R_{\text{merge}}^{\dagger}$	0.077 (0.491)	0.075 (0.505)
$\langle I/\sigma(I) \rangle$	14.3 (2.7)	18.3 (4.4)
Completeness (%)	98.3 (95.7)	97.4 (94.4)
Multiplicity	3.9 (3.9)	7.6 (7.5)
Wilson <i>B</i> factor (Å ²)	70.1	39.7
Mosaicity (°)	0.162	0.216

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

2.3. Crystallization

Crystallization conditions were screened by the sitting-drop vapour-diffusion method at 293 K. We tested several screening kits, Crystal Screen, Crystal Screen 2, Crystal Screen Lite, Crystal Screen Cryo, Natrix, PEG/Ion, Index and SaltRx (Hampton Research), for the initial crystallization experiment. Sitting drops were prepared by mixing 1 µl reservoir solution with 1 µl sample solution and were equilibrated against 100 µl reservoir solution. Crystals of the *Chi*CmrΔ1–ssDNA complex grew in condition No. 89 [15%(w/v) PEG 3350, 100 mM succinic acid pH 7.0] from the Index screen. The crystallization conditions were then optimized by changing the concentration of the precipitant using the hanging-drop vapour-diffusion method. Hanging drops were prepared by combining 1 µl reservoir solution with 1 µl sample solution and were equilibrated against 500 µl reservoir solution. Finally, crystals suitable for X-ray diffraction analysis were obtained under conditions consisting of 13–15%(w/v) PEG 3350, 100 mM succinic acid pH 7.0. The crystals of the complex grew within one week to maximum dimensions of 200 × 200 × 20 µm.

2.4. X-ray data collection

For data collection under cryogenic conditions, the crystals of the *Chi*CmrΔ1–ssDNA complex were transferred into a cryoprotectant solution consisting of 20%(v/v) glycerol in the reservoir solution (cryo-condition I). The crystals were immediately mounted in a cryoloop and flash-cooled in a nitrogen stream at 95 K. X-ray diffraction data were collected on beamline BL-17A at the Photon Factory, Ibaraki, Japan using an ADSC Q270 CCD detector. We only obtained ~3.1 Å resolution diffraction data using the crystals cryo-protected using cryo-condition I. To improve the diffraction quality of the crystals, we optimized the cryo-conditions by changing the cryoprotection method and the cryoprotectant solution. To gradually increase the concentration of the cryoprotectant, we added 1 µl of the cryoprotectant solution

Table 2Sequence identities of the Cmr proteins from *P. furiosus* and *A. fulgidus*.

	Sequence identity (%)
Cmr1	27.1
Cmr2	29.6
Cmr3	33.3
Cmr4	42.2
Cmr5	27.5
Cmr6	37.5

to the crystallization droplet and then removed 1 µl of solution from the resultant droplet. We repeated this procedure several times over 2–4 min. Finally, we obtained higher resolution (~2.1 Å) diffraction data by using a cryoprotectant solution consisting of 30%(v/v) PEG 400 and 2%(w/v) trehalose in the reservoir solution (cryo-condition II). Diffraction data were processed with *XDS* (Kabsch, 2010). The data-processing statistics are summarized in Table 1.

3. Results and discussion

Since we found that the Cmr1-deficient *P. furiosus* Cmr complex (*Pf*CmrΔ1) exhibits crRNA-dependent RNA cleavage activity (Osawa *et al.*, unpublished work), we tried to crystallize *Pf*CmrΔ1 and the *Pf*CmrΔ1–ssDNA complex. However, we were unable to obtain any crystals of these complexes. We then tried to reconstitute the *A. fulgidus* Cmr complex, but we could not prepare the *Af*Cmr2–Cmr3 complex because of its low expression level in *E. coli* cells. Therefore, we sought to reconstitute the chimeric Cmr1-deficient Cmr complex (*Chi*CmrΔ1) composed of *Pf*Cmr2dHD–Cmr3, *Af*Cmr4–Cmr5–Cmr6 and *Pf*7.01-crRNA. Since the Cmr proteins from *P. furiosus* and *A. fulgidus* exhibit sequence identities of 27.1–42.2% (Table 2) and the 5'-tag sequence of the crRNA is the same in these two archaeal species, we expected the hybrid complex to adopt the physiologically relevant architecture. The *Pf*Cmr2dHD–Cmr3 and *Af*Cmr4–Cmr5–Cmr6 complexes were individually over-expressed in *E. coli* cells and were purified using several chromatographic steps. The purified *Pf*Cmr2dHD–Cmr3 and *Af*Cmr4–Cmr5–Cmr6 complexes were mixed in a 1.3:1 molar ratio, and the resulting *Chi*Cmr23456 complex was purified by gel-filtration chromatography, confirming the interaction between these two subcomplexes. The purified *Chi*Cmr23456 complex was mixed with *Pf*7.01-crRNA to reconstitute *Chi*CmrΔ1 (Fig. 1). We confirmed that *Chi*CmrΔ1 degraded the target RNA of the crRNA guide region in a sequence-specific manner and that *Chi*CmrΔ1 produced the same cleavage product as those generated by *Pf*CmrΔ1 (Osawa *et al.*, unpublished work). These findings clearly showed that the hybrid complex is functional and that the Cmr proteins from *P. furiosus* and *A. fulgidus* are interchangeable. We will report these results in more detail in the near future.

For crystallization, *Chi*CmrΔ1 was mixed with ssDNA complementary to the guide region of the *Pf*7.01-crRNA to form the *Chi*CmrΔ1–ssDNA complex. Crystallization screening was performed by the sitting-drop vapour-diffusion

method at 293 K. The crystals were obtained under conditions consisting of 15% (w/v) PEG 3350, 100 mM succinic acid pH 7.0. We subsequently optimized the crystallization conditions by the hanging-drop vapour-diffusion method and obtained complex crystals suitable for X-ray diffraction experiments using the reservoir conditions 13–15% (w/v) PEG 3350, 100 mM succinic acid pH 7.0. The crystals of the *ChiCmr* Δ 1–ssDNA complex grew to maximum dimensions of $200 \times 200 \times 20 \mu\text{m}$ within a week (Fig. 2*a*). Electrophoretic analysis of the crystals by SDS–PAGE revealed that the crystals contained all of the Cmr protein components (Fig. 2*b*). Although we did not confirm the presence of the crRNA and the ssDNA at this stage, these nucleic acids were also present in the crystals, as described below.

The crystals were initially cryoprotected under cryo-condition I, but the resulting crystals only diffracted to a maximum resolution of 3.1 Å (Fig. 3*a*). Furthermore, these crystals did not always diffract to ~ 3.1 Å resolution. Therefore, it was difficult to prepare consistently well diffracting crystals under cryo-condition I. To improve the quality and resolution of the diffraction data, we screened and optimized the cryo-conditions. Finally, we found that the crystals cryoprotected using PEG 400 and trehalose as cryoprotectants (cryo-condition II) consistently diffracted to ~ 2.1 Å resolution (Fig. 3*b*), a much higher resolution than that obtained with cryo-condition I. These findings demonstrated that exploration of the cryo-conditions is crucial to obtain high-quality diffraction data. The crystals of the *ChiCmr* Δ 1–ssDNA complex belonged to the triclinic space group *P*1 (Table 1). The unit-cell parameters of the crystals cryoprotected under cryo-condition I were $a = 79.0$, $b = 79.6$, $c = 142.9$ Å, $\alpha = 90.6$, $\beta = 103.4$, $\gamma = 115.4^\circ$ (Table 1). In contrast, those of the crystals cryoprotected

under cryo-condition II were $a = 75.5$, $b = 76.2$, $c = 139.2$ Å, $\alpha = 90.3$, $\beta = 104.8$, $\gamma = 118.6^\circ$ (Table 1). Therefore, soaking the crystals in the cryo-condition II solution caused lattice shrinking, which led to the significant improvement of the resolution limit during the diffraction experiments.

crRNAs of two different lengths (a 39-mer and a 45-mer) have been observed in the *P. furiosus* Cmr complex (Hale *et al.*, 2009, 2012). A recent study suggested that the 39-mer and 45-mer crRNAs are components of complexes with protein stoichiometries Cmr1₁–Cmr2₁–Cmr3₁–Cmr4₃–Cmr5₂–Cmr6₁ and Cmr1₁–Cmr2₁–Cmr3₁–Cmr4₄–Cmr5₃–Cmr6₁, respectively (Benda *et al.*, 2014). Therefore, we assumed that the stoichiometry of the *ChiCmr* Δ 1–ssDNA complex is 1:1:3:2:1:1:1 Cmr2dHD:Cmr3:Cmr4:Cmr5:Cmr6:crRNA:ssDNA, with a molecular mass of 331 kDa, on the basis of the molecular masses of Cmr2dHD (78.0 kDa), Cmr3 (36.3 kDa), Cmr4 (39.8 kDa), Cmr5 (17.4 kDa), Cmr6 (40.2 kDa), *Pf*7.01-crRNA (12.6 kDa) and ssDNA (9.4 kDa). Therefore, the

Figure 1
Schematic diagram of the reconstituted *ChiCmr* Δ 1 complex and its functional activity.

Figure 2
(*a*) Crystals of the *ChiCmr* Δ 1–ssDNA complex. (*b*) Electrophoretic analysis of the crystals by SDS–PAGE. Since the molecular mass of *AfCmr*4 (39.8 kDa) is almost the same as that of *AfCmr*6 (40.2 kDa), it is difficult to distinguish these protein bands by SDS–PAGE analysis. Lane MK contains molecular-mass markers (labelled in kDa).

Figure 3
Diffraction patterns of the *ChiCmrΔ1*-ssDNA complex cryoprotected under cryo-condition I (a) and cryo-condition II (b).

asymmetric unit of the crystal is expected to contain one *ChiCmrΔ1*-ssDNA complex, with a Matthews coefficient of $2.03 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 39.5%. Since the crystal structures of *PfCmr2dHD*, *PfCmr3* and *AfCmr5* have been determined (Cocozaki *et al.*, 2012; Park *et al.*, 2013; Shao *et al.*, 2013; Osawa *et al.*, 2013b), the *ChiCmrΔ1*-ssDNA complex structure is expected to be solved by a combination of the molecular-replacement and single-wavelength anomalous dispersion methods, with selenium as the anomalous scattering atom. Quite recently, we have succeeded in determining the complex structure by this method. We confirmed that the crystals contained the crRNA and the ssDNA, as well as all of the Cmr protein subunits in the stoichiometry described above. Intriguingly, the subunit interfaces of the *P. furiosus* and *A. fulgidus* proteins contain many residues that are conserved between these two archaeal species, thus supporting their interchangeability in the formation of the active complex. These results will be reported in detail in the near future.

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