A crystallization and preliminary X-ray diffraction study of the Arabidopsis thaliana proliferating cell nuclear antigen (PCNA2) alone and in a complex with a PIP-box peptide from Flap endonuclease 1*

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DNA replication is an important event for all living organisms and the mechanism is essentially conserved from archaebacteria to euarkyotes. Proliferating cell nuclear antigen (PCNA) acts as the universal platform for many DNA transacting proteins. Flap endonuclease 1 (FEN1) is one such enzyme whose activity is largely affected by the interaction with PCNA. To elucidate the key interactions between plant PCNA and FEN1 and possible structural change of PCNA caused by binding of FEN1 at the atomic level, crystallization and preliminary studies of X-ray diffraction of crystals of Arabidopsis thaliana PCNA2 (AtPCNA2) alone and in a complex with a peptide derived from AtFEN1, which contains a typical PCNA-interacting protein (PIP)-box motif, were performed. Both peptide-free and peptide-bound AtPCNA2s were crystallized using the same reservoir solution but in different crystal systems, indicating that the peptide affected the intermolecular interactions in the crystals. Crystals of AtPCNA2 belonged to the hexagonal space group P6₃, while those of the peptide-bound AtPCNA2 belonged to the rhombohedral space group H3, both of which could contain the functional homo-trimers.

Key words: Plant DNA replication, PCNA, FEN1, PIP-box
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INTRODUCTION

Genomic DNA replication requires over 50 kinds of protein which work in a highly coordinated manner by forming a large assembly called a replisome (Masai et al., 2010). The mechanism of DNA replication is conserved from archaebacteria to euarkyotes. PCNA is one of the most essential proteins involved in DNA metabolic processes such as replication, repair and recombination. PCNA recruits and regulates the DNA metabolizing proteins by tethering them onto DNA (Moldevan et al., 2007). PCNA possesses a ring structure formed by the three identical subunits suitable for encircling DNA. PCNA is topologically linked to DNA by the ATP-dependent action of clamp-loader called RFC (replication factor C) (Yao & O'Donnell, 2012). PCNA binds different proteins involved in DNA metabolism by recognizing the canonical motif called the PCNA-interacting protein (PIP)-box, which is usually located at the C-terminus of the partner protein (Warbrick, 1998). Although the conservation of the PIP-box motif sequence is moderate, the binding mode of PIP-box peptides to PCNA is similar, as shown by many crystallographic studies (Gulbis et al., 1996; Matsumiya et al., 2002; Bubeck et al., 2011). Recently, other PCNA-binding motifs have been identified and characterized by structural biology, which showed the unique binding mode of these non-canonical motifs to PCNA (Hishiki et al., 2009; Sebesta et al., 2017).

Previously, we studied the functional and structural aspects of Arabidopsis thaliana PCNA proteins. They are designated as AaPCNA1 (molecular mass Mₐ 30 508 Da) and AaPCNA2 (Mₐ 30 335 Da), which are in 96% identical to each other, and show significant homology to human and archaeal PCNAs (Strzalka et al., 2009). We determined the atomic structure of the two Arabidopsis PCNAs complexed with the canonical PIP-box peptide derived from human cyclin-dependent kinase inhibitor, p21/WAF-1, using X-ray crystallography. The structure of the plant PCNAs showed the basic conservation in comparison to human and archaeal PCNAs (Strzalka et al., 2009). We determined the atomic structure of the two Arabidopsis PCNAs complexed with the canonical PIP-box peptide derived from human cyclin-dependent kinase inhibitor, p21/WAF-1, using X-ray crystallography. The structure of the plant PCNAs showed the basic conservation in comparison to human and archaeal PCNAs (Strzalka et al., 2009). We determined the atomic structure of the two Arabidopsis PCNAs complexed with the canonical PIP-box peptide derived from human cyclin-dependent kinase inhibitor, p21/WAF-1, using X-ray crystallography. The structure of the plant PCNAs showed the basic conservation in comparison to human and archaeal PCNAs (Strzalka et al., 2009). We determined the atomic structure of the two Arabidopsis PCNAs complexed with the canonical PIP-box peptide derived from human cyclin-dependent kinase inhibitor, p21/WAF-1, using X-ray crystallography. The structure of the plant PCNAs showed the basic conservation in comparison to human and archaeal PCNAs (Strzalka et al., 2009). We determined the atomic structure of the two Arabidopsis PCNAs complexed with the canonical PIP-box peptide derived from human cyclin-dependent kinase inhibitor, p21/WAF-1, using X-ray crystallography. The structure of the plant PCNAs showed the basic conservation in comparison to human and archaeal PCNAs (Strzalka et al., 2009). We determined the atomic structure of the two Arabidopsis PCNAs complexed with the canonical PIP-box peptide derived from human cyclin-dependent kinase inhibitor, p21/WAF-1, using X-ray crystallography. The structure of the plant PCNAs showed the basic conservation in comparison to human and archaeal PCNAs (Strzalka et al., 2009). We determined the atomic structure of the two Arabidopsis PCNAs complexed with the canonical PIP-box peptide derived from human cyclin-dependent kinase inhibitor, p21/WAF-1, using X-ray crystallography. The structure of the plant PCNAs showed the basic conservation in comparison to human and archaeal PCNAs (Strzalka et al., 2009). We determined the atomic structure of the two Arabidopsis PCNAs complexed with the canonical PIP-box peptide derived from human cyclin-dependent kinase inhibitor, p21/WAF-1, using X-ray crystallography. The structure of the plant PCNAs showed the basic conservation in comparison to human and archaeal PCNAs (Strzalka et al., 2009). We determined the atomic structure of the two Arabidopsis PCNAs complexed with the canonical PIP-box peptide derived from human cyclin-dependent kinase inhibitor, p21/WAF-1, using X-ray crystallography. The structure of the plant PCNAs showed the basic conservation in comparison to human and archaeal PCNAs (Strzalka et al., 2009).
NA2, which allows revealing the possible structural change of AtPCNA2 caused by the binding of DNA metabolizing proteins such as AtFEN1.

**MATERIALS AND METHODS**

**Overexpression and Purification.** The *Escherichia coli* BL21 (DE3) Rosetta strain (Novagen) was used for AtPCNA2 protein production. Cells transformed with pET15b vector coding for AtPCNA2 (Strzalka et al., 2009) fused with histidine tag were grown at 310 K using LB medium supplemented with 30 mg l−1 chloramphenicol and 100 mg l−1 ampicillin. Protein overexpression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD₆₀₀ of the culture reached ~0.5. Bacteria were cultured by shaking vigorously at 310 K for 4 h then followed by centrifugation (10 min, 12000×g, 277 K). The pellet from 1 l of culture was resuspended in 40 ml of binding buffer A (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 7.5) which contained an EDTA-free protease inhibitor cocktail (Roche). Bacteria were sonicated for 15 min (5 s pulse followed by a 15 s pause, QSonica Q700 sonicator, USA). The cell lysate containing the AtPCNA2 protein was supplemented with 200 units of DNase I (Takara Bio, Japan) and incubated for 20 min on ice. Then, the lysate was centrifuged (20 min, 21000×g, 277 K) and the supernatant was loaded onto HisTrap FF Ni Sepharose (5 ml, GE Healthcare, Sweden). The protein was purified according to the protocols supplied by the manufacturers. After being eluted from the nickel resin, the AtPCNA2 was dialyzed against buffer B (50 mM Tris–HCl pH 7.6) and loaded onto the anion exchange HiTrap Q column (5 ml, GE Healthcare, Sweden). The protein elution was performed using a linear NaCl gradient from 0 to 1 M (10 volumes of the column). The purified AtPCNA2 was dialyzed against buffer C (50 mM Tris–HCl, 200 mM NaCl, 0.2 mM EDTA, pH 8.0), supplemented with glycerol (final concentration of 30%).

**Table 1. Data collection and processing**

| Protein complex | AtPCNA2 | AtPCNA2-Fen1 PIP complex |
|-----------------|---------|--------------------------|
| Wavelength (Å)  | 1.5418  | 1.5418                   |
| Temperature (K) | 100     | 100                      |
| Detector        | R-AXIS VII | R-AXIS VII              |
| Crystal-to-detector distance (mm) | 200 | 175                      |
| Rotation range per image (°) | 0.5 | 0.5                      |
| Exposure time per image (s) | 300 | 270                      |
| Space group     | P6₃     | H3                       |

| Unit cell constants | AtPCNA2 | AtPCNA2-Fen1 PIP complex |
|---------------------|---------|--------------------------|
| a, b, c (Å)         | 93.95, 93.95, 63.54 | 224.17, 224.17, 199.73 |
| a, β, γ (°)         | 90, 90, 120 | 90, 90, 120               |
| Mosaicity (°)       | 0.30     | 0.10                      |
| Resolution range (Å) | 50.0-2.65 (2.78-2.65) | 50.0-2.85 (2.90-2.85) |
| Total No. of observed reflections | 63 486 (8383) | 366 082 (18 559) |
| No. of unique reflections | 9385 (1228) | 87 329 (4497) |
| Completeness (%)    | 99.8 (99.5) | 99.9 (100.0)             |
| Multiplicity        | 6.8 (6.8) | 4.2 (4.1)                |
| I/σ(I)              | 22.2 (3.1) | 9.5 (2.2)                |
| Rmerge              | 0.068 (0.735) | 0.185 (0.863) |
| Rfree               | 0.057 (0.623) | 0.142 (0.658) |
| Half-set correlation CC₁/₂ | 0.999 (0.791) | 0.990 (0.654) |
| Overall B factor from Wilson plot (Å²) | 57.6 | 31.9 |

Values for the outer shell are given in parentheses.
Crystallization of a PIP-box peptide-free and -bound plant PCNA2

RESULTS AND DISCUSSION

Crystallization

We previously succeeded in crystallizing the human p21-derived peptide-bound plant PCNAs which were purified using HisTrap as the only column chromatography method (Strzalka et al., 2009). In this study, using two-step column chromatography we purified AIPCNA2 (Fig. 1). The genomic DNA from the E. coli cells was degraded into small fragments during sonication and DNase I treatment. Next, most of these fragments were removed at the first stage of the protein purification when the HisTrap column was used. The DNA remaining in the protein sample eluted from the HisTrap column was separated from AIPCNA2 using the HiTrap Q column (data not shown). Using a protein sample of higher purity than previously (Strzalka et al., 2009) we were able to crystallize AIPCNA2 free of the ligand as well as complexed with the PIP-box peptide derived from the p21FEN1. Interestingly, both the peptide-free and peptide-bound AIPCNA2 crystallized under the same reservoir conditions but in different crystal forms (Fig. 2), as revealed by the following crystallographic analysis. The peptide used for the present study affected the packing interactions of AIPCNA2 in the crystals.

X-ray crystallographic study

The AIPCNA2 crystals diffracted the X-rays from an in-house generator to a resolution of 2.65 Å, and the AIPCNA2-p21FEN1 PIP-box peptide complex to 2.85 Å (Fig. 3 and Table 1). The AIPCNA2 crystals alone belong to the hexagonal space group P6_3, with unit cell constants of a=b=93.95 Å, c=63.54 Å, and probably contain the one subunit in the asymmetric unit with a Matthews coefficient (V_M) of 2.68 Å³ Da⁻¹ and a solvent content of 54%. These crystallographic parameters are similar to those for homologous PCNAs (Matsumiya et al., 2001; Ladner et al., 2011) which suggests that in the AIPCNA2 crystals monomers form homotrimers with crystallographic symmetry-related molecules. On the other hand, the AIPCNA2-p21FEN1 PIP-box peptide complex crystals belong to the space group H3 with unit cell constants of a=b=224.17 Å, c=199.73 Å. The space group and unit cell constants of the crystal of this complex are similar to those of the previous AIPCNA2-human p21 peptide complex crystal which contains eight complex molecules in the asymmetric unit with two monomers that form the symmetry-related homotrimers and the two independent homotrimers. If the crystal of the AIPCNA2-p21FEN1 PIP-box peptide complex has a molecular arrangement in the crystal very similar to that...
of the peptide-bound PCNA2 complex, it leads to a V_M of 3.66 Å³Da⁻¹ and a solvent content of 66%. Despite the fact that both the peptide-free and peptide-bound AIPCPNA2 proteins were crystallized using the same reservoir solution, two different forms of crystals were obtained, one type for AIPCPNA2 and the other for peptide-bound AIPCPNA2. Determining the structure of these complexes at the atomic level would give us an answer whether the peptide-bound AIPCPNA2 complex displays unique and different inter-homotrimer interactions in the crystal, as compared to those in the ligand-free AIPCPNA2 crystal. This information would be significant in the context of a detailed understanding of the role of PCNA in FEN1-dependent DNA replication in plants. Although we solved both crystal structures by the molecular replacement method, the quality of the electron density maps was too poor to build accurate atomic models. Therefore, the preparation of the better quality crystals is in progress to obtain high-quality X-ray diffraction data at synchrotron radiation facility beamlines.

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