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Expression, purification, crystallization and preliminary X-ray crystallographic analysis of Zucchini from *Drosophila melanogaster*

PIWI-interacting RNAs (piRNAs) bind PIWI proteins and silence transposons to maintain the genomic integrity of germ cells. Zucchini (Zuc), a phospholipase D superfamily member, is conserved among animals and is implicated in piRNA biogenesis. However, the underlying mechanism by which Zuc participates in piRNA biogenesis remains elusive. *Drosophila melanogaster* Zuc (DmZuc) was expressed in *Escherichia coli*, purified and crystallized. X-ray diffraction data were collected to 1.75 Å resolution. The crystal belonged to space group $P2_1$, with unit-cell parameters $a = 55.0$, $b = 71.2$, $c = 56.3$ Å, $\beta = 107.9^\circ$.

1. Introduction

PIWI-interacting RNAs (piRNAs), which are germline-specific small noncoding RNAs, bind PIWI Argonaute proteins and silence transposons in animal germ cells to maintain genomic integrity (Malone & Hannon, 2009; Senti & Brennecke, 2010; Siomi *et al.*, 2011; Pillai & Chuma, 2012). piRNAs are produced through two distinct biogenetic pathways: the primary processing pathway and the secondary ping-pong cycle (Vagin *et al.*, 2006; Gunawardane *et al.*, 2007; Brennecke *et al.*, 2007; Malone *et al.*, 2009; Li *et al.*, 2009; Saito *et al.*, 2009). *Drosophila melanogaster* has three PIWI proteins: Piwi, Aubergine (Aub) and Argonaute 3 (Ago3). Piwi is expressed in ovarian somatic cells and participates in the primary pathway, whereas Aub and Ago3 are expressed in ovarian germ cells and function in the ping-pong cycle. In the primary processing pathway, piRNAs are transcribed as long single-stranded precursors from intergenic repetitive elements known as piRNA clusters (Brennecke *et al.*, 2007; Malone *et al.*, 2009; Li *et al.*, 2009). Genetic and biochemical studies revealed that the putative nuclease Zucchini (Zuc), the RNA helicase Armitage and the Tudor domain-containing RNA helicase Yb are involved in the primary processing pathway (Olivieri *et al.*, 2010; Saito *et al.*, 2010; Haase *et al.*, 2010; Qi *et al.*, 2011).

Zuc is conserved among animals and is composed of an N-terminal mitochondrial localization sequence (MLS) and a catalytic domain belonging to the phospholipase D (PLD) superfamily (Pane *et al.*, 2007; Fig. 1). *D. melanogaster* Zuc (DmZuc) and the mouse homologue MmZuc (also known as Pld6 and MitoPLD) are localized on the mitochondrial surface and have been implicated in piRNA biogenesis (Pane *et al.*, 2007; Malone *et al.*, 2009; Saito *et al.*, 2009, 2010; Olivieri *et al.*, 2010; Haase *et al.*, 2010; Huang *et al.*, 2011; Watanabe *et al.*, 2011). The PLD superfamily is characterized by a conserved HKD (His-Lys-Asp) motif and includes various proteins with diverse functions, such as PLD, cardiolipin synthases, phosphatidylserine synthases, nucleases, toxins and viral envelope proteins (Ponting & Kerr, 1996). Most of the superfamily members have two domains with a similar fold containing the HKD motif, whereas the bacterial nuclease Nuc has only one copy of the HKD motif and dimerizes to create the active site at the dimer interface (Stuckey & Dixon, 1999). Like Nuc, Zuc has one copy of the HKD motif, suggesting that Zuc functions as a dimer. Whereas MmZuc reportedly hydrolyzes the mitochondrial lipid cardiolipin to generate the signalling lipid phosphatidic acid (Choi *et al.*, 2006), Zuc has been considered to be a candidate for the piRNA-processing nuclease, since it shares the highest sequence similarity with Nuc among the

superfamily members (Pane *et al.*, 2007). However, the mechanism by which Zuc participates in piRNA biogenesis remains elusive, since its enzymatic activity has not been fully explored. Here, we report the expression, purification, crystallization and preliminary X-ray crystallographic analysis of DmZuc. The structural analysis, together with a biochemical analysis using the purified enzyme, will help in elucidating the molecular mechanism by which Zuc participates in piRNA biogenesis.

2. Materials and methods

2.1. Construction

We constructed expression plasmids encoding residues 41–253, 89–250 and 89–253 of DmZuc (DmZuc_{41–253}, DmZuc_{89–250} and DmZuc_{89–253}, respectively) and residues 35–221 and 74–221 of MmZuc (MmZuc_{35–221} and MmZuc_{74–221}, respectively). The DNA fragments encoding these regions were amplified by PCR from the cDNAs of DmZuc (UniProt accession code Q9VKD7) and MmZuc (UniProt accession code Q5SWZ9) using PrimeSTAR MAX DNA Polymerase (Takara Bio Inc.). The PCR products were cloned between the *SacI* and *XhoI* sites of the pCold-GST vector (Hayashi & Kojima, 2008) and thus all of the constructs contained the sequence **LEVLFGQPGHME**L (the Turbo3C protease-recognition sequence is indicated in bold) between the His₆-GST tag and the Zuc protein. The DmZuc_{41–253} K171A mutant was generated by a PCR-based method using pCold-DmZuc_{41–253} as the template. The insert sequences were verified by DNA sequencing.

2.2. Expression

DmZuc_{41–253}, DmZuc_{89–253} and MmZuc_{35–221} were overexpressed in *Escherichia coli* Rosetta2 (DE3) cells (Novagen). The *E. coli* cells were cultured at 310 K in LB medium containing 100 g l⁻¹ ampicillin. When the culture reached an OD₆₀₀ of 0.5–0.8, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.1 mM. The cells were further cultured at 293 K for 24 h and were harvested

by centrifugation. Selenomethionine (SeMet) labelled DmZuc_{89–250} was overexpressed in *E. coli* B834 (DE3) cells in SeMet core medium (Wako) supplemented with 10 g l⁻¹ D-glucose, 1 × MEM Vitamin Solution (Sigma), 0.25 g l⁻¹ MgSO₄, 4 mg l⁻¹ FeSO₄, 100 g l⁻¹ ampicillin and 25 mg l⁻¹ L-SeMet (Wako).

2.3. Purification

E. coli cells expressing DmZuc_{89–253} were resuspended in buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole, 3 mM β-mercaptoethanol), lysed by sonication and centrifuged at 40 000g for 30 min. The supernatant was incubated with 4 ml Ni-NTA Superflow resin (Qiagen) and the mixture was loaded into an Econo-Column (Bio-Rad). The resin was washed with buffer A and the protein was eluted with buffer B (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 300 mM imidazole, 3 mM β-mercaptoethanol). The eluted protein was dialyzed against buffer A and treated with Turbo3C protease (Nacalai Tesque) at 277 K overnight in order to cleave the N-terminal His₆-GST tag. The dialyzed protein was passed through an Ni-NTA column and was concentrated using an Amicon Ultra 3K filter (Millipore). The protein was further purified by chromatography on a HiLoad Superdex 75 16/60 column (GE Healthcare) equilibrated with buffer C (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT). The purified protein was concentrated using an Amicon Ultra 3K filter and was stored at 193 K until use. SeMet-labelled DmZuc_{89–250} was purified using a protocol similar to that used for DmZuc_{89–253}.

E. coli cells expressing wild-type (WT) DmZuc_{41–253} were resuspended in buffer D (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 3 mM β-mercaptoethanol), lysed by sonication and centrifuged at 20 000g for 30 min. The supernatant was incubated with 2 ml Glutathione Sepharose Fast Flow resin (GE Healthcare) and the mixture was loaded into an Econo-Column. The resin was washed with buffer D and the protein was eluted with buffer D supplemented with 10 mM glutathione. The eluted protein was treated with Turbo3C protease at 277 K overnight to cleave the N-terminal His₆-GST tag and was then

Figure 1 Multiple sequence alignment of DmZuc, MmZuc and Nuc. The predicted secondary structure of DmZuc is shown above the sequences and the predicted disordered regions are indicated by dashed lines. The secondary structure of Nuc (PDB entry 1byr) is shown below the sequences. The HKD motif is indicated by red triangles. The alignment was prepared using *ClustalW* (Larkin *et al.*, 2007) and *ESPrpt* (Gouet *et al.*, 1999).

passed through an Ni-NTA column. The protein was diluted with a fivefold volume of buffer *E* (20 mM Tris-HCl pH 8.0, 1 mM DTT) to reduce the NaCl concentration to 50 mM and was loaded onto a Resource S column (GE Healthcare) equilibrated with buffer *F* (20 mM Tris-HCl pH 8.0, 20 mM NaCl, 1 mM DTT). The column was washed with buffer *F* and the protein was eluted with a linear gradient of 20–600 mM NaCl in buffer *F*. The protein was concentrated using an Amicon Ultra 10K filter (Millipore) and was further purified by chromatography on a Superdex 200 10/300 column (GE Healthcare). The purified protein was concentrated using an Amicon Ultra 10K filter and was stored at 193 K until use. MmZuc_{35–221} and the DmZuc_{41–253} K171A mutant were purified using a protocol similar to that used for WT DmZuc_{41–253}. All of the purified proteins were stored in buffer *C* at 193 K until use. The Superdex 200 10/300 column was calibrated using a Gel Filtration Calibration Kit LMW (GE Healthcare).

2.4. Crystallization

Initial crystallization screening was performed at 293 K by the sitting-drop vapour-diffusion method in a 96-well MRC Crystallization Plate (Hampton Research) using screening kits including Crystal Screen, Crystal Screen 2, PEG/Ion, Natrix, Index (Hampton Research), PACT, JCSG+ (Qiagen), MemGold (Molecular Dimensions), Wizard (Emerald BioSystems), JBScreen Classic and JBScreen Membrane (Jena Bioscience). Crystallization drops were prepared by mixing 100 nl protein solution and 100 nl reservoir solution using a Mosquito crystallization robot (TTP LabTech). Initial crystallization conditions were optimized at 293 K by varying the volumes of the drops, the protein concentration, the pH and the type and concentration of PEG using the sitting-drop or hanging-drop vapour-diffusion methods. Crystallization conditions were also optimized with the Additive Screen kit (Hampton Research).

2.5. Data collection and preliminary crystallographic analysis

Crystals were cryoprotected in reservoir solution supplemented with 30% ethylene glycol and flash-cooled in a nitrogen-gas stream at 100 K. X-ray diffraction experiments were performed on BL32XU at SPring-8, Hyogo, Japan using an MX225HE detector. X-ray diffrac-

tion data were collected using a helical data-collection strategy involving a microbeam (1 × 15 µm) and were processed using *HKL-2000* (HKL Research Inc.). Molecular replacement was performed with *MOLREP* (Vagin & Teplyakov, 2010).

3. Results and discussion

3.1. Construction

Secondary-structure prediction using the *Phyre2* server (Kelley & Sternberg, 2009) suggested that DmZuc and MmZuc have an N-terminal MLS and a transmembrane helix followed by a β -strand (residues 52–56 of DmZuc and residues 38–42 of MmZuc) which corresponds to the first β -strand of Nuc (Fig. 1). In contrast to Nuc, DmZuc and MmZuc are predicted to have a disordered region (residues 59–88 of DmZuc and residues 57–72 of MmZuc) between the β 1 strand and the α 2 helix in the PLD core fold, suggesting that this predicted disordered region is a conserved feature of Zuc proteins. Thus, we prepared DmZuc_{41–253} and MmZuc_{35–221}, which contain the first β -strand and the disordered region, as well as DmZuc_{89–253} and MmZuc_{74–221}, which lack these regions.

3.2. Expression and purification

DmZuc_{89–253}, DmZuc_{41–253} and MmZuc_{35–221} were expressed in *E. coli* and purified to homogeneity (Fig. 2*a*), whereas MmZuc_{74–221} precipitated during purification. DmZuc_{89–253} (19.4 kDa), DmZuc_{41–253} (24.7 kDa) and MmZuc_{35–221} (21.8 kDa) eluted from the size-exclusion column at volumes corresponding to molecular weights of 16, 39 and 34 kDa, respectively (Fig. 2*b*). These results suggested that DmZuc_{89–253} may be monomeric in solution and DmZuc_{41–253} and MmZuc_{35–221} may be dimeric in solution, which is consistent with the prediction that DmZuc_{89–253} lacks the first β -strand that contributes to dimerization in Nuc (Stuckey & Dixon, 1999). The expression level of DmZuc_{41–253} was much lower (about 0.02 mg protein was purified from 1 l culture) than that of DmZuc_{89–253} (about 2 mg protein was purified from 1 l culture), making it difficult to perform extensive crystallization screening. During the preparation of several DmZuc_{41–253} mutants for functional analyses, we fortuitously found

Figure 2

Preparation of DmZuc and MmZuc. (*a*) SDS-PAGE analysis of DmZuc_{89–253}, DmZuc_{41–253} and MmZuc_{35–221}. The purified proteins were analyzed by 10–20% SDS-PAGE and stained with SimplyBlue SafeStain (Invitrogen). (*b*) Calibration curve of the Superdex 200 10/300 column. The column was calibrated with a Gel Filtration Calibration Kit LMW (GE Healthcare).

that the DmZuc_{41–253} K171A mutant, in which Lys171 in the HKD motif is replaced by alanine, was highly expressed in *E. coli*. The DmZuc_{41–253} K171A mutant was also purified to homogeneity (about 1 mg protein was purified from 1 l culture).

3.3. Crystallization and preliminary X-ray crystallographic analysis

We obtained initial crystals of DmZuc_{89–253} by mixing 0.1 µl protein solution (6 mg ml^{−1}) with 0.1 µl reservoir solution [condition No. A2 of JBScreen Classic 1; 15% (v/v) PEG 400, 100 mM MES pH 6.5; Fig. 3a]. The DmZuc_{89–253} crystal diffracted to 2.5 Å resolution and belonged to space group *P*₃₂1 (where *x* may be 0, 1 or 2), with unit-cell parameters *a* = 60.4, *b* = 60.4, *c* = 254.1 Å (Table 1). Assuming two protein molecules per asymmetric unit, the Matthews coefficient (*V*_M) was estimated to be 3.5 Å³ Da^{−1}, corresponding to a solvent content of 64%. We were unable to determine the structure by molecular replacement using the Nuc structure (PDB entry 1byr; Stuckey & Dixon, 1999) as a search model, even though DmZuc shares 26% sequence identity with Nuc. Neither native nor SeMet-labelled DmZuc_{89–253} crystallized reproducibly, possibly owing to the presence of a C-terminal disordered region (residues 242–253; Fig. 1). Therefore, we prepared a series of constructs lacking C-terminal residues, performed crystallization screening and obtained initial crystals of SeMet-labelled DmZuc_{89–250} under condition No. A9 of

Natrix [10% (v/v) PEG 400, 50 mM MES pH 6.0, 100 mM KCl, 10 mM MgCl₂]. We obtained diffraction-quality crystals of SeMet-labelled DmZuc_{89–250} by mixing 0.4 µl protein solution (2.5 mg ml^{−1}) with 0.4 µl reservoir solution consisting of 8% (v/v) PEG 400, 50 mM MES pH 6.0, 100 mM KCl, 10 mM MgCl₂, 3% (w/v) D-(+)-trehalose (Fig. 3b). The SeMet-labelled crystal diffracted to 2.2 Å resolution and belonged to space group *P*₂₁, with unit-cell parameters *a* = 35.3, *b* = 52.4, *c* = 38.8 Å, *β* = 104.9° (Table 1). We are currently performing the structure determination.

We obtained initial crystals of DmZuc_{41–253} K171A under condition No. 42 of PEG/Ion screen [20% (w/v) PEG 3350, 200 mM KH₂PO₄]. We obtained plate-shaped crystals by mixing 1 µl protein solution (3 mg ml^{−1}) with 1 µl reservoir solution consisting of 16% (w/v) PEG 3350, 200 mM KH₂PO₄. The crystal of DmZuc_{41–253} K171A diffracted to 2.2 Å resolution and belonged to space group *P*₂₁, with unit-cell parameters *a* = 55.9, *b* = 70.2, *c* = 56.9 Å, *β* = 108.8° (Table 1). We also obtained crystals of WT DmZuc_{41–253} under conditions similar to those used for the K171A mutant (Fig. 3c). The crystal of WT DmZuc_{41–253} diffracted to 1.75 Å resolution (Fig. 4) and belonged to space group *P*₂₁, with unit-cell parameters *a* = 55.0, *b* = 71.2, *c* = 56.3 Å, *β* = 107.9° (Table 1). Assuming the presence of two protein molecules per asymmetric unit, the *V*_M was estimated to be 2.1 Å³ Da^{−1}, with a solvent content of 42%. MmZuc_{35–221} failed to crystallize, although it eluted as a single monodisperse peak from the size-exclusion column.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	DmZuc _{89–253}	DmZuc _{41–253}	DmZuc _{41–253} K171A	SeMet DmZuc _{89–250}	
				Peak	Edge
Wavelength (Å)	1.0000	1.0000	1.0000	0.9793	0.9800
Space group	<i>P</i> ₃ ₂ 1†	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	
Unit-cell parameters					
<i>a</i> (Å)	60.4	55.0	55.9	35.3	
<i>b</i> (Å)	60.4	71.2	70.2	52.4	
<i>c</i> (Å)	254.1	56.3	56.9	38.8	
<i>α</i> (°)	90	90	90	90	
<i>β</i> (°)	90	107.9	108.8	104.9	
<i>γ</i> (°)	120	90	90	90	
Resolution (Å)	50.0–2.5 (2.54–2.50)	50.0–1.75 (1.78–1.75)	50.0–2.2 (2.24–2.20)	50.0–2.2 (2.24–2.20)	50.0–2.5 (2.54–2.50)
Unique reflections	19391	39875	19694	6871	4707
<i>R</i> _{merge} ‡	0.15 (0.49)	0.064 (0.34)	0.083 (0.27)	0.14 (0.93)	0.13 (0.90)
<i>I</i> / <i>σ</i> (<i>I</i>)	16.0 (2.0)	27.5 (2.2)	16.6 (2.6)	22.9 (2.7)	24.5 (3.1)
Completeness (%)	99.3 (98.4)	97.2 (91.2)	95.0 (87.7)	100 (100)	100 (100)
Multiplicity	7.0 (4.9)	5.8 (3.7)	2.8 (2.2)	8.4 (8.2)	8.3 (8.3)

† *x* may be 0, 1 or 2. ‡ *R*_{merge} = $\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the average intensity of the symmetry-equivalent reflections of *I*(*hkl*).

Figure 3
Crystals of DmZuc. (a) Crystals of DmZuc_{89–253}. (b) Crystals of SeMet-labelled DmZuc_{89–250}. (c) Crystals of WT DmZuc_{41–253}.

Figure 4
X-ray diffraction pattern of WT DmZuc_{41–253}. The ring indicates 1.75 Å resolution.

Our functional analysis revealed that both DmZuc_{41–253} and MmZuc_{35–221} exhibited enzymatic activity but that DmZuc_{89–253} and DmZuc_{41–253} K171A did not (data not shown), supporting the notion that Zuc functions as a dimer. In addition, a DmZuc_{41–253} mutant lacking the predicted disordered region (residues 63–88) showed reduced activity compared with WT DmZuc_{41–253} (data not shown), indicating the functional significance of the predicted disordered region. These results affirmed the importance of designing crystallization constructs based not only on bioinformatics predictions but also on functional experiments.

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