**Tal Keren-Kaplan* and
Gali Prag***

Department of Biochemistry and Molecular
Biology and The Institute for Structural Biology,
George S. Wise Faculty of Life Sciences, Tel Aviv
University, Tel Aviv 69978, Israel

Correspondence e-mail: talker@post.tau.ac.il,
prag@post.tau.ac.il

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Purification and crystallization of mono-ubiquitylated ubiquitin receptor Rpn10

Protein ubiquitylation controls nearly all cellular pathways in eukaryotes. A repertoire of proteins named ubiquitin (Ub) receptors harbouring ubiquitin-binding domains (UBDs) recognize ubiquitylated proteins. These Ub receptors decode the Ub signal by tethering a UBD or UBDs to a functional domain or domains, thus linking the ubiquitylated target to a specific function. The rapid dynamics of ubiquitylation/deubiquitylation has impeded the characterization of ubiquitylated proteins. To bypass this obstacle, a recently developed synthetic system that reconstructs the entire eukaryotic ubiquitylation cascade in *Escherichia coli* was used to purify the mono-ubiquitylated form of the regulatory proteasomal non-ATPase subunit (Ub-Rpn10) from *Saccharomyces cerevisiae*. Here, the first crystallization and data collection of Ub-Rpn10 is reported. Purified Ub-Rpn10 was crystallized in 12%(w/v) PEG 20 000, 0.1 M MES pH 6.5 and yielded thin rhombus-shaped crystals. X-ray analysis revealed that these crystals belonged to the monoclinic system C2, with unit-cell parameters $a = 107.3$, $b = 49.7$, $c = 81.3$ Å, $\alpha = \gamma = 90.0$, $\beta = 130.5^\circ$. A full synchrotron data set has been collected, merged and scaled with a diffraction limit of 3.14 Å.

1. Introduction

Ubiquitin (Ub) receptors are proteins that decrypt the information conferred by Ub modification into a cellular response (Hicke *et al.*, 2005; Hurley *et al.*, 2006; Prag *et al.*, 2003; Shih *et al.*, 2003). They do so by their multifunctional structure, in which a ubiquitin-binding domain (UBD) is tethered to other functional domain. Depending on the cellular context, Ub receptors specifically bind to ubiquitylated targets and thus bring their other functional domains *in situ* to the ubiquitylation site. Interestingly, it has been shown that Ub receptors are themselves ubiquitylated (Hoeller *et al.*, 2006; Prag *et al.*, 2003; Shih *et al.*, 2003). Moreover, it has been demonstrated for several Ub receptors that this ubiquitylation acts as a mechanism for auto-regulation by blocking the UBD from binding to ubiquitylated cargo (Hoeller *et al.*, 2006; Isasa *et al.*, 2010). Consequently, Ub receptors can be found in three states: (i) apo (unbound form), (ii) trans (bound to ubiquitylated cargo) and (iii) cis (auto-ubiquitylated). While apo and trans structures of many Ub receptors have been determined, no cis structures have been reported. This is probably owing to difficulties in purifying proteins in their ubiquitylated form, since a large number of deubiquitylating enzymes efficiently remove the Ub moiety from modified proteins. This lability of the Ub signal makes it difficult to purify reasonable amounts of the ubiquitylated (cis) state. To circumvent this limitation, we recently developed a novel synthetic biology approach that reconstitutes the entire ubiquitylation cascade in bacteria (Keren-Kaplan *et al.*, 2011). Plasmids expressing His₆-Ub, E1 activating enzyme, E2 conjugating enzyme, E3 ligase and the cognate substrate are transformed into *Escherichia coli*. Since *E. coli* does not possess deubiquitylating enzymes, the modified protein is accumulated and can be purified in fairly large amounts sufficient for crystallography and other biophysical and biochemical studies.

Rpn10 acts as a Ub receptor that decodes the Lys48 poly-ubiquitylation signal on cytosolic proteins into a proteasomal degradation response (Matiuhin *et al.*, 2008). It can be found free in the cytosol, where it can bind ubiquitylated substrates, or as a part of the 26S proteasome complex where protein degradation occurs (Fu *et*

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et al., 1998). In Rpn10 and its human orthologue S5a, a proteasome-associated Von Willebrand factor type A (VWA) domain is tethered by a flexible linker to a Ub-interaction motif (UIM; in the human orthologue there are two UIMs, while in yeast only one UIM has been identified). NMR studies of the trans-complex structures of Rpn10-UIM with Ub and Ub-like domains (UBLs) of other proteasome receptors, including radiation-sensitive protein 23 (Rad23) and Dsk2, demonstrated the affinity, specificity and mechanism of poly-Ub chain-length sensing. It has also been shown that Rpn10 presents increased affinity towards tetra-Ub chains compared with shorter Ub chains or mono-Ub (Matiuhin *et al.*, 2008; Zhang *et al.*, 2009; Wang *et al.*, 2005).

Surprisingly, it has been reported that many nonrelated E3 ligases can ubiquitylate S5a (Uchiki *et al.*, 2009), possibly owing to its two UIMs. Nevertheless, it has been demonstrated that Rsp5 is the cognate E3 ligase of yeast Rpn10 (Lu *et al.*, 2008). Crosas and coworkers showed that yeast Rpn10 is mono-ubiquitylated *in vivo* mainly at Lys84, which is located in the VWA domain, and that Ub-Rpn10 cannot bind ubiquitylated substrates (Isasa *et al.*, 2010). However, it is still not fully understood how auto-ubiquitylation regulates the activity of Rpn10. Moreover, to date there is no structural information on Rpn10 in the ubiquitylated (*cis*) state (or for any other ubiquitylated substrate).

We recently demonstrated that co-expression of Ub, E1 (Uba1) and E2 (Ubc4) with E3 (Rsp5) and the substrate (Rpn10) results in the accumulation of mono-ubiquitylated Rpn10 and that the modified protein could be purified to homogeneity. We also verified the ubiquitylation site as Lys84 by mass spectrometry, as previously found *in vivo* in yeast (Keren-Kaplan *et al.*, 2011).

Here, we report the first crystallization, diffraction and data collection of the ubiquitylated Ub receptor Rpn10.

2. Experimental procedures

2.1. Cloning of the yeast Rpn10 gene

The open reading frame (ORF; encoding residues 1–268) of RPN10 from *Saccharomyces cerevisiae* (NCBI Reference Sequence NM_001179333.2) was amplified by polymerase chain reaction (PCR) from pRS424-Rpn10. The PCR product was cloned into the *SacII*–*NotI* restriction-endonuclease sites of a modified pCDF-Duet vector lacking the N-terminal His₆ tag but harbouring a maltose-binding protein (MBP) tag followed by a human rhinovirus type 14-3C protease-recognition site. The resulting vector expresses an MBP-Rpn10 fusion protein from the T7 promoter of the pCOG31 vector (Keren-Kaplan *et al.*, 2011). The ubiquitylation-cascade components His₆-Ub (human), E1 activating enzyme Uba1 (wheat) and E2 conjugating enzyme Ubc4 (yeast) were expressed from the T7 promoter of the pGEN6 vector in a polycistronic fashion. The E3 ligase NusA-Rsp5 (yeast) was expressed from the T7 promoter of pCOG30 (Keren-Kaplan *et al.*, 2011). A tobacco etch virus protease recognition site between the His₆ tag and Ub allowed tag removal at the end of the purification process.

2.2. Protein expression and purification

E. coli Rosetta2 (λDE3) BL21 cells were co-transformed with the pGEN6, pCOG30 and pCOG31 vectors (Keren-Kaplan *et al.*, 2011) and grown in Terrific Broth medium supplemented with 12.5 µg ml^{−1} streptomycin, 17 µg ml^{−1} chloramphenicol, 12 µg ml^{−1} tetracycline and 50 µg ml^{−1} ampicillin at 310 K. The culture (9 l) was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and was grown for a further ~20 h at 289 K with shaking at 180 rev min^{−1}.

Cells were harvested by centrifugation (5 min, 6000 rev min^{−1}, 277 K) and were resuspended in buffer [20 mM Tris–HCl pH 7.0, 200 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediamine-tetraacetic acid (EDTA)] supplemented with the protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; 0.1 mM), DNase (10 µg ml^{−1}) and lysozyme (0.2 mg ml^{−1}). Complete lysis and DNA shearing were achieved by sonication (3 min action, 5 s pulse, 25 s rest, amplitude 50 W). The soluble fraction was isolated by centrifugation. Ub-Rpn10 (molecular mass of 39.1 kDa after tag removal) was purified using amylose resin (New England Biolabs) according to the manufacturer's instructions. The binding buffer consisted of 20 mM Tris–HCl pH 8, 200 mM NaCl, 1 mM DTT, 1 mM EDTA. The protein was eluted with 150 mM Tris–HCl pH 8, 50 mM NaCl, 10 mM maltose, 20 mM imidazole. The elution fraction contained ubiquitylated and apo forms of Rpn10. The protein was further purified by nickel Sepharose high-performance (GE Healthcare) affinity chromatography to separate ubiquitylated protein from non-ubiquitylated protein. The resin was extensively washed with 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 2% Triton X-100, 20 mM imidazole followed by washes with 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 2% Triton X-100, 40 mM imidazole. Triton X-100 was removed from the buffer in the final washing step. Elution was carried out with

Figure 1 Purification of mono-ubiquitylated Rpn10. (a) Ion-exchange UV_{280 nm} chromatogram showing the peak corresponding to Ub-Rpn10, with relevant fractions indicated below. The NaCl elution gradient is indicated by a dashed line. (b) Concentrated samples of Ub-Rpn10 and apo Rpn10.

50 mM Tris-HCl pH 7.5, 150 mM NaCl, 500 mM imidazole. MBP-His₆-Ub-Rpn10 was digested overnight with His₆-rhinovirus and His₆-tobacco etch virus (His₆-TEV) proteases to remove the MBP tag fused to Rpn10 and the His₆ tag fused to Ub. The affinity tags and proteases were removed using amylose and nickel columns. The buffer was exchanged to 50 mM Tris-HCl pH 7, 50 mM NaCl using Centricon (Amicon Ultra) filter devices to remove the imidazole before the nickel column. Residual remaining MBP tag was removed by ion-exchange chromatography (Q-Sepharose Fast Flow, GE Healthcare). The sample was diluted 1:10 to a final volume of 30 ml in 10% glycerol, 20 mM Tris-HCl pH 8. The column was pre-equilibrated with 25 mM NaCl, 20 mM Tris-HCl pH 8. The column was washed and an NaCl gradient (25–500 mM) was applied. Two peaks

were observed in the the UV_{280nm} spectrum. The first peak (at approximately 180 mM NaCl) corresponded to the MBP tag and the second peak (at approximately 240 mM NaCl) corresponded to Ub-Rpn10. Fractions from the second peak were collected (Fig. 1). Finally, DTT was added to a final concentration of 5 mM and the protein was concentrated to 12 mg ml⁻¹. 100 µl aliquots were flash-frozen in liquid nitrogen and kept at 193 K.

2.3. Crystallization of Ub-Rpn10

Crystallization experiments were carried out at 292 K using 96-well sitting-drop plates with commercially available crystallization kits (Qiagen and Hampton Research). Each well contained 70 µl reservoir volume and a 2 µl drop consisting of 1 µl protein sample in 25 mM Tris-HCl pH 8, 250 mM NaCl, 5 mM DTT mixed with 1 µl reservoir solution. After three weeks, crystals appeared using 12%(w/v) PEG 20 000, 0.1 M MES pH 6.5 (condition No. 96 of The Classics Suite from Qiagen).

2.4. Screening for cryoprotection conditions

Several cryoprotectant materials were screened in order to identify one that would prevent icing but that would not interfere with X-ray diffraction. Compared with Paratone, glycerol and PEG 400, we found that adding ~25% ethylene glycol to the crystallization solution generally yielded excellent cryoprotection.

2.5. Data collection

Following a short soak in cryoprotection solution, the crystals were cooled by immersion in liquid nitrogen. Diffraction was measured on the ID14-4 beamline at the European Synchrotron Radiation Facility (ESRF), Grenoble. Probably owing to the extreme thinness of the crystals (~1–3 µm), they were found to be highly sensitive to radiation damage. Nevertheless, the *BEST* software (Bourenkov & Popov, 2010) precisely predicted a data-collection strategy to achieve data sets with full completeness. Specifically, *BEST* suggested the collection of 95 images with an oscillation of 1.75°. In attempts to increase the redundancy, more images were collected. However, we found that after collecting three to five additional images the diffraction quality dramatically decreased exactly as predicted. Therefore, the algorithm was key to defining the data-collection strategy. Images were indexed and integrated using *MOSFLM* (Leslie, 1992) and were scaled using *SCALA* (Evans, 2006) from *CCP4* (Winn *et al.*, 2011).

3. Results and discussion

We have recently demonstrated the dependence of Rpn10 ubiquitylation on the concerted action of E1, E2 and E3 enzymes in a bacterial system that reconstitutes the entire ubiquitylation cascade (Keren-Kaplan *et al.*, 2011). Specifically, we co-expressed His₆-Ub, Uba1, Ubc4 and Rsp5 together with MBP-Rpn10. The mono-ubiquitylated Rpn10 that accumulates in the bacteria was purified using standard affinity-chromatography techniques as described in §2 (Fig. 1).

The purified protein was subjected to crystallization trials using the sitting-drop vapour-diffusion method. Very thin (~1–3 µm) rhombus-shaped crystals grew to ~220 µm in their longest dimension (Fig. 2*a*). Diffraction data were collected from the tip of a single crystal (Fig. 2*a*) at the ESRF (Fig. 2*b*). We obtained a complete data set with $d_{\min} = 3.14$ Å. Images were indexed, merged and scaled using *MOSFLM* and *SCALA*, respectively (Table 1). The diffraction pattern suggested a monoclinic C121 space group. Matthews

Figure 2
Crystal and diffraction of ubiquitylated Rpn10. (*a*) A typical rhombus-shaped thin crystal of ubiquitylated Rpn10. A black circle marks the site at which diffraction data were obtained. (*b*) A diffraction pattern obtained from a single ubiquitylated Rpn10 crystal on the ID14-4 beamline at ESRF, Grenoble.

Table 1

Data-collection statistics.

Values in parentheses are for the last resolution shell.

Wavelength (Å)	0.979
Space group	C2
Unit-cell parameters (Å, °)	$a = 107.3$, $b = 49.7$, $c = 81.3$, $\alpha = \gamma = 90$, $\beta = 130.5$
Resolution range (Å)	61.8–3.14 (3.31–3.14)
Observed reflections	18898 (2875)
Unique reflections	5824 (857)
Multiplicity	3.2 (3.4)
Completeness (%)	99.8 (99.9)
$\langle I/\sigma(I) \rangle$	9.8 (5.1)
$R_{\text{merge}}^{\dagger}$ (%)	8.4 (22.5)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of reflection hkl .

coefficient calculations yielded a V_M value of $2.11 \text{ \AA}^3 \text{ Da}^{-1}$ considering a single molecule in the asymmetric unit.

The crystal structure of the VWA domain of Rpn10 from *S. pombe* has been determined at 1.3 Å resolution (PDB entry 2x5n; Riedinger *et al.*, 2010). The structure of Ub at 1.8 Å resolution (PDB entry 1ubq; Vijay-Kumar *et al.*, 1987) is also known. Given the 52% amino-acid sequence identity between the VWA domains of Rpn10 from *S. cerevisiae* and *S. pombe*, a molecular-replacement (MR) search is predicted to find the Ub and VWA folds and will provide initial calculated phases. Together, these two folds comprise about 77% of the total Ub-Rpn10 structure, consequently providing sufficient phasing power to determine the entire structure.

To the best of our knowledge, this is the first report of the crystallization of a ubiquitylated substrate other than Ub itself, *i.e.* poly-Ub. It is noteworthy that poly-Ub, as well as Ub conjugates with ubiquitylating enzymes, can be produced *in vitro* and structures of such conjugates have been reported (Varadan *et al.*, 2004; Phillips *et al.*, 2001; Huang *et al.*, 2005; Eddins *et al.*, 2006). However, no crystals of a ubiquitylated substrate (downstream of E3 in the ubiquitylation cascade) have been reported. The recently reported system for the purification of naturally ubiquitylated substrates in amounts sufficient for crystallographic studies enabled us to crystallize an ubiquitylated substrate for the first time. Consequently, these crystals provide a breakthrough in the field of Ub structural biology. Moreover, the structures derived from these crystals should significantly contribute to our understanding of the regulation mechanisms of Ub receptors by auto-ubiquitylation.

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