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Structure Report

Crystal structures and putative interface of *Saccharomyces cerevisiae* mitochondrial matrix proteins Mmf1 and Mam33

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ABSTRACT

The yeast *Saccharomyces cerevisiae* mitochondrial matrix factor Mmf1, a member in the YER057c/Yigf/ Uk114 family, participates in isoleucine biosynthesis and mitochondria maintenance. Mmf1 physically interacts with another mitochondrial matrix protein Mam33, which is involved in the sorting of cytochrome b_2 to the intermembrane space as well as mitochondrial ribosomal protein synthesis. To elucidate the structural basis for their interaction, we determined the crystal structures of Mmf1 and Mam33 at 1.74 and 2.10 Å, respectively. Both Mmf1 and Mam33 adopt a trimeric structure: each subunit of Mmf1 displays a chorismate mutase fold with a six-stranded β -sheet flanked by two α -helices on one side, whereas a subunit of Mam33 consists of a twisted six-stranded β -sheet surrounded by five α -helices. Biochemical assays combined with structure-based computational simulation enable us to model a putative complex of Mmf1–Mam33, which consists of one Mam33 trimer and two tandem Mmf1 trimers in a head-to-tail manner. The two interfaces between the ring-like trimers are mainly composed of electrostatic interactions mediated by complementary negatively and positively charged patches. These results provided the structural insights into the putative function of Mmf1 during mitochondrial protein synthesis via Mam33, a protein binding to mitochondrial ribosomal proteins.

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1. Introduction

Members in the YjgF/YER057c/UK114 family are highly conserved in archaea, bacteria and eukarya. They share a highly conserved primary sequence with an identity from 47% to 78%, but possess diverse biological functions (Leitner-Dagan et al., 2006; Manjasetty et al., 2004; Parsons et al., 2003; Sinha et al., 1999; Volz, 1999). The human homolog hp14.5 has been proposed as a putative translation inhibitor that can inhibit cell-free protein synthesis in the rabbit reticulocyte lysate system (Oka et al., 1995; Schmiedeknecht et al., 1996), whereas the rat homolog rp14.5 has endoribonuclease activity (Morishita et al., 1999), and the goat homolog UK114 has tumor antigen activity (Ceciliani et al., 1996). Biological functions of other homologs include the calpain activation in the bovine (Melloni et al., 1998), the purine regulation activity in Bacillus subtilis (Sinha et al., 1999), and photosynthesis and chromoplastogenesis in plants (Leitner-Dagan et al., 2006). A line of evidences suggested that subtle sequence changes attribute the functional divergence among the members (Thakur et al., 2010). To date, about twenty structures of this family have been

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determined, all of which adopt a trimeric structure with three clefts, each of which is characterized by 6–9 signature residues. These conserved clefts are able to bind diverse ligands, including L-threonine, L-serine, 2-ketobutyrate, ethylene glycol, or propionate (Kim et al., 2001; Parsons et al., 2003; Sinha et al., 1999). However, the physiological significance of these members remains unclear. The homolog from the yeast *Saccharomyces cerevisiae* was named Mmf1, for mitochondrial matrix factor. Mmf1 was proposed to be involved in isoleucine biosynthesis and intact mitochondria maintenance (Burman et al., 2007; Kim et al., 2001; Oxelmark et al., 2000).

Tandem affinity purification assays indicated that Mmf1 physically interacted with a mitochondrial acidic matrix protein Mam33 (Krogan et al., 2006), suggesting their putative functional relevance. Mam33 was found to be involved in sorting cytochrome b₂ to the mitochondrial intermembrane space via binding to its signal peptide (Seytter et al., 1998). Deletion of *MAM33* gene would lead to slower growth of yeast in glycerol medium but not in glucose medium, suggesting that Mam33 may participate in the mitochondrial oxidative phosphorylation (Muta et al., 1997; Seytter et al., 1998). Mam33 was identified as a homo-trimer or tetramer and shared a sequence identity of approximately 24–35% with the homologs from human, *Leishmania major, Trypanosoma brucei*,

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and *Caenorhabditis elegans* (Seytter et al., 1998). The human homolog of Mam33 was termed splicing factor 2-associated protein p32, which was proposed to be involved in the maintenance of mitochondrial oxidative phosphorylation (Muta et al., 1997). The human p32 executes diverse functions via binding to various partners (Jiang et al., 1999; Joseph et al., 1996; Matthews and Russell, 1998; Okagaki et al., 2000). Up to date, a total of 82 protein partners were identified to have physical or genetic interactions with yeast Mam33 (http://www.yeastgenome.org/). Among them, 23 partners are mitochondrial ribosomal proteins (Collins et al., 2007; Gavin et al., 2006; Krogan et al., 2006). This led us to presume that, with the assistance of Mmf1, Mam33 may take part in mitochondrial ribosomal protein synthesis.

To figure out the structural insights into the interaction pattern between Mmf1 and Mam33, we attempted to solve the crystal structure of their complex. We purified the complex of Mam33– Mmf1, but we were unable to obtain its crystal. Nevertheless, we solved the crystal structures of individual Mmf1 and Mam33 at 1.74 and 2.10 Å, respectively. In vitro biochemical assays demonstrated that Mmf1 and Mam33 can form a complex with 2:1 M ratio. Structural analysis revealed the positively charged surfaces of Mmf1 trimer are complementary to the negatively charged patches of Mam33 trimer. In addition, a docking model of Mmf1–Mam33 complex provides the structural basis for the putative binding mode of these two proteins.

2. Protein expression, purification and crystallization

The gene encoding Mmf1 and Mam33 were PCR amplified from the genomic DNA of S. cerevisiae S288C. The coding sequence of an additional His₆-tag was introduced at the 5' end of the gene. The PCR product were cloned into pET28a-derived vectors and expressed at 37 °C using the transformed Escherichia coli BL21 (DE3) strains and $2 \times$ YT medium (OXOID LTD) supplemented with $30 \mu g/ml$ kanamycin. When the cell culture reached an OD_{600nm} of 0.6-1.0, the expression of the protein was induced with 0.2 mM isopropyl-B-D-1-thiogalactopyranoside and the cells were grown for a further 20 h at 16 °C. Cells were collected by centrifugation, resuspended in 15 ml buffer containing 100 mM NaCl, 20 mM Tris-Cl, pH 8.0. His-tagged proteins were purified using Ni²⁺ affinity columns. Eluted protein were further purified by gel filtration using a Superdex[™] 200 column (GE Healthcare Bioscience) equilibrated in 100 mM NaCl, 20 mM Tris-Cl, pH 8.0 and 20 mM βmercaptoethanol. The purity of the pooled fraction was checked by electrophoresis. Crystals of Mmf1 and Mam33 were obtained by the hanging drop diffusion method at 16 °C for 2 weeks. In each drop of crystallization, 1 μ l protein sample at 10–15 mg/ml in the buffer of 50 mM NaCl, 20 mM Tris–Cl, pH 8.0 and 20 mM β mercaptoethanol was mixed with 1 µl reservoir solution and equilibrated against 500 µl reservoir solution (25% polyethylene glycol 3550 for Mmf1 and 20% polyethylene glycol 4000 for Mam33).

3. Data collection, processing, structure determination and refinement

X-ray diffraction data were collected at 100 K in a liquid nitrogen stream using beamline 17 U with an MX225 CCD (MARresearch, Germany) at the Shanghai Synchrotron Radiation Facility (SSRF). The data were integrated and scaled with the program HKL2000 (Otwinowski and Minor, 1997).

The structures of Mmf1 and Mam33 were determined by molecular replacement method with the program MOLREP (Vagin and Teplyakov, 2010) from the CCP4 suite using the coordinates of 1JD1 and 1P32, respectively. The initial models were refined by using the maximum likelihood method implemented in REFMAC5 (Murshudov et al., 1997) as part of CCP4 program suite and rebuilt interactively in the program COOT (Emsley and Cowtan, 2004). The final models were evaluated using the programs MOLPROBITY (Davis et al., 2007) and PROCHECK (Laskowski et al., 1996). Crystallographic parameters are listed in Table 1. All structural figures were created by PyMOL (DeLano, 2002).

4. In vitro assays of the complex between Mmf1 and Mam33

The cells expressing Mmf1 and Mam33, respectively, are mixed and lysed by sonication after three cycles of freezing/thawing. The mixed proteins were purified using Ni²⁺ affinity columns. Eluted protein were further purified by gel filtration using a HiLoad 16/ 60 Superdex 200 column (GE Healthcare) equilibrated in 100 mM NaCl, 20 mM β -mercaptoethanol and 20 mM Tris–Cl, pH 8.0, and with a flow rate of 1.0 ml/min. The purity and the components of the pooled fraction were checked by electrophoresis. The buffer was filtered with a 0.22-mm membrane and thoroughly degassed.

Isothermal titration calorimetry assays were carried out with Microcal iT-200 isothermal titration calorimeter (GE Healthcare, Germany). According to manufacturer's instructions, about 0.2 ml Mmf1 protein (150 μ M) in the Tris–Cl buffer, pH 8.0 was loaded into the cell. The titration syringe was loaded with Mam33 at a concentration of about 400 μ M. Titrations were carried out at 25 °C with 20 injections at an interval of 2 min. A protein-free buffer was set as control. Results of the titration curves were analyzed using Origin 7.5 software (Northampton, MA).

Sedimentation velocity assays were performed on a Proteomelab XL-A/XL-I analytical ultracentrifuge (Beckman Coulter Instruments) with an An-60 Ti rotor, one cell assembled by sapphire windows, a double-sector 12 mm length charcoal-filled Epon centerpiece and UV/vis absorbance optical system. All assays were conducted at 60,000 rpm and 20 °C. Four hundred microliters of Mmf1–Mam33 (C = 1.3 mg/ml) and Mmf1 (C = 4.0 mg/ml) were loaded for measurement with 420 µl of buffer solution as the ref-

Table 1	
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Data collection and refinement statistics.

Data processing	Mmf1	Mam33
Space group	НЗ	I2 ₁ 3
Unit cell (Å)	77.62, 77.62, 43.91	118.76, 118.76, 118.76
Resolution range (Å)	50.00-1.74 (1.80-1.74) ^a	50.00-2.10 (2.18-2.10)
Unique reflections	9,969 (929)	16,404 (1,605)
Completeness (%)	99.3 (94.5)	99.9 (100)
< <i>l</i> / <i>σ</i> (<i>I</i>)>	14.8 (7.2)	37.7 (10.3)
R _{merge} ^b (%)	10.4 (25.0)	8.1(39.3)
Average redundancy	11.0 (7.8)	22.0 (21.3)
Refinement statistics		
Resolution range (Å)	38.81-1.75	41.99-2.10
R-factor ^c /R-free ^d (%)	18.1/22.2	22.7/25.2
Number of protein atoms	957	1,442
Number of water atoms	77	87
RMSD ^e bond lengths (Å)	0.011	0.010
RMSD bond angles (°)	1.139	1.040
Mean B factors (Å ²)	22.8	18.4
Ramachandran plot ^f		
(residues, %)		
Most favored (%)	98.4	100
Additional allowed (%)	1.6	0
PDB entry	3QUW	3QV0

^a The values in parentheses refer to statistics in the highest bin.

^b $R_{merge} = \sum_{hkl} \sum_{i} |I_i(hkl) - \langle I(hkl) \rangle |/\sum_{hkl} \sum_{i} I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl) \rangle$ is the mean value for its unique reflection; Summations are over all reflections.

^c R-factor = $\sum_h |Fo(h) - Fc(h)| / \sum_h Fo(h)$, where Fo and Fc are the observed and calculated structure-factor amplitudes, respectively.

^d R-free was calculated with 5% of the data excluded from the refinement.

Root-mean-square deviation from ideal values.

f Categories were defined by Molprobity.

erence, respectively; 90–300 scans of data for each sample were acquired at a time interval of 1 min at wavelength 280–292 nm to ensure the absorbance lower than 1. Solvent densities and viscosities were computed using the program SEDNTERP (http://www.rasmb.bbri.org/). Partial specific volumes (\bar{v}) of Mmf1–Mam33 (0.735 ml g⁻¹) and Mmf1 (0.743 ml g⁻¹) were calculated from amino acid composition. In principle, the change of solute concentration with time and radius during sedimentation were calculated by the Lamm equation (Colfen et al., 2006).

5. Structure of Mmf1

The crystal structure of Mmf1 was determined at 1.74 Å by molecular replacement using *S. cerevisiae* homologous mitochon-

drial matrix factor 1 Hmf1 (PDB 1JD1) as a search model. It belongs to the space group H3 with unit cell dimensions of a = b = 77.62 Å, c = 43.91 Å, and $\alpha = \beta = 90.00^{\circ}$, $\gamma = 120.00^{\circ}$. Three subunits from the three symmetric units form a tight trimer with a total interface area of 2800 Å². Gel filtration also confirmed the presence Mmf1 trimer as the major fraction in solution, compared to a small fraction of Mmf1 oligomer (data not shown). Similar to other structure-known members in the YjgF/YER057c/UK114 family, the core structure of the trimer resembles a triangular barrel-like shape, which is formed by 12 β -strands (β 3– β 6 from each subunit). The barrel is surrounded by six α -helices on the outer side, and filled with water molecules on the highly hydrophilic inner side (Fig. 1a). At the interfaces of the adjacent subunits of Mmf1 trimer, there are three narrow and deep clefts, mainly composed of seven



Fig.1. Structural analysis of Mmf1. (a) Overall structure of Mmf1 trimer, viewed from the head (left) and the tail (right) surface, respectively. Three subunits are rendered in cyan, green and yellow, respectively. The second structure elements are labeled sequentially in one subunit. (b) Charge distribution on the head (left) and the tail (right) surface of Mmf1 trimer; P1, P2, P3 and N1, N2, N3 represent three positively and three negatively charged surfaces of the head surface. N1', N2', N3' and P1', P2', P3' represent three negatively and three negatively charged surfaces of Mmf1 and homologs. The seven conserved residues at each inter-subunit cleft are labeled with black triangles.

conserved residues Tyr36, Gly50, Asn75, Asn107, Arg121, Pro130 and Glu136 (Fig. 1c). These clefts have been proved to bind various ligands, such as L-threonine, L-serine, 2-ketobutyrate, ethylene glycol, or propionate (Burman et al., 2007; Parsons et al., 2003; Sinha et al., 1999). The cluster of these small molecules strongly suggests these clefts are functionally important for binding or transporting these molecules.

Each subunit of Mmf1 adopts a chorismate mutase fold (Chook et al., 1994; Volz, 1999), which consists of six-stranded β -sheet and two α -helices with the topology of $\beta 1-\beta 2-\beta 3-\alpha 1-\beta 4-\alpha 2-\beta 5-\beta 6$ (Fig. 1a). The consecutive β -sheets, designated through $\beta 1$ to $\beta 6$, form a twisted β -sheet ($\beta 2$ and $\beta 6$ antiparallel to the other four strands), with two α -helices ($\alpha 1$ and $\alpha 2$) flanked at one side. The overall structure of Mmf1 is very similar to its homologs. Superposition of Mmf1 monomer against that of the 53% sequence-identical homolog *B. subtilis* YabJ (PBD 1QD9) revealed an overall root-mean-square deviation (RMSD) of 0.50 Å over 115 C α atoms.

At the head of the barrel, there are three positively charged patches (P1, P2 and P3) separated by three negatively charged patches (N1,N2 and N3) (Fig. 1b, left). The positively charged patches are centralized with Lys41, whereas the negatively charged patches with a center of Asn43 and Asn44. At the tail of the barrel, we can find three positively charged patches (P1', P2' and P3') separated by three negatively charged patches (N1', N2' and N3') in a similar pattern (Fig. 1b, right). The patches on the head can somewhat perfectly complement in charge the patches on the tail. This complementation also provides the structural basis for the formation of a head-to-tail dimer of Mmf1 trimers.

6. Structure of Mam33

The recombinant Mam33 without the N-terminal 46-residue signal peptide was subjected to crystallization. The crystal structure was determined at 2.10 Å by molecular replacement using human p32 (PDB 1P32) as the search model. It belongs to the space group $I2_13$ with unit cell dimensions of a = b = c = 118.76 Å, and $\alpha = \beta = \gamma = 90.00^\circ$. In the final model, each asymmetric unit con-



Fig.2. Overall structure of Mam33 trimer. Three subunits are rendered in cyan, magenta and red, respectively. The secondary structure elements are labeled sequentially in one subunit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tains one subunit and shows good geometry with the residues Thr67–Thr72 not modeled due to their flexibilities. Three subunits from the symmetric units form a trimer by the crystallographic 3-fold axis with a total interface area of 3500 Å². The trimer structure resembles a bowl, with a hydrophilic inner wall composed of 12 β -strands from three subunits (Fig. 2). In addition, three α 3 helices form the bottom, while the other 12 α -helices (four from each subunit) pack against the outside wall of the bowl.

Each subunit of Mam33 consists of six β -strands and five α -helices with the topology of $\alpha 1-\alpha 2-\beta 1-\beta 2-\beta 3-\beta 4-\beta 5-\beta 6-\alpha 3-\alpha 4-\alpha 5$ (Fig. 2). The six β -strands form a highly twisted antiparallel β -sheet, with $\beta 6$ nearly perpendicular to $\beta 1$. The β -sheet is flanked by four α -helices ($\alpha 1, \alpha 2, \alpha 4$ and $\alpha 5$) on outer side and $\alpha 3$ on inner side. The structure of Mam33 resembles human p32 (PDB 1P32) with an overall RMSD of 1.7 Å over 157 C α atoms. The remarkable difference concerns the substitution of $\alpha 2$ and $\alpha 3$ in Mam33 by two loops in human p32, respectively.

Electrostatic potential surface demonstrates the asymmetric charge distribution on the mouth and bottom of the bowl. The mouth of the bowl contains a large content of acidic residues and forms a continuous acidic patch. By contrast, the bottom of the bowl is much less negatively charged. This polarity in charge distribution suggests asymmetric functional roles for the two sides of this structure.

7. The Mmf1-Mam33 complex in solution

Previous report identified that Mmf1 may physically interact with Mam33 (Krogan et al., 2006). To prove this, size-exclusion chromatography assay was performed with a SuperdexTM 200 column for purification the complex of Mmf1–Mam33. The complex was eluted at about 66 ml, corresponding to a molecular mass of approximately 153 kDa. Electrophoresis showed the eluted peak was composed of Mmf1 and Mam33, at a molar ratio of about 2:1 (data not shown). Furthermore, isothermal titration calorimetry was applied to evaluate the interaction between Mmf1 and Mam33, which gives an equilibrium dissociation constant (K_d) of 46.9 μ M. The isothermal titration calorimetry result also indicates that the molar ratio of Mmf1 versus Mam33 is 2:1 (Fig. 3a).

To further prove this, sedimentation velocity assays was performed using the co-purified protein complex. Sufficient data were fitted to Lamm equation by using program SEDFIT (Schuck, 1998). The continuous c(s) and c(M) distribution model with maximum entropy regularization was chosen to fit sedimentation coefficient (*s*) and molar mass (*M*). The result shows a typical sedimentation profile of the complex sample with a molecular weight of about 175 kDa (Fig. 3b), which is close to the theoretical mass of six Mmf1 plus three Mam33 subunits. The sedimentation profile of Mmf1 reveals two distinct peaks, corresponding to the trimer of the major fraction and the dimer of trimers of the small fraction, respectively (Fig. 3c). Taken together, we can infer that the complex is composed of two trimers of Mmf1 and one trimer of Mam33.

8. A putative model of Mmf1-Mam33

The biochemical assays suggested the molar ratio of Mmf1 to Mam33 was about 2:1. Thus based on the structures of Mmf1 and Mam33, we generated a model of Mmf1–Mam33 (Fig. 3d) by using the program HADDOCK (Dominguez et al., 2003). Docking was started with the trimeric structures of Mam33 and Mmf1, and was driven by interaction restraints with the active residues, as defined by the program WHISCY (de Vries et al., 2006). Cluster 1 with seven members satisfied best with interaction restraints. The model consists of a dimer of Mmf1 trimers in a head-to-tail

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Fig.3. The complex of Mmf1–Mam33. (a) Isothermal titration calorimetry titration of Mmf1 with Mam33. Sedimentation coefficient distribution of (b) Mmf1–Mam33 complex and (c) Mmf1. The inset shows the molar mass distribution of the complex or Mmf1. (d) A putative model of the Mmf1–Mmf1–Mam33 complex. Mmf1 and Mam33 are colored yellow and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

manner and a Mam33 trimer. The complex had a buried interface areas of about 4000 Å² between Mmf1 and Mam33, and 3200 Å² between the two Mmf1 trimeric rings. At the Mmf1-Mmf1 interface, three negatively charged surfaces (N1', N2' and N3') and three positively charged surfaces (P1', P2' and P3') from the tail of one trimer are finely complementary to the three positively (P1, P2 and P3) and negatively (N1, N2 and N3) charged surfaces on the head of another trimer, respectively. At the interface between Mmf1 and Mam33, negatively charged surfaces from the mouth of Mam33 bowl could compensate to the positively charged surfaces from the head of Mmf1, by a couple of polar interactions. The model reveals a unique binding mode of Mam33 and Mmf1. The sedimentation profile of Mmf1 reveals a large fraction of Mmf1 trimers and a small fraction of the dimer of trimers, indicating that Mmf1 exists as an equilibrium of two oligomeric forms in solution. Upon binding to Mam33, Mmf1 seems to exist as the dimer of trimers, as shown by the sedimentation profile of Mmf1-Mam33 complex.

In summary, here we reported the crystal structures of *S. cerevisiae* Mmf1 and Mam33 at 1.74 and 2.10 Å, respectively. A simulated model of Mmf1 in complex with Mam33 was proposed based on the structures and in vitro biochemical assays. However, more investigations are needed to elucidate the mechanism of why and how these mitochondrial proteins work together.

Accession numbers

The coordinates and the structure factors Mmf1 and Mam33 have been deposited in the RCSB Protein Data Bank under the accession codes of 3QUW and 3QV0, respectively.

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