Structure of *Saccharomyces cerevisiae* mitochondrial Qri7 in complex with AMP

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*N*⁶-Threonylcarbamoyladenosine (*t*⁶A) is a modified tRNA base required for accuracy in translation. Qri7 is localized in yeast mitochondria and is involved in *t*⁶A biosynthesis. In *t*⁶A biosynthesis, threonylcarbamoyl-adenylate (TCA) is synthesized from threonine, bicarbonate and ATP, and the threonyl-carbamoyl group is transferred to adenine 37 of tRNA by Qri7. Qri7 alone is sufficient to catalyze the second step of the reaction, whereas the Qri7 homologues YgjD (in bacteria) and Kae1 (in archaea and eukaryotes) function as parts of multi-protein complexes. In this study, the crystal structure of Qri7 complexed with AMP (a part of TCA) has been determined at 2.94 Å resolution in a new crystal form. The manner of AMP recognition is similar, with some minor variations, among the Qri7/Kae1/YgjD family proteins. The previously reported dimer formation was also observed in this new crystal form. Furthermore, a comparison with the structure of TobZ, which catalyzes a similar reaction to *t*⁶A biosynthesis, revealed the presence of a flexible loop that may be involved in tRNA binding.

1. Introduction

After they are transcribed, tRNAs are modified by numerous specific enzymes. Among these modifications, those in the anticodon loop are important for proper codon–anticodon interactions, translocation and frameshift prevention in ribosomes (reviewed in Agris, 2008). *N*⁶-Threonylcarbamoyladenosine (*t*⁶A) is present at position 37 in the anticodon stem loop of tRNAs decoding ANN codons (where N is A, U, C or G) and is widely distributed in all three kingdoms of life as well as in intracellular organelles such as chloroplasts and mitochondria (Ishikura *et al.*, 1969; Powers & Peterkofsky, 1972; Grosjean *et al.*, 1995). The *t*⁶A modification stabilizes the structure of the anticodon stem loop and the codon–anticodon interaction, thus preventing frameshifts and promoting translational fidelity (Weissenbach & Grosjean, 1981; Murphy *et al.*, 2004; Stuart *et al.*, 2000). It was recently reported that *t*⁶A is further modified into cyclic *t*⁶A by dehydration in some bacteria and eukaryotes (Miyauchi *et al.*, 2013).

The biosynthesis of *t*⁶A is catalyzed by the YrdC/Sua5 and YgjD/Kae1/Qri7 protein families (El Yacoubi *et al.*, 2009, 2011; Srinivasan *et al.*, 2011). *t*⁶A biosynthesis occurs in two steps. Firstly, threonyl-carbamoyl-adenylate intermediates (TCA) are synthesized from threonine, bicarbonate and ATP by the YrdC/Sua5 family proteins. Secondly, the threonyl-carbamoyl group of TCA is transferred to adenine 37 of tRNA by the YgjD/Kae1/Qri7 family proteins (Wan *et al.*, 2013; Lauhon, 2012; Srinivasan *et al.*, 2011; Perrochia, Crozat *et al.*, 2013). The first step catalyzed by the YrdC/Sua5 family proteins is common in all cases among the kingdoms, while the second step is different. In yeast mitochondria Qri7 alone, together with Sua5, is sufficient for *t*⁶A biosynthesis (Wan *et al.*, 2013). On the other hand, in archaea and eukaryotes Kae1 forms the KEOPS complex composed of five protein components (Kae1, Bud32, Cgi121, Pcc1 and Gon7; the latter is only found in fungi), which is required for the

Table 1

Data-collection and refinement statistics for Qri7–AMP.

Values in parentheses are for the outer shell.

Data-collection statistics	
Wavelength (Å)	1.0000
Space group	P_{432}
Unit-cell parameters (Å, °)	$a = b = c = 180.32$, $\alpha = \beta = \gamma = 90$
Molecules in asymmetric unit	2
Resolution (Å)	50–2.94 (3.00–2.94)
$\langle I/\sigma(I) \rangle$	84.4 (4.36)
Multiplicity	40.2 (33.2)
Unique reflections	22014 (1062)
Completeness (%)	95.1 (92.1)
$R_{\text{merge}}^{\dagger}$	0.103 (over 1.00)
$R_{\text{p.i.m.}}^{\ddagger}$	0.017 (0.239)
Refinement statistics	
$R_{\text{work}}^{\S}$ (%)	22.29
$R_{\text{free}}^{\P}$ (%)	27.34
No. of atoms	
Total	5273
Protein	5223
Water	2
Other molecules/ions	48
B factor (Å ²)	
Protein	97.99
Water	92.07
Zinc	97.58
AMP	116.13
Ramachandran statistics (%)	
Preferred regions	93.64
Allowed regions	6.36
Outliers	0
R.m.s.d., bond angles (°)	0.667
R.m.s.d., bond lengths (Å)	0.002

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. $\ddagger R_{\text{p.i.m.}} = \sum_{hkl} [1/(n-1)]^{1/2} \times \sum_{j=1}^n |I_{hkl,j} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_j I_{hkl,j}$. $\S R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$ for reflections in the work set. $\P R_{\text{free}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$ for reflections in the test set (5% of the total reflections).

threonyl-carbamoyl transferase activity (Perrochia, Crozat *et al.*, 2013; Kisseleva-Romanova *et al.*, 2006). KEOPS mutants have the phenotype of slow growth and shortened telomeres (Downey *et al.*, 2006; Kisseleva-Romanova *et al.*, 2006). Similarly, in bacteria, YgjD forms the DEZ complex composed of three proteins (YgjD, YjeE, and YeaZ), which is required for transferase activity (El Yacoubi *et al.*, 2011; Deutsch *et al.*, 2012). Thus, although the Qri7 homologues of YgjD and Kae1 exhibit threonyl-carbamoyl transferase activity only in the presence of the other subunits, Qri7 forms a homodimer which has the full threonyl-carbamoyl transferase activity (Wan *et al.*, 2013; Deutsch *et al.*, 2012). To date, structures of YgjD in complexes with AMP and with ATP γ S, of Kae1 in complex with ATP and of the apo form of Qri7 have been determined (Wan *et al.*, 2013; Hecker *et al.*, 2007; Nichols *et al.*, 2013). In the apo structure, Qri7 forms a homodimer related by crystallographic symmetry.

In this study, we determined the crystal structure of Qri7 in complex with AMP (a part of TCA) at 2.94 Å resolution in a new crystal form. A comparison of the structure of Qri7 with those of Kae1 and YgjD revealed that their manners of AMP recognition are almost the same, with some minor differences. Furthermore, a comparison with the structure of TobZ, which catalyzes a reaction similar to the t⁶A biosynthesis reaction, revealed the presence of a flexible loop that may be involved in tRNA binding.

2. Materials and methods

2.1. Cloning and expression of Qri7

The gene encoding *Saccharomyces cerevisiae* Qri7 (residues 30–407) was amplified by PCR using PrimeSTAR MAX DNA

polymerase (Takara Bio Inc.) and cloned into a modified pET-28a vector (Novagen) bearing the Turbo3C protease-recognition site. The protein was overexpressed in *Escherichia coli* Rosetta2 (DE3) cells (Novagen). The cells were cultured in LB medium containing kanamycin (25 µg ml^{−1}) at 310 K, and protein expression was induced at an OD₆₀₀ of ~0.8 with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 18 h at 293 K.

2.2. Protein purification and tRNA preparation

Cells were harvested by centrifugation at 5000g and the cell pellet was disrupted by sonication in 100 ml buffer A [50 mM Tris–HCl pH 8.0 containing 300 mM NaCl, 10 mM imidazole, 0.1 mM PMSF, 1 mM β-mercaptoethanol (β-ME)]. The crude cell extract was centrifuged for 40 min at 37 000g to remove the cell debris. The supernatant was incubated with 5 ml nickel–nitrilotriacetic acid (Ni–NTA) resin (Qiagen) for 1 h and the mixture was loaded onto an Econo Column (Bio-Rad). The resin was washed with 75 ml buffer B (50 mM Tris–HCl pH 8.0 containing 300 mM NaCl, 10 mM imidazole, 1 mM β-ME) and the proteins were eluted with 25 ml buffer C (50 mM Tris–HCl pH 8.0 containing 300 mM NaCl, 300 mM imidazole, 1 mM β-ME). The eluted proteins were dialyzed against 1 l buffer D (50 mM Tris–HCl pH 8.0 containing 100 mM NaCl, 20 mM imidazole, 1 mM β-ME) in the presence of Turbo3C Protease (Accelagen) for 18 h to cleave the N-terminal histidine tag. The dialyzed sample was passed through the Ni–NTA column again and the flowthrough fractions were collected. The protein was further purified by gel-filtration column chromatography using HiLoad Superdex 200 (GE Healthcare) equilibrated with buffer E (20 mM Tris–HCl pH 8.0 containing 200 mM NaCl, 1 mM DTT). The purity of the protein was monitored by 10.0% SDS–PAGE. The purified protein was concentrated to 12.8 mg ml^{−1} with an Amicon Ultra 10K filter and stored at 193 K until use. All of the purification procedures described above were conducted at 277 K.

S. cerevisiae mitochondrial tRNA^{lle} was transcribed *in vitro* with T7 polymerase and purified by denaturing urea PAGE.

2.3. Crystallization

In the initial crystallization screen, the protein solution [5 mg ml^{−1} in 20 mM Tris–HCl pH 8.0, 50 mM NaCl, 5 mM MgCl₂, 5 mM AMP, 5 mM threonine, 3.3 mg ml^{−1} tRNA^{lle} (yeast mitochondria)] was prepared using an Amicon Ultra 10K filter and 0.1 µl of the solution was mixed with 0.1 µl mother liquor. Initial screening for crystallization was performed with several commercially available screening kits, including Crystal Screen, Crystal Screen 2, PEG/Ion and Natrix (Hampton Research), The PACT and JCSG Suites (Qiagen), and MemGold and MemGold2 (Molecular Dimensions), with a Mosquito Crystal crystallization robot (TTP Labtech) by the sitting-drop vapour-diffusion method at 293 K. Initial crystals appeared in a day from MemGold2 condition No. 89 [33% polyethylene glycol (PEG) 400, 0.1 M HEPES pH 7.5]. After optimization of the conditions, crystals were grown by the hanging-drop vapour-diffusion method in about a week by mixing 2 µl protein solution with an equal volume of the reservoir solution [25% PEG 400, 3% (w/v) dextran sulfate sodium salt (molecular weight 5000), 0.1 M HEPES pH 7.5].

2.4. X-ray data collection and processing

The crystals were soaked for a few seconds in the cryoprotection solution [35% PEG 400, 3% (w/v) dextran sulfate sodium salt (molecular weight 5000), 0.1 M HEPES pH 7.5] and were flash-cooled in liquid nitrogen. The diffraction data set was collected at

100 K on beamline BL-5A at the Photon Factory-KEK, Tsukuba, Japan using an ADSC Quantum 315r detector. A total of 180 diffraction images were collected with 1° oscillation per image for the data set. The resulting data set was processed with *HKL-2000* (HKL Research, Otwinowski & Minor, 1997) and the data-collection statistics are summarized in Table 1. The crystals belonged to space group $P4_332$, with unit-cell parameters $a = b = c = 180.32$ Å, and diffracted to 2.94 Å resolution.

2.5. Structure solution and refinement

The structure was solved by the molecular-replacement method with *Phaser* (McCoy *et al.*, 2007) using the previously determined *S. cerevisiae* Qri7 structure (PDB entry 4k25; Wan *et al.*, 2013) as a search model. Manual model building and the addition of water molecules and AMP were performed with *Coot* (Emsley & Cowtan, 2004) and the structure was refined with *PHENIX* (Afonine *et al.*, 2012). The structure of Qri7 in complex with AMP was refined to 2.94 Å resolution, with R_{work} and R_{free} values of 22.3 and 27.3%, respectively. The final model contained 693 residues, 93.6% of which were in the preferred region of the Ramachandran plot. Refinement statistics are summarized in Table 1.

3. Results and discussion

3.1. The overall structure of Qri7-AMP

We have determined the structure of *S. cerevisiae* Qri7 complexed with AMP at 2.94 Å resolution (PDB entry 3wuh). The cubic crystals contained two chains (*A* and *B*) and each coordinates a metal ion (Figs. 1 and 2*a*). Although we could not identify the source of the metal ions, they might be zinc ions, as in the case of the YgjD-ATPγS structure (Nichols *et al.*, 2013). Both refined chains *A* and *B* consist of residues 31–406. Residues 70–80, 241–249, 275–279 and 341–348 of chain *A* and residues 70–80, 241–247 and 341–348 of chain *B* were disordered and therefore were not included in the model. The crystallization experiments were performed in the presence of Qri7, AMP, threonine and tRNA^{Ile}; however, there was no electron density corresponding to threonine and tRNA^{Ile}.

Although the space group of Qri7-AMP in this study (cubic, $P4_332$) differs from that of Qri7 in the previous study (orthorhombic, $P2_12_12_1$; PDB entry 4k25; Wan *et al.*, 2013), the two structures are similar to each other (r.m.s.d. of 1.13 Å for the C $^\alpha$ atoms of residues

31–406). In addition, the two molecules in the asymmetric unit of the Qri7-AMP structure form a similar dimer to that observed in the previous study (Wan *et al.*, 2013), which was formed by crystallographic twofold symmetry. The previous study reported that Qri7 forms a homodimer in solution using a sedimentation-velocity analysis (Wan *et al.*, 2013), and our gel-filtration chromatography experiments confirmed this result (data not shown). Therefore, the observed dimer formation is not an artifact of the crystallization but reflects the solution status of Qri7. Indeed, the residues involved in the dimer formation were shown to be important for Qri7 activity by mutational analyses (Wan *et al.*, 2013). In contrast, the homodimeric interface of Qri7 is not conserved in Kae1 and YgjD. Kae1 and YgjD do not form a homodimer but form the KEOPS and DEZ complexes, respectively. Using the interface corresponding to the Qri7 dimeric

Figure 2
(*a*) The $F_o - F_c$ OMIT electron-density map of AMP and zinc ion (2.95 σ). (*b*) The dimerization interface of the Qri7 homodimer between chain *A* (green) and chain *B* (red). Hydrogen bonds are depicted by black dashed lines. The residues forming the hydrogen bonds are shown as stick models. (*c*) Superimposition of the crystal structures of apo Qri7 (orange) and Qri7-AMP (green). A zinc ion in the Qri7-AMP structure is depicted by a grey sphere.

Figure 1
Overall structure of the Qri7-AMP dimer. Chains *A* and *B* are coloured green and red, respectively. The two zinc ions are depicted as grey spheres and the two AMP molecules are shown as orange stick models.

interface, Kae1 and YgjD interact with Pcc1 and YeaZ, respectively, in the complexes (Mao *et al.*, 2008; Nichols *et al.*, 2013).

On the dimerization surface of Qri7, the side chains of Trp136 form hydrogen bonds to the carbonyl groups of the main chains of Ala135 and Arg104, and Gln87 forms hydrogen bonds to the side chains of Asp127 and Lys130 (Fig. 2*b*). Moreover, Ala88, Ile90, Gly124, Phe128, Gly131 and Val134 of chains *A* and *B* form a hydrophobic surface and contribute to hydrophobic interactions. Thus, these data indicated that the Qri7 dimer is formed by hydrogen bonding and hydrophobic interactions of the $\alpha 1$ and $\alpha 2$ helices. The superposition of the $\alpha 1$ and $\alpha 2$ helices of chains *A* and *B* of Qri7 shows that the interacting residues involved in the Qri7 homodimerization form almost the same structure between chains *A* and *B* (r.m.s.d. of 0.242 Å for the C α atoms of residues 81–104 and 118–136). The superposition of $\alpha 1$ and $\alpha 2$ helices of the apo Qri7 and Qri7–AMP structures shows that the interacting residues involved in the dimerization form almost the same structure in the apo and AMP-bound forms of Qri7 (r.m.s.d. of 0.563 Å for the C α atoms of residues 81–104 and 118–136). Therefore, AMP binding to Qri7 has no effect on Qri7 dimerization.

The Qri7 monomer forms two lobes consisting of a β -sheet and flanking α -helices. The AMP molecule binds to the interface between the two lobes. This region might be the catalytic cleft, where TCA was

predicted to bind in the previous study (Wan *et al.*, 2013). Compared with the apo Qri7 structure determined previously, the $\alpha 4$, $\alpha 6$ and $\alpha 7$ helices are slightly closer to the inside of Qri7, probably owing to AMP binding to the catalytic cleft in our Qri7–AMP structure (Fig. 2*c*). However, the catalytic cleft is distant from the dimer interface and it remains unclear why the dimerization is important for the activity of Qri7.

3.2. Structural comparison of Qri7 with Kae1 and YgjD

The overall structure of Qri7–AMP is similar to those of Kae1–ATP (PDB entry 2ivp, r.m.s.d. 2.02 Å for the C α atoms of residues 1–321; Hecker *et al.*, 2007), YgjD–ATP γ S (PDB entry 3zeu, r.m.s.d. 2.25 Å for the C α atoms of residues 1–327; Nichols *et al.*, 2013) and YgjD–AMP (PDB entry 3zet, r.m.s.d. 2.33 Å for the C α atoms of residues 1–335; Nichols *et al.*, 2013). In the Qri7–AMP structure, the 2' and 3' hydroxyl groups of the ribose of AMP hydrogen bond to the side chain of Asp203. The adenine moiety of AMP hydrogen bonds to the side chains of Glu221, Ser328 and Asn329, while the phosphate group of AMP hydrogen bonds to the main chains of Gly325 and Gly173, as well as the side chain of Ser360 (Fig. 3*a*). In the Qri7–AMP structure, additional hydrogen bonds are formed between the AMP molecule and the Ser328 and Ser360 residues compared with the

Figure 3 The interactions of (a) Qri7 (green) with AMP and Zn (grey), (b) Kae1 (violet) with ATP and Fe³⁺ (violet), (c) YgjD (yellow) with AMP and Cd (light blue) and (d) YgjD with ATP γ S and Zn (grey) are shown. The hydrogen bonds and coordinated bonds are depicted by black dashed lines. Red spheres indicate water molecules. (e), (f) Superimposition of the crystal structures of Qri7–AMP (green) and Kae1–ATP (e) or YgjD–AMP (f). The superimposition was performed between the AMP of Qri7 and the AMP part of ATP of Kae1 or the AMP of YgjD. The positions of the metal ions relative to the bound nucleotides vary among Qri7, Kae1 and YgjD.

Figure 4

Superimposition of the crystal structures of TobZ (blue) with Qri7 (green) (*a*), TobZ with Kae1 (violet) (*b*) and TobZ with YgjD (yellow) (*c*). The disordered loop (residues 72–79) in Qri7 is depicted by a dashed line. The loop of Kae1 (residues 33–40) or YgjD (residues 37–44) in the crystal structure, shown in red, is overlapped with tobramycin. The binding of tobramycin, ADP and Fe to TobZ in the crystal structure is shown.

complex of Kae1 and ATP (Fig. 3*b*). In contrast, there is no hydrogen bond corresponding to that formed by Glu221 of Qri7 in the YgjD–ATP γ S and YgjD–AMP structures (Figs. 3*c* and 3*d*). Thus, the manner of AMP recognition by Qri7 is slightly different from that in Kae1 and YgjD.

While the phosphate group(s) of the bound nucleotides directly coordinate cations in Kae1 and YgjD, the phosphate group of AMP in the Qri7–AMP structure coordinates the metal ion through a water molecule (Figs. 3*a*–3*d*). The distance between the metal ion and the water molecule is 3.23 and 2.33 Å in chains *A* and *B*, respectively. Therefore, the water molecule might coordinate the metal ion, although the precise positions of the water molecules are uncertain owing to the low resolution. Molecular-docking experiments indicated that the main-chain carbonyl group of the threonine of TCA could coordinate the metal ion, similarly to ATP or ATP γ S in Kae1 or YgjD (Perrochia, Guetta *et al.*, 2013). In the Qri7–AMP structure the side chains of His145, His149, His175 and Asp361 and the water molecule coordinate the putative zinc ion (Fig. 3*a*). In the Kae1–ATP structure the side chains of His107, His111, Trp127 and Asp285 and two O atoms of the γ -phosphate of ATP coordinate an Fe³⁺ ion (Fig. 3*b*; Hecker *et al.*, 2007). In the YgjD–AMP structure the side chains of His111, His115 and Asp300, the O atom of the α -phosphate of AMP and two water molecules coordinate a cadmium ion (Fig. 3*c*; Downey *et al.*, 2006). In the YgjD–ATP γ S structure, the side chains of His111, His115 and Asp300 and the O atom of the β -phosphate of ATP γ S coordinate a zinc ion (Fig. 3*d*; Downey *et al.*, 2006). Thus, the positions of the metal ions relative to the bound nucleotides differ among Qri7, Kae1 and YgjD, although they can bind to the same substrate, TCA (Figs. 3*e* and 3*f*). In particular, although Qri7–AMP and YgjD–AMP bind the same compound, TCA, the distance between the metal ion and the O atom of the α -phosphate of AMP is quite different between them (5.81 Å in Qri7 and 2.34 Å in YgjD). These results indicated that in the Qri7–TCA, Kae1–TCA and YgjD–TCA complex structures the position of TCA relative to the metal ion might vary among the three homologues.

3.3. Structural comparison with the *O*-carbamoyltransferase TobZ

TobZ is an *O*-carbamoyltransferase involved in nebramycin synthesis (Wehmeier & Piepersberg, 2009; Kharel *et al.*, 2004). It catalyzes a carbamoyl transferase reaction which is similar to the

threonyl-carbamoyl transferase reaction in t⁶A biosynthesis. TobZ has a YrdC-like domain, which synthesizes carbamyladenylate from carbamoylphosphate and ATP, and a Kae1-like domain, which transfers the carbamoyl group of the synthesized carbamyladenylate to tobramycin to produce nebramycin (Kharel *et al.*, 2004). The TobZ structure (PDB entry 3vet; Parthier *et al.*, 2012) is similar to the Qri7/Kae1/YgjD structures (*C α* r.m.s.d.s with TobZ are 3.01 Å for residues 31–371 of Qri7–AMP, 2.26 Å for residues 1–299 of Kae1–ATP and 2.70 Å for residues 1–306 of YgjD–ATP γ S; Parthier *et al.*, 2012). In the TobZ structure, the ADP molecule binds to the Kae1-like domain at the corresponding sites to where AMP/ATP/ATP γ S bind in the Qri7/Kae1/YgjD structures (Parthier *et al.*, 2012). The region binding to tobramycin, which corresponds to tRNA in t⁶A biosynthesis, is occupied by a loop composed of residues 33–40 in Kae1 and residues 37–44 in YgjD, while the corresponding loop in Qri7, composed of residues 72–79, is disordered (Fig. 4). In addition, the *B* factors of this loop of Kae1 are also high (the average *B* factors of *C α* atoms are 32.9 and 54.8 Å² overall and for the loop, respectively), although those of YgjD are low (the average *B* factors of *C α* atoms are 24.3 and 22.0 Å² overall and for the loop, respectively) probably owing to crystallographic contacts with neighbouring molecules (Nichols *et al.*, 2013; Hecker *et al.*, 2007). These data suggested that the loop of the Qri7/Kae1/YgjD family is flexible in the absence of the substrate tRNA. The loop is conserved among Qri7, Kae1 and YgjD, and has been proposed to be involved in tRNA binding (Wan *et al.*, 2013).

Our structure of Qri7–AMP and the structural comparisons with the Qri7 homologues and TobZ provided insights into the manner of TCA binding and revealed the presence of the flexible loop. To elucidate the detailed recognition manner and the tRNA modification mechanism, the structure of the Qri7–tRNA complex is required.

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