Crystallization of mutants of *Turnip yellow mosaic virus* protease/ubiquitin hydrolase designed to prevent protease self-recognition

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Processing of the polyprotein of *Turnip yellow mosaic virus* is mediated by the protease PRO. PRO cleaves at two places, one of which is at the C-terminus of the PRO domain of another polyprotein molecule. In addition to this processing activity, PRO possesses an ubiquitin hydrolase (DUB) activity. The crystal structure of PRO has previously been reported in its polyprotein-processing mode with the C-terminus of one PRO inserted into the catalytic site of the next PRO, generating PRO polymers in the crystal packing of the trigonal space group. Here, two mutants designed to disrupt specific PRO–PRO interactions were generated, produced and purified. Crystalline plates were obtained by seeding and cross-seeding from initial ‘sea urchin’-like microcrystals of one mutant. The plates diffracted to beyond 2 Å resolution at a synchrotron source and complete data sets were collected for the two mutants. Data processing and analysis indicated that both mutant crystals belonged to the same monoclinic space group, with two molecules of PRO in the asymmetric unit.

1. Introduction

Turnip yellow mosaic virus (TYMV) encodes a replication polyprotein of 206 kDa (206K) harbouring an RNA polymerase (POL) and an RNA helicase (HEL). TYMV replication depends on POL and HEL being successively cleaved from the C-terminus of 206K in an orchestrated sequence of events (Jakubiec *et al.*, 2007). Proteolysis is mediated by a 17 kDa protease domain PRO encoded within 206K just upstream of HEL (Fig. 1*a*). Thus, cleavage at the PRO–HEL junction entails specific recognition of one PRO by another PRO and the engagement of the C-terminus of the first PRO by the catalytic site of the other (*trans* cleavage; Fig. 1*a*). Despite its small size, PRO is a multifunctional protein that also displays ubiquitin hydrolase (DUB) activity *in vivo* and *in vitro* (Chenon *et al.*, 2012), a feature of PRO that allows regulation of the amount of POL in plant cells infected by TYMV (Camborde *et al.*, 2010). Thus, PRO can specifically recognize several distinct substrates: both PRO–HEL and HEL–POL endopeptide cleavage sites, as well as POL–ubiquitin isopeptide bonds. We previously shed light on the self-recognition of PRO thanks to the atomic structure of PRO in a crystal form in which the C-terminus of one molecule inserts into the catalytic cleft of the next molecule (Lombardi *et al.*, 2013). However, we could not obtain diffraction-quality crystals of disengaged PRO (Robin *et al.*, 2012), *i.e.* PRO not engaged in this self-interaction, which would have allowed assessment of the conformational adjustments upon PRO self-recognition. Here, we report the

Table 1
Macromolecule-production information.

Source organism	<i>Turnip yellow mosaic virus</i>
Expression vector	Modified pGEX with an N-terminal His ₆ tag instead of a GST tag
Expression host	<i>E. coli</i> strain BL21(DE3)
Complete amino-acid sequence of the PRO I847A construct	MHHHHHGSSQLLPAPLTNDPTAIGPVLPEELH- PRYPENTATFLTRLRLSLPSNHLQPQTLNCLL- SAVSDQTKVSEEHLESWESLQTLPLDSQLSNEET- NTLGLSTEHLTALAHLYNFQATVYSDRGPALF- GPSDTIKRIDITHTTGPPSHFSPGKRLGSG
Complete amino-acid sequence of the PRO ΔC5 construct	MHHHHHGSSQLLPAPLTNDPTAIGPVLPEELH- PRYPENTATFLTRLRLSLPSNHLQPQTLNCLL- SAVSDQTKVSEEHLESWESLQTLPLDSQLSNEET- NTLGLSTEHLTALAHLYNFQATVYSDRGPILF- GPSDTIKRIDITHTTGPPSHFSPGK

production and crystallization of mutants of PRO designed to impair self-recognition (Fig. 1*b*). The first mutant (PRO I847A) alters a hydrophobic patch prominently used by PRO to recognize its substrates (Lombardi *et al.*, 2013). The second mutant is a five-residue C-terminal deletion (PRO ΔC5) that prevents engagement by the active site. PRO I847A should be severely impaired mostly in DUB activity, while PRO ΔC5 will not allow cleavage at the PRO–HEL junction. These mutants should thus be useful in disentangling the functional importance of the two distinct activities of PRO.

2. Methods and materials

2.1. Macromolecule production

PRO I847A was generated by QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, California, USA) according to the manufacturer’s instructions. The coding sequence of the PRO domain (residues 728–879 of 206K; Fig. 1*b*) previously inserted into a modified pGEX plasmid was used as a template to generate the mutant. In this modified vector the protein sequences harbour a six-histidine tag at their N-terminus (Table 1). PRO ΔC5 lacking the last five residues was generated similarly by ShineGene Biotech (Shanghai, People’s Republic of China). Expression conditions in *Escherichia coli* and purification were as previously reported (Robin *et al.*, 2012; Lombardi *et al.*, 2013). The purified proteins were then concentrated to 12 mg ml^{−1} for PRO I847A and 17 mg ml^{−1} for PRO ΔC5, frozen and stored at 193 K. The concentrations were estimated from the absorbance at 280 nm assuming extinction coefficients of 9919 and 9975 M^{−1} cm^{−1} calculated from the sequences of PRO I847A and PRO ΔC5, respectively.

2.2. Crystallization

Screening for crystallization conditions was performed by robotics using commercially available kits (The Classics and PEGs Suites from Qiagen; 192 conditions in all). Experiments were set up with a Cartesian robot using the sitting-drop vapour-diffusion method. Equal volumes (100 nl) of protein solution and well solution were mixed. Fine screening around the crystallization conditions of PRO I847A was performed in larger drop volumes (1 μl protein solution and 1 μl crys-

tallization reagent equilibrated against a 0.5 ml reservoir volume) using the hanging-drop vapour-diffusion setup. We obtained ‘sea urchin’-like microcrystals in a few days. In order to slow down crystal growth from the initial nuclei, we used the streak-seeding technique as described previously (Stura & Wilson, 1991). We also cross-seeded using the same technique from the PRO I847A microcrystals into hanging drops pre-equilibrated with PRO ΔC5 (Table 2).

2.3. Data collection and processing

Crystals of PRO I847A and PRO ΔC5 were transferred into cryoprotected solutions following the protocols and using the cryosolutions of Vera & Stura (2014). After a 1 min soak, crystals were flash-cooled by plunging into liquid nitrogen.

Figure 1
(a) TYMV 206K polyprotein processing. Blue arrows indicate the two successive *cis* and *trans* proteolysis events. Note that in the second, *trans* proteolysis, the C-terminus of one PRO is engaged and cleaved by the catalytic site of another PRO (the star locates the catalytic cysteine). (b) PRO constructs crystallized previously (wild type; Robin *et al.*, 2012) and in this report (PRO I847A and PRO ΔC5). Residue numbers refer to 206K numbering. ‘His’ denotes the N-terminal six-histidine tag. (c) Purification of PRO mutants. The SDS–PAGE analysis of successive fractions from the Superdex 75 size-exclusion chromatography of PRO ΔC5 is displayed (17.1 kDa). The 10.3 kDa *E. coli* ribosomal protein S15 is also indicated. Fractions 2–4 were pooled for crystallization. Lane MW contains molecular-weight markers (labelled in kDa).

Table 2
Crystallization.

	PRO I847A	PRO Δ C5
Method	Hanging drop with streak-seeding	Hanging drop with cross-streak-seeding
Temperature (K)	293	293
Protein concentration (mg ml ⁻¹)	12	17
Buffer composition of protein solution	0.01 M Tris-HCl pH 8, 0.35 M ammonium acetate, 1 mM DTT	0.01 M Tris-HCl pH 8, 0.35 M ammonium acetate, 1 mM DTT
Composition of reservoir solution	0.1 M trisodium citrate pH 5.6, 0.2 M ammonium acetate, 16.3%(w/v) PEG 4000	0.1 M trisodium citrate pH 5.6, 0.2 M ammonium acetate, 12%(w/v) PEG 4000
Initial volume and ratio of drop	2 μ l, 1:1	2 μ l, 1:1
Volume of reservoir (ml)	0.5	0.5

The plate-shaped crystals diffracted to beyond 2 Å resolution on the PROXIMA2 microfocus beamline at the SOLEIL synchrotron. The programs *XDS* and *XSCALE* (Kabsch, 2010) were used for data processing.

3. Results and discussion

PRO mutants were produced and purified by nickel-affinity and size-exclusion chromatography essentially as for the wild-type PRO (Robin *et al.*, 2012). The similarity extended to a small but persistent contamination by the bacterial ribosomal protein S15 that co-purified with the mutants and, as for the wild type, was only partially removed by the gel-filtration polishing step (Fig. 1c).

Initial screening of crystallization conditions yielded polycrystalline ‘sea urchins’ within a few days for PRO I847A with a well solution consisting of 0.1 M sodium acetate pH 4.7, 0.2 M ammonium acetate, 30%(w/v) PEG 4000. We easily reproduced the sea urchin-like microcrystals manually (Fig. 2a), but could not obtain single crystals in a first round of

optimization. Seeding is often a good way to circumvent this difficulty by bypassing the nucleation step and thus preventing multiple or secondary nucleation. Indeed, streak-seeding proved to be successful at the first attempt and yielded small but single-looking plate-like crystals that became visible overnight and grew to maximum size within a week (Fig. 2b, Table 2). We therefore tried cross-seeding from PRO I847A crystals, and in this way also obtained plate-shaped crystals of PRO Δ C5 using essentially the same crystallization conditions (Table 2) and in the same time frame (Fig. 2c).

The plate-shaped crystals were cryoprotected using cryo-solutions consisting of the crystal-growth solution supplemented with mixtures of cryoprotectants (Vera & Stura, 2014). Specifically, cryomixes C5 (final concentrations 5% diethylene glycol, 10% ethylene glycol, 5% MPD, 5% 1,2-propanediol, 5% glycerol, 5 mM NDSB-201) and C6 (5% ethylene glycol, 10% MPD, 5% 1,2-propanediol, 5% DMSO, 5% glycerol) proved especially effective at cryoprotecting the PRO I847A and PRO Δ C5 crystals. The plate-shaped crystals were tested for diffraction, and diffraction data were collected on the

Figure 2

Crystals of PRO I847A and PRO Δ C5. (a) Sea urchin-like microcrystals initially obtained for PRO I847A. (b) Final PRO I847 crystals obtained through streak-seeding. (c) Final PRO Δ C5 crystals obtained by cross-streak-seeding from the sea urchin-like microcrystals of PRO I847A. For both mutants, typical crystal sizes after seeding were 100 $\times$ 40–60 $\times$ 10–15 μ m.

Table 3
Data collection and processing.

	PRO I847A	PRO ΔC5
Diffraction source	PROXIMA2, SOLEIL	PROXIMA2, SOLEIL
Wavelength (Å)	0.9763	0.9763
Temperature (K)	100	100
Detector	ADSC CCD Q315	ADSC CCD Q315
Crystal-to-detector distance (mm)	238.4	237.6
Rotation range per image (°)	0.5	0.5
Total rotation range (°)	180	180
Exposure time per image (s)	0.5	0.5
Space group	C2	C2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	132.71, 39.92, 73.07	134.07, 39.63, 72.86
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90, 121.85, 90	90, 122.30, 90
Mosaicity (°)	0.213	0.313
Resolution range (Å)	37.63–1.89 (2.01–1.89)	36.43–1.99 (2.11–1.99)
Total No. of reflections	84255	80161
No. of unique reflections	25940	21954
Completeness (%)	98.5 (95.0)	97.0 (88.3)
Average multiplicity	3.25 (3.20)	3.65 (3.62)
$\langle I/\sigma(I) \rangle$	10.08 (2.37)	10.48 (2.61)
R_{merge}	0.110 (0.700)	0.095 (0.602)
$R_{\text{r.i.m.}}^{\dagger}$	0.132 (0.844)	0.111 (0.708)
Overall <i>B</i> factor from Wilson plot (Å ²)	29.32	34.29

[†] Estimated $R_{\text{r.i.m.}} = R_{\text{merge}}[N/(N-1)]^{1/2}$, where *N* is the data multiplicity.

Figure 3
First diffraction image of the PRO I847A data set described in Table 3.

PROXIMA2 microfocus beamline of the SOLEIL synchrotron. Complete data sets were collected to 1.9 Å resolution

and processed from a single plate-shaped crystal each of PRO I847A (Fig. 3) and PRO ΔC5 (Table 3).

The crystal plates obtained for both mutants belong to the same monoclinic space group. The space group and unit cell indicate the presence of two molecules per asymmetric unit with a solvent content of 45% and a Matthews coefficient of 2.3 Å³ Da⁻¹. Indeed, preliminary molecular replacement using *Phaser* (McCoy *et al.*, 2007) with the wild-type PRO structure (chain A of PDB entry 4a5u) trimmed of its last five residues unambiguously confirmed this. S15 is not present in the crystals as it was for the wild type (Robin *et al.*, 2012). Crystal packing shows that neither PRO molecule in the asymmetric unit has its C-terminus oriented towards the catalytic cleft of another molecule (not shown).

In our previous work, a crystal form with a single molecule of PRO in the asymmetric unit yielded insights into self-recognition by PRO (Lombardi *et al.*, 2013). These new crystal structures will provide views of PRO in environments that are not constrained by self-recognition. In turn, these may provide further hints as to how PRO switches between its protease and ubiquitin hydrolase substrates.

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