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Purification, crystallization and preliminary X-ray diffraction analysis of the non-ATPase subunit Nas6 in complex with the ATPase subunit Rpt3 of the 26S proteasome from *Saccharomyces cerevisiae*

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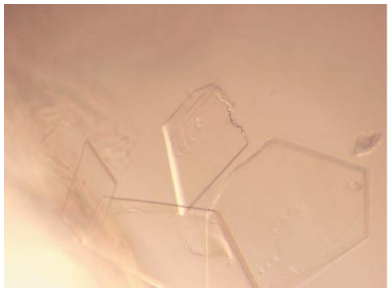

The non-ATPase subunit Nas6, which is the human orthologue of gankyrin, was co-expressed with the C-terminal domain of the ATPase subunit Rpt3 of the yeast 26S proteasome in *Escherichia coli*, purified to near-homogeneity and crystallized using the hanging-drop vapour-diffusion method. The protein crystallized in space group $P2_1$, with unit-cell parameters a = 60.38, b = 100.22, c = 72.20 Å, β = 94.70° and with three Nas6–Rpt3C molecules per asymmetric unit. The crystal diffracted to beyond 2.2 Å resolution using synchrotron radiation.

1. Introduction

Regulation of protein degradation through the 26S proteasome is an evolutionarily conserved cellular process (Tanaka, 1998; Wollenberg & Swaffield, 2001). The 26S proteasome is composed of the 20S core and 19S regulatory particles, which form a cylindrical organelle (Tanaka, 1998; Glickman & Raveh, 2005). The 20S core particle adopts a barrel-like structure with four rings, while the 19S regulatory particle forms a base and a lid structure (Wollenberg & Swaffield, 2001). The base structure of the 19S regulatory particle consists of heterohexameric proteasomal ATPases (Rpt1–Rpt6 in yeast), each belonging to the AAA (ATPases associated with diverse cellular activity) superfamily. Cellular proteins to be degraded are marked with a polyubiquitin tag and are then assumed to become unfolded within the ATPase base and proteolyzed inside the barrel of the 20S core particle (Tanaka, 1998). Structural analyses of the 20S core particles from various species have been reported (Lowe *et al.*, 1995; Groll *et al.*, 1997; Tomisugi *et al.*, 2000; Unno *et al.*, 2002*a*,*b*). In contrast, little is known about the structure of the 19S regulatory particle.

Importantly, the mammalian orthologue of yeast Nas6 (non-ATPase subunit 6) of the 19S regulatory particle was identified as the oncoprotein gankyrin, which controls the degradation of the tumour-suppressor proteins pRb and p53 (Hori *et al.*, 1998; Higashitsuji *et al.*, 2000, 2005; Dawson *et al.*, 2006). The tertiary structures of the apo forms of Nas6 and gankyrin have recently been determined (Padmanabhan *et al.*, 2004; Krzywda *et al.*, 2004; Manjasetty *et al.*, 2004; Yuan *et al.*, 2004). Since human gankyrin interacts with human S6 ATPase (Rpt3 in yeast) of the 19S regulatory particle (Dawson *et al.*, 2002) and yeast Nas6 has been shown to interact with yeast Rpt3 both *in vivo* and *in vitro* (Uetz *et al.*, 2000; Ho *et al.*, 2002), there should be a similar regulatory mechanism in yeast that controls cellular proliferation through the interaction between Nas6/gankyrin and the proteasomal ATPase Rpt3/S6. To understand the functional role of Nas6 in protein degradation in yeast as well as its phylogenetical relationship, we sought to determine the structure of the complex between Nas6 and the proteasomal ATPase Rpt3. We obtained the Nas6 complex with the C-terminal domain of Rpt3, which is essential for the interaction with Nas6 (Dawson *et al.*, 2002), by a co-expression method. In the present study, we report the purification, crystallization and preliminary crystallographic studies of the complex of the non-ATPase subunit Nas6 and the C-terminal domain of the ATPase subunit Rpt3.

2. Materials and methods

2.1. Cloning

The open reading frame of the gene encoding full-length *Saccharomyces cerevisiae* Nas6 (residues 1–231) was subcloned into cassette 1 of pETDuet1 at the *Bam*HI and *Hin*dIII restriction sites in order to obtain the expression plasmid pETDuet1-6HisNas6. In addition, the cDNA encoding the C-terminal region of Rpt3 (residues 348–428; hereafter referred to as Rpt3C) from *S. cerevisiae* was subcloned into the second cassette of pETDuet1-6HisNas6 at the *Nde*I and *Xho*I restriction sites in order to generate the expression plasmid pETDuet1-6HisNas6-Rpt3C.

2.2. Protein expression

To overproduce the Nas6–Rpt3C complex, *Escherichia coli* Rosetta (DE3) cells (Novagen) were transformed with the pETDuet1-6HisNas6-Rpt3C co-expression plasmid and grown at 310 K in LB medium containing 50 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol. At an OD_{600} of about 0.5, protein expression was induced by the addition of IPTG to a final concentration of 0.4 m*M*. After 6 h of culture at 310 K, the cells were harvested by centrifugation (9600*g* for 5 min) and resuspended in 50 ml 20 m*M* Tris–HCl buffer pH 8.0 containing 500 m*M* NaCl.

2.3. Purification

The *E. coli* cells were homogenized using a sonicator (Tomy) and the homogenate was centrifuged at 20 000*g* for 1 h. The resultant supernatant, containing the Nas6–Rpt3C complex, was applied onto a HisTrap HP column (Amersham Biosciences) and the target proteins were eluted with buffer containing 0.5 *M* imidazole. For further purification of the Nas6–Rpt3C complex, the proteins were fractionated on a HiTrapQ anion-exchange column (Amersham Biosciences), which was eluted with a linear gradient of NaCl from 0 to 1.0 *M*. The fractions containing the Nas6–Rpt3C complex were fractionated on a HiLoad 16/60 Superdex 75 gel-filtration column (Amersham Biosciences). The Nas6 in the protein complex has a six-His tag at its N-terminus which could not be removed from the protein. The purity of the Nas6–Rpt3C complex was greater than 95% as judged by an SDS–PAGE analysis (Fig. 1*a*).

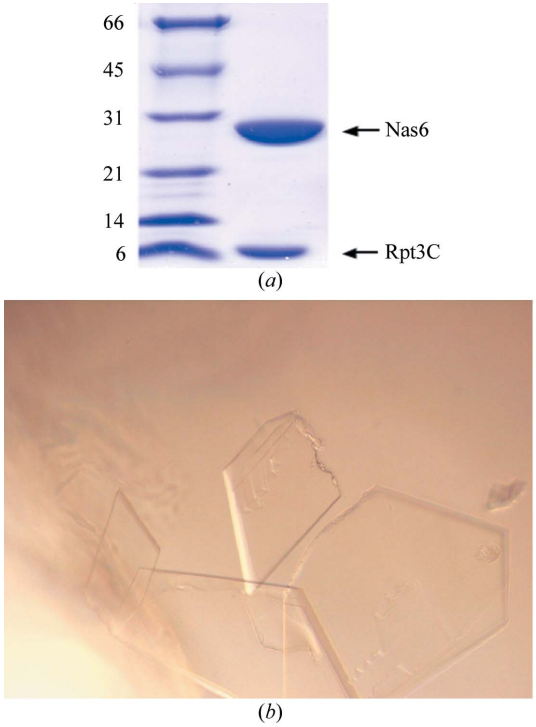

Figure 1
Purification and crystallization of the Nas6–Rpt3C complex. (*a*) SDS–PAGE profile of the Nas6–Rpt3C complex. Lane 1, molecular-weight markers. The size of each protein (in kDa) is shown on the left. Lane 2, purified Nas6–Rpt3C complex fraction. Electrophoresis was performed with a 17.5% SDS–polyacrylamide gel. (*b*) Crystals of the Nas6–Rpt3C complex.

2.4. Crystallization

The purified protein complex was concentrated to about 8 mg ml^{-1} in 20 m*M* Tris–HCl buffer pH 8.0 containing 150 m*M* NaCl. Crystallization screening was carried out at 298 K using the hanging-drop vapour-diffusion method. Hampton Research Crystal Screen, Crystal Screen 2 and Index Screen (Hampton Research, CA, USA) were used for the initial screening. The drops were prepared by mixing 1.0 µl protein complex solution with 1.0 µl reservoir solution on siliconized cover slides and were equilibrated against 500 µl reservoir solution. After one month, small crystals of the protein complex were initially obtained in the drops containing PEG 4000 as the major precipitant. After optimization, plate-shaped crystals were obtained in a drop containing 20% PEG 6000, 100 m*M* MES buffer pH 6.8 and 2% glycerol (Fig. 1*b*).

2.5. Data collection

X-ray diffraction data sets were collected at beamline NW12, KEK, Tsukuba, Japan with a Quantum 210 detector. The wavelength used was 1.0 Å and the incident beam was collimated to a 0.1 mm diameter. The crystal-to-detector distance was set to 180 mm and the oscillation range was 1°, with an exposure time of 15 s. The crystal was first flash-cooled without any cryoprotectant and was maintained at

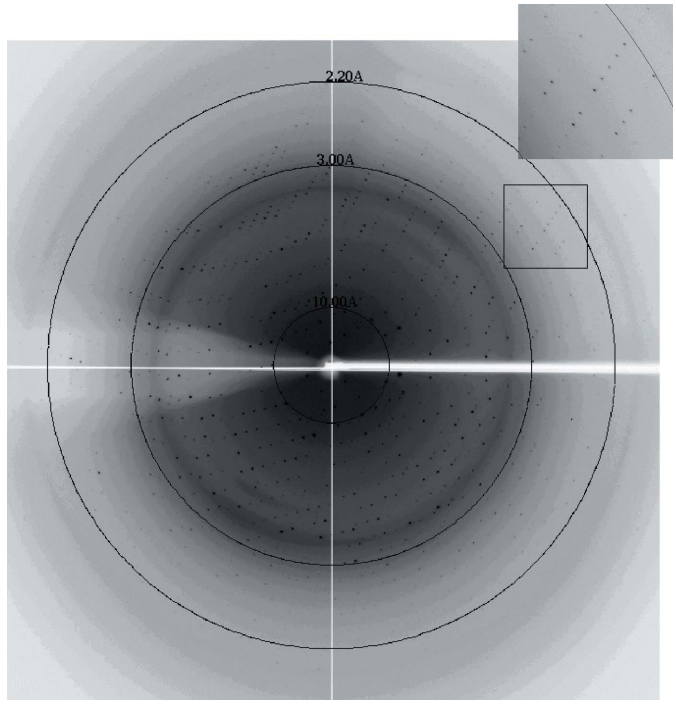

Figure 2
X-ray diffraction by the Nas6–Rpt3C complex crystal showing resolution rings and a magnified view. The crystal-to-detector distance was 180 mm, the oscillation angle was 1.0° and the exposure time was 15 s.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Source	NW12, KEK
Wavelength (Å)	1.00
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 60.38, b = 100.22, c = 72.20, γ = 94.70
Resolution range (Å)	50.0–2.2 (2.28–2.2)
No. of measured reflections	163820
No. of unique reflections	43936
Completeness (%)	99.6 (99.5)
Redundancy	3.7 (3.8)
$R_{\rm merge}$† (%)	5.6 (20.3)
$I/\sigma(I)$	35.2 (7.3)

† $R_{\rm merge} = \sum |I_{\rm obs} - \langle I \rangle| / \sum \langle I \rangle$ summed over all observations and reflections.

100 K using nitrogen gas during data collection. A typical X-ray diffraction pattern of the Nas6–Rpt3C complex is shown in Fig. 2. All data were integrated and scaled using the *HKL*-2000 suite (Otwinowski & Minor, 1997).

3. Results and discussion

Expression of the Rpt3C protein by itself (*i.e.* in the absence of Nas6) yielded only an insoluble form (data not shown). In contrast, the Rpt3C protein was soluble when co-expressed with the partner protein Nas6. The complex of Nas6 and Rpt3C of the yeast 26S proteasome was purified to near-homogeneity (Fig. 1*a*) and was crystallized. After optimizing the crystallization conditions, plate-shaped crystals with dimensions of approximately 0.025 × 0.5 × 0.5 mm were obtained (Fig. 1*b*). The crystals diffracted to beyond 2.2 Å resolution (Fig. 2) using synchrotron radiation. The crystals belong to space group $P2_1$, with unit-cell parameters a = 60.38, b = 100.22, c = 72.20 Å, β = 94.70°. Data-collection statistics are summarized in Table 1.

Assuming that three molecules of the Nas6–Rpt3C complexes (molecular weight of approximately 35 kDa per complex) are present in the asymmetric unit, the Matthews coefficient $V_{\rm M}$ and the solvent content were estimated to be 2.1 Å^3 Da^{-1} and 40%, respectively (Matthews, 1968). Structure determination of the Nas6–Rpt3C complex by molecular replacement using the native Nas6 structure (PDB code 1ixv; Padmanabhan *et al.*, 2004) is in progress.

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