Zhenzhen Zhang,^a Yicui Wang,^a
Zhubing Shi^{b*} and Min Zhang^{a*}

^aSchool of Life Sciences, Anhui University,
Hefei, Anhui 230039, People's Republic of
China, and ^bInstitute of Biochemistry and Cell
Biology, Shanghai Institutes for Biological
Sciences, Chinese Academy of Sciences,
Shanghai 200031, People's Republic of China

Correspondence e-mail: zbshi@sibcb.ac.cn,
zhmin07@ustc.edu

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Structure of zebrafish MO25

MO25, a conserved scaffold protein, activates the tumour suppressor LKB1 with the pseudokinase STRAD. MO25 also promotes the activities of the STE20-family kinases MST3, MST4, STK25, SPAK and OSR1. Zebrafish MO25 was purified and crystallized, and a crystal of zebrafish MO25 diffracted to 2.9 Å resolution and belonged to space group $P3_221$, with unit-cell parameters $a = b = 156.665$, $c = 221.251$ Å. The structure of zebrafish MO25 was determined by molecular replacement. It is constituted of seven helical repeats. Structural comparison indicates that the overall structures of zebrafish and human MO25 are very similar, suggesting that MO25 has conserved functions in zebrafish. This work provides a structural basis for further functional and evolutionary studies of MO25.

1. Introduction

Mouse protein 25 (MO25) was first identified in the process of mouse embryogenesis (Miyamoto *et al.*, 1993). It is widely expressed in human tissues. As a scaffold protein, MO25 forms a regulatory complex with the pseudokinase STE20-related adaptor (STRAD) to activate the tumour suppressor liver kinase 1 (LKB1) (Boudeau *et al.*, 2003; Zeqiraj, Filippi, Deak *et al.*, 2009). Many germline mutations in LKB1 cause the inherited Peutz–Jeghers cancer syndrome (PJS; Hemminki *et al.*, 1998; Jenne *et al.*, 1998). LKB1 is a key player in growth control and cell polarity (Alessi *et al.*, 2006; Shackelford & Shaw, 2009). The LKB1–MO25–STRAD complex phosphorylates and activates AMP-activated protein kinase (AMPK), as well as other AMPK-related kinases such as NUA1/2, SIK and MARK1–4 (Hawley *et al.*, 2003; Lizcano *et al.*, 2004). Besides STRAD, MO25 also interacts with and stimulates the kinase activity of a group of STE20 kinases, including MST3, MST4, STK25, SPAK and OSR1 (Filippi *et al.*, 2011). The MST4–MO25 complex induces apoptosis in HEK293 cells (Shi *et al.*, 2013). Biochemical and structural studies showed that MO25 binds STRAD, MST3 and MST4 via the concave surface and a hydrophobic pocket in the convex surface (Mehellou *et al.*, 2013; Shi *et al.*, 2013; Zeqiraj, Filippi, Goldie *et al.*, 2009). MO25 binding results in conformational change of the α C helix in these STE20 kinases and promotes the formation of the conserved Lys–Glu salt bridge to activate these kinases. MO25 association also induces the specific dimerization of the MST4 kinase domain which is required for MST4 autophosphorylation (Shi *et al.*, 2013).

MO25 has also been identified in other species such as fruit fly, yeast and *Aspergillus nidulans*. *Drosophila* MO25 and the germinal centre (GC) kinase Fray together regulate asymmetric cell division. The *Saccharomyces cerevisiae* orthologue Hym1p functions in cellular morphogenesis and promotes the daughter-cell-specific localization of the Ace2p transcription factor (Bidlemaier *et al.*, 2001; Dorland *et al.*, 2000). Hym1p also plays a regulatory role in cell-cycle progression, and together with Ace2p and Cln3p contributes to the establishment of asynchronous mother–daughter cell divisions (Bogomolnaya *et al.*, 2004). In *Schizosaccharomyces pombe*, Pmo25 constitutes a morphogenesis network with the GC kinase Nak1, Mor2 and Orb6, which play an important role in polarity control and cell separation (Kanai *et al.*, 2005; Mendoza *et al.*, 2005). Another GC

kinase, Ppk11, interacts with Pmo25 and plays an auxiliary role in cell morphogenesis (Goshima *et al.*, 2010). In *A. nidulans*, the MO25 homologue HymA is required for conidiophore development (Karos & Fischer, 1999). MO25 is also present in plants, although its function has not been determined. Sequence alignment suggests that MO25 is a highly conserved protein (Fig. 1).

The structure of human MO25 has been determined, but structures of MO25 from other species are not known. Here, we purified and crystallized MO25 from zebrafish. The sequences of human and zebrafish MO25 have 92% identity (Fig. 1). The crystal structure of zebrafish MO25 was determined by molecular replacement. Zebrafish MO25 is constituted of seven helical repeats, the same as human MO25. Our work is beneficial for further functional study of MO25.

2. Materials and methods

2.1. Gene cloning and expression

Zebrafish MO25 (amino acids 12–334) was obtained by PCR using zebrafish whole cDNA as the template and was inserted into the *Nco*I and *Xho*I sites in HT-pET28a, which was modified from pET28a (Novagen) and adds an N-terminal 6×His tag. The recombinant plasmid HT-pET28a-MO25 was validated by sequencing.

The HT-pET28a-MO25 plasmid was transformed into *Escherichia coli* BL21(DE3) CodonPlus competent cells. The cells were incubated in 750 ml Terrific Broth medium with 30 µg ml⁻¹ kanamycin and 34 µg ml⁻¹ chloramphenicol at 310 K until the absorbance at 600 nm reached 1.0. The temperature was lowered to 289 K and isopropyl

Figure 1

Sequence alignment of MO25 from different species, *Homo sapiens* (HUMAN), *Danio rerio* (DANRE; zebrafish), *Drosophila melanogaster* (DROME), *Caenorhabditis elegans* (CAEEL), *Schizosaccharomyces pombe* (SCHPO) and *Arabidopsis thaliana* (ARATH), was performed with *ClustalW2* (Goujon *et al.*, 2010; Larkin *et al.*, 2007) and *ESPrpt* (Gouet *et al.*, 1999). The secondary structure is shown according to the human MO25 structure. Identical residues among these species are highlighted with a red background, highly conserved residues are shown in red and all of these residues are boxed. Residues from MO25 involved in interaction with its partners are indicated with asterisks.

β -D-1-thiogalactopyranoside was added to a final concentration of 0.25 mM; the culture was then incubated at 289 K for 18 h.

2.2. Protein purification

The following procedures were carried out at 277 K. The *E. coli* cells were collected at 6300 rev min⁻¹ for 8 min and suspended in five volumes of lysis buffer consisting of 20 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 5% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride. The cells were then broken by five passages through a high-pressure homogenizer (JNBIO) at 130 MPa. The debris was removed by centrifugation at 20 000g for 40 min. The supernatant was mixed with pre-equilibrated Ni Sepharose (GE Healthcare) for 1 h and the beads were washed with lysis buffer without PMSF. The proteins were eluted with 300 mM imidazole in lysis buffer. The sample was desalted to 20 mM HEPES pH 7.5, 250 mM NaCl, 5% glycerol, 1 mM DTT, 10 mM imidazole. The 6 $\times$ His-tagged zebrafish MO25 was digested with TEV protease. The 6 $\times$ His tag and TEV protease were then removed using Ni Sepharose. The proteins were concentrated using a 10 kDa cutoff Amicon Ultra-15 (Millipore) and applied onto a HiLoad 16/60 Superdex 200 column (GE Healthcare) with 20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT. The purity of the proteins was monitored by SDS-PAGE. Purified zebrafish MO25 was concentrated to 10 mg ml⁻¹, aliquoted and stored at 193 K.

2.3. Crystallization

Crystallization trials were carried out at 289 K by the sitting-drop vapour-diffusion method. The 2 μ l sitting drops consisted of 1 μ l protein solution and 1 μ l reservoir solution and were equilibrated against 100 μ l reservoir solution. Crystals were optimized using sitting-drop and hanging-drop vapour diffusion. The crystals were soaked in different cryoprotection solutions and flash-cooled in liquid nitrogen.

2.4. Data collection, structure determination and refinement

Diffraction data were collected on beamline 1W2B at Beijing Synchrotron Radiation Facility (BSRF), People's Republic of China and were processed using *HKL-2000* (Otwinowski & Minor, 1997). The structure of zebrafish MO25 was solved by molecular replacement with *Phaser* (McCoy *et al.*, 2007) in the *PHENIX* suite (Adams *et al.*, 2010) using human MO25 (PDB entry 1upl; Milburn *et al.*, 2004) as a search model. A solution with eight zebrafish MO25 molecules in the asymmetric unit was found. The structure was refined using

phenix.refine (Adams *et al.*, 2010) with NCS restraints and model building was performed in *Coot* (Emsley *et al.*, 2010).

2.5. Structure deposition

The coordinate file and structure factors for the crystal structure of zebrafish MO25 have been deposited in the RCSB Protein Data Bank under accession code 4kzg.

3. Results and discussion

3.1. Determination of the zebrafish MO25 structure

We purified the fragment 12–334 of zebrafish MO25 by nickel-affinity and gel-filtration chromatography (Fig. 2). The gel-filtration chromatography results showed that zebrafish MO25 eluted as two peaks, in which it mainly existed as a monomer with only a small proportion as a dimer. We chose the monomer to perform crystal screening. Crystallization experiments were set up by the sitting-drop and hanging-drop vapour-diffusion methods. In the initial crystallization trials, crystals appeared in several conditions in which the reservoir solutions contained PEG 3350 and a variety of salts. The best crystal was grown in a condition consisting of 0.2 M lithium sulfate monohydrate, 20% (w/v) polyethylene glycol 3350 using hanging-drop vapour diffusion. The diffraction resolution was 2.9 Å and the crystal belonged to space group *P*3₂21, with unit-cell parameters *a* = *b* = 156.665, *c* = 221.251 Å. The structure of zebrafish MO25 was solved by molecular replacement using human MO25

Figure 2
Elution profile of zebrafish MO25 from a HiLoad 16/60 Superdex 200 column. Inset, SDS-PAGE. Lane *M* contains molecular-mass markers (labelled in kDa).

Figure 3
Overall structure of zebrafish MO25 shown as cartoon (*a*, *b*) and surface (*c*, *d*) representations. Zebrafish MO25 is constituted of seven helical repeats. It forms a concave and a convex surface.

Figure 4

Structural comparison of zebrafish and human MO25. Zebrafish and human MO25 are coloured green and yellow, respectively. Their overall structures are very similar. The key residues from zebrafish and human MO25 involved in interactions with their partners are identical and are shown in stick representation.

Table 1

Data-collection and refinement statistics for zebrafish MO25.

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	$P3_221$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = b = 156.665$, $c = 221.251$, $\alpha = \beta = 90.0$, $\gamma = 120.0$
Wavelength ($\text{\AA}$)	0.9795
Resolution range ($\text{\AA}$)	50.0–2.90 (2.95–2.90)
Total reflections	359967
Unique reflections	70049
Completeness (%)	99.8 (100.0)
Multiplicity	5.1 (4.6)
$R_{\text{merge}}^\dagger$	0.172 (0.467)
$\langle I/\sigma(I) \rangle$	12.9 (3.9)
Mosaicity ($^\circ$)	1.016
Wilson B factor ($\text{\AA}^2$)	33.8
Refinement	
Resolution ($\text{\AA}$)	50.0–2.90
No. of reflections	69358
$R_{\text{work}}/R_{\text{free}}^\ddagger$	0.288/0.327
No. of atoms	
Protein	20035
Water	39
R.m.s. deviations	
Bond lengths ($\text{\AA}$)	0.003
Bond angles ($^\circ$)	0.881
Ramachandran favoured (%)	96
Ramachandran outliers (%)	1.2
Average B factor ($\text{\AA}^2$)	33.7

$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. $^\ddagger R_{\text{work}} = \frac{\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}||}{\sum_{hkl} |F_{\text{obs}}|}$. R_{free} was computed identically except that all reflections belonged to a test set consisting of a randomly selected 10% of the data.

(PDB entry 1upl; Milburn *et al.*, 2004) as a search model. Details of the refinement statistics are summarized in Table 1.

3.2. Overall structure of zebrafish MO25

There were eight molecules in the asymmetric unit of the zebrafish MO25 crystals and the structures of the eight copies were essentially the same, with root-mean-square deviations (r.m.s.d.s) of 0.220–0.368 $\text{\AA}$ on C^α atoms (Fig. 3). Although a small part exists as a dimer in solution, the structure shows that zebrafish MO25 is a monomer. Zebrafish MO25 is a helical-repeat protein with seven helical repeats named R0–R6. The R0 repeat contains two helices, while the R1–R6 repeats have three helices (H1–H3) and the structures of these six repeats are very similar. Zebrafish MO25 has a twisted shape and

forms obvious concave and convex surfaces. The concave surface is constituted by H3 of each repeat and the convex surface is formed by H1 and H2 of each repeat.

3.3. Structural comparison of zebrafish and human MO25

Structures of human apo MO25 and MO25–partner complexes have been determined (Mehellou *et al.*, 2013; Milburn *et al.*, 2004; Shi *et al.*, 2013; Zeqiraj, Filippi, Deak *et al.*, 2009; Zeqiraj, Filippi, Goldie *et al.*, 2009). The overall structures of zebrafish and human MO25 are very similar, with an r.m.s.d. of 0.755 $\text{\AA}$ (Fig. 4). Like other helical-repeat proteins, the MO25 concave surface forms an extensive interaction network with its partners STRAD, MST3 and MST4. Residues Glu93 and Lys96 in H3 of R1, Phe178 in H3 of R3 and Tyr223 and Arg227 in H3 of R4 are involved in the interactions. On the C-terminal convex surface of MO25, a hydrophobic pocket formed by H2 of R5 and H1 and H2 of R6 embeds the conserved WxF (Trp-*x*-Phe) motif from its partners. The residues involved in the interactions are highly conserved in zebrafish and human MO25, as well as in other species (Figs. 1 and 4), suggesting that zebrafish MO25 has similar functions to human MO25, such as activating STE20 kinases. Collectively, our research provides a structural basis for further functional and evolutionary studies of MO25.

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