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Purification, crystallization and preliminary X-ray data collection of the N-terminal domain of the 26S proteasome regulatory subunit p27 and its complex with the ATPase domain of Rpt5 from *Mus musculus*

The protein 26S proteasome regulatory subunit p27 is one of the four chaperones that help in the assembly of the 19S regulatory particle (RP) of the 26S proteasome. In the present work, the N-terminus of p27 (residues 1–128) from *Mus musculus* was cloned, expressed, purified and crystallized alone and in complex with the C-terminal ATPase domain of Rpt5 (residues 173–442). The crystals of p27^(1–128) diffracted to 1.7 Å resolution and belonged to space group *P*2₁2₁2₁, with unit-cell parameters *a* = 26.79, *b* = 30.39, *c* = 145.06 Å. Resolution-dependent Matthews coefficient probability analysis suggested the presence of only one molecule per asymmetric unit, with 40.5% solvent content and a *V_M* value of 2.02 Å³ Da^{−1}. The crystal of the p27^(1–128)–Rpt5^(173–442) complex diffracted to 4 Å resolution and belonged to space group *P*222, with unit-cell parameters *a* = 75.93, *b* = 76.08, *c* = 336.85 Å. The presence of four heterodimers in the asymmetric unit with 53.2% solvent content and a *V_M* value of 2.63 Å³ Da^{−1} or five heterodimers in the asymmetric unit with 41.5% solvent content and a *V_M* value of 2.10 Å³ Da^{−1} is assumed.

1. Introduction

Nearly all of the functions of a cell require proteins, and the types and amounts of those proteins will determine the fate of the cell. The level of a protein is regulated by its synthesis and degradation. In eukaryotic cells, many intracellular proteins that are destined for degradation are covalently tagged with a polyubiquitin chain and are then recognized and degraded by the 26S proteasome in an ATP-dependent manner (Hochstrasser, 1996; Coux *et al.*, 1996; Heinemeyer *et al.*, 2004). If the proteasome malfunctions, the level of the proteins in the cell will be abnormal and the functions of the cell will subsequently be disrupted (Debigaré & Price, 2003). It has been found that defects in the proteasome system result in many human diseases, including hypoxia, inflammation, neurodegenerative diseases, muscle-wasting disorders and human papillomaviruses (Schwartz & Ciechanover, 2009; Debigaré & Price, 2003).

The 26S proteasome is a large protein complex consisting of more than 30 proteins that compose two subcomplexes: the cylindrical catalytic 20S core particle (CP) and the regulatory particle (RP). The CP is barrel-shaped and is formed by two outer α -rings (α 1– α 7) and two inner β -rings (β 1– β 7). Target proteins are degraded within the chamber. However, the reorganization, unfolding and translocation of the ubiquitinated proteins are related to the RP, which binds on one or both sides of the CP (Marques *et al.*, 2009).

The RP is composed of two subcomplexes: the base and lid. Three non-ATPase subunits, Rpn1, Rpn2 and Rpn13, and six AAA-ATPase subunits, Rpt1–6, which contain four helices at their C-termini, form the base that is attached to the CP α -ring, and other non-ATPase subunits form the lid (Glickman *et al.*, 1998; Hamazaki *et al.*, 2006; Jørgensen *et al.*, 2006; Qiu *et al.*, 2006; Rosenzweig *et al.*, 2008). The assembly mechanism of the RP has been preliminarily described in mammalian cells and yeast, and it was revealed that four assembly chaperones, including Nas6 (p28, gankyrin), Rpn14 (PAAF1), Hsm3 (S5b) and Nas2 (p27, bridge1), formed three modulator complexes with base subunits (p28–Rpt3–Rpt6–Rpn14, S5b–Rpt1–Rpt2–Rpn1 and p27–Rpt4–Rpt5) and dissociated from the RP during proteasome assembly (Funakoshi *et al.*, 2009; Kaneko *et al.*, 2009; Saeki *et al.*, 2009).

Structure information on some of the chaperones has been reported. Partial structures of gankyrin and Rpt3 showed that gankyrin interacts with the C-terminal domain of Rpt3 through its six ankyrin repeats (Nakamura *et al.*, 2007). The structure of yeast Rpn14 has been solved, and it was predicted that it interacted with the C-terminal domain of Rpt6 through complementary charge interactions (Kim *et al.*, 2010). The structures of yeast Hsm3 and the heterodimer Hsm3–Rpt1 (C-terminal) have also been solved, and indicated that Hsm3 has 11 Arm/Heat repeats and interacts with the C-terminal domain of Rpt1 at its central domain (Barrault *et al.*, 2012; Takagi *et al.*, 2012). However, no structural data concerning the p27–Rpt4–Rpt5 complex have been published to date.

The 26S proteasome regulatory subunit p27 is also known as 26S proteasome non-ATPase regulatory subunit 9. This subunit contains an N-terminal helices domain, which interacts with the C-terminal ATPase domain of Rpt5 (also known as 26S protease regulatory subunit 6A) to facilitate RP assembly, and a predicted C-terminal PDZ domain, which has been reported to regulate insulin expression by acting as a co-activator of p300 (Lee *et al.*, 2005), E2A (Thomas *et al.*, 1999) and PDX-1 (Stanojevic *et al.*, 2005). Sequence alignment reveals that Rpt5 also has a coiled-coil segment and an OB-fold domain at its N-terminus in addition to a C-terminal ATPase domain (Zhang *et al.*, 2009).

Here, we describe the crystallization of the N-terminus of p27 and the complex of N-terminal p27 with the ATPase domain of Rpt5, and report the data that were collected from these crystals.

2. Materials and methods

2.1. Cloning, expression and purification

The DNA encoding residues 1–128 of p27 [p27^(1–128)] (Fig. 1) and 173–442 of Rpt5 [Rpt5^(173–442)] (Fig. 1) was amplified using a mouse liver cDNA library and polymerase chain reaction (PCR). The DNA fragments were cloned into the pET-32a vector (Novagen) in which the S-tag and the thrombin recognition site were substituted by a PreScission protease (3C)-cleavable sequence (LEVLFQGP).

The recombinant plasmid of p27^(1–128) was transformed into *Escherichia coli* BL21 (DE3) cells, and the *E. coli* cells were cultured in LB medium at 310 K until the A_{600} reached 0.6, whereupon a final concentration of 0.3 mM isopropyl β -D-1-thiogalactopyranoside was added to induce the expression of the protein at 289 K for 16 h. After centrifugation at 5000g for 15 min, the supernatant was removed and the pellet was resuspended in T₅₀N₅₀₀I₅ buffer (50 mM Tris–HCl pH 7.9, 500 mM NaCl, 5 mM imidazole) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 μ M leupeptin, 1 μ M antipain. The cells were lysed *via* sonication and centrifuged at 20 000g for 30 min at 277 K to obtain the supernatant. The cell supernatant was loaded onto a 5 ml HisTrap HP column (GE Healthcare) that was equilibrated with T₅₀N₅₀₀I₅ buffer. The column was washed with 20 column

volumes of T₅₀N₅₀₀I₅ buffer and eluted with a 40 column-volume linear gradient by changing the concentration of imidazole from 5 to 500 mM over 60 min at a flow rate of 3 ml min^{−1}. After identification using SDS–PAGE, the preferred fractions were collected and loaded onto a HiLoad 26/60 Superdex 200 size-exclusion column (GE Healthcare) and eluted using T₅₀N₅₀₀I₅ buffer. After identification using SDS–PAGE, the preferred fractions were collected, 3C protease was added to cleave the Trx–His₆ tag and the protein was made to flow through a HisTrap HP column (GE Healthcare) to remove the tag and the protease. Finally, the protein was loaded onto another HiLoad 26/60 Superdex 200 size-exclusion column and eluted with T₅₀N₅₀₀E₁D₁ buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT).

To create a p27^(1–128)–Rpt5^(173–442) complex, cells expressing p27^(1–128) and cells expressing Rpt5^(173–442) were mixed together in an estimated molar ratio of 1:1; the purification was then similar to that of p27^(1–128). Finally, the complex with no tag was loaded onto a HiLoad 26/60 Superdex 200 size-exclusion column and eluted with H₁₀N₅₀E₁D₁ (10 mM HEPES pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) buffer.

Figure 2
The elution profile and the SDS–PAGE analysis of p27^(1–128) (a) and p27^(1–128)–Rpt5^(173–442) complex (b) after gel filtration on a Superdex 200 16/60 HiLoad column.

Figure 1
Schematic representation of the domain organization of p27 and Rpt5. The grey dashed box shows the heterotrimer, which consists of p27^(1–128) and Rpt5^(173–442).

Table 1

X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

	p27 ^(1–128)	p27 ^(1–128) –Rpt5 ^(173–442)
X-ray source	BL17U1, SSRF	BL17U1, SSRF
Space group	<i>P</i> ₂ ₁ ₂ ₁	<i>P</i> 222
Unit-cell parameters (Å)	<i>a</i> = 26.79, <i>b</i> = 30.39, <i>c</i> = 145.06	<i>a</i> = 75.93, <i>b</i> = 76.08, <i>c</i> = 336.85
Wavelength (Å)	0.9795	0.9795
Resolution range (Å)	50–1.70 (1.76–1.70)	50–4.0 (4.14–4.00)
No. of unique reflections	75858	77628
Multiplicity	5.7 (2.6)	5.3 (5.2)
<i>R</i> _{merge} [†] (%)	5.6 (16.1)	14.2 (48.4)
<i>R</i> _{meas} [‡] (%)	6.0 (19.5)	15.8 (51.9)
<i>I</i> / <i>σ</i> (<i>I</i>)	28.2 (5.3)	8.7 (4.3)
Completeness (%)	95.5 (74.5)	84.8 (85.4)

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th reflection and $\langle I(hkl) \rangle$ is the average intensity. [‡] $R_{\text{meas}} = \sum_{hkl} \{N(hkl) / [N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$ where $N(hkl)$ is the multiplicity, $I_i(hkl)$ is the intensity of the *i*th measurement of reflection *hkl* and $\langle I(hkl) \rangle$ is the average value over multiple measurements.

2.2. Crystallization

p27^(1–128) was concentrated to 15 mg ml^{−1} and the complex p27^(1–128)–Rpt5^(173–442) was concentrated to 4 mg ml^{−1}. For crystallization, 1 μl protein solution was mixed with 1 μl precipitant solution in 48-well sitting-drop plates at 277 and 293 K, respectively. As many as 14 commercial screens were utilized, including Index, Crystal

Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2, SaltRx, SaltRx 2, PEGRx and PEGRx2 (Hampton Research) as well as Wizard I and II (Emerald Bio). Optimization was also performed in 48-well sitting-drop plates at 277 K. The crystals were recovered in 0.2–0.3 mm nylon loops (Hampton Research) and immediately flash-cooled in liquid nitrogen.

2.3. Data collection and processing

X-ray data were collected on beamline BL17U1 at the Shanghai Synchrotron Radiation Facility. Diffraction experiments were conducted at 100 K, and images of the p27^(1–128) and p27^(1–128)–Rpt5^(173–442) crystals were recorded using a 225 mm MAR CCD detector (MAR Research, Norderstedt, Germany) and a Quantum 315r CCD detector (ADSC, Poway, California, USA), respectively. The data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997) and *Novel_R* (Diederichs & Karplus, 1997).

3. Results and discussion

3.1. Preparation of the proteins

The proteins were highly overexpressed in *E. coli* BL21 (DE3) and were purified as described in §2. The elution peaks of the final HiLoad 26/60 Superdex 200 size-exclusion columns corresponded to the expected molecular weight of p27^(1–128) when acting as a

Figure 3

Crystals of p27^(1–128) before (a) and after optimization (b), and crystals of the p27^(1–128)–Rpt5^(173–442) complex after optimization (c). (d) SDS–PAGE resolving the crystal of the complex. Lane *M* contains molecular-weight marker (labelled in kDa).

monomer (Fig. 2a). Meanwhile, p27^(1–128) formed a stable heterodimer with Rpt5^(173–442) (Fig. 2b). Samples from these peaks were loaded onto a reducing SDS–PAGE gel and it was observed that the proteins were sufficiently pure for crystallization.

3.2. Crystallization

The p27^(1–128) crystal was obtained in the well corresponding to condition No. 9 (0.1 M ammonium citrate, 0.2 M ammonium acetate, 30% polyethylene glycol 4000) of Crystal Screen (Hampton

Research) at a protein concentration of 15 mg ml^{–1} at 277 K (Figs. 3a and 3b). p27^(1–128) crystals for X-ray analysis were flash-cooled using liquid nitrogen in a cryoprotectant which consisted of the reservoir solution supplemented with 20% glycerol, and diffracted to 1.7 Å resolution on beamline BL17U1 at the Shanghai Synchrotron Radiation Facility.

The crystal of the p27^(1–128)–Rpt5^(173–442) complex was obtained in condition No. 1 (2.0 M NaCl, 10% polyethylene glycol 6000) of Crystal Screen 2 (Hampton Research) at a complex concentration of 4 mg ml^{–1}. The crystallization conditions were optimized by varying

Figure 4 Representative diffraction images from the crystal of p27^(1–128) (a) and the p27^(1–128)–Rpt5^(173–442) complex (b). The inset shows weak spots at the edge. Complete data sets of the p27^(1–128) and p27^(1–128)–Rpt5^(173–442) crystals were collected to 1.7 and 4.0 Å resolution, respectively.

the concentrations of polyethylene glycol 4000 and ammonium acetate and the buffer pH, and by using the Additive Screen, Detergent Screen and Silver Bullet Kits (Hampton Research). The final condition was 13.5% (w/v) PEG 6000, 1.8 M NaCl, 0.2 M LiNO₃, 0.1 M bis-tris pH 5.7 (Fig. 3c). Some crystals were dissolved and loaded onto a reducing SDS–PAGE gel to identify the proteins that were included in the crystals (Fig. 3d). Crystals of the p27^(1–128)–Rpt5^(173–442) complex for X-ray analysis were flash-cooled using liquid nitrogen in Paratone-N (Hampton Research) and diffracted to 4.0 Å on beamline BL17U1 at the Shanghai Synchrotron Radiation Facility.

3.3. Data collection and processing

A full native diffraction data set of the p27^(1–128) crystal was collected and processed to a resolution of 1.7 Å. One of the diffraction patterns is shown in Fig. 4(a). The crystal belonged to space group *P*2₁2₁2₁, with unit-cell parameters *a* = 26.79, *b* = 30.39, *c* = 145.06 Å. Resolution-dependent Matthews coefficient probability analysis (Kantardjieff & Rupp, 2003; Matthews, 1968) suggested the presence of only one molecule per asymmetric unit, with a 40.5% solvent content and a *V*_M value of 2.02 Å³ Da^{−1}.

A full native diffraction data set of the p27^(1–128)–Rpt5^(173–442) crystal was collected and processed to a resolution of 4.0 Å. One of the diffraction patterns is shown in Fig. 4(b). The crystal belonged to space group *P*222, with unit-cell parameters *a* = 75.93, *b* = 76.08, *c* = 336.85 Å. The calculated Matthews coefficient (*V*_M) and solvent content for four heterodimers were 2.63 Å³ Da^{−1} and 53.2%, respectively, and those calculated for five heterodimers were 2.10 Å³ Da^{−1} and 41.5%, respectively, suggesting the presence of either four or five heterodimers of the complex in the asymmetric unit. The data-collection statistics are listed in Table 1.

The ATPase domain of yeast Rpt5 (Unverdorben *et al.*, 2014; PDB entry 4cr2, chain *M*), which shares 78% sequence identity with Rpt5 from *Mus musculus*, was used as a template for molecular-replacement studies using *Phaser* (McCoy, 2007) in the *CCP4* suite (Winn *et al.*, 2011), but no correct solution was obtained. Expression of selenomethionine-labelled protein in *E. coli* B834 (DE3) cells is in progress to obtain the phases.

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References

- Barraut, M.-B., Richet, N., Godard, C., Murciano, B., Le Tallec, B., Rousseau, E., Legrand, P., Charbonnier, J.-B., Le Du, M.-H., Guérois, R., Ochsenbein, F. & Peyroche, A. (2012). *Proc. Natl Acad. Sci. USA*, **109**, E1001–E1010.
- Coux, O., Tanaka, K. & Goldberg, A. L. (1996). *Annu. Rev. Biochem.* **65**, 801–847.
- Debigaré, R. & Price, S. R. (2003). *Am. J. Physiol. Renal Physiol.* **285**, F1–F8.
- Diederichs, K. & Karplus, P. A. (1997). *Nature Struct. Mol. Biol.* **4**, 269–275.
- Funakoshi, M., Tomko, R. J., Kobayashi, H. & Hochstrasser, M. (2009). *Cell*, **137**, 887–899.
- Glickman, M. H., Rubin, D. M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V. A. & Finley, D. (1998). *Cell*, **94**, 615–623.
- Hamazaki, J., Iemura, S., Natsume, T., Yashiroda, H., Tanaka, K. & Murata, S. (2006). *EMBO J.* **25**, 4524–4536.
- Heinemeyer, W., Ramos, P. C. & Dohmen, R. J. (2004). *Cell. Mol. Life Sci.* **61**, 1562–1578.
- Hochstrasser, M. (1996). *Annu. Rev. Genet.* **30**, 405–439.
- Jørgensen, J. P., Lauridsen, A. M., Kristensen, P., Dissing, K., Johnsen, A. H., Hendil, K. B. & Hartmann-Petersen, R. (2006). *J. Mol. Biol.* **360**, 1043–1052.
- Kaneko, T., Hamazaki, J., Iemura, S., Sasaki, K., Furuyama, K., Natsume, T., Tanaka, K. & Murata, S. (2009). *Cell*, **137**, 914–925.
- Kantardjieff, K. A. & Rupp, B. (2003). *Protein Sci.* **12**, 1865–1871.
- Kim, S., Saeki, Y., Fukunaga, K., Suzuki, A., Takagi, K., Yamane, T., Tanaka, K., Mizushima, T. & Kato, K. (2010). *J. Biol. Chem.* **285**, 15159–15166.
- Lee, J. H., Volinich, J. L., Banz, C., Yao, K.-M. & Thomas, M. K. (2005). *J. Endocrinol.* **187**, 283–292.
- Marques, A. J., Palanmurugan, R., Matias, A. C., Ramos, P. C. & Dohmen, R. J. (2009). *Chem. Rev.* **109**, 1509–1536.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J. (2007). *Acta Cryst.* **D63**, 32–41.
- Nakamura, Y., Nakano, K., Umehara, T., Kimura, M., Hayashizaki, Y., Tanaka, A., Horikoshi, M., Padmanabhan, B. & Yokoyama, S. (2007). *Structure*, **15**, 179–189.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Qiu, X.-B., Ouyang, S.-Y., Li, C.-J., Miao, S., Wang, L. & Goldberg, A. L. (2006). *EMBO J.* **25**, 5742–5753.
- Rosenzweig, R., Osmulski, P. A., Gaczynska, M. & Glickman, M. H. (2008). *Nature Struct. Mol. Biol.* **15**, 573–580.
- Saeki, Y., Toh-E, A., Kudo, T., Kawamura, H. & Tanaka, K. (2009). *Cell*, **137**, 900–913.
- Schwartz, A. L. & Ciechanover, A. (2009). *Annu. Rev. Pharmacol. Toxicol.* **49**, 73–96.
- Stanojevic, V., Yao, K.-M. & Thomas, M. K. (2005). *Mol. Cell. Endocrinol.* **237**, 67–74.
- Takagi, K., Kim, S., Yukii, H., Ueno, M., Morishita, R., Endo, Y., Kato, K., Tanaka, K., Saeki, Y. & Mizushima, T. (2012). *J. Biol. Chem.* **287**, 12172–12182.
- Thomas, M. K., Yao, K.-M., Tenser, M. S., Wong, G. G. & Habener, J. F. (1999). *Mol. Cell. Biol.* **19**, 8492–8504.
- Unverdorben, P., Beck, F., Sledz, P., Schweitzer, A., Pfeifer, G., Plitzko, J.M., Baumeister, W., Foerster, F. (2014). *Proc. Natl Acad. Sci. USA*. doi:10.1073/pnas.1403409111.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Zhang, F., Hu, M., Tian, G., Zhang, P., Finley, D., Jeffrey, P. D. & Shi, Y. (2009). *Mol. Cell.* **34**, 473–484.