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Purification, crystallization and preliminary X-ray diffraction analysis of yeast regulatory particle non-ATPase subunit 6 (Nas6p)

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The regulatory particle non-ATPase subunit, Nas6p, from *Saccharomyces cerevisiae* has been crystallized by the hanging-drop vapour-diffusion method using PEG 4000 as precipitant. The crystals belong to the space group $P2_12_12_1$, with unit-cell parameters a = 41.43 (2), b = 61.74 (1), c = 98.09 (2) Å, and contain one molecule in the asymmetric unit. A complete diffraction data set using synchrotron radiation was collected to 2.6 Å resolution.

1. Introduction

The activation of the major proteolytic system in various cellular processes is carried out by the ubiquitin–proteosome pathway (Hershko & Ciechanover, 1998). The ubiquitin–proteosome system also plays important roles including cell-cycle control, antigen presentation, signal transduction, DNA repair, transcriptional silencing, neuronal pathfinding and long-term facilitation of withdrawal reflexes in *Aplysia*. Target proteins which are to be degraded by this pathway are first conjugated with ubiquitins. The multi-ubiquitin chains attached to the proteins are recognized by the 26S proteosome, which degrades the target proteins in an ATP-dependent manner and releases ubiquitin for the next cycle. The ubiquitin-machinery system is a multi-component system in which three enzymes are essentially involved in ubiquitination: ubiquitin is activated by an ubiquitin-activating enzyme (E1 enzyme), transferred to ubiquitin-conjugating enzymes (E2 enzymes) and then finally transferred to the target proteins. The ubiquitin ligase (E3 enzyme) needed for the final step of ubiquitination depends upon the proteins to be ubiquitinated (Hershko & Ciechanover, 1998).

Recent studies have reported that the specificity of proteolysis by the ubiquitin–proteosome pathway is modulated by the 26S proteosome (Coux *et al.*, 1996; Glickman, Rubin, Coux *et al.*, 1998; Glickman, Rubin, Fried *et al.*, 1998). The 26S proteosome (2000 kDa) consists of two subcomplexes: the 20S proteosome and the 19S regulatory particle. The 19S regulatory particle is attached to one or both ends of the 20S proteosome. In *S. cerevisiae*, 14 genes encoding subunits (seven α-subunits and seven β-subunits) of the 20S proteosome have been identified (Coux *et al.*, 1996; Groll *et al.*, 1997). The X-ray structure of 20S proteosome (Groll *et al.*, 1997) suggests that the protease activity is sequestered inside the β-ring and there is no opening on the α-ring for protein substrates to enter the lumen of the 20S proteosome. The 19S regulatory particle is composed of regulatory particle triple ATPase subunits (Rpt) and regulatory particle non-ATPase subunits (Rpn; Glickman, Rubin, Fried *et al.*, 1998). Most of the Rpt subunits are located near the 20S core particle, whereas Rpn subunits are located far from the 19S regulatory particle (Glickman, Rubin, Coux *et al.*, 1998).

Nas6p (non-ATPase subunit 6), which is found both in human and yeast, contains six copies of the ankyrin repeat. Human Nas6p was identified as a subunit of the human 19S regulatory cap, also known as PA700 (Hori *et al.*, 1998). The yeast Nas6p homologue (Ygr232w), which is dispensable for viability (Hori *et al.*, 1998), was identified as an Rpt3-interacting protein (Uetz *et al.*, 2000). Since the function of Nas6p is not yet clearly known in the 26S proteosome assembly, we have initiated a structural study to understand its functional role in the proteosome–ubiquitin pathway. Here, we report the purification, crystallization and preliminary crystallographic studies of *S. cerevisiae* Nas6p.

2. Materials and methods

2.1. Protein expression and purification

The open reading frame of the gene encoding Nas6p was amplified using PCR with primers including a 5′ *Nde*I site and 3′ *Sal*I site to facilitate cloning into the expression vector pET28b (Novagen). For overproduction of Nas6p protein, *Escherichia coli* BL21-CodonPlus (DE3) pLys-RIL (Stratagene) cells were transformed with the pET28b-Nas6p recombinant plasmid and grown at 291 K in TBG-M9 medium containing 30 µg ml^{-1} kanamycin and 34 µg ml^{-1} chloramphenicol until OD_{600} reached 0.8–1.0 (Munakata *et al.*, 2000). Plasmid expression was then induced by addition of 0.4 m*M* IPTG. After 16 h of culture at 291 K, the cells harvested by centrifugation

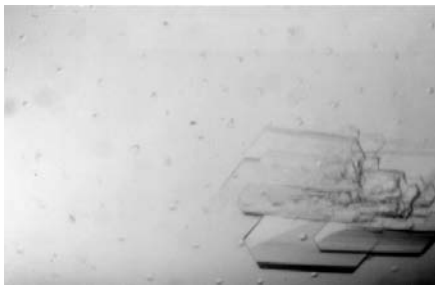

Figure 1
Crystals of *S. cerevisiae* Nas6p.

(3000 rev min^{-1}, 10 min, 277 K) were resuspended in buffer containing 20 m*M* Tris–HCl (pH 7.9 at 277 K), 10% glycerol, 500 m*M* KCl, 50 m*M* β-mercaptoethanol, 1 m*M* PMSF, 20 µg ml^{-1} leupeptin and 20 µg ml^{-1} pepstatin A and lysed using EmulsiFlex-C5 (Avestin). The cell lysate was centrifuged at 24 000 rev min^{-1} for 30 min at 277 K. The resultant supernatant was then applied to ProBond Resin (Invitrogen) and eluted with a buffer containing 0.2 *M* imidazole. For further purification, the eluted protein, which was concentrated with a Centriprep YM-10 (Millipore), was fractionated by gel-filtration Superdex 200 (Amersham Pharmacia). The pooled Nas6p protein was concentrated to 4 mg ml^{-1} with a Centriprep YM-10 (Millipore). The purity of Nas6p was checked by SDS–PAGE and mass spectrometry. The purity was higher than 95%.

2.2. Crystallization

Initial crystallization trials used the hanging-drop vapour-diffusion method and a wide range of conditions (including Hampton Research Screens I and II). Drops containing equal volumes (2 µl) of protein (5 mg ml^{-1}) and reservoir solution were equilibrated against 0.5 ml of reservoir solution. Small crystals were obtained with polyethylene glycol as precipitant. With optimized crystallization conditions, thin plate-like crystals were obtained in the presence of 15–20% PEG 4000, 0.1 *M* MES pH 6.5. Crystals of approximate dimensions 0.25 × 0.2 × 0.07 mm were obtained after 10 d (Fig. 1).

2.3. Data collection

Diffraction data were collected from a single crystal on beamline BL18B using the ADSC Quantum-4 CCD detector at the Photon Factory, Tsukuba, Japan. The wavelength used was 1.00 Å and the incident beam was collimated to a diameter of 0.1 mm. The crystal-to-detector distance was set to 200 mm. A complete data set was collected at room temperature to a maximum resolution of 2.6 Å (Fig. 2). All data were processed and scaled using the programs *DPS*/*MOSFLM* (Rossmann & van Beek, 1999) and *SCALA* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

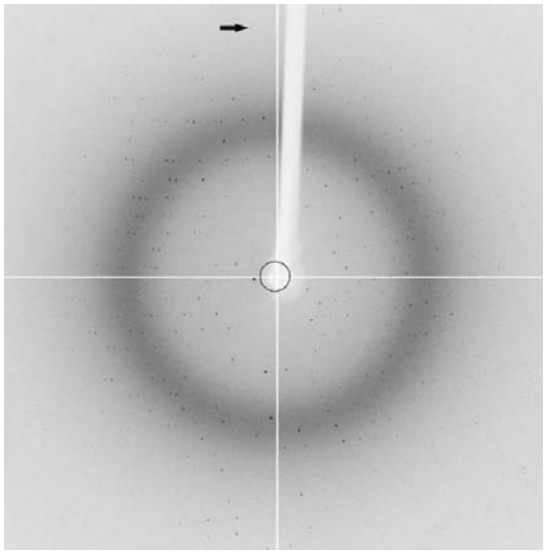

Figure 2
An X-ray diffraction pattern from a *S. cerevisiae* Nas6p crystal (oscillation range 2.0°). The arrow indicates a resolution of 2.54 Å.

3. Results

Examination of diffraction data from Nas6p crystals revealed that these crystals diffract beyond 2.6 Å resolution and belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 41.43 (2), b = 61.74 (1), c = 98.09 (2) Å. Data-collection statistics are summarized in Table 1. A total of 36 269 measured reflections were merged into 8146 unique reflections with an $R_{\rm merge}$ of 7.8%. The merged data set is 99.4% complete to 2.6 Å resolution. A value for the Matthews coefficient of 2.41 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 49% were obtained assuming one molecule in the asymmetric unit and a molecular weight of 26 000 Da. Primary-structure studies showed that Nas6p belongs to the ankyrin family and contains six ankyrin-repeat domains. Nas6p has 25% sequence identity with ankyrin-repeat domains 1–6 and 23% sequence identity with the ankyrin-repeat domains 2–7 of Bcl-3, which contains seven ankyrin-repeat domains. An attempt to solve the structure by the molecular-replacement method using the Bcl-3 structure (Michel *et al.*, 2001) as a model is in progress.

Table 1
Data-collection and processing statistics.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 41.43 (2), b = 61.74 (1), c = 98.09 (2)
Resolution range (Å)	30.0–2.6
No. of measured reflections	36269
No. of unique reflections	8146
$R_{\rm merge}$ †‡ (%)	7.1 (13.8)
Completeness‡ (%)	99.4 (99.4)
Average $I/\sigma(I)$‡	7.8 (5.0)

† $R_{\rm merge} = \sum|I(h) - \langle I(h)\rangle|/\sum I(h)$, where $I(h)$ is the observed intensity and $\langle I(h)\rangle$ is the mean intensity of reflection h over all measurements of $I(h)$. ‡ Values in parentheses refer to the last shell, 2.74–2.60 Å.

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