Expression, purification, crystallization and preliminary crystallographic study of the cytoplasmic domain of the mitochondrial dynamics protein MiD51

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Mitochondria play central roles in many cellular and physiological processes. They are highly dynamic organelles and continually undergo fusion and fission. Mitochondrial dynamics protein 51 kDa (MiD51), an integral mitochondrial outer membrane protein, recruits dynamin-related protein 1 (Drp1; a mitochondrial fission protein) to mitochondria and facilitates Drp1-directed mitochondrial fission. In this study, the cytoplasmic domain of MiD51 was overexpressed in *Escherichia coli*, purified and crystallized. An X-ray diffraction data set was collected to a resolution of 3.1 Å and the crystal belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 90.1$, $c = 124.7$ Å, $\alpha = \beta = \gamma = 90^\circ$. The asymmetric unit had the highest probability of containing one molecule, with a Matthews coefficient of $3.32 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 63.0%.

1. Introduction

Mitochondria are important organelles and are involved in many cellular and physiological processes such as energy metabolism, amino-acid and lipid metabolism, apoptosis and mitophagy, calcium homeostasis and innate antiviral immunity (Westermann, 2010). Mitochondria are also highly dynamic organelles that continually undergo fusion, fission and movement along the cytoskeleton (Westermann, 2010; Chan, 2012). Beyond the primary function of mitochondrial dynamics in controlling their shape, size, number and distribution, mitochondrial dynamics are also crucial for maintaining their biological functions (Nunnari & Suomalainen, 2012; Westermann, 2010). The molecular mechanisms of mitochondrial dynamics have yet to be elucidated.

Previous studies have revealed that mitochondrial fusion and fission are predominantly mediated by dynamin-related GTPases (Chan, 2012). The fusion of the mitochondrial outer membrane is mediated by Mfn1/Mfn2 in mammals and Fzo1 in yeast. The fusion of the mitochondrial inner membrane is mediated by OPA1 in mammals and Mgm1 in yeast. Mitochondrial fission is mediated by dynamin-related protein 1 (Drp1) in mammals and Dnm1p in yeast. Drp1/Dnm1p is mainly present in the cytosol and is recruited onto the mitochondria outer membrane *via* their receptors (Otera & Mihara, 2011; Chan, 2012). After targeting mitochondria, Drp1/Dnm1p self-assembles into large spirals in a GTP-dependent manner and then causes mitochondrial membrane fission *via* GTP hydrolysis (Westermann, 2010; Chan, 2012). In yeast, Dnm1p is recruited by Mdv1p/Caf4p and Fis1p to form the Dnm1p–Mdv1p/Caf4p–Fis1p fission complex (Chan, 2012). In mammals, three integral outer-membrane proteins Mff (Gandre-Babbe & van der Bliek, 2008; Otera *et al.*, 2010), MiD51 (mitochondrial dynamics protein 51 kDa) and MiD49 (Palmer *et al.*, 2011; Zhao *et al.*, 2011) have been identified as the receptors recruiting Drp1 to mitochondria and thereby facilitating mitochondrial fission independently (Koirala *et al.*, 2013; Losón *et al.*, 2013; Palmer *et al.*, 2013).

To date, besides structural studies of yeast Fis1p (Zhang & Chan, 2007) and Mdv1p/Caf4p (Koirala *et al.*, 2010; Zhang *et al.*, 2012), there is a lack of structural information on mammalian Drp1 receptors, which is an obstacle to elucidating the molecular mechanism of mammalian mitochondrial fission. MiD51 is one of the Drp1 receptors and anchors the mitochondrial outer membrane *via* its N-terminal transmembrane helix. Full-length human MiD51 contains

Figure 1
Purification of MiD51 (129–463). (a) Purification on a Resource Q anion-exchange column. (b) Purification on a Superdex 75 16/60 HiLoad gel-filtration column. (c) SDS-PAGE analysis. Lane M, protein molecular-weight markers (Fermentas; labelled in kDa); lane 1, purified protein after Glutathione Sepharose 4B affinity column; lane 2, purified protein after anion-exchange column; lane 3, purified protein after gel-filtration column. The gel was stained with Coomassie Blue G-250.

463 residues, and it has been predicted that the transmembrane domain contains residues 23–48 and that the membrane-proximal region (residues 49–128) is likely to be disordered, with only the cytoplasmic domain MiD51 (residues 129–463) having a compact, globular structure (Palmer *et al.*, 2011; Zhao *et al.*, 2011). Here, we report the cloning, expression, purification, crystallization and preliminary crystallographic study of the cytoplasmic domain of MiD51 (residues 129–463).

2. Materials and methods

2.1. Cloning and protein expression

The nucleotide sequence of the human MiD51 (GenBank accession No. NM_019008) cytoplasmic domain (residues 129–463) was amplified from a human spleen cDNA library using polymerase chain reaction (PCR) with the forward primer 5'-TAAGGATCCTCAGACTCCGCATGTCCCT-3' and the reverse primer 5'-TATACTC-GAGCTACGTCTGCAGCAGCACCT-3'. The amplicon was double-digested with *Bam*HI and *Xho*I and then ligated into the vector pGEX-6P-1 (GE Healthcare) that encodes an upstream glutathione S-transferase (GST) tag followed by a PreScission protease (GE Healthcare) cleavage site. *Escherichia coli* strain DH5 α (Novagen) was used for plasmid cloning and the insertion of the gene for MiD51 cytoplasmic domain was confirmed by DNA sequencing (Invitrogen). The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) for protein expression. The transformed cells were cultured at 310 K in 2 $\times$ YT medium containing 100 μ g ml⁻¹ ampicillin. When the optical absorption density at 600 nm reached 1.0, the protein expression was induced by the addition of 0.3 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The culture was incubated for a further 16–20 h at 289 K. The cells were then harvested by centrifugation at 6000 rev min⁻¹ for 10 min at 277 K.

2.2. Purification

The harvested cells were resuspended in a lysis buffer consisting of 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl, 1 mM DTT pH 7.4 and disrupted using a high-pressure homogenizer. Cell debris was discarded after centrifugation at 16 000 rev min⁻¹ for 45 min at 277 K. The clarified supernatant containing the target

Figure 2
Crystal of MiD51 (129–463) obtained in 0.1 M L-proline, 0.1 M HEPES pH 7.0, 7% PEG 3350.

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

X-ray source	BL17U, SSRF
Wavelength (Å)	0.9196
Detector	ADSC Q315
Space group	$P4_12_12$
Unit-cell parameters (Å, °)	$a = b = 90.1$, $c = 124.7$ $\alpha = \beta = \gamma = 90$
Resolution range (Å)	45.07–3.11 (3.32–3.11)
Observed reflections	130882
Unique reflections	9741
Completeness (%)	99.7 (100)
Multiplicity	13.14 (14.1)
Average $I/\sigma(I)$	16.5 (4.1)
$R_{\text{meas}}/R_{\text{merge}}^{\dagger}$ (%)	10.6 (91.8)/10.2 (88.5)
Mosaicity (°)	0.48
Matthews coefficient (Å ³ Da ⁻¹)	3.32
Molecules per asymmetric unit	1
Solvent content (%)	63.0

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation and $\langle I(hkl) \rangle$ is the mean intensity of reflection hkl .

protein was loaded onto a Glutathione-Sepharose 4B affinity column (GE Healthcare) pre-equilibrated with the lysis buffer. The resin was washed with the lysis buffer, and the GST fusion protein was then digested using PreScission protease (GE Healthcare) at 277 K overnight. The digested MiD51 protein was eluted using the lysis buffer and concentrated using Amicon Ultra-4 centrifugal filter units (10 kDa cutoff, Millipore). A HiTrap Desalting column (5 ml, GE Healthcare) was used to change the buffer to 20 mM Tris–HCl pH 8.0, 50 mM NaCl. The protein was further purified by anion-exchange chromatography on a Resource Q column (GE Healthcare) with an NaCl linear gradient of 50–600 mM in 20 mM Tris–HCl pH 8.0. The eluted fractions containing MiD51 were pooled and concentrated, and finally purified by size-exclusion chromatography using a Superdex 75 column (GE Healthcare) pre-equilibrated with 20 mM HEPES pH 7.5, 150 mM NaCl. All fractions at each step were examined by SDS–PAGE. Purified MiD51 was concentrated to a final concentration of 30 mg ml⁻¹ and stored in liquid nitrogen for crystallization. Protein concentration was determined from the

absorbance at 280 nm based on an extinction coefficient of 47 245 M⁻¹ cm⁻¹.

2.3. Crystallization

The initial crystallization screening was performed with the Index, Crystal Screen, Crystal Screen 2, PEG/Ion and Natrix kits (Hampton Research) using the hanging-drop vapour-diffusion method in 24-well plates at 289 K. The hanging drops were prepared by mixing 1 µl protein solution with an equal volume of reservoir solution and the mixture was equilibrated against 200 µl aliquots of reservoir solution. Crystals appeared from various conditions including 0.2 M L-proline, 0.1 M HEPES pH 7.5, 10% PEG 3350. Optimization was carried out *via* altering the pH, the protein concentration and the concentrations of L-proline and PEG 3350. The final optimized condition was 0.1 M L-proline, 0.1 M HEPES pH 7.0, 7% PEG 3350. Crystals appeared within 2 d and reached their maximum dimensions after one week.

2.4. Data collection and processing

Prior to data collection, all crystals were transferred into a cryo-protectant solution (mother liquor supplemented with 25% glycerol) and flash-cooled in a nitrogen-gas stream. Preliminary diffraction data were collected at 100 K using an ADSC Q315 detector on beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF) using X-rays of wavelength 0.9196 Å. 180 diffraction images were collected with a 400 mm crystal-to-detector distance and 1° oscillation and 1 s exposure time per image. The diffraction data were indexed, integrated and scaled using *iMosflm* v.7.0.9 (Battye *et al.*, 2011), *POINTLESS* v.1.6.19 and *SCALA* v.3.3.20 from the *CCP4* program suite (Winn *et al.*, 2011).

3. Results and discussion

The cytoplasmic domain of human MiD51 (129–463) was expressed and purified to homogeneity in a three-step procedure by affinity, ion-exchange (Fig. 1*a*) and size-exclusion (Fig. 1*b*) chromatography with a final yield of 1 mg purified protein per litre of 2×YT culture medium. The final purified sample was at least 95% pure as estimated

Figure 3

A typical X-ray diffraction pattern collected using an ADSC Q315 detector on beamline BL17U at the SSRF. The maximum observed resolution is 3.1 Å.

by SDS–PAGE (Fig. 1c). The best diffracting crystals (Fig. 2) were obtained using the hanging-drop vapour-diffusion method at 289 K with a reservoir solution consisting of 0.1 M L-proline, 0.1 M HEPES pH 7.0, 7% PEG 3350.

A complete data set was collected on beamline BL17U at the SSRF. Details of the data-collection statistics are summarized in Table 1. The crystals diffracted to 3.1 Å resolution (Fig. 3) and belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 90.1$, $c = 124.7$ Å, $\alpha = \beta = \gamma = 90^\circ$. Calculations based on the unit-cell parameters and the protein molecular weight (37 512 Da) deduced from the sequence suggested the presence of one molecule per asymmetric unit to have the highest probability ($P_{\text{tot}} = \sim 0.98$), with a Matthews coefficient (Matthews, 1968; Winn *et al.*, 2011) of $3.32 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 63.0%.

The crystal structure of the human MiD51 cytoplasmic domain MiD51 (129–463) was eventually solved using the single-wavelength anomalous diffraction (SAD) method (Terwilliger, 2003). While this manuscript was under review, crystal structures of the cytoplasmic domain of MiD51 were reported (Losón *et al.*, 2014; Richter *et al.*, 2014). However, the crystallization condition, space group and crystal packing reported in our study are different and our crystal structure should provide different information.

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