Structural basis for Dicer-like function of an engineered RNase III variant and insights into the reaction trajectory of two-Mg\(^{2+}\)-ion catalysis

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ABSTRACT

The RNase III family of dsRNA-specific endonucleases is exemplified by prokaryotic RNase III and eukaryotic Rnt1p, Drosha, and Dicer. Structures of *Aquifex aeolicus* RNase III (AaRNase III) and *Saccharomyces cerevisiae* Rnt1p (ScRnt1p) show that both enzymes recognize substrates in a sequence-specific manner and propel RNA hydrolysis by two-Mg\(^{2+}\)-ion catalysis. Previously, we created an *Escherichia coli* RNase III variant (EcEEQ) by eliminating the sequence specificity via protein engineering and called it bacterial Dicer for the fact that it produces heterogeneous small interfering RNA cocktails. Here, we present a 1.8-Å crystal structure of a postcleavage complex of EcEEQ, representing a reaction state immediately after the cleavage of scissile bond. The structure not only establishes the structure-and-function relationship of EcEEQ, but also reveals the functional role of a third Mg\(^{2+}\) ion that is involved in RNA hydrolysis by bacterial RNase III. In contrast, the cleavage site assembly of ScRnt1p does not contain a third Mg\(^{2+}\) ion. Instead, it involves two more amino acid side chains conserved among eukaryotic RNase III. We conclude that the EcEEQ structure (this work) represents the cleavage assembly of prokaryotic RNase III and the ScRnt1p structure (PDB: 4OOG), also determined at the postcleavage state, represents the cleavage assembly of eukaryotic RNase III. Together, these two structures provide insights into the reaction trajectory of two-Mg\(^{2+}\)-ion catalysis by prokaryotic and eukaryotic RNase III enzymes.

Introduction

Discovered in 1968, *Escherichia coli* RNase III (EcRNase III) is the founding member of the RNase III family of dsRNA-specific endoribonucleases found in all kingdoms of life [1,2]. Representative members of the family include bacterial RNase III, yeast Rnt1p, human Drosha, and human Dicer. A common type of substrate for all RNase III enzymes is stem-loop RNA. Also known as hairpin RNA, stem-loop RNA is an essential secondary structure of primary importance [3]. A less common type of substrate for some RNase III is long duplex RNA. Among the four representative family members, Rnt1p and Drosha process stem-loop RNAs only, whereas RNase III and Dicer also process long dsRNAs. In a successive manner, the processing starts from one end of a long dsRNA and produces small duplex RNAs [4–6]. Dicer typically measures 22 nucleotides for cleavage, producing small interfering RNAs (siRNAs) (Figure 1(a)). In contrast, RNase III typically measures 11 nucleotides for cleavage, resulting in small duplex RNAs of half the length of siRNAs (Figure 1(b)) [7]. In addition to this end-in manner of processing, long dsRNA can also be processed by RNase III in an inside-out scheme, where multiple RNase III molecules bind consecutively to a long dsRNA and cleave the substrate simultaneously [8]. Since the distance between consecutive active centres of adjacent RNase III molecules is 22 nucleotides, the inside-out processing produces siRNA-like small duplex RNAs, indistinguishable from the siRNAs produced by Dicer (Figure 1(b)). This mechanism has been observed for EcRNase III under special conditions [9].

Since the discovery of EcRNase III, tremendous progress has been made in studies of bacterial enzymes [10,11]. Genetic and functional studies have been performed mainly with EcRNase III, whereas structural and mechanistic studies have been carried out mainly with *Aquifex aeolicus* RNase III (AaRNase III). Crystal structures of AaRNase III have been determined at several distinct catalytic states, providing insights into the mechanisms of substrate recognition, scissile bond selection, two-Mg\(^{2+}\)-ion catalysis, phosphoryl transfer, and product release [6,11–13]. In addition, a hypothesis that the E38A mutant of EcRNase III (EcE38A) could promote the inside-out processing of long dsRNA led to the discovery of a reagent for the preparation of siRNA cocktails to be used in gene silencing studies (Figure 1(c)) [14]. It offers an economic way for siRNA cocktail preparation since making EcE38A costs much less than the production of human Dicer. Residue E38 is conserved among bacterial RNase III and is located on the RNA-contacting surface distant from the cleavage site. Bacterial RNase III functions as a dimer, and thus removing two negatively charged side chains between the protein and RNA promotes consecutive binding and cleavage of long dsRNA by multiple RNase III dimers (Figure 1(c)).

By next generation sequencing, we previously demonstrated that both EcRNase III and AaRNase III cleave long dsRNAs at preferred sites [7]. The AaRNase III structures

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suggest that the specificity for a guanine nucleotide at the +3 position near the scissile bond could be eliminated by an alanine substitution at Q165 of EcRNase III. Like E38, residue E65 of EcRNase III is also located on the RNA-contacting surface. Therefore, the E65A mutation in EcRNase III could also assist in promoting the inside-out mechanism. These two predictions prompted us to create a triple mutant (E38A/E65A/Q165A) of EcRNase III (EcEEQ, Figure 1(c)), which indeed produces heterogeneous siRNA cocktails [7]. Therefore, we call this EcRNase III variant bacterial Dicer. It is remarkable that following distinct mechanisms, human Dicer (end-in) and bacterial Dicer (inside-out) produce indistinguishable products (Figure 1). Here, we report the crystal structure of EcEEQ in complex with a stem-loop RNA (RNA6) at 1.80-Å resolution (EcEEQ:RNA6), showing how the E38A and E65A mutations stabilize the protein:RNA complex and how the Q165A mutation eliminates the sequence specificity of EcRNase III.

To enhance substrate specificity and catalytic efficiency, two-Mg²⁺-ion catalysis is used by all RNA and DNA polymerases, and most nucleases and recombinases [15]. The two-Mg²⁺-ion catalysis by RNase III was established by structures of AaRNase III and ScRnt1p, in the form of a postcleavage complex determined at a stage immediately after the cleavage of the scissile bond [12,16]. Intriguingly, the EcEEQ:RNA6 structure reveals the functional role of a third Mg²⁺ ion that is involved in the mechanism of two-Mg²⁺-ion catalysis by bacterial RNase III.

In parallel with EcEEQ, we performed crystallization trials again with wild-type EcRNase III in this study, but only the EcEEQ:RNA6 complex crystallized, underscoring the significant impact of removing four negatively charged amino acid side chains from RNA-contacting surface of the protein on the stability of the protein:RNA complex. Solving the phase problem by molecular replacement (MR), we first utilized the AaRNase III:RNA6 structure, a protein-product complex containing one Mg²⁺ ion in each cleavage site [6], as the search model, which resulted in multiple solutions of low log-likelihood gain (LLG) and final translation function Z (TFZ) scores (top solution: LLG = 71; TFZ = 6.0). Then, we tested the postcleavage complex of AaRNase III (AaRNase III:RNA9), containing two Mg²⁺ ions in each cleavage site [12], which resulted in a unique solution of much higher LLG and TFZ scores (LLG = 445; TFZ = 15.9). Hence, the EcEEQ:RNA6 structure resembles the postcleavage complex of AaRNase III, indicating that the EcEEQ:RNA6 complex is also a postcleavage complex. The structure is indeed a postcleavage complex with an additional feature. It reveals that a third Mg²⁺ ion is integrated in the cleavage site assembly of two-Mg²⁺-ion catalysis by bacterial RNase III.

The EcEEQ:RNA6 structure is schematically illustrated in Figure 2(a). The complex is composed of two EcEEQ subunits (each containing 226 amino acid residues), two RNA6 molecules (each containing 28 nucleotide residues in the form of a hairpin with a 4-nucleotide capping loop and a 2-nucleotide 3’ overhang), six Mg²⁺ ions, 418 water oxygen atoms, and several other ions and molecules from the solvent and cryo-protectant. All three Mg²⁺ ions in the cleavage site of each subunit are well defined with full occupancy (Figure 2(b)). Each EcEEQ molecule contains a specialized endonuclease domain (RIIID, residues 1–147) and a dsRNA-binding domain (dsRBD, residues 156–226, Figure 2(c)). The root-mean-square deviation (RMSD) between the two EcEEQ subunits is 0.17 Å for 196 out of 226 pairs of Ca atoms, and the

**Figure 1. Mechanisms of long dsRNA processing by RNase III enzymes.** (a) Human Dicer recognizes the dsRNA termini with a 2-nucleotide 3’ overhang, cleaves both strands, and produces a duplex RNA of 22 nucleotides in each strand (The end-in mechanism). (b) An EcRNase III dimer recognizes the dsRNA termini, especially those featuring a 2-nucleotide 3’ overhang, cleaves both strands, and produces a short duplex RNA of 11 nucleotides in each strand (The end-in mechanism). Under special conditions, however, two EcRNase III dimers bind to and cleave dsRNA in a cooperative manner, which produces a duplex RNA of 22 nucleotides in each strand. (The inside-out mechanism). (c) Two EcEEQ (the E38A/E65A/Q165A triple mutant of EcRNase III) or EcE38A (the EcE38A single mutant of EcRNase III) dimers bind to and cleave dsRNA in a cooperative manner, which produces a duplex RNA of 22 nucleotides in each strand (The inside-out mechanism). A similar version of this figure was previously published in [7].

**Results and discussion**

**The EcEEQ:RNA6 is highly homologous to the postcleavage complex of AaRNase III**

We started working on EcRNase III in 1996, but we were not able to crystallize the protein, either full-length or truncated.
RMSD between the two RNA6 molecules is 0.14 Å for 587 out of 598 pairs of atoms, underscoring the highly symmetric nature of the EcEEQ-RNA6 complex (Figure 2(a)).

The EcEEQ:RNA6 and AaRNase III:RNA9 structures are highly homologous. The EcEEQ structure exhibits 15 secondary structure elements, of which 13 are shared with AaRNase III (Figure 2(c)). Eleven out of the 13 shared secondary structure elements are of the same length in the two structures, whereas two (α7 and α8) are one residue longer in AaRNase III. Superposition of the EcEEQ:RNA6 and AaRNase III:RNA9 complexes shows that the two structures align very well (Figure 3(a)). The RMSD for 404 out of 417 pairs of Ca atoms between the two RNase III dimers is 1.7 Å. Including RNA, the RMSD for 382 out of 458 pairs of Ca and P atoms is 1.6 Å. The high homology of the two structures offers the convenience of using just the numbering scheme of the focus, i.e. the residue numbers in EcRNase III.

**Structural basis for Dicer-like function of EcEEQ**

The first glimpse at the RNase III active site was provided by our structure of A. aeolicus RIIID dimer 33 years after the discovery of the enzyme [17,18]. The structure showed that the dimerization of RIIID creates a catalytic valley about 20 Å
wide and 50 Å long, which can accommodate a dsRNA substrate. Five years later, our AaRNase III:dsRNA structure showed that the proposed catalytic valley is indeed the binding site of a dsRNA substrate [6]. In the middle of the valley are located two cleavage sites, each featuring a cluster of four negatively charged side chains (E41, D45, D114, and E117) [12]. At each end of the valley, two more negatively charged side chains (E38 and E65) are located. These negatively charged side chains are conserved among bacterial RNase III enzymes.

As depicted in Figure 3, panels A and B, residues E38 and E65 are located on the RNA-contacting surface. Therefore, the alanine mutation of both E38 and E65 stabilizes the resulting protein:RNA complex and thereby promotes the inside-out processing of long dsRNAs. This structural implication was validated by in vitro cleavage products analysis [7]. The third mutation in EcEEQ is Q165A that is critical for the production of heterogeneous siRNA cocktails. As shown in Figure 3 (c), the Q165 side chain forms three hydrogen bonds with the +3 G nucleotide, two with the base and one with the O2'-hydroxyl group [12]. The length of these hydrogen bonds ranges between 2.9 and 3.1 Å. Hence, the Q165A mutation eliminates these strong hydrogen bonds and thereby abolishes its specificity for the +3 G nt. This structural implication was also validated by in vitro cleavage products analysis [7]. The loss of three hydrogen bonds between the protein and RNA results in a less stable protein:RNA complex. This negative impact on the stability is offset by the overwhelming stabilization effect of the E38A and E65A mutations as demonstrated previously by product analysis of in vitro cleavage [7].

As mentioned above, the RNA-contacting surface of the catalytic valley is coated by a total of 12 negatively charged side chains. The E38A and E65A mutations neutralize four of them. The remaining eight are catalytic side chains, forming two cleavage sites in the catalytic valley. Interactions between these catalytic side chains and dsRNA are bridged by catalytic Mg2+ ions that mitigate electrostatic repulsion between the enzyme and the RNA. No other negatively charged side chains exist between the catalytic valley and the bond dsRNA. Therefore, the impact of E38A and E65A mutations on the stability of the protein:RNA complex is profound. Taken together, these features revealed by the EcEEQ:RNA6 and AaRNase III:RNA9 complexes are structural basis for the function of EcEEQ, the bacterial Dicer that is most suitable for producing heterogeneous siRNA cocktails.

The EcEEQ:RNA6 represents the postcleavage state of RNA hydrolysis by bacterial RNase III

The RIIID dimer hydrolyzes both strands of dsRNA simultaneously with two identical cleavage sites. In each cleavage site of the EcEEQ:RNA6 structure, three Mg2+ ions are well defined and fully occupied. As depicted in Figure 4(a), these Mg2+ ions (MgA, MgB and MgC) are ‘organizers’ of the cleavage site assembly by coordinating with four catalytic side chains (E41, D45, D114, and E117), three nucleotide residues (R-1, R 0, and R+1), and eight water molecules around the scissile bond. MgA coordinates with two oxygen atoms of the scissile phosphate group (R 0), two oxygen atoms
of the E41 and E117 side chains, and two oxygen atoms of water molecules. MgB coordinates with one oxygen atom of the scissile phosphate group (R 0), the oxygen atom of 3'-OH group (R-1), two oxygen atoms of the D45 and E117 side chains, and two oxygens of water molecules. MgC coordinates with one oxygen of the scissile phosphate group (R 0), one phosphate oxygen of the R+1 nucleotide, and four oxygen atoms of water molecules. Among the four catalytic side chains, only D114 does not coordinate with Mg$^{2+}$ directly. Bridging negatively charged components, the three Mg$^{2+}$ ions make the cleavage site assembly as compact as needed for catalysis.

The compactness of the assembly can be measured with two contact distances (CDs), one between MgA and MgB (CD$^\text{MgA,MgB}$) and the other between 3'-oxygen of nucleotide R-1 and phosphorus of nucleotide R 0 (CD$^\text{O3,P}$). The phosphorus of nucleotide R 0 represents the scissile phosphate group after RNA hydrolysis. Before the reaction, it is covalently bonded to the 3'-oxygen of nucleotide R-1. In the EcEEQ:RNA6 structure, the CD$^\text{O3,P}$ measures 2.9 Å (Figure 4(a)), which is smaller than the sum of van der Waals (vdW) radii of O (1.52 Å) and P (1.80 Å) [19]. As shown in Figure 4(b), the cleavage site assembly in the AaRNase III:RNA9 structure exhibits two distinct conformations, a transformation of 25% occupancy (minor-AaRNase III:RNA9) and a transformation of 75% occupancy (major-AaRNase III:RNA9) [12]. The CD$^\text{O3,P}$ in minor-AaRNase III:RNA9 measures 3.0 Å, indicating that 25% of the population represents the postcleavage state, whereas it measures 3.5 Å in major-AaRNase III:RNA9, indicating that 75% of the population represents a state of product release. Whereas two Mg$^{2+}$ ions were observed in minor-AaRNase III:RNA9, three Mg$^{2+}$ ions were observed in major-AaRNase III:RNA9. Neither MgC nor its four coordination water molecules were observed in minor-AaRNase III:RNA9 (Figure 4(b)). It has been previously shown that CD$^\text{MgA,MgB}$ is about 3.5 Å at the intermediate state for the two-Mg$^{2+}$ ion catalysis by either RNase H1 or DNAPγ [20,21]. The CD$^\text{MgA,MgB}$ in the EcEEQ:RNA6 structure is 3.7 Å (Figure 4(a)), suggesting that the EcEEQ:RNA6 structure represents the postcleavage state immediately after the intermediate state of RNA hydrolysis.

**Structural insights into two-Mg$^{2+}$-ion catalysis by bacterial and yeast RNase III enzymes**

Nucleophilic attack on phosphorus could produce a relatively long-lived pentacovalent intermediate. And all phosphoryl transfer reactions in DNA and RNA involve such an intermediate and inversion of the stereo configuration at the phosphorus [15]. Based on the EcEEQ:RNA6 structure, models of reaction intermediate and precleavage complex could be readily derived by adjusting the torsion angles along the C4'-C5'-O5'-P-O1P chain and breaking or making the P-O bonds. At the postcleavage state, a scissile-phosphate oxygen is coordinated with both MgA and MgC (Figure 5(a)). This special oxygen atom is in fact the nucleophilic water oxygen at the intermediate (Figure 5(b)) and precleavage states (Figure 5(c)). It appears that MgC plays three important roles in catalysis. First, MgC teams with MgA and MgB to optimize the cleavage site geometry for the formation of the pentacovalent intermediate (Figure 5(b)). Second, MgC synergizes with MgA to activate the nucleophilic water molecule (Figure 5(c)). Third, MgC facilitates the nucleophilic attack and subsequent electron transfer (Figure 5(b)). Coordinated with the scissile phosphate, MgC has been observed not only at the postcleavage state (EcEEQ:RNA6, Figure 4(a)), but also at two distinct snapshots during product release, one after the scissile phosphate moves away from the cleavage site (major-AaRNase III:RNA9, Figure 4(b)) and the other after the scissile hydroxyl also moves away from the cleavage site [12]. We predict that all prokaryotic RNase IIIIs employ the third Mg$^{2+}$ ion to assist two-Mg$^{2+}$-ion catalysis of RNA hydrolysis.
Unlike bacterial RNase IIIs that use four catalytic side chains (E1, D2, D3, and E4) in each event of two-Mg$^{2+}$-ion catalysis, yeast Rnt1p uses two more (N5 and K6) that are conserved among eukaryotic RNase IIIs (Figure 5d)). As revealed by the Rnt1p postcleavage structure, the side chains of E1, D2, D3, and E4 in Rnt1p assume identical positioning as in bacterial RNase IIIs, whereas those of N5 and K6 are unique for eukaryotes [16]. The side chain of N5 interacts with two water molecules and one oxygen of the 5’ phosphate while the ε-amino group of K6 side chain interacts with one oxygen of the 5’ phosphate and another from the carboxylic group of D3 (Figure 5e). As shown, in the presence of N5 and K6, the CD$^{\text{MgA-MgB}}$ is 3.6 Å at the postcleavage state of Rnt1p. Like the MgC in the two-Mg$^{2+}$-ion catalysis of prokaryotic RNase IIIs, the N5 and K6 side chains also play important roles. First, the K6 side chain recognizes the scissile phosphate group and teams with MgA and MgB to optimize the cleavage site geometry for the formation of the pentacovalent intermediate (Figure 5f)). Second, K6 side chain synergizes with MgA to activate the nucleophilic water molecule (Figure 5g)). Third, K6 and N5 side chains facilitate the nucleophilic attack and subsequent electron transfer (Figure 5f)). Since K6 is not conserved in one RIIID among eukaryotic RNase III enzymes [8], we predict that all eukaryotic RNase IIIs employ the N5 and most also employ K6 side chains to assist two-Mg$^{2+}$-ion catalysis of RNA hydrolysis. In the presence of K6, the positively charged ε-amino group prevents a positively charged metal ion from binding at the MgC site. In the absence of K6, however, whether a third metal ion would bind at the MgC site remains to be elucidated.

Materials and methods

Protein expression and purification

The expression vector of EeEQ was constructed and His$_6$-MBP tagged EeEQ (His$_6$-MBP-EeEQ) was overproduced in E. coli BL21(DE3) Codon Plus-RIL cells (ThermoFisher Scientific, Waltham, MA) as described [7] with limited
modifications. Briefly, the cells were cultivated in Luria-Bertani (LB) broth containing 100 µg ml⁻¹ ampicillin and 30 µg ml⁻¹ chloramphenicol at 37°C, induced by the addition of isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and shaken for 4–6 hr at 37°C. The cells were harvested by centrifugation at 4000 g for 10 min at 4°C and lysed in 30 mM Tris (pH 7.4), 1 M NaCl, and 100 µl⁻¹ 2-mercaptoethanol by sonication at 45 kHz. After removal of insoluble cell debris by centrifugation at 12,000 rpm for 30 min, the supernatant was filtered through a 0.45-µm cellulose acetate membrane and applied to a HisTrap FF column (GE Healthcare Life Sciences, Pittsburgh, PA). Equilibration and washing were performed using a buffer containing 30 mM Tris (pH 7.4), 1 M NaCl, 20 mM imidazole, and 100 µl⁻¹ 2-mercaptoethanol, and elution was carried out in 30 mM Tris (pH 7.4), 1 M NaCl, 400 mM imidazole, and 100 µl⁻¹ 2-mercaptoethanol. Fractions containing His₆-MBP-EcEEQ were pooled. The His₆-MBP tag was removed by cleaving the fused protein with 0.5 mg ml⁻¹ TEV protease [22] at a designed site in the linker and passing the digested protein through a reverse HisTrap FF column to yield the recombinant EcEEQ protein. The EcEEQ protein was further purified with a HiLoad (26/60) Superdex 200 size exclusion column (GE Healthcare Life Sciences). The final product, in 25 mM Tris (pH 7.4), 200 mM NaCl, and 100 µl⁻¹ 2-mercaptoethanol, was concentrated to 12 mg ml⁻¹ (determined spectrometrically using a molar extinction coefficient of 14,440 M⁻¹ cm⁻¹), aliquoted, flash frozen in liquid nitrogen, and stored at −80°C.

Crystallization and X-ray diffraction data collection

Previously, a total of 12 RNA oligos (RNA1 through RNA12) were used and/or observed in the structures of AaRNase III:RNA complexes [11]. RNA6, a 28-nucleotide stem-loop RNA derived from a canonical substrate of EcRNase III [6], was purchased from Dharmacon RNA Technologies (Chicago, IL) for this study. Prior to crystallization, the protein and RNA were incubated at room temperature for 30 min in a solution consisting of 7.6 mg ml⁻¹ EcEEQ, 0.4 mM RNA6, 50 mM MgCl₂, 300 mM NaCl, and 25 mM Tris-HCl (pH 7.4). The crystallization screening was carried out with a Mosquito crystallization robot (SPT Labtech Ltd., Hertfordshire, UK) by sitting drop vapour diffusion and the plates were incubated at 19 ± 1°C. Micro crystals appeared after 3 days in drops containing the protein-RNA solution and an equal volume of well solution (25% PEG 3350 and 0.2 M KBr in 100 mM Heps buffer, pH 7.5) and reached suitable size for X-ray diffraction after 1–2 weeks. The crystals were flash-frozen after being soaked briefly in a cryoprotection solution containing 75% (v/v) reservoir solution and 25% (v/v) ethylene glycol. X-ray diffraction data were collected at 100 K at the Southeast Regional Collaborative Access Team (SER-CAT) insertion device beamline 22 (22-ID) of the Advanced Photon Source, Argonne National Laboratory. The data was indexed, integrated, and scaled with the HKL3000 suite [23]. Data collection and processing statistics are summarized in Table 1.

| Table 1. Data collection and structure refinement statistics. |
|---------------------------------------------------------------|
| **Data Collection**                                           |
| Space group                                                  | P2₁ |
| Cell constants                                               | a, b, c (Å) | 56.93, 65.75, 94.52 |
|                                                             | α, β, γ (°)  | 90.0, 102.0, 90.0 |
| Resolution (Å)                                               | 30.00–1.80 (1.86–1.80)* |
| Completeness (%)                                             | 99.5 (98.4) |
| Total/Unique reflections                                     | 362,165/55,901 |
| Redundancy                                                   | 6.5 (6.1) |
| I/σ(I)                                                       | 14.4 (3.3) |
| Rmerge                                                      | 0.121 (0.881) |
| Rfree                                                        | 0.053 (0.504) |
| CC₁/₂                                                       | 80.6 (60.4) |
| **Refinement**                                               |
| Resolution (Å)                                               | 28.31–1.80 (1.90–1.80) |
| No. of reflections                                           | 55,856 (7,711) |
| Rwork/Rfree                                                 | 0.171/0.202 |
| No. of atoms/B-factors (Å³)                                   | 3,617/26.4 |
| Protein                                                     | 1,196/27.84 |
| Water                                                       | 418/32.6 |
| Mg²⁺, K⁺, Cl⁻                                                | 15/29.6 |
| Ethylene glycol, Tris                                       | 72/40.5 |
| R.m.s. deviations                                            | Bond lengths (Å) | 0.008 |
|                                                             | Bond angles (°)  | 1.018 |
| Ramachandran plot (%)                                        | Favoured       | 98.88 |
|                                                             | Allowed        | 1.12 |
|                                                             | Outliers       | 0 |

*Values in parentheses are for the highest-resolution shell.

Structure solution and refinement

The structure of the EcEEQ:RNA6 complex was solved by molecular replacement (MR) using PHASER [24]. As mentioned above, the search model was the AaRNase III:RNA9 structure [12] after solvent molecules and ions were removed. The sequence identity between AaRNase III and EcEEQ is 33%. Each RNA3:RNA complex contains two RNA3 and two RNA molecules. RNA9 contains 22 nucleotide residues. Therefore, 44 nucleotides are present in the AaRNase III:RNA9 search model, whereas 56 nucleotides are present in the EcEEQ:RNA6 structure. Nonetheless, the MR solution was unique with high LLG and TFZ scores (LLG = 445; TFZ = 15.9). Starting with the MR solution, the phases were improved with phenix_mr_rosetta [25] by density- and energy-guided model optimization and iterative model rebuilding. Although the current version of phenix_mr_rosetter did not work on nucleic acids structures, the phasing power of the EcEEQ protein were so strong that the difference Fourier electron density revealed the structure of two RNA6 molecules in their entirety. The initial RNA6 molecules that we built with COOT [26] were remodelled with ERRASER (Enumerative Real-Space Refinement ASsisted by Electron density under Rosetta) maintained at the ROSIE server [27,28]. Further adjustment and refinement of the structure were carried out with COOT [26] and PHENIX [29]. The quality of the final structure was validated on the Worldwide PDB (wwPDB) Validation Server [30]. The structure refinement statistics are summarized in Table 1. Illustrations were prepared using PyMOL (Schrödinger, LLC.).

Based on the EcEEQ:RNA6 structure, model complexes for the intermediate and precleavage states of EcRNase III were derived by adjusting the torsion angles along the C4’-C5’-OS’
-P-O1P chain and breaking or making the P-O bonds [12]. Similarly, model complexes for the intermediate and precleavage states of ScRnt1p were derived based on the structure of its postcleavage complex [16]. No adjustments were made to other components of the cleavage assemblies because the CD\textsuperscript{2}Mg\textsubscript{2}A,Mg\textsubscript{2}B values (3.6 or 3.7 Å) mimic that at the intermediate state (3.5 Å) observed for RNase H1 and DNAPt [20,21].

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Disclosure statement
No potential conflict of interest was reported by the authors.

Data availability statement
The atomic coordinates and structure factors for the EcEEQ:RNA\textsubscript{6} complex have been deposited with the Protein Data bank (PDB: 7R97). https://www.rcsb.org/

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