Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Preparation and crystallization of human flap endonuclease FEN-1 in complex with proliferating-cell nuclear antigen, PCNA

Shigeru Sakurai,[a] Ken Kitano,[a] Kengo Okada,[a] Keisuke Hamada,[a] Hiroshi Morioka[b] and Toshio Hakoshima[a]*

[a]Structural Biology Laboratory, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan, and [b]Department of Structural Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060-0812, Japan

Correspondence e-mail: hakosima@bs.aist-nara.ac.jp

Received 13 December 2002
Accepted 3 March 2003

Flap endonuclease-1 (FEN-1) is a structure-specific nuclease that removes 5′-overhanging flaps in DNA repair and replication. FEN-1 binds proliferating-cell nuclear antigen (PCNA), a DNA-clamp protein, when processing Okazaki fragments during lagging-strand DNA synthesis. Here, the crystallization of the complex between human FEN-1 and PCNA is reported. The crystals were found to belong to space group $P2_12_12_1$, with unit-cell parameters a = 82.2, b = 143.4, c = 246.7 Å, and contained one complex in the crystallographic asymmetric unit. A diffraction data set was collected to a resolution of 3.0 Å.

1. Introduction

DNA replication in eukaryotes is a highly coordinated process involving many proteins that work cooperatively to ensure the accurate and efficient replication of DNA (Waga & Stillman, 1998). During this process, flap endonuclease-1 (FEN-1) plays a crucial role in the removal of RNA primers during Okazaki fragment maturation in lagging-strand DNA synthesis (Goulian *et al.*, 1990; Harrington & Lieber, 1994*a*,*b*). FEN-1 belongs to the XPG-like family of structure-specific nucleases, which includes bacteriophage and bacterial 5′-nucleases (Lieber, 1997). Flap-DNA removal by FEN-1 is also essential during long-patch base-excision repair (Klungland & Lindahl, 1997). Thus, FEN-1 and its homologues are central to processes influencing genome stability and early events that modulate cancer susceptibility and tumorigenesis. Proliferating-cell nuclear antigen (PCNA), which is well known as the 'sliding clamp', binds eukaryotic DNA polymerase δ and plays a key role in processive DNA synthesis (Kelman, 1997). PCNA also binds FEN-1, leading to the positioning of FEN-1 onto the DNA and a 10–50-fold increase in FEN-1 nuclease activity (Cox, 1997; Lieber, 1997; Kelman, 1997). Mutations in FEN-1 that disrupt the PCNA interaction reduce the efficient cleavage of flap DNA at the replication fork, thus leading to the generation of long flap strands during DNA replication. These unexcised flap strands may form FEN-1-resistant secondary structures, as would be expected for short repeat DNA sequences. The inability of FEN-1 endonuclease activity to remove these sequences provides a specific molecular basis for the observed expansion of such short repeats in certain human cancers and heritable genetic diseases. Consequently, an understanding of the interactions between FEN-1 and PCNA are critical from biomedical perspectives.

Here, we report the first crystallization and preliminary crystallographic studies of human FEN-1 complexed with human PCNA. Human FEN-1 consists of 379 amino-acid residues (42 kDa) and is believed to exist as a monomer in solution (Kim *et al.*, 1999). Human PCNA, which consists of 261 amino-acid residues (29 kDa), is known to form a trimeric ring that contains a central hole used in its interaction with DNA (Gulbis *et al.*, 1996).

2. Materials and methods

2.1. Protein preparation

The FEN-1 expression plasmid pT7-hFEN-1 was transformed into *Escherichia coli* strain BL21(DE3). Cells were grown at 310 K in Luria–Bertani (LB) medium containing 100 µg ml^{-1} ampicillin. When the absorbance at 660 nm (OD_{660}) of the cell culture reached 0.8, isopropyl-β-D-thio-galactopyranoside (IPTG) was added to a concentration of 0.5 mM to induce expression of the gene. Cells were grown for an additional 5 h following IPTG induction and were then harvested by centrifugation at 6000 rev min^{-1} (Beckman J2-M1 JA10 rotor) for 20 min at 277 K. The expression plasmid for PCNA, pT7-PCNA, was also transformed into *E. coli* strain BL21(DE3). At an OD_{660} of 0.5, IPTG was added to a concentration of 1 mM. After an additional 12 h incubation, cells were then harvested in the same manner as described above.

Recombinant human FEN-1 was purified as follows. Firstly, the soluble portion of the cell

extract was loaded onto a DEAE anion-exchange column (Pharmacia) to remove nucleic acids. The flowthrough fraction was loaded onto a heparin column (Pharmacia) and eluted using a linear NaCl concentration gradient. Following gel filtration on a Sephacryl S-100 column (Pharmacia), protein fractions were applied to a hydroxylapatite column. Finally, collected fractions were loaded onto a MonoS cation-exchange column (Pharmacia) and eluted using a linear NaCl concentration gradient. 60 mg of purified FEN-1 was obtained from 3 l of culture.

Recombinant human PCNA was also purified by sequential column chromatography. The soluble portion of the cell extract was loaded onto a DEAE anion-exchange column and eluted using a linear NaCl concentration gradient. Protein fractions were further purified by Q-Sepharose (Pharmacia), Sephacryl S-200 (Pharmacia) and hydroxylapatite column chromatography. Finally, protein fractions were loaded onto a MonoQ anion-exchange column (Pharmacia) and eluted using a linear NaCl concentration gradient. 3 mg of purified PCNA was obtained from 3 l of culture.

The purified samples were verified using N-terminal sequence analysis (M492; Applied Biosystems) and matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI–TOF MS; PerSpective). Peaks of 42 440 Da (calculated MW 42 462 Da) and 28 738 Da (calculated MW 28 769 Da) were observed for FEN-1 and PCNA, respectively. Purified proteins were stored at 193 K.

2.2. Crystallization

Crystallization screening was carried out by the hanging-drop vapour-diffusion method (McPherson, 1990) using commercial crystallization screening solutions from Hampton Research, USA: Crystal Screen kits (1, 2 and Cryo) and Grid Screen kits (ammonium sulfate, 2-methyl-2,4-pentanediol, polyethylene glycol 6000, PEG/LiCl and sodium chloride). The protein-complex solution was mixed in a 1:1 ratio with the reservoir solution. One PCNA trimer has three identical FEN-1-binding sites. We attempted to crystallize 1:1, 2:1 and 3:1 (FEN-1:PCNA trimer) complexes, but failed to obtain crystals from the 1:1 and 2:1 complexes. The 3:1 complex (hereafter referred to as the FEN-1–PCNA complex) was prepared by mixing protein solutions containing 10 m*M* Na HEPES pH 7.8, 100 m*M* NaCl, 1 m*M* DTT and final concentrations of 0.36 m*M* FEN-1 and 0.12 m*M* PCNA. Complex formation was confirmed by two methods. Gel filtration using a Sephacryl S-300 column (Pharmacia) yielded a single peak corresponding to 230 kDa as estimated by protein size markers. This value is close to the calculated value (213 kDa) for the 3:1 complex. Furthermore, dynamic light-scattering measurements (DynaPro-801, Protein Solutions) suggested that the solution was monodisperse with a 220 kDa protein complex estimated from the hydrodynamic radius (60 Å).

Using the Grid Screen kit, initial crystals were obtained at pH 6 and 7 with 5% polyethyleneglycol 6000 (PEG 6K) as precipitant at 277 K. The conditions were then refined by changing the pH (5.0–8.0 in steps of 0.5) and the temperature (277, 283, 288 and 293 K) and using different PEGs (PEG 200, PEG 400, PEG 1K, PEG 2K, PEG 4K, PEG 6K, PEG 8K and PEG 20K). Relatively large plate-shaped crystals (100 × 100 × 10 µm) were found to grow at pH 6.5 using 12% PEG 1K at 283 K. However, these crystals were still small and clustered by stacking onto each other. To improve the conditions, we tried adding metal ions, organic compounds and detergents to the crystallization solutions using Additive Screen kits I–III and Detergent Screen kits I–III (Hampton Research, USA). We found that the crystal size and morphology could be improved by adding *n*-hexadecyl-*β*-D-maltoside or *n*-decyl-*β*-D-thiomaltoside. After optimization of the crystallization conditions, single crystals appeared in drops containing 12.8 mg ml^{-1} protein complex (0.06 m*M*), 50 m*M* Na MES pH 6.5, 50 m*M* NaCl, 6%(*w*/*v*) PEG 1000 and 0.6 µ*M* *n*-hexadecyl-*β*-D-maltoside at 283 K. Crystals grown under optimized conditions reached their maximum size within a week and had dimensions of 600 × 80 × 20 µm (Fig. 1). The crystals obtained were washed and dissolved in an aliquot of water in preparation for analysis by 12.5%(*w*/*v*) SDS–PAGE (Laemmli, 1970). SDS–PAGE gels stained with Coomassie Brilliant Blue confirmed that the crystals contained both FEN-1 and PCNA (Fig. 2). The crystals diffracted to 2.8 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 82.2, b = 143.4, c = 246.7 Å.

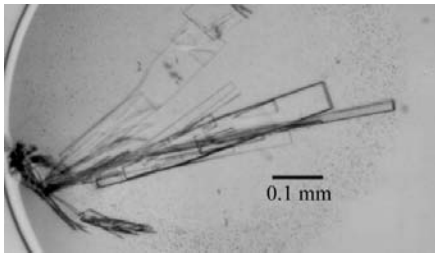

Figure 1
Orthorhombic crystals of the FEN-1–PCNA complex in 6%(*w*/*v*) PEG 1000, 50 m*M* Na MES pH 6.5, 50 m*M* NaCl and 0.6 µ*M* *n*-hexadecyl-*β*-D-maltoside.

Table 1
X-ray diffraction data of the FEN-1–PCNA complex.

Beamline	SPring-8/BL38B1
Detector	ADSC Quantum Q4
Wavelength (Å)	1.00
Temperature (K)	100
Oscillation range (°)	180 (1° × 180 images)
Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	82.2 (3)
b (Å)	143.4 (10)
c (Å)	246.7 (15)
Resolution† (Å)	50–3.0 (3.1–3.0)
Reflections, total/unique [$I/\sigma(I) > 1.0$]	228402/52176
Completeness† (%)	87.9 (64.5)
Mosaicity	0.3–0.5
$\langle I/\sigma(I)\rangle$†	13.7 (3.3)
$R_{\rm merge}$†‡ (%)	7.9 (26.5)

† Values for the outer resolution shell are given in parentheses. ‡ $R_{\rm merge} = \sum|I_i - \langle I_i\rangle|/\sum\langle I_i\rangle$, where I_i is the observed intensity and $\langle I_i\rangle$ is the average intensity over symmetry-equivalent measurements.

2.3. Data collection and processing

For X-ray data collection (Table 1), crystals were transferred into a cryoprotective solution containing 20%(*v*/*v*) PEG 200 and reservoir solution [100 m*M* Na MES pH 6.5, 100 m*M* NaCl and 12%(*w*/*v*) PEG 1000] and then mounted on a rayon loop for flash-cooling to 100 K using a Rigaku cryostat with liquid-nitrogen vapour. A set of X-ray

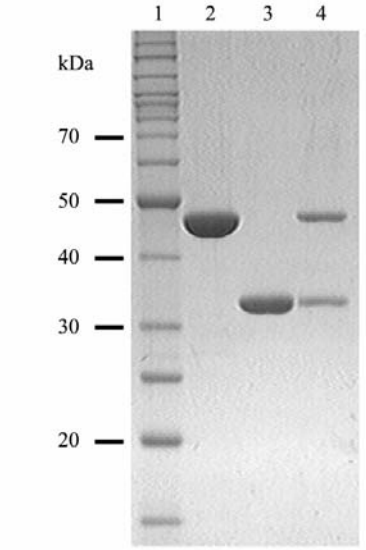

Figure 2
SDS–PAGE of the purified proteins and the complex crystals. A stained 12.5% polyacrylamide gel is shown with molecular-weight markers (lane 1), purified human FEN-1 (lane 2), purified human PCNA (lane 3) and the FEN-1–PCNA crystal complex (lane 4).

diffraction data for the FEN-1–PCNA complex crystal was collected at SPring-8 on beamline BL38B1 using an ADSC Quantum detector system with a total oscillation range of 180° and a step size of 1.0° with a frame exposure time of 40 s. The wavelength was set to 1.00 Å. Diffraction data were indexed, scaled and merged using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The total number of observed reflections was 228 402, which gave 52 176 unique reflections [$I/\sigma(I) > 1$]. The resulting data gave an $R_{\rm merge}$ of 7.9% (26.5% for the outer shell, 3.1–3.0 Å) with a completeness of 87.9% (64.5% for the outer shell). The averaged $I/\sigma(I)$ value was 13.7 (3.3 for the outer shell). The estimated mosaicity of the crystal was in the range 0.3–0.5°. A Matthews coefficient (Matthews, 1968) of 3.4 Å^3 Da^{-1} was calculated assuming one PCNA trimer and three FEN-1 monomers in the asymmetric unit, which corresponds to 64% solvent content by volume.

The PCNA trimer has pseudo-threefold symmetry perpendicular to the trimer ring plane (Krishna *et al.*, 1994; Gulbis *et al.*, 1996; Matsumiya *et al.*, 2001). We calculated self-rotation functions (Rossmann & Blow, 1962) to search for non-crystallographic symmetry with several Patterson sphere radii. However, we failed to find significant peaks indicating threefold symmetry on the maps. It might be that the positions of the three PCNA subunits and/or the three FEN-1 molecules bound to them deviate strongly from the ideal equivalent positions of threefold symmetry.

Human FEN-1 exhibits a relatively high sequence identity to hyperthermophilic archaeon FEN-1s: 38% identity to that from *Pyrococcus furiosus* and 34% to that from *Methanococcus jannaschii*. It may be possible to build a starting model of human FEN-1 by homology modelling based on the crystal structures of these archaeal FEN-1s (PDB codes 1b43 and 1a76; Hosfield *et al.*, 1998; Hwang *et al.*, 1998). Moreover, the structure of human PCNA (PDB code 1axc; Gulbis *et al.*, 1996) is also amenable to molecular replacement, although the PCNA structure in our complex may be significantly different to that in an uncomplexed state. Structural analyses by molecular-replacement and/or MAD methods are in progress.

We are grateful to Ms J. Tsukamoto for her assistance with the mass spectroscopy. This work was supported by a Grant-in-Aid for Scientific Research on Priority Area (B) Metal Sensor and a Protein 3000 project on Signal Transduction to TH, and by a Grant-in-Aid for the Encouragement of Young Scientists to KO from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The work was also supported by the CREST project from Japan Science and Technology Corporation to TH.

References

Cox, L. S. (1997). *Trends Cell Biol.* **7**, 493–497.
Goulian, M., Richards, S. H., Heard, C. J. & Bigsby, B. M. (1990). *J. Biol. Chem.* **265**, 18461–18471.
Gulbis, J. M., Kelman, Z., Hurwitz, J., O'Donnell, M. & Kuriyan, J. (1996). *Cell*, **87**, 297–306.
Harrington, J. J. & Lieber, M. R. (1994*a*). *Genes Dev.* **8**, 1344–1355.
Harrington, J. J. & Lieber, M. R. (1994*b*). *EMBO J.* **13**, 1235–1246.
Hosfield, D. J., Mol, C. D., Shen, B. & Tainer, J. A. (1998). *Cell*, **95**, 135–146.
Hwang, K. Y., Beak, K., Kim, H. Y. & Cho, Y. (1998). *Nature Struct. Biol.* **5**, 707–713.
Kelman, Z. (1997). *Oncogene*, **14**, 629–640.
Kim, C. Y., Shen, B., Park, M. S. & Olah, G. A. (1999). *J. Biol. Chem.* **274**, 1233–1239.
Klungland, A. & Lindahl, T. (1997). *EMBO J.* **16**, 3341–3348.
Krishna, T. S. R., Kong, X. P., Gary, S., Burgers, P. M. & Kuriyan, J. (1994). *Cell*, **79**, 1233–1243.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Lieber, M. R. (1997). *Bioessays*, **19**, 233–240.
McPherson, A. (1990). *Eur. J. Biochem.* **189**, 1–23.
Matsumiya, S., Ishino, Y. & Morikawa, K. (2001). *Protein Sci.* **10**, 17–23.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rossmann, M. G. & Blow, D. M. (1962). *Acta Cryst.* **15**, 24–31.
Waga, S. & Stillman, B. (1998). *Annu. Rev. Biochem.* **67**, 721–751.