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Crystallization of the coiled-coil domain of Atg16 essential for autophagy

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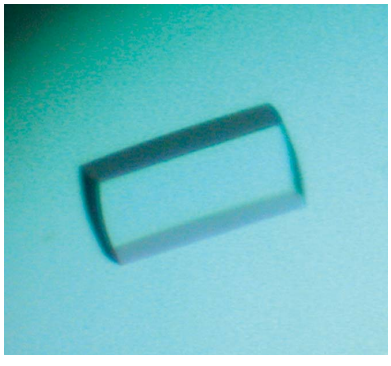


Atg16 is a scaffold protein that interacts with Atg12–Atg5 protein conjugates *via* its N-terminal domain and self-assembles *via* its coiled-coil domain, thus forming a multimeric Atg12–Atg5–Atg16 complex that is essential for autophagy. The coiled-coil domain of Atg16 was expressed, purified and crystallized. The crystal belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters a = 127.7, c = 77.8 Å. Self-rotation functions and volume-to-weight ratio values suggested that the crystal may contain six molecules per asymmetric unit. Since the domain does not contain a methionine residue, selenomethionine-labelled crystals were prepared with a leucine-to-methionine substitution in the coiled-coil domain and these crystals were used for the collection of single-wavelength anomalous dispersion data to 2.5 Å resolution.

1. Introduction

Autophagy is a starvation-induced response that mediates the bulk degradation of cytoplasmic components in the lysosome/vacuole (Seglen & Bohley, 1992; Takeshige *et al.*, 1992) and plays a critical role in fundamental biological processes such as intracellular clearance, differentiation, development, programmed cell death and antigen representation (Mizushima, 2007). In autophagy, a double-membrane structure called an autophagosome sequesters a portion of cytoplasm and fuses with the lysosome/vacuole to deliver its contents into the organelle lumen. The autophagosome-formation step requires at least 18 Atg proteins (Suzuki & Ohsumi, 2007; Kawamata *et al.*, 2008), of which eight constitute two ubiquitin-like conjugation systems, the Atg12 and Atg8 systems (Ohsumi, 2001).

In the Atg12 system, Atg12 is covalently linked to Atg5 by sequential reactions catalyzed by Atg7 (an E1-like enzyme) and Atg10 (an E2-like enzyme) (Mizushima *et al.*, 1998; Shintani *et al.*, 1999). The Atg12–Atg5 conjugate further interacts noncovalently with the N-terminal domain of Atg16 (Mizushima *et al.*, 1999). Since Atg16 self-assembles *via* the predicted coiled-coil motif (residues 67–123; Mizushima *et al.*, 1999), the Atg12–Atg5–Atg16 ternary complex behaves like a huge protein complex that has been shown by gel-filtration chromatography to be as large as 350 kDa (Kuma *et al.*, 2002). The Atg12–Atg5–Atg16 complex is essential for the formation of autophagosomes, for which the multimerization of the complex *via* the coiled-coil region of Atg16 is required (Kuma *et al.*, 2002). Little is known of the specific functions of the Atg12–Atg5–Atg16 complex in autophagosome formation; however, recent studies have shown that the complex functions as an E3-like enzyme in the Atg8 system (Hanada *et al.*, 2007; Fujioka *et al.*, 2008; Fujita *et al.*, 2008). In the Atg8 system, Atg8 is conjugated to phosphatidylethanolamine (PE) by sequential reactions catalyzed by Atg7 and Atg3 (another E2-like enzyme; Ichimura *et al.*, 2000), in which the Atg12–Atg5 conjugate has been shown to promote the transfer of Atg8 from Atg3 to PE by direct interaction with both Atg3 and PE (Hanada *et al.*, 2007). Furthermore, the mammalian Atg12–Atg5–Atg16L complex has been shown to specify the lipidation site of LC3, a mammalian Atg8 orthologue, through interaction with Atg3 and some unidentified factor(s) on the membrane (Fujita *et al.*, 2008).

We have previously reported the structures of plant Atg12 (Suzuki *et al.*, 2005) and Atg5 complexed with the N-terminal domain of

Atg16 (Matsushita *et al.*, 2007) and have shown that both Atg12 and Atg5 are comprised of ubiquitin fold(s). However, the structure-determined region of Atg16 (residues 1–57) did not contain the predicted coiled-coil motif (residues 67–123). Furthermore, the predicted coiled-coil motif only has weak sequence homology to other coiled-coil proteins of reported structure. Therefore, in order to obtain structural information on the multimeric state of the Atg12–Atg5–Atg16 complex, structural study on the coiled-coil motif of Atg16 is required. In this report, we describe the expression, purification and crystallization of the coiled-coil domain (residues 50–123) of Atg16.

2. Experimental methods

2.1. Expression and purification

A fragment coding residues 50–123 of *Saccharomyces cerevisiae* Atg16 (NCBI Gene ID 855194) was PCR-amplified from the plasmid pGEX6P-1-ATG16 (Matsushita *et al.*, 2007) using 5′-GGGGATCCG-GCGCCATTGGTGGCAACATTG-3′ as the forward primer and 5′-GGGGATCCTTATTCTTTTTTCAGATCCGAGAG-3′ as the reverse primer. The PCR product was cloned into the *Bam*HI site of a pGEX4T-1 vector (GE Healthcare). We hereafter refer to this region as the coiled-coil domain (CCD). The constructs were transformed into *Escherichia coli* BL21 (DE3) and expressed as a GST-fusion protein. After cell lysis, the GST-fused Atg16 CCD was purified by affinity chromatography using a glutathione-Sepharose 4B column (GE Healthcare). After cleaving a GST tag from the Atg16 CCD with thrombin protease (GE Healthcare; an artificial glycine remained at the N-terminus of the Atg16 CCD), the Atg16 CCD was again applied onto a glutathione-Sepharose 4B column in order to remove the excised GST. The Atg16 CCD was then applied onto a HiTrap DEAE FF column (GE Healthcare) equilibrated with 20 m*M* Tris–HCl pH 8.5 and eluted with a 0–300 m*M* NaCl gradient in the same buffer. Final purification was performed using a Superdex200 gel-filtration column (GE Healthcare) equilibrated with 500 m*M* NaCl, 20 m*M* Tris–HCl pH 8.5. The purified Atg16 CCD was concentrated and used for crystallization. For single-wavelength anomalous dispersion (SAD) phasing, a selenomethionine-labelled protein was prepared as follows. Since the Atg16 CCD lacked a methionine residue for selenomethionine labelling, one was introduced by PCR-mediated site-directed mutagenesis. Sequence alignment of the Atg16 CCD with other structure-determined coiled-coil proteins with weak sequence homology suggested that Leu103 of Atg16 would have an ordered conformation but that its substitution with a methionine would not destroy the Atg16 CCD structure. Therefore, Leu103 of the Atg16 CCD was chosen to be replaced with a methionine and the mutant protein (L103M) was expressed in methionine-auxotroph *E. coli* B834 (DE3) using an amino-acid medium containing selenomethionine instead of methionine. The selenomethionine-labelled mutant protein was purified using the same procedure as that described for the native protein.

Table 1
Diffraction data statistics for Atg16 CCD crystals.

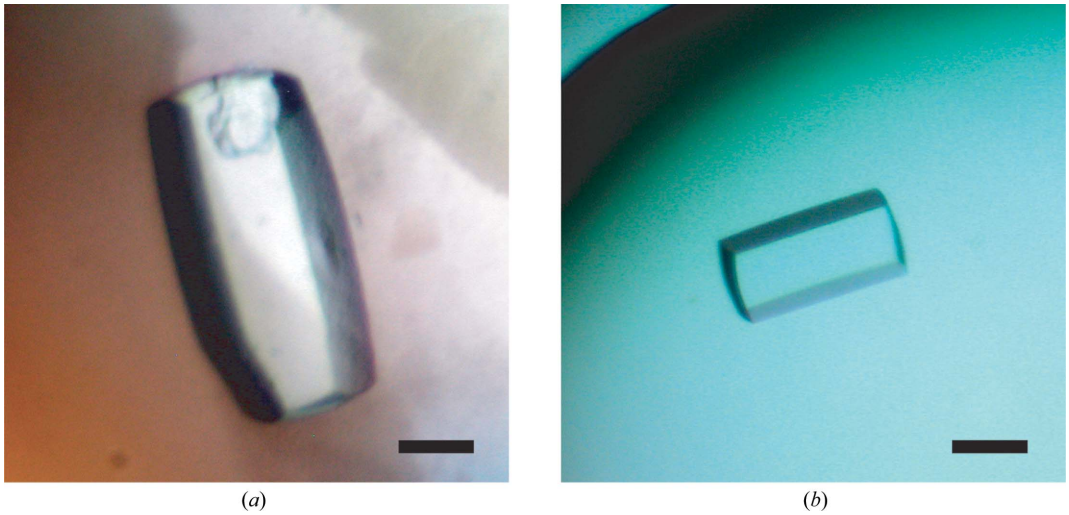
Values in parentheses are for the outer shell.

	Native	Selenomethionine
Beamline	BL-5A (KEK)	BL41XU (SPring-8)
Wavelength (Å)	1.1000	0.9791
Resolution range (Å)	50–2.70 (2.80–2.70)	50–2.50 (2.59–2.50)
Observed reflections	240090	640275
Unique reflections	17961	42101
Completeness (%)	98.8 (94.8)	99.8 (99.9)
$R_{\rm merge}(I)$†	0.060 (0.375)	0.054 (0.406)
Mean $I/\sigma(I)$	43.9 (4.3)	83.4 (10.6)
Matthews coefficient (Å^3 Da^{-1})	3.17	3.14
Wilson *B* factor (Å^2)	64.6	59.5

† $R_{\rm merge}(I) = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observed amplitude of reflection *hkl* and $\langle I(hkl)\rangle$ is the mean amplitude for all observed reflections.

2.2. Crystallization

Crystallization trials were performed using the sitting-drop vapour-diffusion method at 293 K. Initial screening was performed using Crystal Screen and Crystal Screen 2 (Hampton Research), Wizard Screens I and II (Emerald BioStructures) and Structure Screens 1 and 2 (Molecular Dimensions Ltd) as reservoir solutions. Drops (0.3 µl) of 8 mg ml^{-1} Atg16 CCD in 20 m*M* Tris buffer pH 8.5 and 500 m*M* NaCl were mixed with equal amounts of each reservoir solution and equilibrated against 100 µl of the same reservoir solution by vapour diffusion. The Atg16 CCD crystallized using a reservoir solution consisting of 0.4–1.2 *M* ammonium chloride and 0.1 *M* acetate buffer pH 5.0 after incubation for a few months. Streak-seeding was used to accelerate the nucleation of the crystals and large



(*a*) (*b*)

Figure 1
Atg16 CCD crystals. (*a*) Native crystal. (*b*) Selenomethionine crystal. The scale bar represents 100 µm.

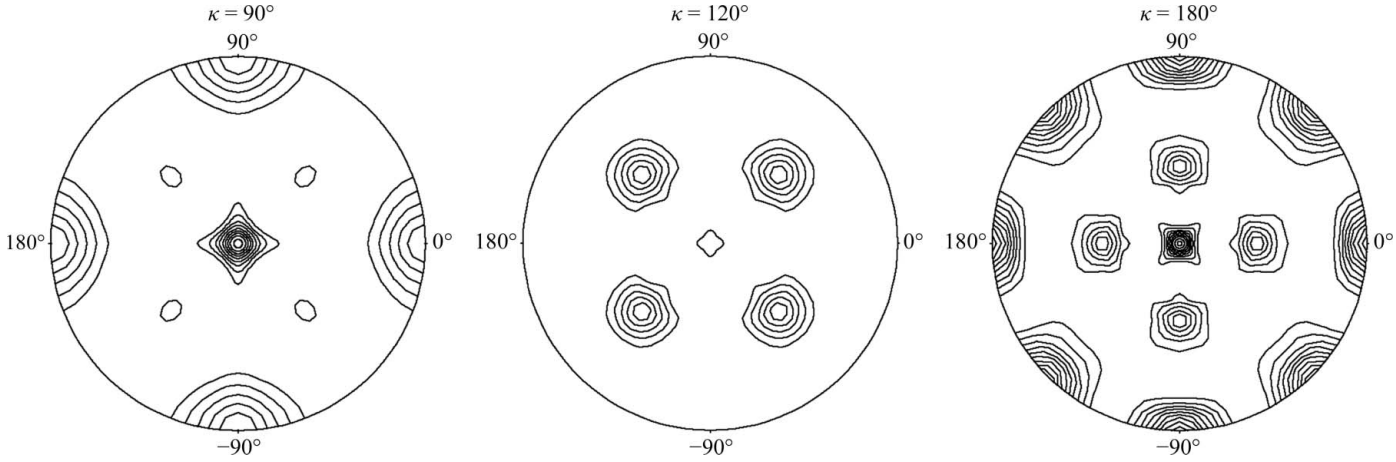


Figure 2
Stereographic projections of the self-rotation functions from the data set for a native crystal. The self-rotation functions were calculated using a 30 Å radius of integration and data in the resolution range 15–4 Å.

crystals of 0.50 × 0.25 × 0.25 mm in size were obtained after incubation for several months (Fig. 1*a*). Since the selenomethionine-labelled L103M mutant did not crystallize using the conditions under which native crystals were obtained, initial screening was again performed using the above-mentioned screening kits. The selenomethionine-labelled L103M mutant in 20 m*M* Tris buffer pH 8.5 and 500 m*M* NaCl was crystallized using a reservoir solution consisting of 1.5 *M* sodium chloride and 10% ethanol. A crystal with typical dimensions of 0.30 × 0.15 × 0.10 mm (Fig. 1*b*) was obtained within a week.

2.3. Diffraction data collection and processing

Crystals were immersed in reservoir solution supplemented with 30% glycerol as a cryoprotectant for several seconds; they were then flash-cooled and kept in a stream of nitrogen gas at 90–100 K during data collection. Diffraction data from a native crystal were collected using an ADSC Quantum 315 charge-coupled device detector on the KEK beamline BL-5A at a wavelength of 1.1000 Å. The crystal belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters a = 127.7, c = 77.8 Å. Diffraction data from a selenomethionine-labelled crystal were collected using a Rayonix MX-225 charge-coupled device detector on SPring-8 beamline BL41XU at a wavelength of 0.9791 Å. The crystal was almost isomorphous to the native crystal, with unit-cell parameters a = 127.3, c = 77.6 Å. All diffraction data were processed using the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1.

3. Discussion

The acceptable range of the volume-to-weight ratio (V_M) value (Matthews, 1968) indicates that the crystal contains 5–10 molecules of Atg16 CCD per asymmetric unit (V_M = 1.90–3.80 Å^3 Da^{-1}). Self-rotation functions were calculated using diffraction data from a native crystal. The sections at κ = 90°, 120° and 180° (Fig. 2) showed that the crystal contains noncrystallographic twofold, threefold and fourfold axes. These data suggest that the crystal presumably contains six molecules per asymmetric unit (V_M = 3.17 Å^3 Da^{-1}, calculated solvent content 61.2%). Using the SAD data, six Se positions were determined and good initial phases were obtained using *SOLVE*/*RESOLVE* (Terwilliger & Berendzen, 1999; Terwilliger, 2000). The number of Se positions and the electron-density map indicate that the asymmetric unit actually contains six Atg16 CCD molecules. Model building and crystallographic refinement are now in progress.

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