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Crystallization and preliminary X-ray analysis of Atg3

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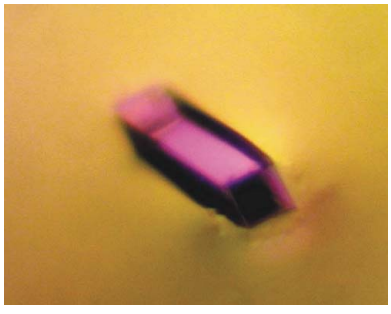

Atg3 is an E2-like enzyme that catalyzes the conjugation reaction between Atg8 and phosphatidylethanolamine (PE). The Atg8–PE conjugate is essential for autophagy, the bulk degradation process of cytoplasmic components by the vacuolar/lysosomal system. Crystals of *Saccharomyces cerevisiae* Atg3 have been obtained by the sitting-drop vapour-diffusion method using ammonium sulfate and lithium sulfate as precipitants. A native data set was collected from a single crystal to 2.5 Å resolution. The crystals belong to space group $P4_1$ or $P4_3$, with unit-cell parameters a = 59.33, c = 115.22 Å, and are expected to contain one protein molecule per asymmetric unit.

1. Introduction

Ubiquitin is conjugated to its target proteins by the following sequential reactions. Firstly, the C-terminus of ubiquitin is processed by a specific protease and a glycine residue is exposed at its C-terminus. The exposed glycine is activated by an E1 enzyme and is then transferred to an E2 enzyme. Finally, usually supported by an E3 enzyme, ubiquitin is conjugated to its target proteins (Varshavsky, 1997). Many ubiquitin-like modifiers have been reported and they all seem to be conjugated to their targets *via* a mechanism similar to ubiquitination (Welchman *et al.*, 2005). A 14 kDa protein Atg8 was also shown to be one such modifier, although its target is not a protein but a phospholipid, phosphatidylethanolamine (PE; Ichimura *et al.*, 2000). In spite of its low sequence homology with ubiquitin, Atg8 was shown to be structurally similar to ubiquitin (Sugawara *et al.*, 2004) and is thought to be conjugated to PE by a mechanism similar to ubiquitination. Firstly, the carboxy-terminal arginine of Atg8 is processed by Atg4, a cysteine protease structurally related to deubiquitinating enzymes (Kirisako *et al.*, 2000; Sugawara *et al.*, 2005). The exposed glycine residue of Atg8 is activated by Atg7, an E1-like enzyme (Tanida *et al.*, 1999), and is then transferred to Atg3, an E2-like enzyme (Ichimura *et al.*, 2000). Finally, Atg8 is conjugated to PE (Ichimura *et al.*, 2000). *In vitro* reconstitution revealed that Atg3, Atg7, Atg8, PE and ATP are sufficient for this conjugation reaction (Ichimura *et al.*, 2004). The formation of the Atg8–PE conjugate is essential for autophagy, the bulk degradation process of cytosolic components by the vacuolar/lysosomal system (Ohsumi, 2001).

Compared with other E2 enzymes, Atg3 is unique in mediating protein lipidation. Furthermore, Atg3 does not require E3 enzymes for the reaction. Structural information on Atg3 would be helpful in understanding the unique protein-lipidation reaction mediated by Atg3. In this report, we describe the purification, crystallization and preliminary crystallographic analysis of Atg3.

2. Experiments

2.1. Expression and purification

The region encoding full-length Atg3 (residues 1–310) was inserted into the pGEX-4T and pGEX-6P vectors (GE Healthcare Bio-Sciences) using the *Bam*HI–*Xho*I restriction sites. pGEX-4T-Atg3 has a thrombin protease-cleavage sequence between glutathione-

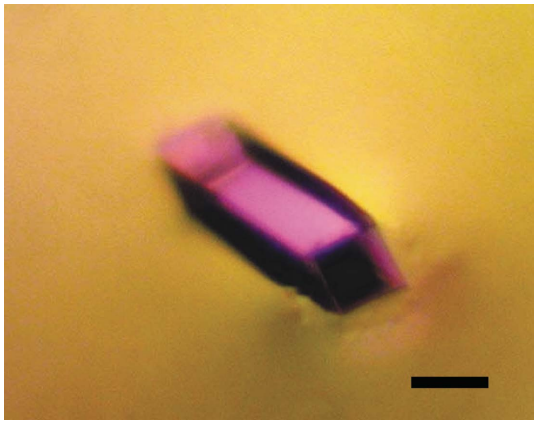

Figure 1
A crystal of Atg3. The black scale bar is 100 µm in length.

S-transferase (GST) and Atg3, whereas pGEX-6P-Atg3 has a PreScission protease-cleavage sequence between GST and Atg3. GST-fused Atg3s with either the thrombin or PreScission cleavage site were expressed in *Escherichia coli* BL21 DE3. After cell lysis, the GST-fusion proteins were first purified by affinity chromatography using a glutathione-Sepharose 4B column (GE Healthcare Biosciences). The GST proteins were then excised from Atg3 with thrombin protease (GE Healthcare Biosciences) at 295 K or PreScission protease (GE Healthcare Biosciences) at 277 K, resulting in two Atg3s: one has Gly-Ser (derived from thrombin digestion) and the other has a Gly-Pro-Leu-Gly-Ser artificial sequence (derived from PreScission digestion) at the N-terminus. We call the former GS-Atg3 (molecular weight 36 030 Da) and the latter GPLGS-Atg3 (molecular weight 36 297 Da). Each protein was applied onto a HiTrap DEAE anion-exchange column (GE Healthcare Biosciences) equilibrated with 20 m*M* Tris buffer pH 8.0, 2 m*M* DTT and was eluted with a 0–500 m*M* NaCl gradient in the same buffer. The eluted protein was further applied onto a Superdex200 gel-filtration column (GE Healthcare Biosciences) and was eluted with 20 m*M* Tris buffer pH 7.4, 150 m*M* NaCl and 2 m*M* DTT. Each purified protein was concentrated to 30–50 mg ml^{-1} for crystallization.

2.2. Crystallization

Crystallization trials were performed using the sitting-drop vapour-diffusion method at 293 K using GS-Atg3 and GPLGS-Atg3. Initial screening was performed using Crystal Screen and Crystal Screen 2 (Hampton Research) and Wizard I, Wizard II, Cryo I and Cryo II (Emerald Biostructures) as reservoir solutions. Typically, 0.3 µl drops of a protein solution were mixed with equal amounts of a reservoir solution and were equilibrated with 100 µl of the same reservoir solution by vapour diffusion. Small crystals of GS-Atg3 were obtained with a reservoir solution consisting of 0.5 *M* ammonium sulfate, 1.0 *M* lithium sulfate, 0.1 *M* citrate buffer pH 5.6. After optimization of the crystallization conditions, rod-shaped crystals of GS-Atg3 were obtained with a reservoir solution consisting of 0.28–0.35 *M* ammonium sulfate, 1.0 *M* lithium sulfate, 0.1 *M* citrate buffer pH 5.8 using 50 mg ml^{-1} GS-Atg3 (Fig. 1). They grew to dimensions of about 0.3 × 0.1 × 0.1 mm after a few weeks. In contrast, GPLGS-Atg3 did not crystallize at all.

Table 1
Summary of crystallographic data.

Values in parentheses refer to the highest resolution shell.

Resolution range (Å)	50.0–2.50 (2.60–2.50)
Observed reflections	83016
Unique reflections	13564
Completeness (%)	98.8 (99.6)
$R_{\rm merge}(I)$	0.060 (0.271)
$I/\sigma(I)$	22.3 (4.1)

2.3. Preliminary crystallographic analysis

Crystals were immersed into reservoir solution supplemented with 25% glycerol as a cryoprotectant for several seconds and then flash-cooled and kept in a stream of nitrogen gas at 100 K during data collection. Diffraction data were collected at 100 K using an ADSC Quantum 315 charge-coupled device detector on beamline BL41XU at SPring-8, Japan at a wavelength of 1.00 Å. Diffraction data were indexed, integrated and scaled with the program *HKL*-2000 (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1. The crystal belongs to the tetragonal space group $P4_1$ or $P4_3$, with unit-cell parameters $a = 59.33$, $c = 115.22$ Å. The acceptable range of the volume-to-weight ratio ($V_{\rm M}$) values (Matthews, 1968) indicates that the crystal contains one protein molecule per asymmetric unit, with a solvent content of 56.4% ($V_{\rm M} = 2.82$ Å^3 Da^{-1}). Structure determination by the multiple isomorphous replacement method is now in progress.

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