

Doctoral Dissertation

**Structural basis for recruitment of flap
endonuclease-1 to PCNA**

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フラップエンドヌクレアーゼ1とPCNAの相互作用 の構造的基礎*

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内容梗概

フラップヌクレアーゼ 1 (flap endonuclease-1; FEN1) は、DNA合成の過程でつくられる RNA-DNAプライマーや、DNAの修復の過程で生じる flap DNAの切除に決定的な役割を演じている。DNAクランプとして知られている増殖細胞核抗原(proliferating cell nuclear antigen; PCNA) はFEN1 と結合して、その酵素活性を大幅に促進する。本研究では、ヒト FEN1 とPCNAとの複合体の結晶構造を決定して、FEN1-PCNA相互作用の構造的基礎を明らかにした。FEN1 のC末端領域は、2本の β ストランドが1本の短いヘリックスで繋がった β A- α A- β Bモチーフを形成して、PCNAとの β シート形成や疎水的相互作用に関与していた。この相互作用は以前に構造解析されたp21^{CIP1/WAF1}とよく似ているが、FEN1 全長を含む今回の構造は、FEN1 のコアドメインを介したPCNAとの付加的な相互作用も明らかにした。分子境界でのこの相互作用は、FEN1 を不活性な状態の配向に維持するとともに、PCNAの中央の穴を保持することによって、DNA上を高速に移動するのに利用されていると考えられる。FEN1 のコアドメインとC末端との間に存在していたヒンジ領域は、FEN1 や他のPCNA結合タンパク質の活性の切替えに重要であろう。

キーワード

フラップエンドヌクレアーゼ, DNAクランプ, 修復, 複製, エックス線

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Structural basis for recruitment of flap endonuclease-1 to PCNA[†]

Shigeru Sakurai

Abstract

Flap endonuclease-1 (FEN1) plays crucial roles in the removal of RNA-DNA primers during DNA synthesis and in the removal of flap DNA during DNA repair. Proliferating cell nuclear antigen (PCNA), the DNA-clamp protein, binds FEN1 and stimulates its endonuclease activity. The structural basis of the FEN1-PCNA interaction was revealed by analysis of the crystal structure of the complex between human FEN1 and PCNA. The main interface involves the C-terminal tail of FEN1, which forms two β -strands connected by a short helix, the β A- α A- β B motif, participating in β - β and hydrophobic interactions with PCNA. These interactions are similar to those previously observed for the p21^{CIP1/WAF1} peptide. However, this structure involving the full-length enzyme has revealed additional interfaces that are involved in the core domain. The interactions at the interfaces maintain the enzyme in an inactive orientation and might be utilized in rapid DNA-tracking by preserving the central hole of PCNA for the purposes of sliding along the DNA. A hinge region present between the core domain and the tail may switch FEN1 and other enzymes bound to the same PCNA ring.

Key words

Flap endonuclease, DNA clamp, repair, replication, X-ray

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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 and Stillman, 1998). In this process, flap endonuclease-1 (FEN1) plays a crucial role in the removal of RNA-DNA primers during Okazaki fragment maturation in lagging strand DNA synthesis (Liu *et al.*, 2004) (Figure 1-1). FEN1 belongs to the XPG-like family of structure-specific nucleases that includes bacteriophage and bacterial 5'-nucleases (Figure 1-3 and Table 1). Flap DNA removal by FEN1 is also essential during long-patch base excision repair (Klungland and Lindahl, 1997) (Figure 1-2A). Moreover, FEN1 has been shown to participate in physical and functional interactions with the Werner Syndrome protein WRN, and prevents aberrant homologous recombination (Brosh *et al.*, 2002; Sharma *et al.*, 2004) (Figure 1-2B). Thus, FEN1 appears to be a key player in maintaining genomic stability by participating in the DNA replication and repair processes and DNA recombination, which are the early events that modulate cancer susceptibility and tumorigenesis (Henneke *et al.*, 2003). Indeed, FEN1 null mutants appear several impaired phenotype in eukaryotic. In yeast, FEN1 mutants display severely impaired phenotypes such as UV sensitivity, deficient chromosome segregation, conditional lethality and accumulation in S phase. Haplo-insufficiency of FEN1 leads to rapid tumor progression in mice (Henneke *et al.*, 2003; Kucherlapati *et al.*, 2002) and hypersensitive to methylating agents and H₂O₂ in chicken cell (Matsuzaki *et al.*, 2002). Furthermore, eukaryotic FEN1 could directly interact with several proteins, which probably cooperates each other (Table 1-2) (Liu *et al.*, 2004). Despite its pronounced importance in biology and medicine, no three-dimensional structure of eukaryotic FEN1 has been elucidated.

In vitro experiments have shown that FEN1 specifically binds flap DNAs and subsequently exerts its nuclease activity against the substrate DNAs. This activity is markedly

stimulated by proliferating cell nuclear antigen (PCNA), which is well known as the ‘DNA sliding-clamp’ (Kelman, 1997; Tsurimoto, 1998). This stimulation is induced by direct binding of FEN1 to PCNA, leading to a 10-50 fold increase in its nuclease activity (Li *et al.*, 1995; Tom *et al.*, 2000; Wu *et al.*, 1996). The interaction between FEN1 and PCNA is an essential prerequisite that provides for the subsequent FEN1 functionality found in cells. Mutations in FEN1 that disrupt the interaction with PCNA decrease the cleavage efficiency of flap DNA at the replication fork (Stucki *et al.*, 2001), thus leading to the generation of unfavorably long flap DNA strands (Gary *et al.*, 1999). PCNA forms a trimeric ring with 3-fold symmetry perpendicular to the ring plane, and possesses a central hole that can be used to clamp DNA (Krishna *et al.*, 1994). Importantly, PCNA binds several enzymes that function in DNA metabolic processes ranging from DNA methylation, base excision repair, nucleotide excision repair, mismatch repair, DNA replication and translesion DNA synthesis (Maga and Hubscher, 2003) (Table 1-3). It appears that PCNA acts as a platform for these enzymes, including FEN1, and facilitates the efficient functioning of these proteins, perhaps in the form of a multi-protein complex *in vivo*. To date, crystal structures of complexes between PCNA and peptides from several PCNA-interacting proteins have been reported since the first structure of human PCNA bound to a peptide from the cyclin-dependent protein kinase inhibitor p21^{CIP1/WAF1} (hereafter referred to as p21) had been determined (Gulbis *et al.*, 1996). However, the precise molecular mechanism by which these entire proteins cooperate with PCNA on the DNA remains unknown. Studying the entire complex adds to previous knowledge of peptide-based interactions and the few cases of domains/proteins bound to clamps such as the little finger (LF) domain of the Y-family DNA polymerase Pol IV bound to the *Escherichia coli* β -clamp processivity factor (Bunting *et al.*, 2003).

PCNA-binding proteins including p21 possess a short PCNA-binding motif, QXX(I/L/M)XXF(F/Y), located either at the N- or C-terminal region (reviewed in

(Matsumoto, 2001; Tsurimoto, 1999; Warbrick, 1998)), while the crystal structure of human PCNA complexed with the C-terminal peptide of p21 (the PCNA-p21 complex) revealed the presence of extensive interactions with over 22 residues of p21 containing the motif (Gulbis *et al.*, 1996). Interestingly, human FEN1 possesses a C-terminal tail consisting of ~45 residues containing the short PCNA-binding motif. This long C-terminal tail represents one of the features common to eukaryotic FEN1s. Flanking residues of the motif may be utilized in interactions with DNA (Stucki *et al.*, 2001).

We now report on the crystal structure of human FEN1 complexed with PCNA. This is the first structure of eukaryotic FEN1 and the structure of the entire complex providing the first evidence pertaining to the presence of protein-protein interactions within the FEN1-PCNA complex. The structure revealed three FEN1 molecules bound to one PCNA trimer, which preserves the central hole that is utilized for the interaction with DNA. The main interaction between FEN1 and PCNA involves the N-terminal half of the C-terminal tail of each FEN1 molecule, forming the β A- α A- β B motif anchored to one PCNA subunit. The second interaction involves the enzyme core domain of FEN1 and preserves the central hole of PCNA for the purposes of DNA-tracking. FEN1 possesses a short linker region containing small residues between the core domain and the C-terminal tail. This linker region acts as a hinge, which endows FEN1 with a degree of freedom to swing the core domain, and may play a role in switching the enzyme activity.

Table 1-1. The structure-specific endonucleases of the XP-G family and the functional related enzymes.

Protein	Organism	Function
FEN1	Eukaryote,	Cleavage RNA-DNA primer
XP-G	Eukaryote	DNA repair (NER)
The 5'-3'-exonuclease fragment of DNA polymerase I	prokaryote	Cleavage RNA-DNA primer
5'-exonuclease	Virus	Cleavage RNA-DNA primer
RNase H	Virus	Cleavage RNA primer

XP-G, xeroderma pigmentosum-G; NER; nucleotide excision repair

Table 1-2. Interaction partners of eukaryotic FEN1

Protein	Function
Dna2	DNA replication
HIV-1 integrase ^d	DNA repair
RP-A	DNA replication
PCNA	DNA replication, DNA repair (BER)
AP endonuclease 1	DNA repair (BER)
DNA polymerase β	DNA repair (BER)
Werner syndrome protein	DNA replication, DNA repair, DNA
Bloom syndrome protein	DNA replication, DNA repair, DNA
p300	DNA repair/ DNA replication?
Cdk1, Cdk2	DNA replication (end of S phase)
Cyclin A	Reduced FEN1 nuclease activity

HIV-1, human immunodeficiency virus-1; RP-A, replication protein A; PCNA, proliferating cell nuclear antigen; AP, apurinic/apyrimidinic; BER, base excision repair

Table 1-3. PCNA-interacting proteins and their functions.

Protein	Function
RFC	DNA replication
DNA polymerase δ	DNA replication
DNA polymerase ϵ	DNA replication
FEN1	DNA replication, Base excision repair
DNA ligase I	DNA replication, Base excision repair
DNA polymerase β	Base excision repair
AP endonucleases (APE1,APE2)	Base excision repair
Uracil-DBA glycosidase	Base excision repair
XPG	Nucleotide excision repair
MSH1, MSH 2/3/6	Mismatch DNA repair
DNA polymerase η	Translesion repair synthesis
DNA polymerase κ	Translesion repair synthesis
DNA polymerase ι	Translesion repair synthesis
DNA polymerase λ	Translesion repair synthesis
UBC9	SUMOylation
p300	Histone acetylation
CAF-1	Chromatin Assembly
HDAC	Histone deacetylation
MCMT	Maintenance of methylation
GADD45	Negative growth control
p21	Cell cycle control
Cyclin/CDKs	Cell cycle control
WRN helicase	Homologous recombination

A

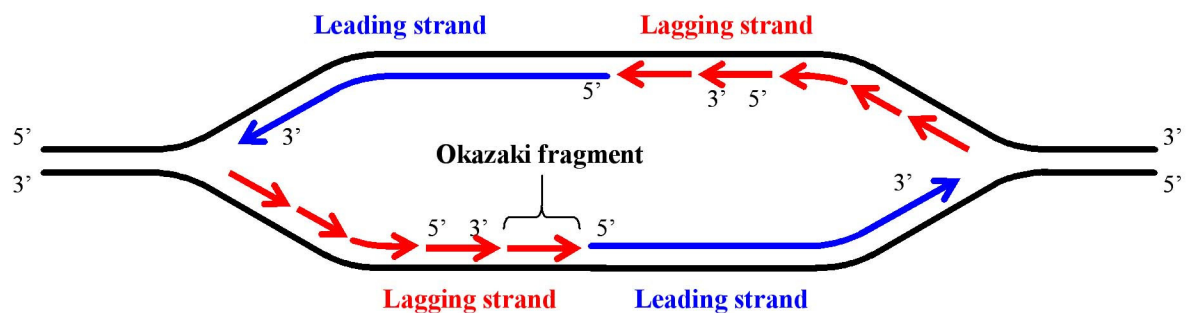


Figure 1-1. A model for DNA replication fork progression and Okazaki fragment maturation.

(A) Because of the antiparallel structure of the genomic duplex DNA, the replication fork moves in the direction from 5' to 3' on one strand and in the direction from 3' to 5' on the other strand. However, all known DNA polymerases could only synthesize DNA from 5' to 3' direction. Therefore, nascent DNA is synthesized continuously in the direction of the opening fork in the former strand, so-called the leading strand. On the other hand, in the later strand, the lagging strand is extended discontinuously in the direction away from fork movement.

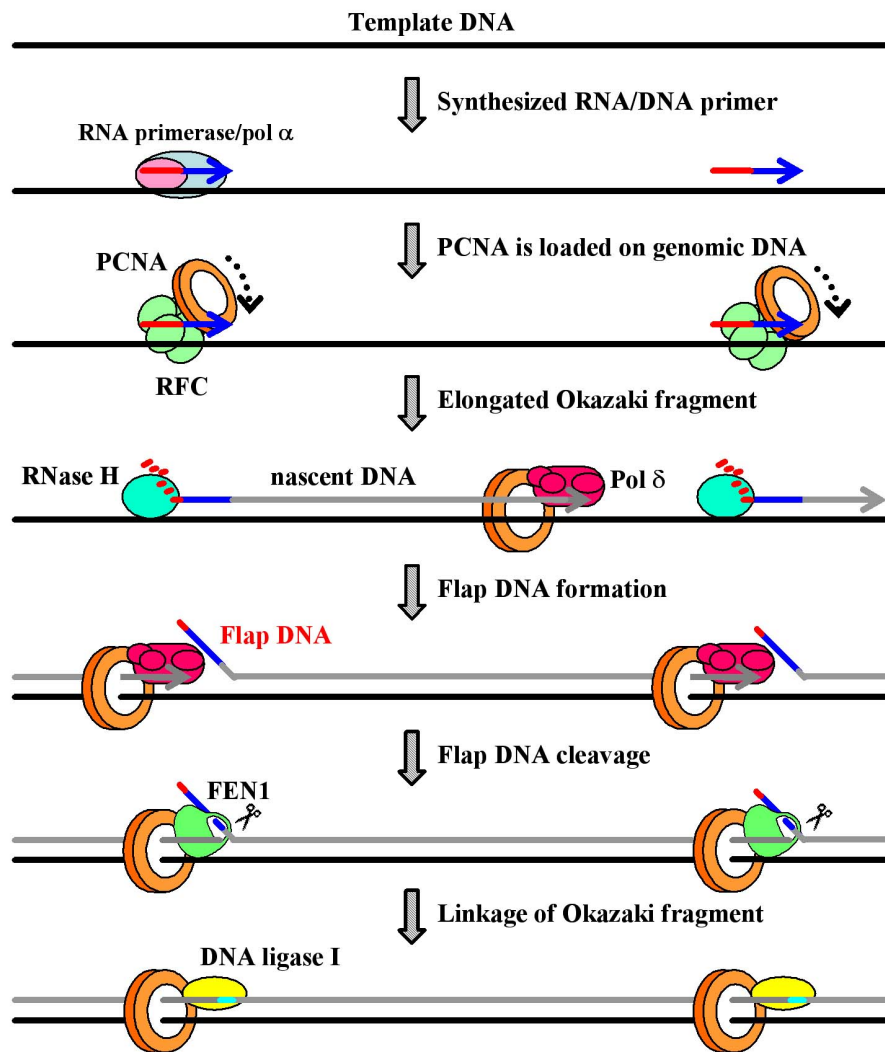
B

Figure 1-1(B) This lagging strand is initially made as a series of short segments (100-150 nucleotides (nt)), named Okazaki fragments, which are later joined. Before DNA polymerase ϵ/δ elongate Okazaki fragment, DNA polymerase α -primase complex must synthesize RNA-DNA primer as a foothold of DNA polymerase ϵ/δ . However, these primers must be finally removed as byproducts of the genomic DNA. The RNase H removes most parts of RNA primers, except for last 1nt of RNA primer. Then, the remainders of RNA-DNA primers are accurately removed by FEN1.

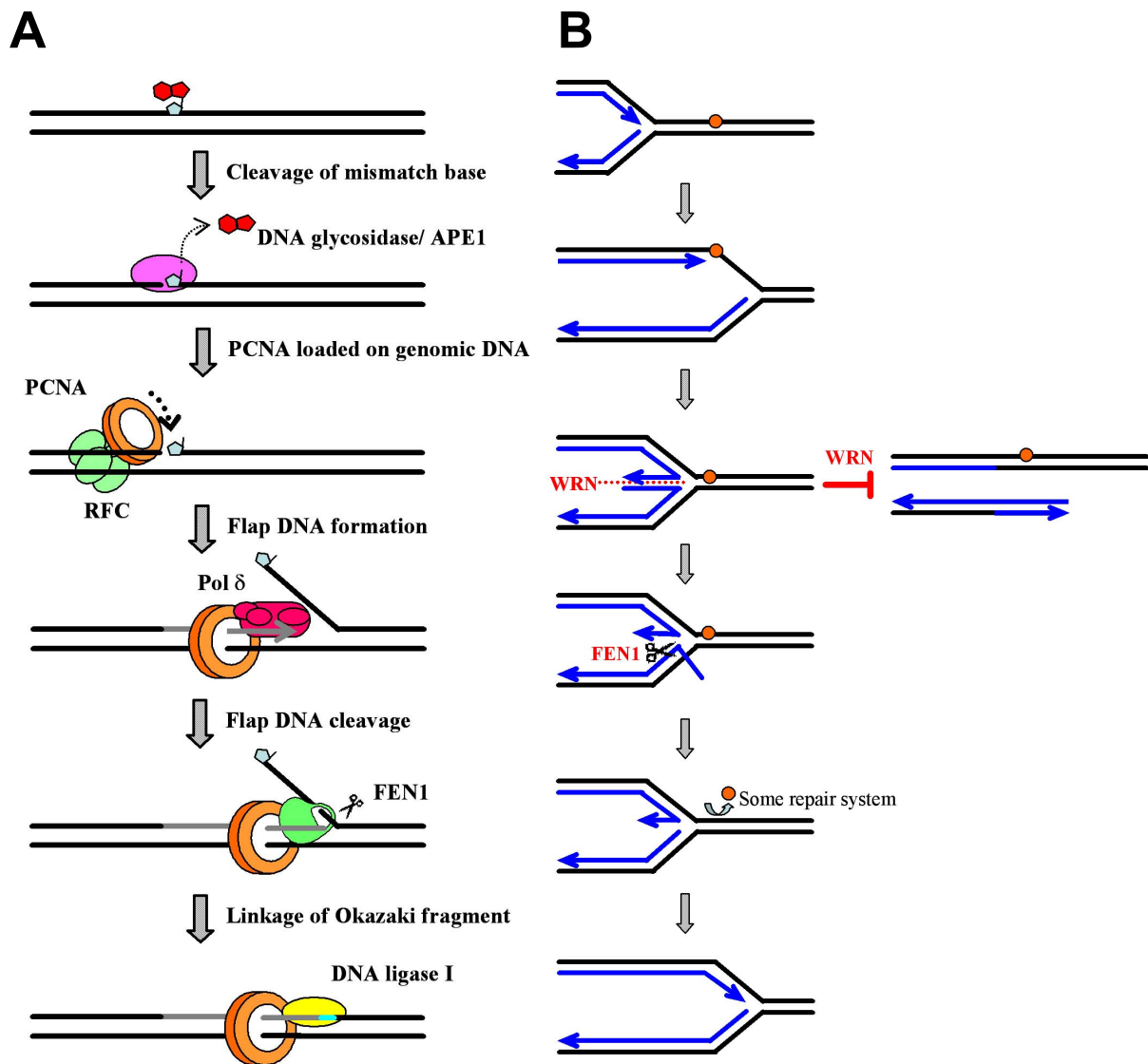


Figure 1-2. A model for DNA repair (long-path base excision repair) and homologous recombination. **(A)** Long-path base excision repair pathway. **(B)** Potential roles of FEN1 and WRN helicase to prevent aberrant homologous recombination during DNA replication. If a fork encounters a blocking lesion (orange circle) in the leading strand template, the fork might move backward and the nascent DNA (blue) might anneal to form the Holiday junction. The leading strand could be extended using the longer lagging strand as a template. WRN unwinds the duplex arm of the Holiday junction (as described red broken line) and stimulates FEN-1 cleavage of the newly synthesized lagging strand to create a region of single-stranded DNA.

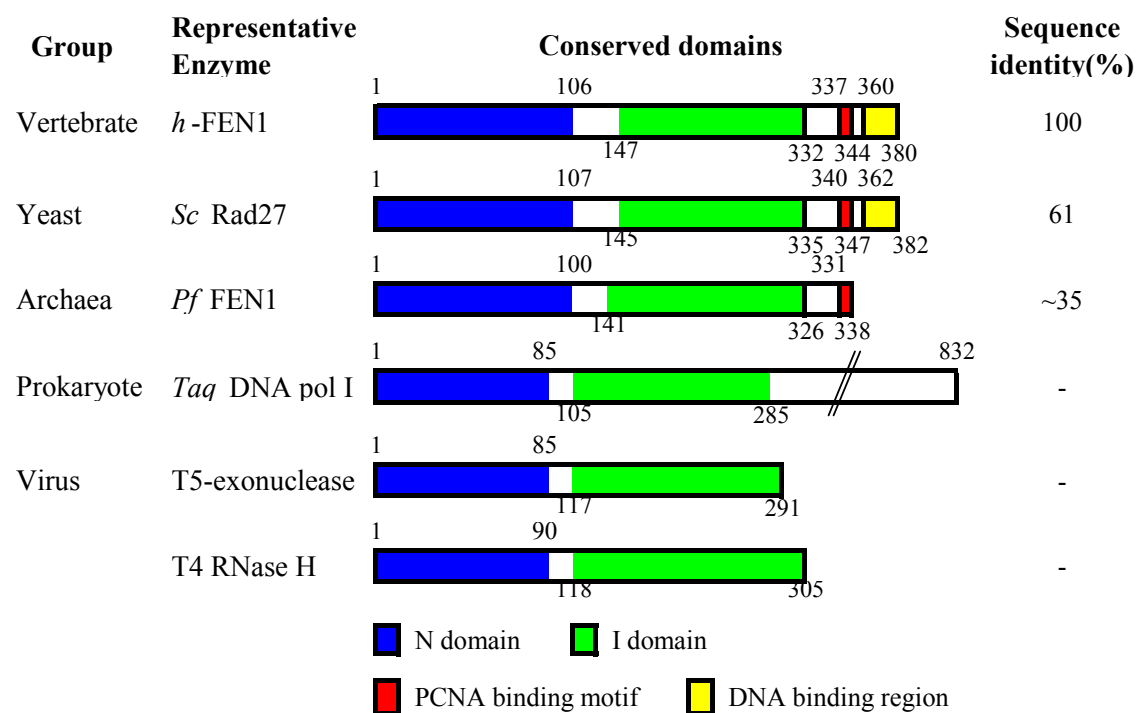


Figure 1-3. Structure-specific endonucleases of the XP-G family and the functional related enzymes. Each protein contains the clamp region (see Results and Discussion) between the N and I domains.

		PCNA binding motif																															
FEN-1	X ₃₂₆ -	R	L	S	K	S	R	Q	G	S	T	Q	G	R	L	D	D	F	F	K	V	T	G	S	L	S	S	A	K	-	X ₂₆		
p21 (Cip1/Waf1)	X ₁₃₃ -	P	G	D	S	Q	G	R	K	R	R	Q	T	S	M	T	D	F	Y	H	S	K	R	R	L	I	F	S	K	-	X ₃		
DNA polymerase δ (3th subunit)	X ₄₄₅ -	G	T	A	A	L	G	K	A	N	R	Q	V	S	I	T	G	F	F	Q	R	K											
DNA polymerase ε	X ₁₁₆₉ -	K	L	L	E	K	N	D	V	Y	K	Q	K	K	I	S	E	L	F	T	L	E	G	R	R	Q	V	T	M	-	X ₁₀₈₉		
DNA polymerase η	X ₆₉₁ -	T	N	K	R	P	R	P	E	G	M	Q	-	T	L	E	S	F	F	K	P	L	T	H									
DNA polymerase ι	X ₅₂₉ -	K	G	S	V	S	C	P	L	H	A	S	R	G	V	L	S	F	F	S	K	K	Q	M	Q	D	I	P	I	-	X ₁₅₈		
DNA polymerase κ	X ₈₅₁ -	T	S	K	K	I	K	P	N	N	P	K	H	T	L	D	I	F	F	K													
DNA polymerase λ	X ₃₂₀ -	H	L	R	K	L	D	H	I	S	E	S	V	P	V	L	E	L	F	S	N	I	W	G	A	G	T	K	T	-	X ₂₂₇		
RFC p140														M	D	I	R	K	F	F	G	V	I	P	S	G	K	K	L	V	-	X ₁₁₃₀	
DNA ligase I												M	Q	R	S	I	M	S	F	F	H	P	K	K	E	G	K	A	K	K	-	X ₉₀₀	
XPG	X ₉₇₉ -	V	L	K	Q	L	D	A	Q	Q	T	Q	L	R	I	D	S	F	F	R	L	A	Q	Q	E	K	E	D	A	-	X ₁₇₉		
MYH	X ₅₁₂ -	P	C	S	R	K	K	P	R	M	G	Q	Q	V	L	D	N	F	F	R	S	H	I	S	T	D	A	H	S	-	X ₆		
MSH3	X ₁₀ -	L	A	A	S	S	S	A	P	A	R	Q	A	V	L	S	R	F	F	Q	S	T	G	S	L	K	S	T	S	-	X ₁₀₉₉		
MSH6											M	S	R	Q	S	T	L	Y	S	F	F	P	K	S	P	A	L	S	D	A	N	-	X ₁₃₃₇
UNG2											M	I	G	Q	K	T	L	Y	S	F	F	S	P	S	P	A	R	K	R	H	A	-	X ₂₉₂
WRN helicase	X ₁₃₃ -	K	K	A	G	V	G	I	E	G	D	Q	W	K	L	L	R	D	F	D	I	K	L	K	N	F	V	E	L	-	X ₁₂₇₁		
MCMT	X ₂₉ -	S	P	R	I	T	R	K	S	T	R	Q	T	T	I	T	S	H	F	A	K	G	P	A	K	R	K	P	Q	-	X ₉₀₆		
												Q	-	-	L	-	-	F	F														
															I																		
															V																		

Q - - L - - F F
I
V

Figure 1-4. Human proteins carrying the conserved PCNA-binding motif. The number attached to left- or right-end indicates the residue count at N- and C-terminal respectively. The most conserved four residues are highlighted in yellow.

Materials and methods

Expression and Purification of FEN1 and PCNA

The pT7-hPCNA plasmid which allows expression of human PCNA, pT7-hFEN1 and pT7-His-D181A hFEN1 plasmids, which allow expression of wild-type and mutant (D181A) human FEN1, respectively, were gift from Dr. Hiroshi Morioka (Hokkaido University). The plasmids were transformed into *Escherichia coli* (*E. coli*) strain BL21(DE3). Cells were grown at 310 K in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin. For expression of wild type (wt) and mutant (D181A) FEN1, when the absorbance at 660 nm (OD₆₆₀) 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 hours following IPTG induction and were then harvested by centrifugation at 6,000 rpm (Beckman J2-M1 JA10 rotor) for 20 min at 4 °C. On the other hand, at an OD₆₆₀ of 0.5, IPTG was added to a concentration of 1 mM to induce expression of the PCNA gene. After 12 hours incubation, cells were then harvested in the same manner as described above. Then cells were suspended with a 150 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% leupepsin, 0.1% (*p*-Amidinopheny) methanesulfonyl fluoride (*p*-APMSF), 1 mM dithiothreitol (DTT)), and sonicated with a 1/2 tip-horn (Misonix Inc., USA) at 0 °C for 15 s, 80 times with 1 min interval. The sonicated solutions were centrifuged at 38,000 rpm for 30 min at 4 °C, and the cleared lysate were obtained (sonic lysate).

Recombinant human wt FEN1 was purified as follows. Firstly, the sonic lysate of FEN1 was loaded onto a DEAE anion-exchange column (Amersham Biosciences) to remove nucleic acids. The flowthrough fraction was loaded onto a Hitrap Heparin HP column (Amersham Biosciences) and eluted with a linear from 0.2 to 0.6 M NaCl in buffer A (20 mM Tris-HCl pH 7.5, 1 mM DTT). Following gel-filtration on a Sephacryl S-100 column (HR

26/60; Amersham Biosciences), protein fractions were applied to a hydroxylapatite Bio-Gel HT column (1.6 x 5 cm; BIO-RAD). Finally, collected fractions were loaded onto a Mono S (HR 10/10; Amersham Biosciences) and eluted with a linear gradient from 0.1 to 0.6 M NaCl in buffer A. 60 mg of purified FEN1 was obtained from 3 l of culture. On the other hand, sonic lysate of His-tagged FEN1(D181A) was loaded onto Ni-NTA agarose column (2.6 x 4 cm; QIAGEN) and eluted with a linear from 0.04 to 0.6 M imidazole in buffer A. Subsequent process is same as wt FEN1. Approximately, 100 mg of purified His-tagged FEN1(D181A) was obtained from 3 l of culture. Recombinant human PCNA was also purified by sequential column chromatography. The sonic lysate of PCNA loaded onto a DEAE anion-exchange column (Amersham Biosciences) and eluted with a linear gradient from 0.2 to 0.6 NaCl in buffer A. Protein fractions were further purified by Hitrap Q HP (Amersham Biosciences), Sephacryl S-200 (HR 26/60; Amersham Biosciences) and hydroxylapatite Bio-Gel HT column (1.6 x 5 cm; BIO-RAD). Finally, protein fractions were loaded onto a Mono Q (HR 10/10; Amersham Biosciences) and eluted with a linear gradient from 0.2 to 0.6 M NaCl in buffer A. The 3 mg of purified PCNA was obtained from 3 l of culture.

The protein samples at each purification step were analyzed by 12.5% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). The purified proteins 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). N-terminal methionine residue derived from initiation codon was digested from wt- and His-tagged FEN1(D181A). Peaks of 42,440 Da (calculated MW 42,462 Da) and 28,738 Da (calculated MW 28,769 Da) were observed for FEN1 and PCNA, respectively. Purified proteins were frozen by liquid nitrogen and stored at -80 °C.

Electrophoretic mobility shift assay (EMSA)

For our EMSA experiments (Figure 2-1), reaction was performed in binding buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT, and 5 mM MgCl₂) in a final reaction volume of 8 µl. After incubation at 4 °C for 1 hour, 50 % glycerol was added to each reaction solution, and then products were loaded into 8% acrylamide gel. The gel was prepared in TB buffer (90 mM Tris, 90 mM boric acid). Pre-run and run were performed in the same buffer. After one hour of pre-run at 100 volts (V), the samples were loaded and run 3 hours at 100 V. The gel was stained by Coomassie Brilliant Blue R-250 (CBB) or SYBER green to detect proteins or DNA, respectively.

Gel-filtration analysis and stoichiometry estimation of the FEN1-PCNA complex

A Sephacryl S-300 column (HR 16/60; Amersham Biosciences) was equilibrated at a flow rate of 0.5 ml/min with binding buffer. The eluted fractions were analyzed by 12.5% (w/v) (Figure 2-2A). The stoichiometry of FEN1 and PCNA was analyzed from band intensity on SDS-PAGE gels using the software ATTO Densitograph Ver. 3.0.2. and a calibration curve for correlation between the band intensity and the protein amount of FEN1 and PCNA on the gel. The column was then calibrated using gel filtration molecular mass markers containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and vitamin B12 (1.35 kDa) (Bio-Rad). The solutions of FEN1, PCNA and FEN1-PCNA (3:3) complex were loaded onto the column, and were estimated MW from a calibration curve (log MW vs. K_{av} ; Figure 2-2B). A partition coefficient, designated K_{av} , relating these values is given in equation 2;

$$K_{av} = (V_e - V_0) / (V_t - V_0) \quad \text{———— eq. (2)}$$

Where V_0 is the void volume, V_t is the total volume, and V_e is the elution volume.

Fluorescence measurement Polarization analysis of the flap DNA- FEN1(D181A)

Fluorescence polarization assays were based on the increase of the rotational correlation time of a fluorescent-labeled DNA molecule upon the binding of a protein. The protein-DNA complex, due to its increased size, tumbles more slowly than does free DNA. The ensuing reduction in the rotational correlation time of the fluorescent causes an increase in polarization, which allows the binding to be monitored (LeTilly and Royer, 1993). As shown in equation 1, polarization (P) is defined as the ratio of the difference between the vertical ($I_{||}$) and horizontal ($I_{\perp}$) emission components to their sum.

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}} \quad \text{--- eq. (1)}$$

Fluorescence polarization measurements were carried out at 10 °C using a Beacon 2000 Fluorescence Polarization System (PanVera, Madison, WI). The excitation wavelength was 488 nm, and its emission was detected through a 520-nm cutoff filter. FEN1(D181A) was diluted into FP assay buffer (10 mM HEPES pH 7.5, 5 mM MgCl₂, 1 mM DTT, and various concentration of KCl (0, 10, 20, 50, 100, 140 or 300 mM)) in Beacon borosilicate test tubes from 2 pM to 20 μM, creating 9 concentrations of FEN1(D181A) in 100 μl volumes. The FITC-labeled flap DNA was added to each tube (100 μl of 200 pM stock solution) for a final DNA concentration of 100 pM. The tubes were incubated at 10 °C for 30 minutes after which the fluorescence polarization (FP) values were measured (Figure 2-3).

Dynamic Light Scattering (DLS) analysis of the FEN1-PCNA complex

DLS measurements were performed with a DynaPro-801 instrument (Protein Solutions, Inc.) at 10 °C. This technique is used for estimating the molecular weight (MW) and polydispersity of the molecule. The output of the DLS is the translational diffusion coefficient, D_T , and it is converted to hydrodynamic radius, R_H . The MW is estimated from linear calibration curves (R_H vs. log MW). In practice, R_H and the MW are calculated using

the software DynaPro Ver. 4.0. The polydispersity is estimated from the standard deviation of the size distribution based on the mean radius. The FEN1-PCNA complex is prepared at a molar ratio of 3:3 (three FEN1 molecules with one PCNA trimer) in binding buffer. The complexes were prepared in the same manner as described above for the binding studies. Protein solutions were passed through a 0.22 μm filter to remove particulates before use. The results were summarized in table 2-1.

Crystallization of the FEN1-PCNA complex

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 FEN1-binding sites. Hence, we attempted to crystallize 1:3, 2:3 and 3:3 (FEN1: PCNA) complexes, but failed to obtain crystals from the 1:3 and 2:3 complexes. The 3:3 complex (hereafter referred to as the FEN1-PCNA complex) was prepared by mixing protein solutions containing 10 mM Na HEPES pH 7.8, 100 mM NaCl, 1 mM DTT and final concentrations of 0.36 mM FEN1 and 0.36 mM PCNA.

Using the Grid Screen kit, initial crystals were obtained at pH 6 and 7 with 5% polyethyleneglycol 6000 (PEG 6K) as precipitant at 4 °C. The conditions were then refined by changing the pH (5.0-8.0 in steps of 0.5) and the temperature (4, 10, 15 and 20 °C) and using various PEGs (PEG 200, PEG 400, PEG 1K, PEG 2K, PEG 4K, PEG 6K, PEG 8K and PEG 20K). Relatively large plate-shaped crystals (100 x 100 x 10 μm) were found to grow at pH 6.5 using 12% PEG 1K at 10 °C. 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 protein complex (0.06 mM), 50 mM Na MES pH 6.5, 50 mM NaCl, 6%(w/v) PEG 1000 and 0.6 mM n-hexadecyl- β -D-maltoside at 10 °C. Crystals grown under optimized conditions reached their maximum size within a week and had dimensions of 600 x 80 x 20 mm (Figure 2-5A). 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 CBB confirmed that the crystals contained both FEN1 and PCNA (Figure 2-5B). Based on the measured band intensities of the SDS-PAGE gels, the molar ratio of FEN1:PCNA was approximately 1:1 in the crystal (data not shown).

Crystallization of the flap DNA-FEN1-PCNA complex

HPLC-purified oligonucleotides (Griner) used for crystallization are listed in Table 2-2, and they were annealed as described in the Figure 2-4. After annealing, their flap DNAs were purified by Mono Q (HR 5/5; Amersham Biosciences) column chromatography. For preparation of the flap DNA-FEN1-PCNA complex, flap DNA, FEN1(D181A) and PCNA were mixed in a molar ratios of 1:1:3, 1:2:3 or 1:3:3. To identify suitable precipitant reagents for the ternary complexes, 38 nt flap DNA was used for preliminary crystallization screening performed with the hanging-drop vapour-diffusion technique using the protein-DNA crystallization reagent kit, Natrix (Hampton Research, USA), and the same screening kits used in the FEN1-PCNA crystallization (see above). Each protein-DNA-complex solution was mixed in a 1:1 ratio with the reservoir solution, and equilibrated at 4 °C and 20 °C.

Using the Grid Screen PEG 6K, single crystals were obtained at pH 7, 8 and 9 with 10% PEG 6K as precipitant at 4 °C and 20 °C in a molar ratio (1:1:3), while spherulites were appeared in other molar ratios. By SDS-PAGE of the crystals, the former crystal appeared to contain the ternary complex of flap DNA: FEN1(D181):PCNA, while other spherulites contain DNA alone. Several cryogenic solutions were attempted to flash-cooling ternary complex crystal. However these crystals poorly diffracted (approximately, 15 Å). The crystallization conditions were then tried to be refined by changing the pH (5.0-8.0 in steps of 0.5) and the temperature (4, 10, 15 and 20 °C) and using various PEGs (PEG 200, PEG 1K, PEG 6K and PEG 20K). We found that several crystals were improved to diffract up to 8-9 Å resolution. We also tried conditions using the Additive Screen kits I-III and Detergent Screen kits I-III (Hampton Research, USA). We obtained crystals in different conditions, although the crystal quality was not quite improved.

Subsequently, we retried further purification of flap DNA by MonoQ or hydroxylapatite column chromatography. The DNA samples gave crystals in the same condition as previously used. We found that the diffraction of crystals could be improved effectively when we use the purified flap DNA using MonoQ column. After optimization of the crystallization conditions, single crystals appeared in drops containing 100 µM the protein-DNA (flap DNA:FEN1(D181A):PCNA = 1:1:3) complex, 50 mM Na-MES (pH 6.5), 25 mM KCl, 6%(w/v) PEG 6K at 20 °C. The crystal diffracted to 6.5 Å resolution. Furthermore, other purified flap DNAs were tried to crystallized with FEN1(D181A) and PCNA for the purpose of improvement of crystal packing (Figure 2-4), while diffraction was not improved for these DNAs.

The crystals of the ternary complex were resolved in an aliquot of water for SDS-PAGE or native-PAGE analyses. The molar ratio of the flap DNA:FEN1(D181A):PCNA was approximately 1:2:3 in the crystals. This molar ratio is different from the ratio in the

crystallization mixture, which contains DNA and protein in a 1:1:3 ratio corresponding to the results of the EMSA and gel-filtration analyses.

X-ray data collection and processing

For preventing the radiation damage during data collection, crystals were transferred into a cryoprotective solution containing 20% (v/v) PEG 200 and reservoir solution [100 mM Na MES pH 6.5, 100 mM NaCl and 12%(w/v) PEG 1000] and then mounted on a rayon loop for flash-cooling to -173 °C using a Rigaku cryostat with liquid-nitrogen vapour (Hope, 1988). The crystal diffracted to 2.9 Å resolution. A set of X-ray diffraction data for the FEN1-PCNA complex crystal was collected at SPring-8 on beam-line 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 seconds. The wavelength was set to 1.00 Å. Diffraction data were indexed, scaled and merged using the computer programs DENZO and SCALEPACK (Otwinowski and Minor, 1997). The total number of observed reflections was 228,402, which gave 52,176 unique reflections [$I/\sigma(I) > 1$]. The crystals possess a primitive orthorhombic lattice with lattice parameters $a = 82.2$, $b = 143.4$, $c = 246.7$ Å and belong to space group $P2_12_12_1$, which was predicted from the systematic absences on each axes. The resulting data gave an R_{merge} of 8.0% (26.5% for the outer shell, 3.0-2.9 Å) with a completeness of 85.1% (58.3% for the outer shell). The averaged $I/\sigma(I)$ value was 13.3 (2.9 for the outer shell). The estimated mosaicity of the crystal was in the range 0.3-0.5° (Table 2-3).

The Matthews volume (V_M) (Matthews, 1968) of 3.4 Å³/Da was calculated assuming one PCNA trimer and three FEN1 monomers in the asymmetric unit, which corresponds to 64% solvent content (V_{solvent}) by volume. The PCNA trimer has pseudo-threefold symmetry perpendicular to the trimer ring plane (Gulbis *et al.*, 1996; Krishna *et al.*, 1994; Matsumiya *et al.*, 2001). We calculated self-rotation functions (Rossmann and Blow, 1962) to search for

non-crystallographic symmetry with several Patterson sphere radii. However, we failed to find significant peaks indicating three-fold symmetry on the maps. It might be that the positions of the three PCNA subunits and/or the three FEN1 molecules bound to them deviate strongly from the ideal equivalent positions of threefold symmetry.

Structure determination

Human FEN1 exhibits a relatively high sequence identity to hyperthermophilic archaeal FEN1s: 38% identity to that from *Pyrococcus furiosus* and 34% to that from *Methanococcus jannaschii*. Hence, it may be possible to performed molecular replacement (MR) using human PCNA and archaeal FEN1s structures as search models. The crystal structures of the human PCNA-p21 complex (Gulbis *et al.*, 1996) and hyperthermophilic archaeal, *P. furiosus* (Hosfield *et al.*, 1998) and *M. jannaschii* (Hwang *et al.*, 1998), FEN1s have been reported.

The flowchart of our MR processes is summarized in Figure 2-9. MR was performed by the program MOLREP (Collaborative Computational Project, 1994) using a PCNA monomer from human p21-PCNA complex, (PDB code: 1AXC) as a search model. Three distinct solutions corresponding to each monomer of the PCNA trimer were obtained (Table 2-5 and 2-6). On the other hand, MR trials using *P. furiosus* or *M. jannaschii* FEN1 monomers (PDB code: 1B43 and 1A76, respectively) failed. Therefore, the initial electron density map was calculated using only the phases of the trimeric PCNA structure (Figure 2-10A). This map was of good quality for PCNA but not interpretable for FEN1, except for the C-terminal PCNA-binding regions.

To search FEN1 molecules on the difference Fourier map, $F_O - F_C$ map, the electron densities for the PCNA trimer were omitted from the map (compare Figures 2-10 A and C). This process was performed using the programs NCSMASK and OVERLAPMAP (Figures 2-9,

2-10 B and C) (Collaborative Computational Project, 1994). Subsequently, structure factors were calculated using inverse Fourier transfer, performed by the program SFALL (Collaborative Computational Project, 1994). Using this set of the modified structure factors, the first FEN1 solution was then obtained by MR with the *P. furiosus* FEN1 structure as a search model (Figure 2-10 D). The second and third FEN1 molecules were also determined as the same manner as that for the first molecule (Figures 2-10E-H). The electron density map was markedly improved by non-crystallographic symmetry (NCS) averaging using the program DM (Collaborative Computational Project, 1994) for the three FEN1 core domains and for the three PCNA monomers. The structure was modeled by the program O (Jones *et al.*, 1991) and finally refined by the program CNS (Brunger *et al.*, 1998) in the absence of NCS-restraints. Superimpositions of human and archaeal FEN1s were performed by the program LSQMAN (Kleywegt and Jones, 1997) and figures were prepared by the programs MOLSCRIPT (Kraulis, 1991), CONSCRIPT (Lawrence and Bourke, 2000), GRASP (Nicholls *et al.*, 1991) and Pymol (Delano, 2002). The atomic coordinates have been deposited in the PDB (accession code 1UL1).

Table 2-1. Summary of DLS measurements

Stoichiometry		Radius (nm)	MW (kDa)	Dispersity (%)
FEN1		3.7	68	11
PCNA		4.8	128	23
FEN1-PCNA	(3 : 3)	6	227	3.3

Table 2-2. Oligonucleotide sequences used for crystallization.

The sequences are listed 5' to 3'.

Templates

T ₁	(36-mer)	ATCACGACTCTGCCTCAAGACGGTAGTCAATTACGT
T ₂	(37-mer)	ATCACGACTCTGCCTCAAGACGGTAGTCAATTACGTG
T ₃	(38-mer)	ATCACGACTCTGCCTCAAGACGGTAGTCAATTACGTGG
T ₄	(39-mer)	ATCACGACTCTGCCTCAAGACGGTAGTCAATTACGTGGC
T ₅	(40-mer)	ATCACGACTCTGCCTCAAGACGGTAGTCAATTACGTGGCC
T ₆	(39-mer)	ATCACGACTCTGCCTCAAGACGGTAGTCAATTACGTGGT
T ₇	(41-mer)	ATCACGACTCTGCCTCAAGACGGTAGTCAATTACGTGGCCT
T ₈	(38-mer)	CGACTCTGCCTCAAGACGGTAGTCAATTACGTGGATCA
T ₉	(38-mer)	CACGACTCTGCCTCAAGACGGTAGTCAATTACGTGGAT
T ₁₀	(38-mer)	GGATCACGACTCTGCCTCAAGACGGTAGTCAATTACGT
T ₁₁	(38-mer)	GTGGATCACGACTCTGCCTCAAGACGGTAGTCAATTAC

Upstream Primers

U ₁	(26-mer)	ACGTAATTGACTACCGTCTTGAGGCA
U ₂	(27-mer)	CACGTAATTGACTACCGTCTTGAGGCA
U ₃	(28-mer)	CCACGTAATTGACTACCGTCTTGAGGCA
U ₄	(29-mer)	GCCACGTAATTGACTACCGTCTTGAGGCA
U ₅	(30-mer)	GGCCACGTAATTGACTACCGTCTTGAGGCA
U ₆	(27-mer)	ACCACGTAATTGACTACCGTCTTGAGGCA
U ₇	(27-mer)	AGGCCACGTAATTGACTACCGTCTTGAGGCA
U ₈	(24-mer)	TGATCCACGTAATTGACTACCGTC
U ₉	(26-mer)	ACGTAATTGACTACCGTCTTGAGGCA
U ₁₀	(30-mer)	ATCCACGTAATTGACTACCGTCTTGAGGCA
U ₁₁	(32-mer)	TGATCCACGTAATTGACTACCGTCTTGAGGCA
U ₁₂	(29-mer)	CCACGTAATTGACTACCGTCTTGAGGCAC

Downstream Primers

D ₁	(13-mer)	ACTGAGTCGTGAT
D ₂	(17-mer)	ACTGAGTCGTGATCCAC
D ₃	(15-mer)	ACTGAGTCGTGATCC
D ₄	(11-mer)	ACTGAGTCGTG
D ₅	(9-mer)	ACTGAGTCG

Hairpin loops

H ₁	(55-mer)	ACTGAGTCGTGCGTTTTTCGCACGACTCTGCCTCAAGACGGTAGTC AATTACGTGG
H ₂	(54-mer)	ACTGAGTCGTGCGGAACGCACGACTCTGCCTCAAGACGGTAGTCA ATTACGTGG

Table 2-3. Crystal data of the human FEN1-PCNA complex

X-ray source	SPring-8 BL38B1
Wavelength (Å)	1.000
Space group	$P2_12_12_1$
Cell parameters, a , b , c (Å)	82.2, 143.4, 246.7
Number of reflections (total/unique)	234,329 / 55,775
Resolution (Å) ^a	50-2.9 (3.0-2.9)
Completeness (%) ^b	85.1 (58.3)
V_M (Å ³ /Da)	3.4
Mosaicity (°)	0.3 – 0.5
$I/\sigma(I)$	13.3 (2.9)
R_{merge} (%)	8.0 (28.0)

^aStatistics for the outer resolution shell are given in parentheses.

^bIntensities ($I/\sigma_I > 1.0$) were merged and used in the refinement.

Table 2-4. Crystal data of the flap DNA-FEN1(D181A)-PCNA complex

X-ray source	SPring-8 BL41XU
Wavelength (Å)	1.000
Crystal system	Primitive tetragonal
Cell parameters, a , b , c (Å)	169.6, 169.6, 207.3
Number of reflections (total/unique)	12,523 / 4,556
Resolution (Å) ^a	30-6.0 (6.2 - 6.0)
Completeness (%) ^b	55.3 (20.6)
V_M (Å ³ /Da)	3.8
Mosaicity (°)	1.7 – 2.2
$I/\sigma(I)$	29.7 (4.6)
R_{merge} (%)	7.8 (22.4)

^aStatistics for the outer resolution shell are given in parentheses.

^bIntensities ($I/\sigma_I > 1.0$) were merged and used in the refinement.

Table 2-5. Solutions of rotation and translation functions of the first PCNA monomer

	θ	ϕ	χ	X	Y	Z	R	C
<i>P2₁2₁2₁</i>	42.89	-152.43	92.96	0.041	0.326	0.330	0.503	0.204
	125.09	-95.02	71.30	0.111	0.093	0.247	0.510	0.180
	148.05	-31.82	165.81	0.349	0.042	0.401	0.509	0.171
	107.51	157.76	61.28	0.411	0.066	0.231	0.525	0.124
<i>P222</i>	42.89	-152.43	92.96	0.504	0.705	0.141	0.519	0.146
	125.09	-95.02	71.30	0.268	0.278	0.037	0.523	0.131
	148.05	-31.82	165.81	0.204	0.986	0.454	0.525	0.119
	106.96	162.80	60.14	0.442	0.378	0.109	0.527	0.115
<i>P222₁</i>	42.89	-152.43	92.96	0.791	0.326	0.150	0.515	0.160
	125.09	-95.02	71.30	0.362	0.093	0.296	0.520	0.141
	148.05	-31.82	165.81	0.099	0.041	0.136	0.519	0.135
	106.96	162.80	60.14	0.613	0.448	0.279	0.524	0.116
<i>P2₁2₁2</i>	42.89	-152.43	92.96	0.791	0.318	0.080	0.515	0.159
	125.09	-95.02	71.30	0.269	0.093	0.247	0.520	0.145
	148.05	-31.82	165.81	0.846	0.042	0.402	0.517	0.132
<i>P2₁22</i>	42.89	-152.43	92.96	0.040	0.076	0.330	0.514	0.160
	125.09	-95.02	71.30	0.165	0.342	0.247	0.520	0.144
	148.05	-31.82	165.81	0.516	0.292	0.402	0.521	0.136
	33.40	-161.76	111.77	0.114	0.795	0.088	0.530	0.114
<i>P22₁2</i>	42.89	-152.43	92.96	0.541	0.059	0.080	0.514	0.164
	125.09	-95.02	71.30	0.610	0.391	0.496	0.519	0.143
	148.05	-31.82	165.81	0.849	0.924	0.151	0.521	0.135
	106.96	162.80	60.14	0.141	0.750	0.419	0.526	0.114
<i>P2₁22₁</i>	42.89	-152.43	92.96	0.041	0.326	0.339	0.516	0.157
	125.09	-95.02	71.30	0.112	0.092	0.401	0.519	0.144
	148.05	-31.82	165.81	0.348	0.041	0.293	0.521	0.134
	33.40	-161.76	111.77	0.328	0.292	0.404	0.526	0.116
<i>P22₁2₁</i>	42.89	-152.43	92.96	0.292	0.076	0.041	0.515	0.162
	125.09	-95.02	71.30	0.110	0.448	0.247	0.517	0.150
	148.05	-31.82	165.81	0.099	0.291	0.102	0.519	0.138
	33.40	-161.76	111.77	0.307	0.193	0.008	0.528	0.117

* This table includes the top four solutions of the molecular replacement sorting by the correlation coefficient C , R -factor (see text) for the respective space groups. (θ , ϕ , χ) are the polar angles and (X , Y , Z) are the functions of the unit cell axes. Space group $P2_12_12_1$ gave the highest C and the lowest R , boxed in blue. These calculations used the reflections between 3-50 Å.

Table 2-6. Solutions of rotation and translation functions of the second and third PCNA monomer

	θ	ϕ	χ	X	Y	Z	C	R
2ndPCNA	125.09	-95.02	71.30	0.111	0.593	0.247	0.478	0.291
	148.05	-31.82	165.81	0.349	0.541	0.401	0.480	0.276
	122.39	-86.28	65.58	0.102	0.600	0.250	0.510	0.180
	134.05	-84.39	72.56	0.112	0.589	0.254	0.510	0.172
	106.96	162.80	60.14	0.165	0.531	0.220	0.511	0.170
3rdPCNA	148.05	-31.82	165.81	0.348	0.541	0.402	0.454	0.360
	106.96	162.80	60.14	0.524	0.951	0.587	0.487	0.257
	33.40	-161.76	111.77	0.177	0.941	0.601	0.488	0.255
	125.09	-95.02	71.30	0.111	0.593	0.400	0.508	0.240
	42.89	-152.43	92.96	0.040	0.325	0.138	0.508	0.239

* This Table includes the top five ranking of the molecular replacement solutions at $P2_12_12_1$. Parameters (θ , ϕ , χ , X , Y , Z , C , R) are the same as Table 2-5. In searching the second PCNA subunit, the first PCNA subunit was fixed. In searching the third PCNA subunit, the first and second PCNAs subunits were fixed. Each top solution gave clearly the highest correlation coefficient and lowest R -factor, boxed in blue. These calculations used the reflections between 3-50 Å.

Table 2-7. Solutions of rotation and translation functions of FEN1

	θ	ϕ	χ	X	Y	Z	TF	C
1st FEN1	100.50	125.35	67.06	0.623	0.105	0.412	38.405	0.046
	53.74	161.14	122.54	0.326	0.528	0.418	10.373	0.025
	101.68	135.26	92.45	0.690	0.114	0.413	8.749	0.024
	128.46	-78.40	119.39	0.306	0.617	0.090	8.957	0.024
	161.62	-43.19	176.43	0.294	0.057	0.442	7.787	0.024
2nd FEN1	177.27	-101.24	67.68	0.146	0.009	0.377	50.302	0.046
	27.32	93.29	93.03	0.302	0.567	0.382	36.195	0.039
	21.68	117.51	95.28	0.276	0.572	0.387	9.532	0.026
	93.39	154.17	29.83	0.777	0.033	0.612	7.883	0.025
	109.09	-137.73	80.12	0.489	0.470	0.539	7.827	0.025
3rd FEN1	28.64	89.43	91.21	0.303	0.567	0.383	71.572	0.048
	27.15	100.47	103.14	0.279	0.572	0.382	20.438	0.033
	20.46	113.89	91.64	0.311	0.567	0.380	17.007	0.032
	20.65	122.39	93.96	0.301	0.567	0.379	14.344	0.031
	19.90	137.67	109.16	0.291	0.611	0.377	13.773	0.031

This table includes the top five solutions of the molecular replacement sorting by peak number of translation function TF. Each top solution gave clearly the highest correlation coefficient and lowest R -factor, boxed in blue. These calculations used the reflections between 3-50Å.

Table 2-8. Crystallographic statistics of the human FEN1-PCNA complex

Refinement	
Resolution (Å) ^a	50-2.9 (3.0-2.9)
Number of residues included	
FEN1 (X, Y, Z)	313, 312, 349 (of 380)
PCNA (A, B, C)	256, 258, 255 (of 261)
Number of atoms	13,162
R / R_{free} (%) ^b	22.0 / 28.4
Ramachandran plot (%)	
Most favored	76.2
Additional allowed	21.4
Generously allowed	1.9
Disallowed	0.5
Average B-factor (Å ²)	
Total	59.2
FEN1 (X, Y, Z)	55.7, 78.8, 85.2
PCNA (A, B, C)	36.1, 40.1, 46.9
R.m.s. bond length (Å), angles (°)	0.007, 1.4

^a Statistics for the outer resolution shell are given in parentheses.

^b $R = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$. R_{free} is the same as R , but for a 5% subset of all reflections that were never used in the crystallographic refinement.

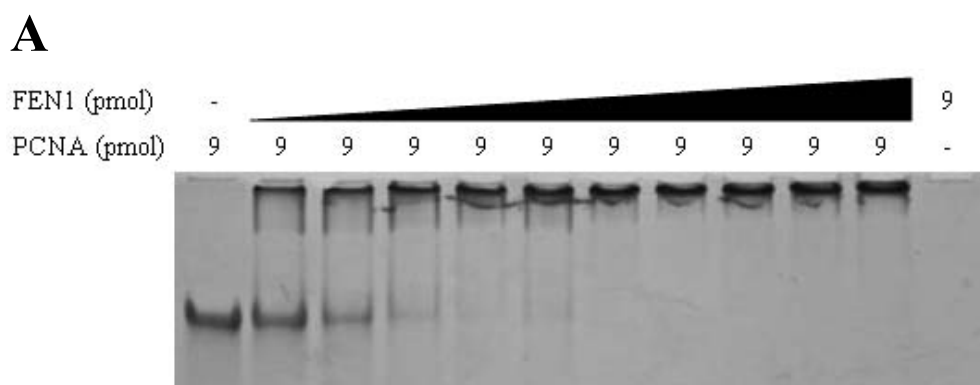


Figure 2-1. Binding assays of FEN1, PCNA and DNA.

(A) The FEN1 titration binding to PCNA in EMSA. Lanes 1-11 contain 9 pmol PCNA and lanes 1-12 also contain 0, 3.0, 4.5, 6, 6.75, 7.5, 8.25, 9.0, 9.75, 10.5, 12 pmol wt FEN1 as denoted by the *triangle*.

B

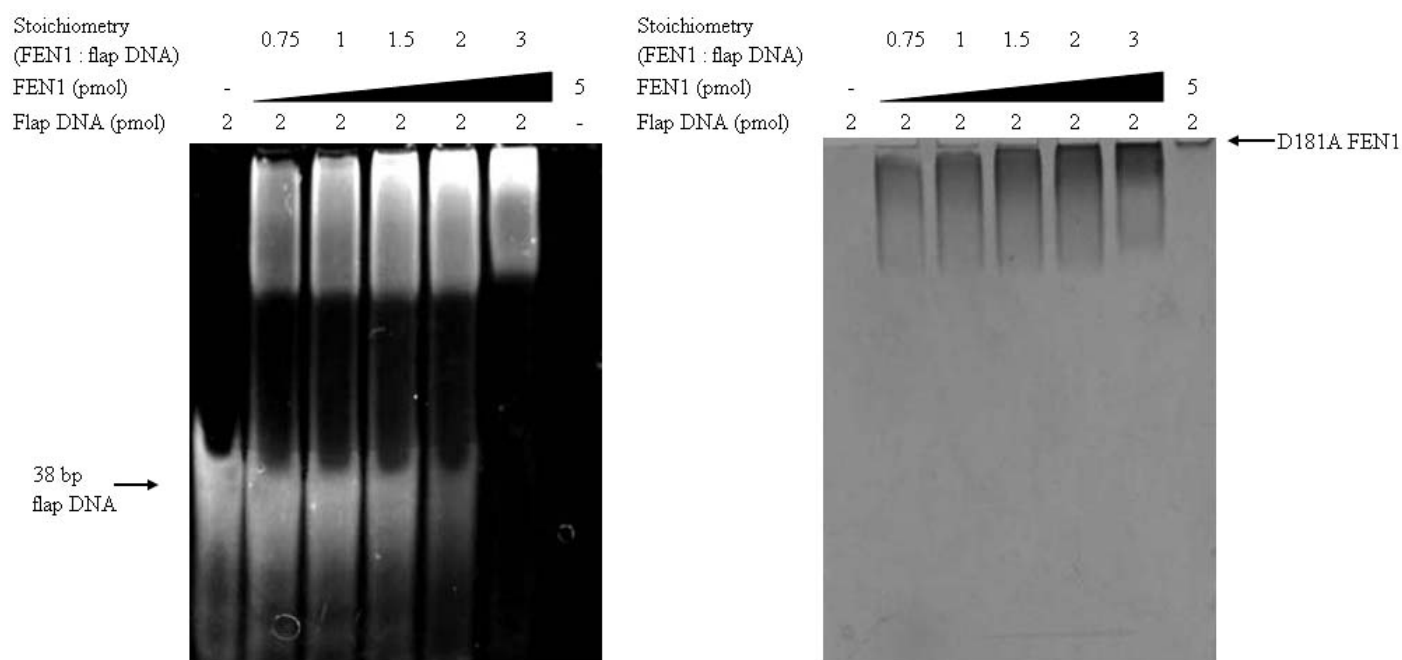


Figure 2-1.

(B) EMSAs of flap DNA with FEN1(D181A) in binding buffer. The lanes 2-6, have increasing concentrations of FEN1(D181A), 1.5, 2, 3, 4, and 6 μ M (corresponding to stoichiometry: 0.75, 1, 1.5, 2, and 3). Left and right gels were stained by SYBER GREEN for DNA and CBB for protein, respectively.

C

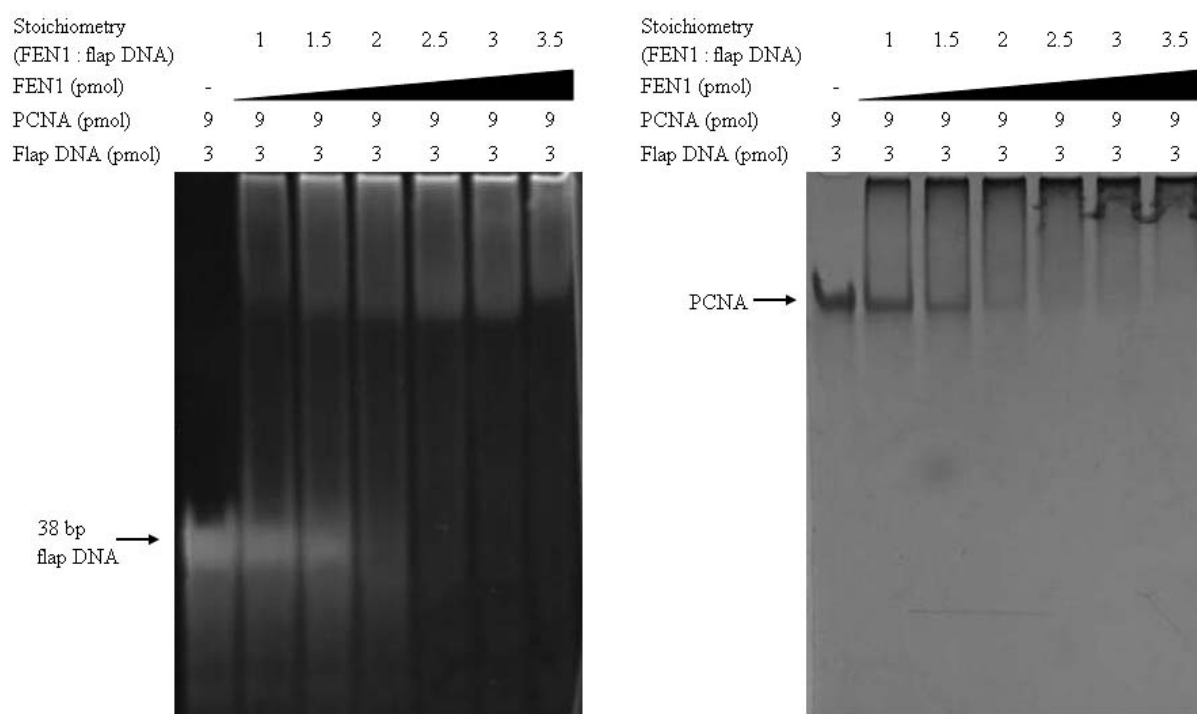


Figure 2-1.

(C) EMSAs of flap DNA with FEN1(D181A) and PCNA. The lanes 2-7 have increasing concentrations of FEN1(D181A), 3, 4.5, 6, 7.5, 9, and 10.5 μ M (corresponding to stoichiometry: 1, 1.5, 2, 2.5, 3, and 3.5). Left and right gels were stained by SYBER GREEN and CBB, respectively.

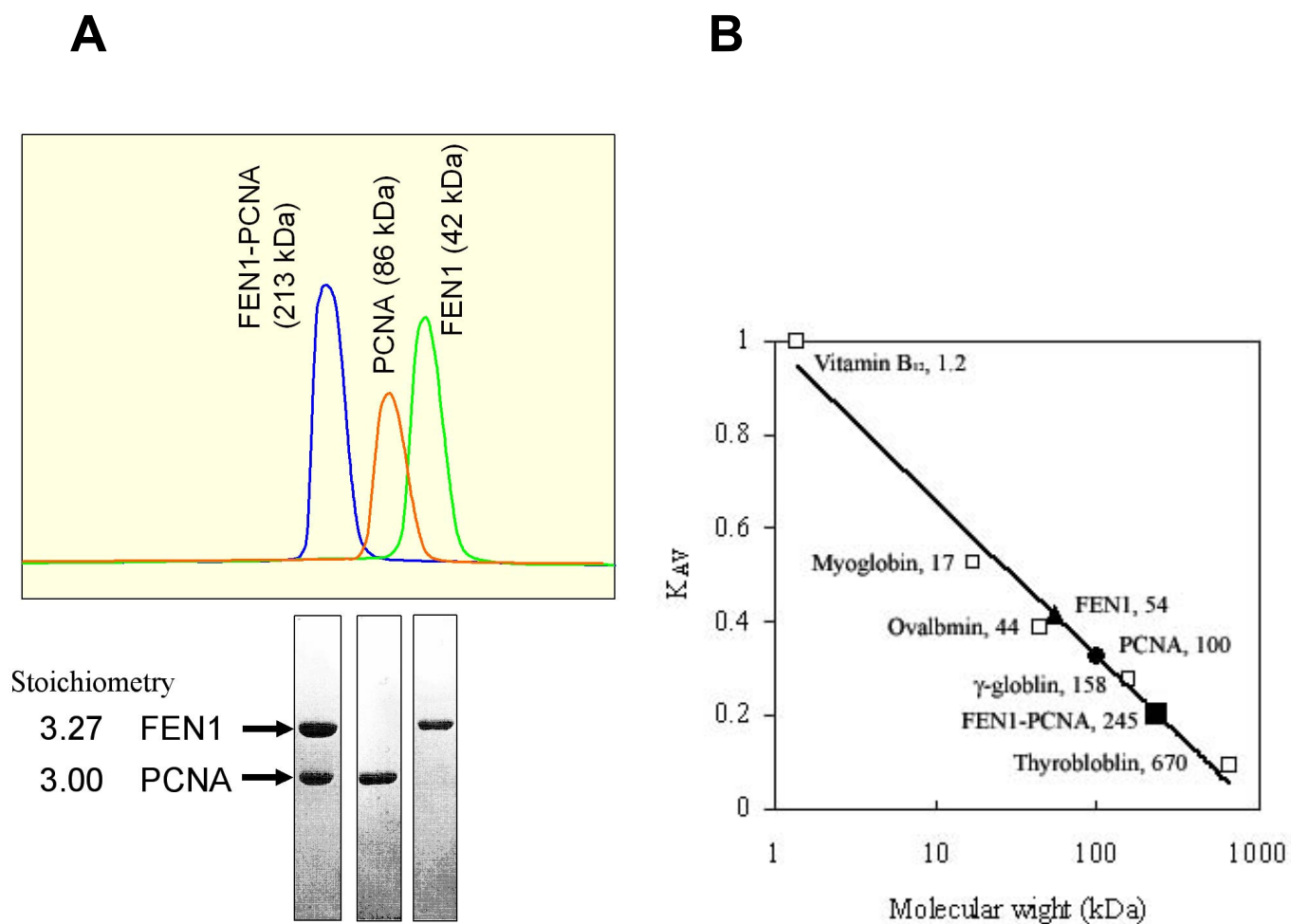


Figure 2-2. Gel-filtration analysis of the wt-FEN1-PCNA complex.

(A) The elution profile of the Sephacryl S-300 column chromatography with monitoring UV absorption. Elution profiles of FEN1, PCNA and the FEN1-PCNA complex were superimposed. Each fraction at elution peaks was analyzed by SDS-PAGE. **(B)** Calibration curve of partition coefficient (K_{av}) versus molecular weight of several protein standards (open squares) for the Sephacryl S300 column. Molecular weights of FEN1 (close square), PCNA (close circle) and the FEN1-PCNA complex (close triangle) were estimated from this curve.

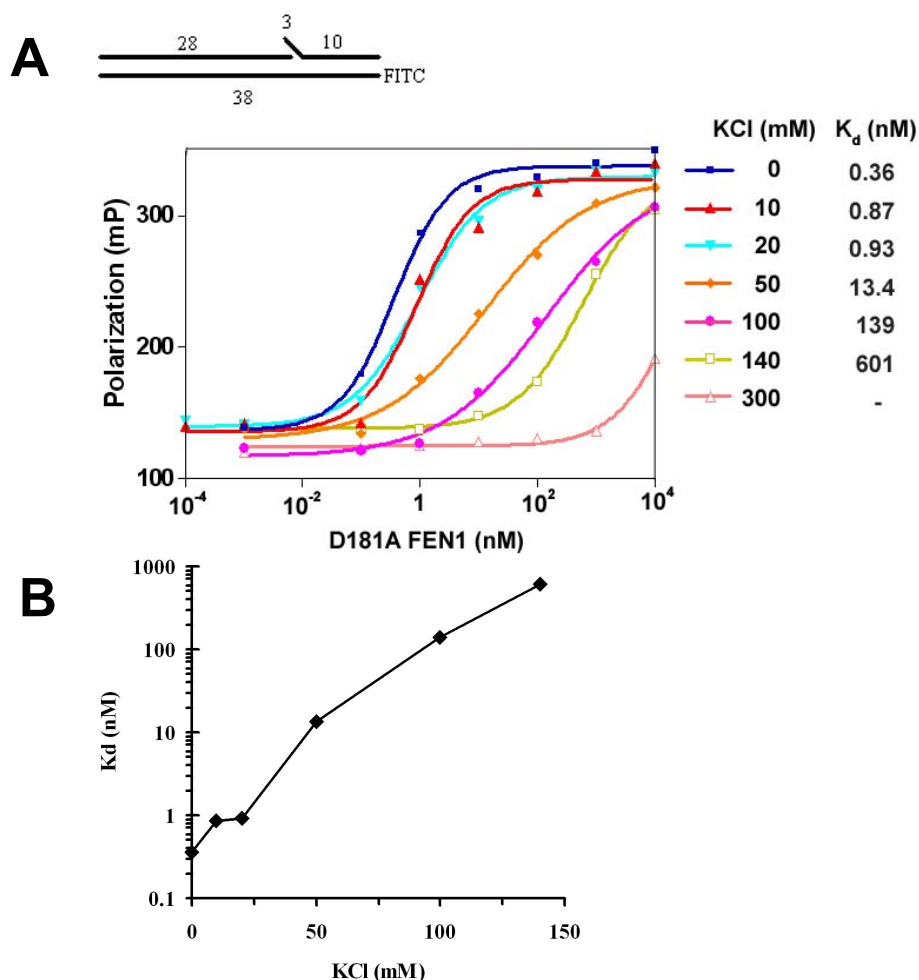


Figure 2-3. Fluorescence polarization analyses of FEN1(D181A) to flap DNA on various KCl concentrations.

(A) Various concentrations of FEN1(D181A) were incubated with 100 pM of FITC-labeled flap DNA as described under “Materials and methods”. Binding reactions were carried out under various concentrations of KCl; blue (0 mM), red (10 mM), cyan (20 mM), orange (50 mM), magenta (100 mM), yellow (140 mM) and pink (300 mM) line. The dissociation constants (K_d) of the flap DNA-FEN1(D181A) complex were estimated from dose-response curves.

(B) The KCl-dependent K_d variation. The K_d values were plotted against KCl concentrations.

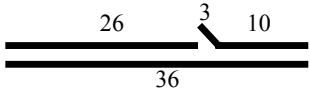
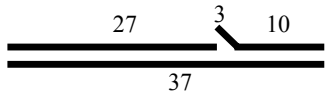
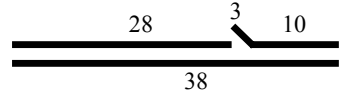
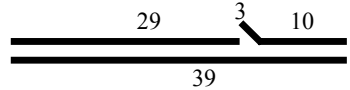
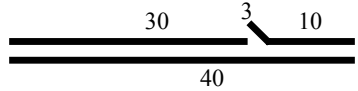
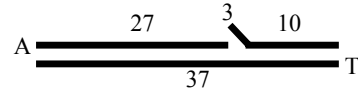
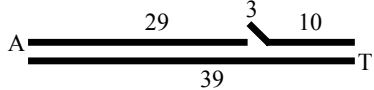
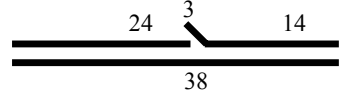
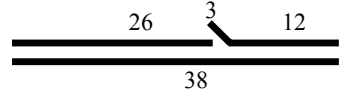
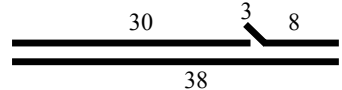
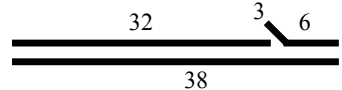
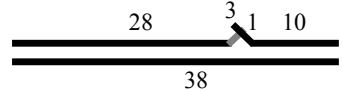
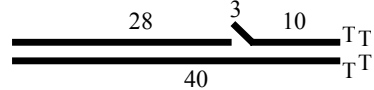
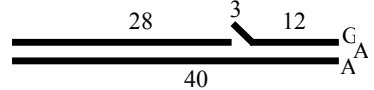
Length	Crystallinity	Oligonucleotide	Flap DNA structure
36 bp	Δ	D ₁ :T ₁ :U ₁	
37 bp	Δ	D ₁ :T ₂ :U ₂	
38 bp	Δ	D ₁ :T ₃ :U ₃	
39 bp	O	D ₁ :T ₄ :U ₄	
40 bp	x	D ₁ :T ₅ :U ₅	
36 bp	x	D ₁ :T ₆ :U ₆	
38 bp	x	D ₁ :T ₇ :U ₇	
38 bp	x	D ₂ :T ₈ :U ₈	
38 bp	x	D ₃ :T ₉ :U ₉	
38 bp	x	D ₄ :T ₁₀ :U ₁₀	
38 bp	x	D ₅ :T ₁₁ :U ₁₁	
38 bp	Δ	D ₅ :T ₃ :U ₁₂	
40 bp	Δ	H ₁ :U ₃	
40 bp	Δ	H ₂ :U ₃	

Figure 2-4. Flap DNA structures used in crystallization with FEN1(D181A) and PCNA.

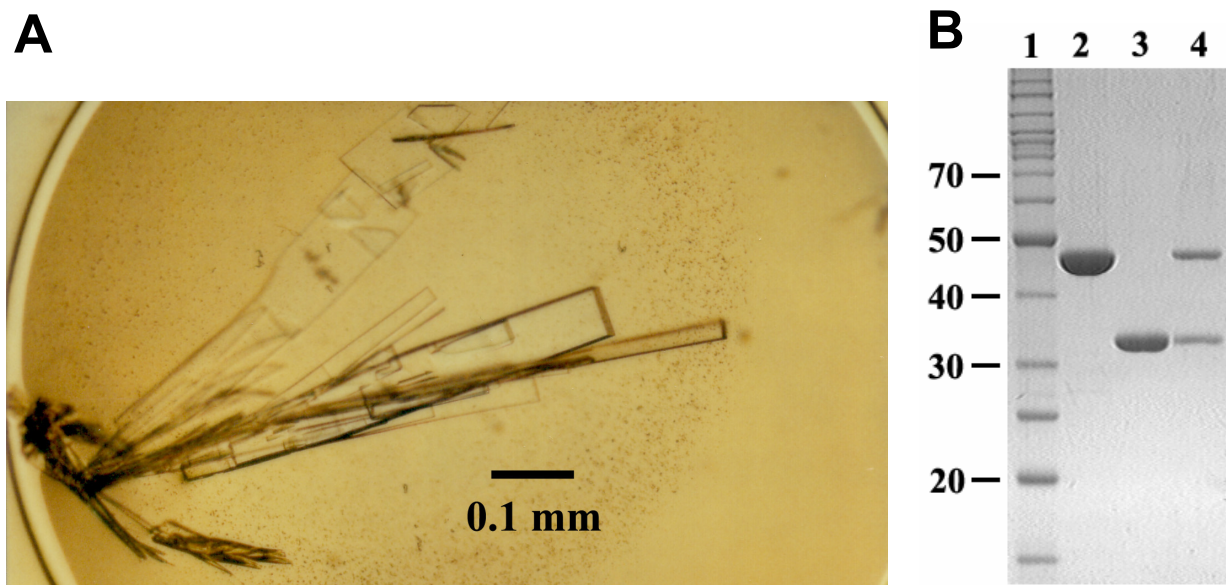


Figure 2-5. The crystal of the FEN1-PCNA complex.

(A) Orthorhombic crystals of the FEN1-PCNA complex. (B) FEN1 and PCNA in the crystals shown by SDS-PAGE. A stained 12.5% polyacrylamide gel is shown with molecular-weight markers (lane 1), purified human FEN1 (lane 2), purified human PCNA (lane 3) and the FEN1-PCNA complex crystal (lane 4).

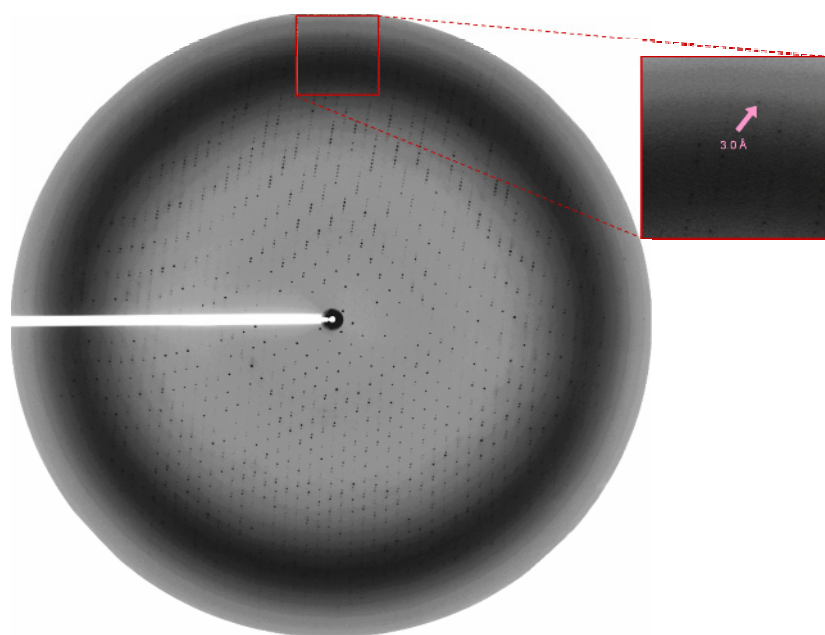


Figure 2-6. A diffraction image of the FEN1-PCNA crystal. The highest resolution was at 2.9Å resolution.

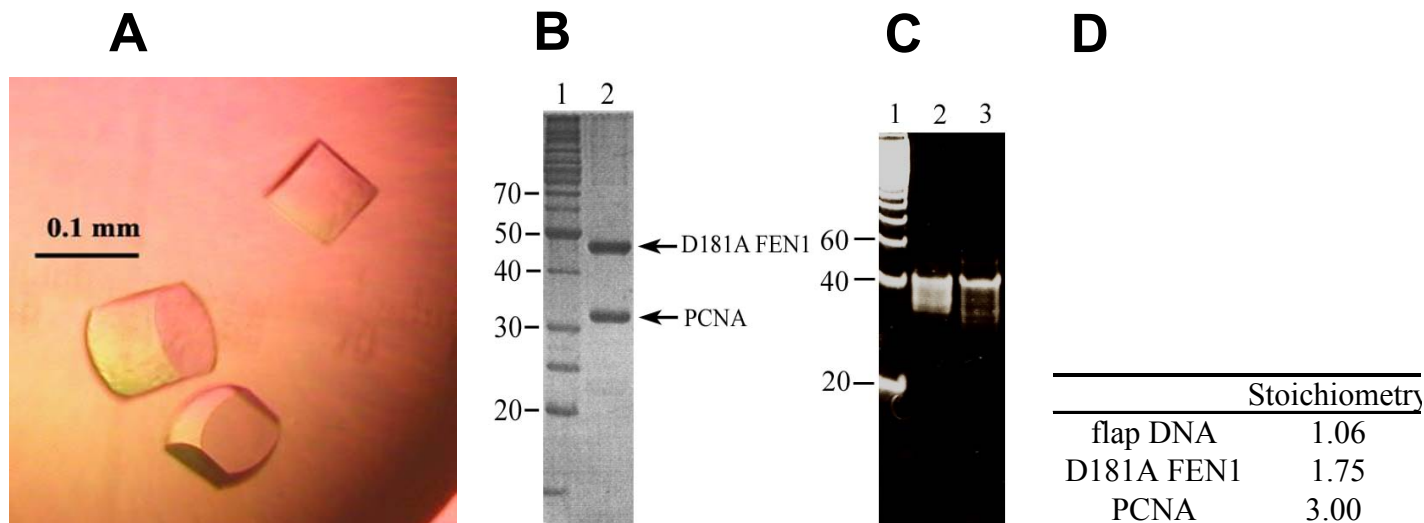


Figure 2-7. (A) Crystals of the flap DNA-FEN1(D181A)-PCNA complex. (B) FEN1(D181A) and PCNA in the crystals shown by SDS-PAGE. A stained 12.5% polyacrylamide gel is shown with molecular-weight markers (lane 1) and the flap DNA-FEN1(D181A)-PCNA crystal complex (lane 2). (C) Flap DNA in the crystals shown by native-PAGE. A stained 8.0% polyacrylamide gel is shown with base-pair markers (lane 1) and the DNA-FEN1(D181A)-PCNA crystal complex (lane 2). (D) Stoichiometry of flap DNA: FEN1(D181A): PCNA was estimated band intensity.

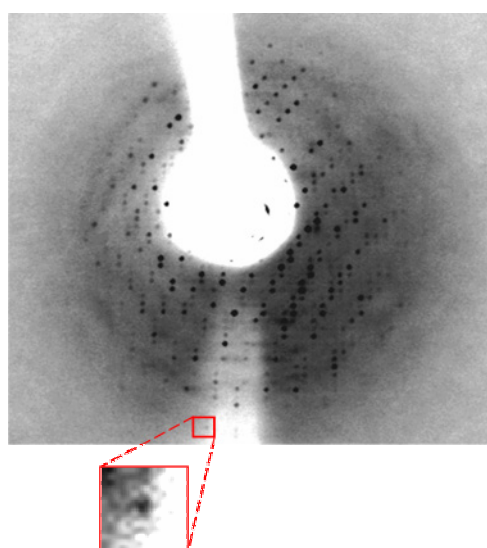


Figure 2-8. A diffraction image of the flap DNA-FEN1(D181A)-PCNA complex crystal. The highest resolution was observed at 6.5Å.

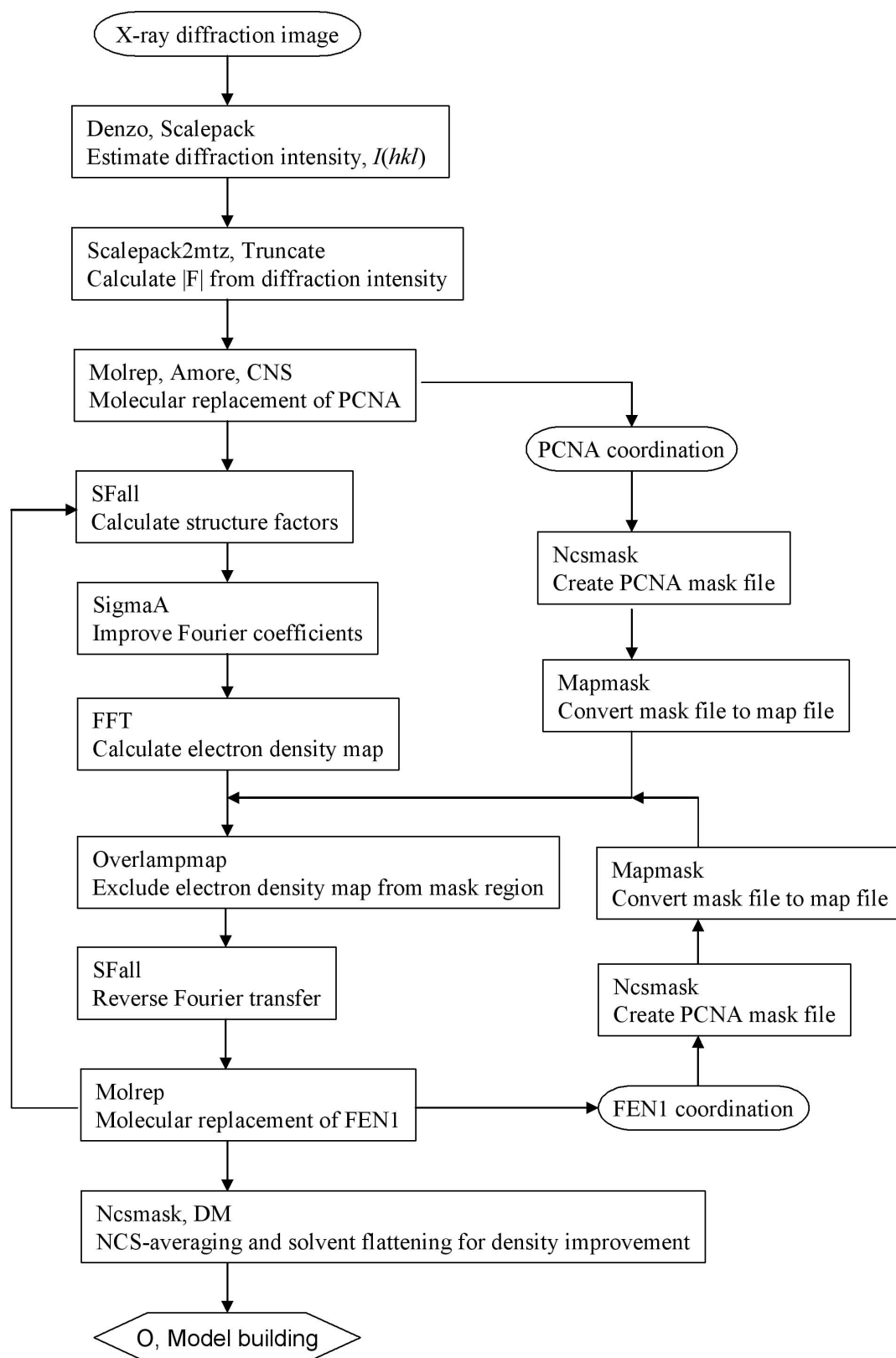


Figure 2-9. Flowchart of our initial phase determination of the human FEN1-PCNA complex by molecular replacement.

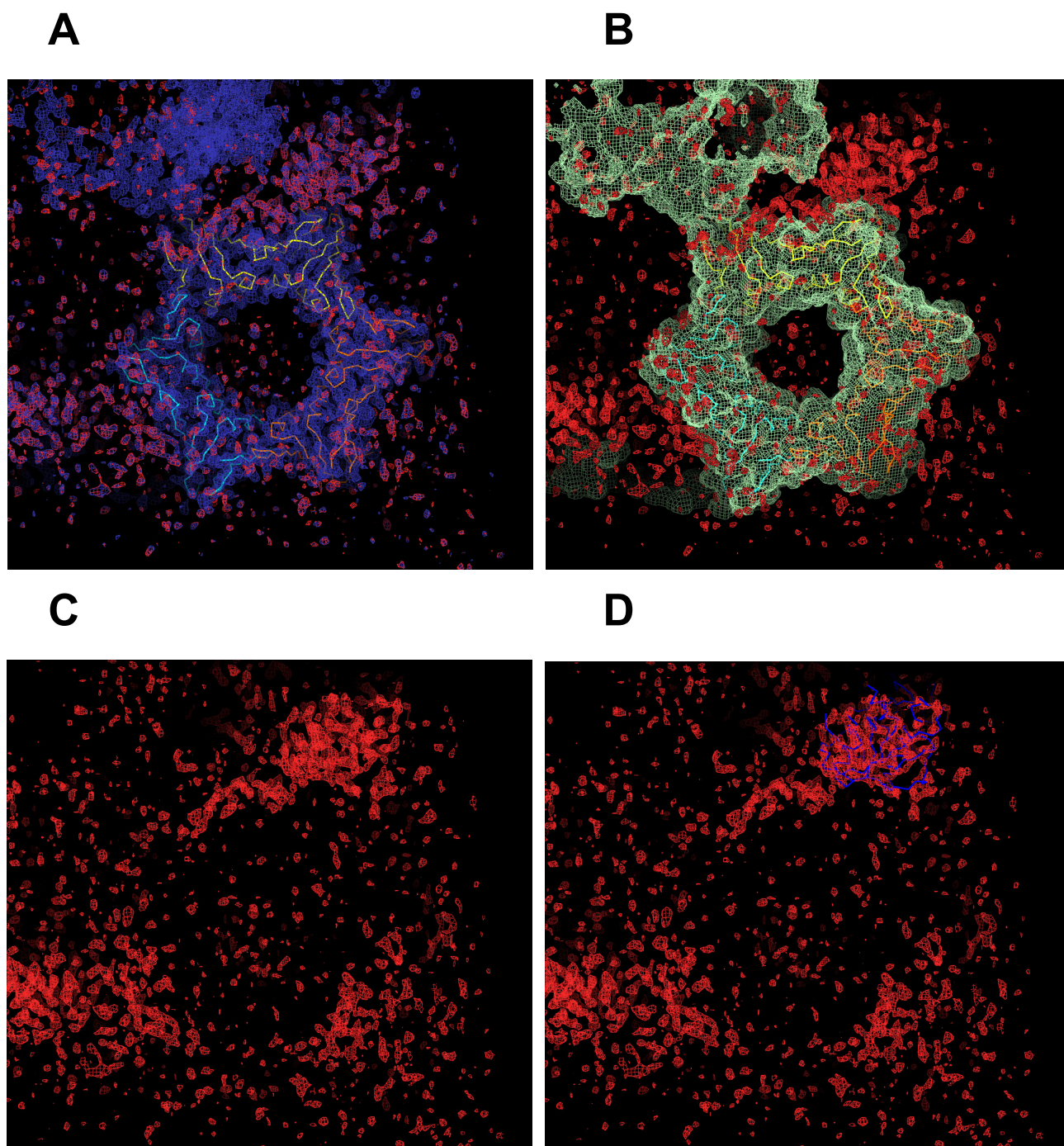


Figure 2-10. The electron density maps in the initial phase determination of the human FEN1-PCNA complex by MR. **(A)** Three PCNA monomers were determined with a conventional MR protocol. The Blue meshes are electron densities from a 2Fo-Fc map and red meshes from a Fo-Fc map, calculated using only the phase of the trimeric PCNA structure. **(B)** The mask region for PCNA was colored in lime. **(C)** The modified Fo-Fc electron density by excluding the mask region. **(D)** The first FEN1 solution was by MR is shown in blue.

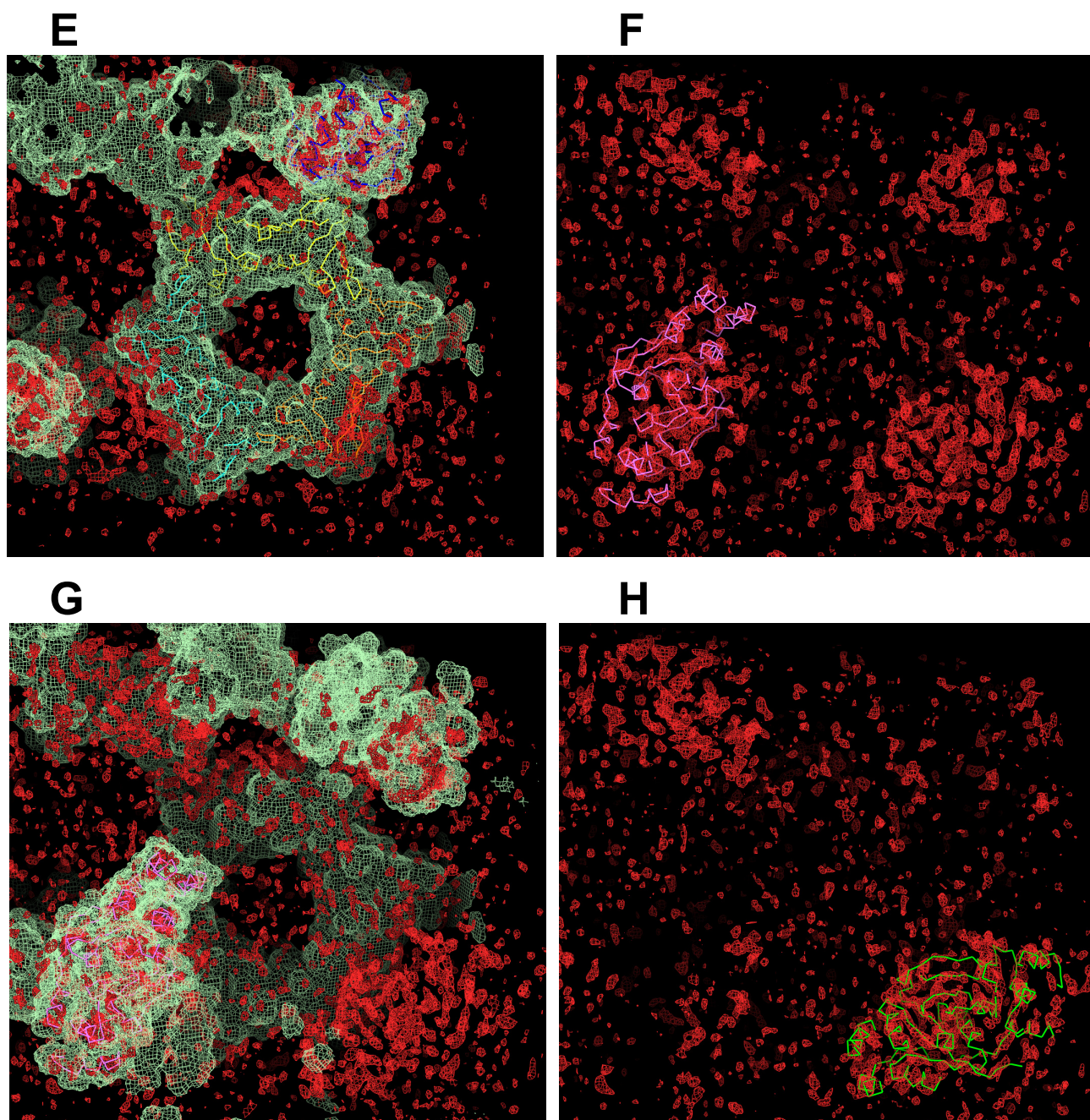


Figure 2-10. (E) The mask region for the PCNA and first FEN1 molecule was shown in lime. (F) The modified Fo-Fc map was recalculated using the phase of the trimer PCNA and the first FEN1 molecules. The second FEN1 solution was determined by MR using the structure factors calculated from this electron density. The second FEN1 molecule is colored in violet. (G) The mask region for the PCNA and two FEN1 molecules is shown by lime color meshes. (H) The final modified Fo-Fc density map recalculated using the phase of the trimer PCNA and two FEN1 molecules. The final FEN1 molecule was determined by MR is colored in green.

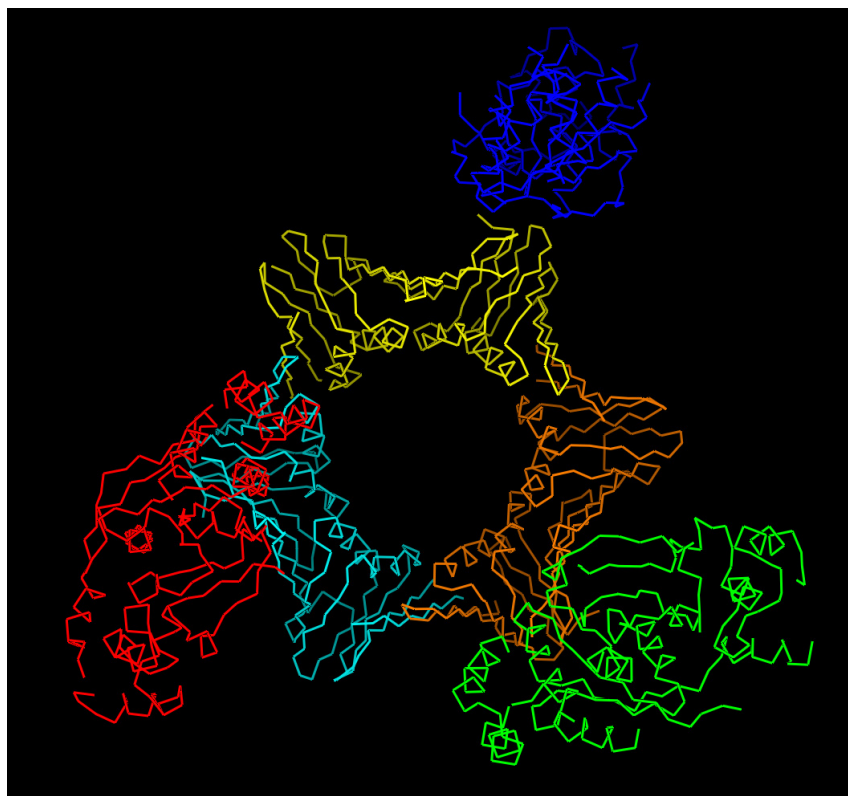


Figure 2-10. (I) The obtained FEN1-PCNA structure. Three FEN1 molecules are colored in blue (X), red (Y) and green (Z), and the three subunits of the PCNA trimer in yellow (A), cyan (B) and orange (C).

Results

Overall structure of the FEN1-PCNA complex

The crystal structure of human FEN1 complexed with human PCNA was determined at 2.9 Å resolution by the molecular replacement method (see Materials and methods and Figure 2-9). The structure revealed three FEN1 molecules (Figure 3-1, molecules X, Y and Z) bound to one PCNA trimer. Human FEN1 and PCNA consist of 380 (42 kDa) and 261 (29 kDa) amino acid residues, respectively. The total mass (213 kDa) of the complex calculated from the structure agreed with the results from EMSA, gel-filtration chromatography and dynamic light scattering, which yielded a monodisperse peak in solution (Figures 2-1A, 2-2 and Table 2-1) (Sakurai *et al.*, 2003).

Three PCNA subunits (subunits A, B and C in Figure 3-1) are tightly associated to form a closed ring. Each subunit contains two topologically identical domains, which are related by a pseudo 2-fold symmetry and are connected by the interdomain connector (IDC) loop (Figure 3-2A). Each domain consists of two β - α - β - β motifs that are also related by a pseudo 2-fold symmetry. Furthermore, these 6 domains are also related by a pseudo 6-fold symmetry in trimeric PCNA ring (Figure 3-2B). In the trimer, 6 β sheets form a circular outer layer that supports 12 α helices forming the inner surface. This architecture is essentially the same as those previously reported for PCNA (Gulbis *et al.*, 1996; Krishna *et al.*, 1994) and all secondary structural elements are preserved in our PCNA complex. The structures of human PCNAs bound to FEN1 and a p21 peptide are closely superimposable over the core secondary structural elements, with a small root-mean-square (rms) deviation (0.6 Å) for 247 C $_{\alpha}$ carbon atom positions in the trimer, while local structural differences are found in the IDC loops, caused by differences in the interactions with PCNA as described later. Other differences are

found within the loop of residues 184-194, which are visible in our complex but were invisible in the p21 complex. Each PCNA subunit binds one FEN1 molecule (molecules A, B and C bind X, Y and Z, respectively).

Structure of human FEN1

The structure of human FEN1 consists of the nuclease core domain (residues 1-332) and the C-terminal tail (residues 333-380) (Figure 3-3A). The N-terminal half (residues 333-359) of the C-terminal tail is visible, whereas the C-terminal half (residues 360-380) was not observed in the density map. It has been suggested that the C-terminal half, which is rich in lysine residues, is involved in DNA binding (Stucki *et al.*, 2001) and might be flexible without DNA. The visible C-terminal tail region forms two β -strands and one helical conformation. This β A- α A- β B motif represents the main interacting interface with PCNA. In fact, strand β A and the flanking helix α A are formed by the conserved short PCNA-binding motif QXXLXXFF among many PCNA binding proteins (Figure 1-4). Details of the FEN1-PCNA interactions are described later.

The core domain is folded into an α/β structure with a groove formed by a twisted seven-stranded mixed β -sheet in the topology β 1- β 7- β 6- β 5- β 2- β 3- β 4 and two helical regions; one formed by helices α 0 and α 5- α 11, and the other by helices α 1- α 4, α 12 and α 13 (Figure 3-3A). These two helical regions are located on both sides of the β -sheet, which forms a central groove. The region between strand β 3 and helix α 4 contains 46 residues that project from the main body of the domain. In the electron density map, 29 (residues 100-128), 31 (103-133) and 6 (114-119) residues of this large looping-out region were not observed for molecules X, Y and Z, respectively. This region contains many charged and hydrophobic residues, which are conserved in eukaryotic FEN1s. Hence, this region is thought to thread

along the single strand of the DNA substrate (Chapados *et al.*, 2004; Storici *et al.*, 2002; Tom *et al.*, 2000) and hereafter is referred to as the “clamp region”. A superimposition of the three FEN1 molecules in the crystal (Figure 3-3B) shows the diverse conformations of the clamp regions. The loop between helices $\alpha 2$ and $\alpha 3$ (loop $\alpha 2$ - $\alpha 3$) also seems to be flexible. Indeed, those for molecules X and Y are disordered. Finally, the C-terminal tails of the three FEN1 molecules project from the core domains in different directions. Excluding these flexible regions, the core domains of the three molecules are superimposed with a rms deviation of 0.8 Å (for 265 C $_{\alpha}$).

Comparison with archaeal FEN1s

The structure of human FEN1 (molecule Z) was compared with those of two archaeal FEN1s from the hyperthermophilic archaea, *Methanococcus jannaschii* (Hwang *et al.*, 1998) and *Pyrococcus furiosus* (Hosfield *et al.*, 1998) (Figures 3-3C). These archaeal FEN1s exhibit 34% and 38% sequence identity to human FEN1, respectively (Figure 3-4). From archaea to humans, the primary structures of the clamp regions used for potential DNA threading are mostly conserved without any deletion or insertion. In archaeal FEN1 and related bacteriophage enzymes, the corresponding regions of the clamp region have been referred to as a “helical arch” in T5 5'-exonuclease (Ceska *et al.*, 1996), or “L1 loop” or “helical clamp” in *M. jannaschii* (Hwang *et al.*, 1998) and *P. furiosus* (Hosfield *et al.*, 1998) FEN1s, respectively. In our crystal, this region contains only two short helices, αa and αb , each consisting of 4 residues. Even single amino acid substitutions in this clamp region of human FEN1 have been reported to significantly decrease the nuclease activity (Storici *et al.*, 2002). Deletion of the region in *M. jannaschii* FEN1 completely abolished the nuclease activity (Hwang *et al.*, 1998). However, no common structure was observed in the FEN1 structures

(Figure 3-3C). We believe that the clamp region of FEN1s would be intrinsically flexible, which could allow for effective tracking of flap DNA.

Compared with the archaeal FEN1s, human FEN1 possesses two major and one minor insertion site. One major insertion is located at the N-terminus (residues 9-15) and the other (residues 267-274) at the loop region between helices $\alpha 10$ and $\alpha 11$ (Figure 3-4). The former insertion forms an additional helix, $\alpha 0$, which packs against helices $\alpha 5$ - $\alpha 8$ and a strand $\beta 7$. The latter insertion induces re-orientation of helix $\alpha 10$ (Figure 3-3C). Helix $\alpha 0$ induces significant changes in the shape of the helical region on the left side of the central β -sheet, narrowing the central groove (Figure 3-3D). A minor insertion (2 residues) is located at loop $\alpha 11$ - $\alpha 12$. Human FEN1 has three one-residue deletion sites at loop $\alpha 2$ - $\alpha 3$, loop $\alpha 8$ - $\alpha 9$ and loop $\alpha 13$ - βA . In *P. furiosus* FEN1, insertion residues between $\beta 6$ and $\beta 7$ form an antiparallel β ribbon that forms part of the groove wall at the active site, whereas no insertion is present in human or *M. jannaschii* FEN1s.

The electrostatic potential surface of human FEN1 is comparable to that of *M. jannaschii* FEN1 (Figure 3-3D). The bottom of the clamp region forming the central cleft is dominated by negatively charged residues for the active site containing metal ions as described in the next section. Both sides of the central cleft contain basic residues (Figure 3-3D), which provide complementarily to the negative charges of the DNA substrate. These residues are highly conserved among eukaryotic FEN1s (Figure 3-3E). In human FEN1, these basic residues are located at helix $\alpha 0$, loop $\alpha 8$ - $\alpha 9$ and loop $\alpha 10$ - $\alpha 11$ on one side and helix $\alpha 3$, loop $\beta 6$ - $\beta 7$ and helix $\alpha 13$ on the other side. Compared with *M. jannaschii* FEN1, the negatively charged cleft of human FEN1 is more closed, partly due to the additional helix $\alpha 0$ at the entrance of the cleft. On the other hand, human FEN1 possesses a hydrophobic groove at the right side of clamp region, which are also highly conserved among eukaryotic FEN1s

(Figures 3-3D and 3-3E). This region corresponds to the 3'-flap binding site found in the crystal structure of *Archaeoglobus fulgidus* FEN1 bound to a 3'-flap DNA fragment (Chapados *et al.*, 2004).

Active site of human FEN1

Like archaeal FEN1s, the active site of human FEN1 is located at the bottom of the groove with two possible metal ions (Figure 3-3A). The active site of human FEN1 is formed by two clusters of conserved acidic residues (Figure 3-3F). Four residues (Asp34, Asp86, Glu158 and Glu160) form the first metal ion binding site (M1). Three residues (Asp179, Asp181 and Asp233) form the second metal ion binding site (M2). These acidic residues are conserved in all known FEN1 enzymes, and the prevailing catalytic mechanism is thought to be universal from archaea to humans. In the case of FEN1 molecules X and Y in our crystal, the electron densities for two metal ions were clearly observed in the difference Fourier map (Figure 3-3F). The density height for M1 is over 5σ in both X and Y, and that for M2 is 3σ and 5σ in X and Y, respectively. In the case of archaeal FEN1s, like our human FEN1, M2 appeared only at a lower electron density level (Hwang *et al.*, 1998, Hosfield, 1998 #2). Since no bivalent cations were added during the purification or crystallization procedures, two metal ions would have been tightly bound to FEN1 during these steps. In the present structure, these metal ions were assigned to Mg^{2+} . M1 is known to play an important role in catalysis, probably in the nucleophilic attack of the phosphodiester bonds of DNA, while it has been suggested that M2 is involved in DNA binding (Shen *et al.*, 1997). In molecule Z, which has a higher overall B-factor compared to the other two molecules (Figure 3-5 and Table 3-1), we failed to identify the high densities for two metal ions. In *P. furiosus* FEN1, the conserved Tyr234 (numbered for human) was also involved in the active site *via* a hydrogen bond to Glu158 and a

water-mediated contact with Asp181 (Hosfield *et al.*, 1998). In the present structure, this Tyr residue was observed at the same position, however the density for the water molecule was not identified, probably due to insufficient quality of the map.

FEN1-PCNA interactions

The interactions between FEN1 and PCNA are schematically summarized in Figure 3-6. Overall, the binding of each FEN1 to PCNA buries 2560 - 3580 Å² of molecular surface area (Table 3-2). The interface between FEN1 and PCNA consists of a peptide-protein interaction involving the extended C-terminal tail of FEN1 (residues 336-356) and a channel on the surface of PCNA, and also a protein-protein interaction involving the FEN1 core domain. The latter interaction is mainly mediated by the PCNA-bound FEN1 tail and the edge of each PCNA monomer. This bipartite interaction is reminiscent of that observed in the crystal structure of the complex between the LF domain of the *E. coli* Y-family DNA polymerase Pol IV and the β-clamp processivity factor (Bunting *et al.*, 2003), while the protein-protein interaction in the Pol IV-β-clamp complex involves two β-clamp monomers.

Peptide-binding interface

The interactions that are mediated by the βA-αA-βB motif located at the C-terminal tail of FEN1 bury about 2,350 - 2,660 Å² of total surface area at the interface and can be divided into three parts (Table 3-2). While the interaction mode resembles that found in the PCNA-p21 complex (Gulbis *et al.*, 1996), many differences are found. The short FEN1 strand βA (residues ³³⁶TQG³³⁸) binds the PCNA C-terminal residues (Ala252-Ile255) in the form of antiparallel β-β-like interactions (βA-C-term interactions) (Figures 3-6 and 3-7). The interactions involve the side chain of FEN1 Gln337, which is the key residue in the consensus

PCNA-binding motif and is conserved in other PCNA-binding proteins including p21 (Figure 1-4 and 3-4). The hydrogen bond from the main chain of PCNA Pro253 to the main chain of FEN1 Gly338 in our complex is replaced by a hydrogen bond to the side chain of p21 Thr145 in the PCNA-p21 complex. Two PCNA residues (Ala252 and Pro253) are completely conserved from archaea to humans. Among these residues, Pro253 might be involved in coordinating two oxygen atoms in the backbone of Ala252 and Pro253 toward β A of FEN1 (Figure 3-7). In fact, a double mutation of P252A and K253A (P253 and K254 in human) in yeast PCNA affected the stimulation of FEN1 activity (Gomes and Burgers, 2000).

Mutational studies showed that the β A-C-term interactions, especially the main-chain interactions, are important for enhancing the nuclease activity of human FEN1. Deletion of PCNA C-terminal residues Δ 8 (254-261) or Δ 7 (255-261) resulted in a substantial reduction in the stimulation of FEN1 nuclease activity without a significant reduction in the binding affinity to FEN1 (data not shown). The β -like structure of the PCNA C-terminal residues, ²⁵²APKI²⁵⁵, could act as a rigid joint that directs the FEN1 core domain toward the DNA substrate. In contrast, deletion of Δ 5 (257-261) did not affect the binding affinity or nuclease activity of FEN1. This C-terminal region of PCNA could not contribute to β A-C-term interactions and the extreme C-terminal residues (2, 3 and 6 residues for molecules A, B and C, respectively) of this region were disordered in our crystal.

Non-polar contacts are found between FEN1 helix α A (³³⁹RLDDFF³⁴⁴) and a large hydrophobic pocket within PCNA. The six FEN1 residues adopt a mixture of 3_{10} and α helical conformations at the present resolution. In the Y FEN1 molecule, the helix is stabilized by intramolecular interactions where the side chain of Asp342 accepts hydrogen bonds from both the main chain and side chain of Arg339. Three non-polar FEN1 residues (Leu340, Phe343 and Phe344) located on one side of the helix dock into the hydrophobic

pocket of the PCNA molecular surface. These interactions are similar to those in the PCNA-p21 complex. Interestingly, on the opposite side of the helix, FEN1 has an acidic residue, Asp341, which forms a salt bridge with PCNA His44 in our complex. The position corresponding to Asp341 in human FEN1 is replaced by threonine in p21, but is restricted to an acidic residue (Asp or Glu) in all FEN1s. While the position corresponding to His44 in human PCNA is restricted to a basic residue (His or Arg) in PCNAs, suggesting that salt bridge formation would represent a characteristic feature of FEN1-PCNA interactions.

Antiparallel β - β association (β B-IDC loop interactions) is formed between the FEN1 strand β B (residues 345-352) and the PCNA IDC loop (121-127). Our observation of this β - β interaction that has been found in the PCNA-p21 complex is somewhat surprising given the lack of sequence homology between FEN1 and p21 strand β Bs. Consequently, side chain-side chain interactions between the associated β strands in our complex are different from those in the PCNA-p21 complex. In our complex, two FEN1 strand β B non-polar residues (Val346 and Leu350) make contact with hydrophobic residues from PCNA: Val346 makes contact with Leu126 of the PCNA IDC loop and Leu350 is packed against PCNA non-polar residues Cys27 and Ala67, located at the small hydrophobic pocket formed at the junction between the IDC loop and the underlying β sheet of the PCNA N-terminal domain. In the PCNA-p21 complex, however, electrostatic complementarity seems to be dominant. Two basic residues located at strand β B of the p21 peptide form salt bridges with negatively charged PCNA residues, while the small hydrophobic pocket of PCNA accommodates a hydrophobic residue of the p21 peptide, which corresponds to Leu350 of FEN1. As previously pointed out (Gulbis *et al.*, 1996), the molecular surface of the human PCNA trimer involved in peptide binding is negatively charged. In our complex, a FEN1 basic residue (Arg355) located at the N-terminal flanking region of strand β B forms a salt bridge with a PCNA acidic residue (Asp120) at the

N-terminal end of the IDC loop. Moreover, a notable difference is found in the main chain-main chain interactions. In the PCNA-p21 complex, strand β B of p21 forms a well aligned β - β interaction with the IDC loop residues, whereas in the FEN1-PCNA complex a one-residue insertion of Gly348 in strand β B interrupts a regular hydrogen bond network between the main chains in the β - β interaction (Figure 3-6A). This distortion results in a slight decrease in the number of interactions with PCNA, which might be one of the reasons why the binding affinity of FEN1 to PCNA ($K_d = 60$ nM) is lower than that of p21 ($K_d = 10$ -15 nM) (Chen *et al.*, 1996). The archaeal FEN1s completely lack the residues to form these interactions (Figure 3-4).

The aforementioned FEN1-PCNA interactions are essentially conserved in all three FEN1 molecules in our complex. Figure 3-8A shows the superimposition of the β A- α A- β B motifs derived from the three FEN1 molecules, resulting in a small rms deviation (0.6 Å), together with the p21 peptide at the PCNA-binding region: the peptide is superimposed on FEN1 with a rms deviation of 1.3 Å. The relatively large rms deviation between FEN1 and p21 is due to the aforementioned one-residue insertion in FEN1 β B. Overlap of the binding sites on the PCNA molecular surface could account for the observed inhibition by p21 of the binding of FEN1 to PCNA by replacing and occupying the entire binding site on PCNA.

Protein-protein interface

The FEN1 core domains are involved in intermolecular contacts with its C-terminal tail bound to the PCNA groove, in addition to intermolecular contacts with PCNA. The average temperature factors of the C-terminal tail are smaller than those of the core domain and approximately comparable to those of PCNA (Table 3-1 and Figure 3-5). Therefore, the C-terminal tail seems to be part of PCNA rather than that of FEN1. The interface involving

the core domain of FEN1 molecule Y resembles to that of molecule Z (Figure 3-9), although the two FEN1 molecules within the complex make different lattice contacts in the crystal. This strongly suggests that their similar orientation with respect to PCNA is determined by the stability of that particular interaction with PCNA and is not an artifact of crystal packing. The interface for A-X contains different areas from those for B-Y or C-Z (Figure 3-10), while residues at the interfaces are conserved among eukaryotic FEN1s and PCNAs (Figure 3-11), implying a potential biological importance of these interfaces.

At the heart of the interface, helix $\alpha 1$ and strand $\beta 2$ from the FEN1 core domain make contact with strand βA and helix αA from the PCNA-bound FEN1 tail (Figures 3-3A and D left): the main chain of Ser25 of the core domain forms hydrogen bonds with the conserved Arg339 of the C-terminal tail. In Z, an additional salt bridge is formed between Arg29 of the core domain and Asp342 of the C-terminal tail helix αA . The hydrophobic side chain of Phe27 of the core domain is stacked on Arg339 of the C-terminal tail. In Y, this interaction is replaced with the hydrophobic interaction with Val45 of PCNA. FEN1 molecules Y and Z display a similar orientation of their core domains relative to the PCNA ring, thus preserving the PCNA central hole that is utilized in the interaction with DNA.

The interface involving the core domain of FEN1 molecule X is much larger than those of molecules Y or Z (Table 3-2 and Figure 3-9A). Thus, the X-position may be the more populated “inactive” form and the other two forms may be “intermediate” on the way to a cleavage-competent position. Strands $\beta 2$ and $\beta 3$ and loop $\alpha 11$ - $\alpha 12$ from the FEN1 core domain make contact with PCNA residues at the C-terminal flanking region of the IDC loop and the C-terminal tail: four FEN1 core domain residues (Arg29, Lys30, Lys80 and Trp298) interact directly with three PCNA residues (Gln131, Asp232 and Glu258). Furthermore, a hydrophobic interaction is formed between Phe27 of the core domain and Arg339 of the

C-terminal tail. The FEN1 molecule X displays a different orientation of its core domain compared to molecules Y and Z, although the PCNA trimer preserves the central hole that is utilized in the interaction with DNA.

The FEN1 hinge region

The FEN1 core domain is linked to the PCNA-binding tail by a short linker (Figures 3-6 and 3-7). We found that this linker, ³³³QGST³³⁶, functions as a hinge (Figure 3-8A). The torsion angles of these residues are markedly different among the three FEN1 molecules (Figure 3-8B). Interestingly, the temperature factors of these residues are markedly higher than those for other regions (Figure 3-5B and Table 3-1). This hinge region connects helix α 13 of the core domain and strand β A of the C-terminal tail, thus providing a degree of freedom that allows for a possible swing motion of the core domain. The QGST motif of the hinge region is conserved in higher eukaryotic FEN1s (Figure 3-8C).

We investigated the importance of the short linker in terms of the nuclease activity of FEN1 bound to PCNA by assaying the ability of PCNA to stimulate the nuclease activity of FEN1 and FEN1 deletion mutants that possess a shorter linker (or no linker) between the core domain and the PCNA-binding tail (data not shown). Deletion of the linker residues resulted in decreased nuclease activity while complete deletion of the linker, Δ QGST, exhibited no stimulation by PCNA. These results clearly indicate the importance of the loop, probably acting as a hinge, to direct the FEN1 core domain toward the DNA substrate.

In our complexed structure, the nuclease core domains of the Z and Y FEN1 molecules swing about $\sim 90^\circ$ and $\sim 100^\circ$, respectively, from that of molecule X around Arg332 toward the front of the PCNA ring (Figure 3-12A). During this swing displacement, the active site tracks on a large spherical surface with a radius of 40-50 Å. FEN1 locates its core domain

in front of the PCNA C-terminal face, while the active site of molecules Y and Z face toward the conceivable position occupied by the DNA substrate. The swing-in motion might be utilized in threading the flap DNA through the clamp region. Suppose that dsDNA is passing through the center of PCNA ring as in Figure 3-12B and that the DNA occupies a linear orientation, approximately 50° more swing-in displacement of the FEN1 core domain would be needed to place its active site on the cleavage site (the junction of dsDNA and flap-ssDNA). A similar displacement would also be needed for a proposed kinked DNA substrate (Chapados *et al.*, 2004) since the upstream duplex of the kinked DNA would run through PCNA just as a linear DNA duplex.

Table 3-1. The average temperature factors of FEN1 and PCNA

	Molecule					
	X	FEN1 Y	Z	A	PCNA B	C
Overall ($\text{\AA}^2$)	55.1	77.3	83.2	35.9	40.0	46.9
Core domain ($\text{\AA}^2$)	54.4	79.0	84.2			
Hinge region ($\text{\AA}^2$)	148.0	72.5	98.4			
C-terminal tail ($\text{\AA}^2$)	44.7	42.8	58.6			

Table 3-2. Buried surface area of FEN1 and PCNA

	Buried surface area		
	A-X	B-Y	C-Z
PCNA – overall FEN1 ($\text{\AA}^2$)	3581	2922	2558
PCNA – the FEN1 C-terminal tail($\text{\AA}^2$)	2352	2660	2511
PCNA* – the FEN1 core domain ($\text{\AA}^2$)	1432	623	601

* PCNA plus the FEN1 C-terminal tail

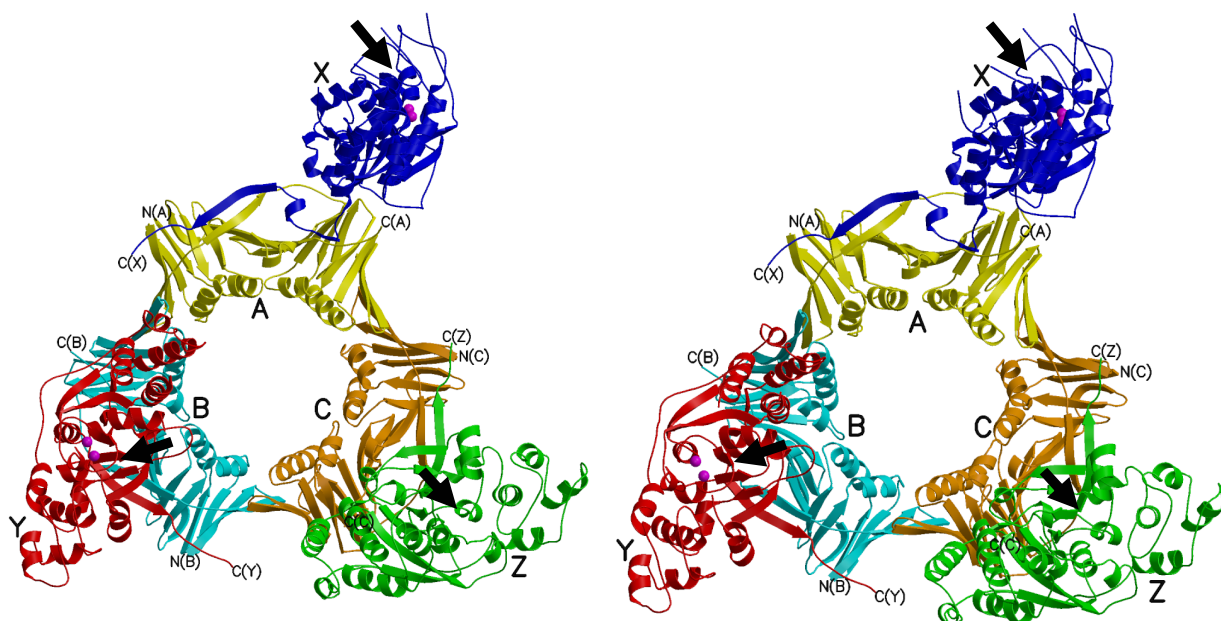


Figure 3-1. A stereo view of the human FEN1-PCNA complex. Three FEN1 molecules are colored in blue (X), red (Y) and green (Z), and the three subunits of the PCNA trimer in yellow (A), cyan (B) and orange (C). The C-termini of FEN1 and PCNA are labeled. Metal ions bound to the active sites of FEN1 (X and Y) are shown in magenta. Proposed catalytic faces of FEN1 are indicated by arrows.

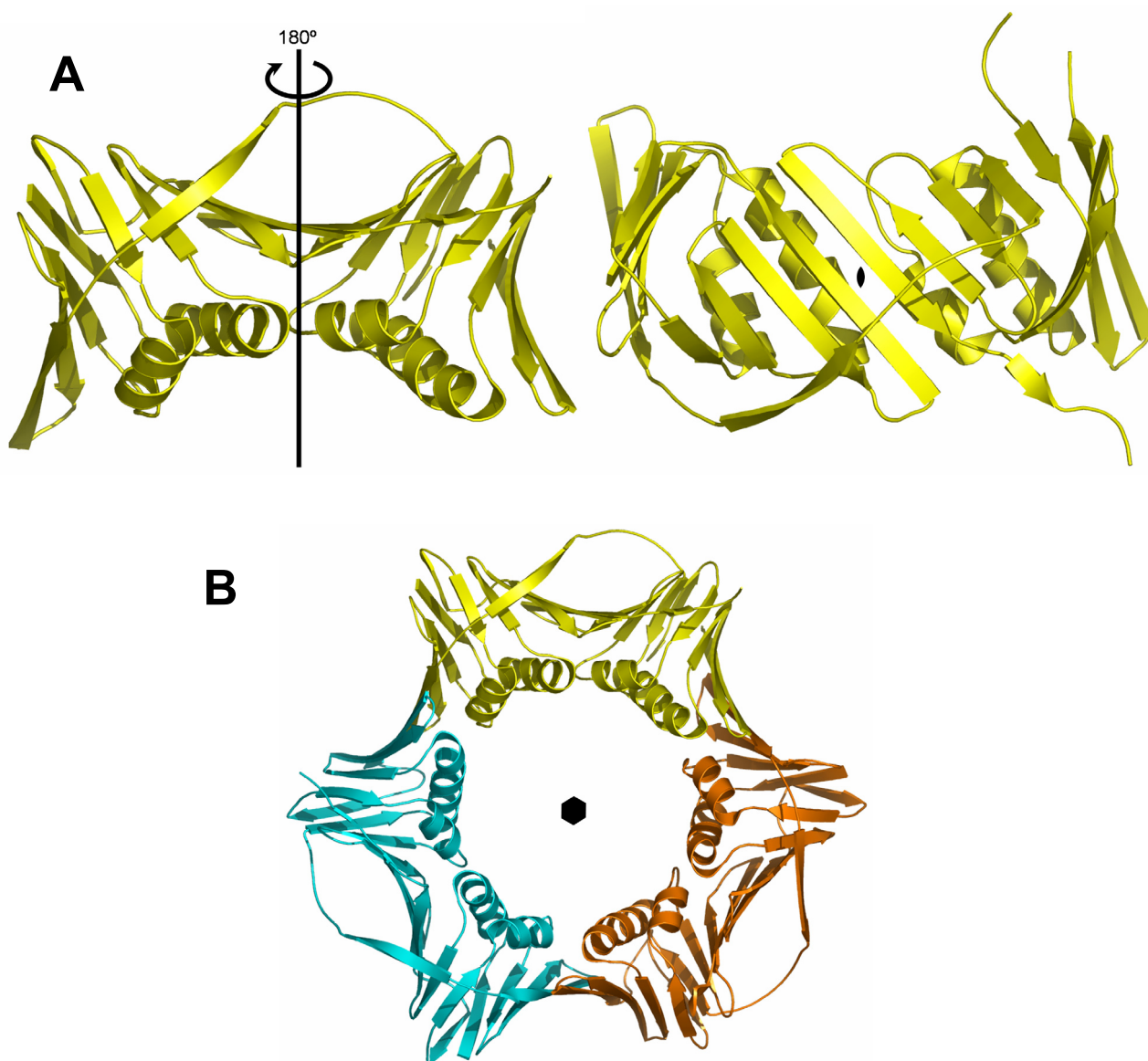


Figure 3-2. Pseudo symmetry in both the PCNA subunit and trimer.

(A) One PCNA subunit has two topologically domains, which are related by a pseudo 2-fold symmetry. The 2-fold symmetry axis is showed by a vertical line (*left*) or a central mark (*right*). The left image is viewed from the same as Figure 3-1. The right image is rotated by 90° around the horizontal axis from the left. **(B)** The 6-fold pseudo symmetry axis is showed with a central hexagonal mark. The orientation viewed and molecules colors are the same as in Figure 3-1.

A

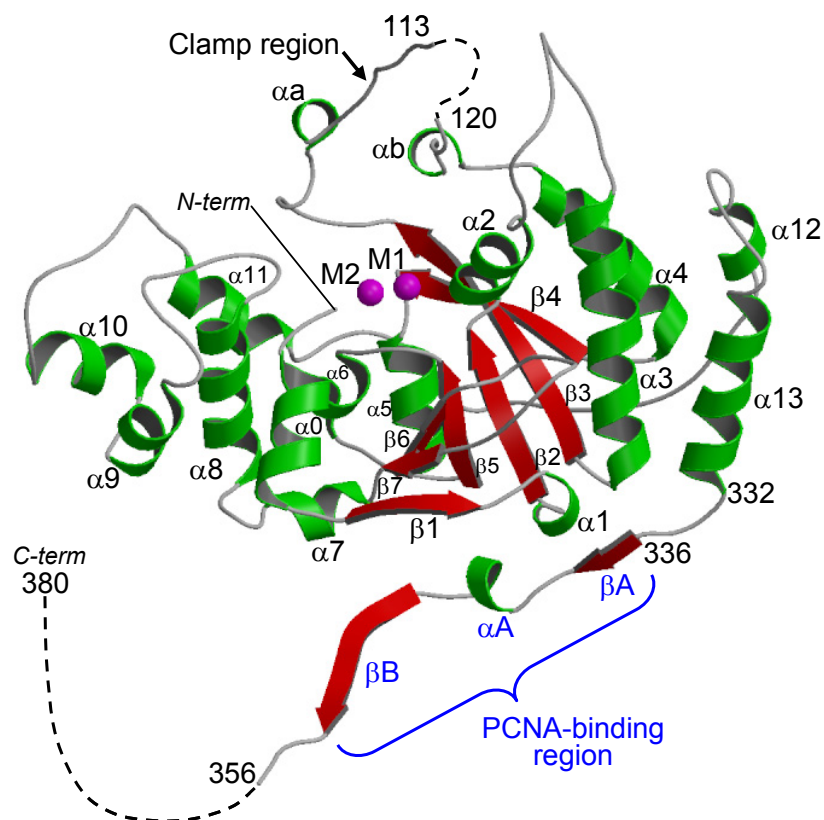


Figure 3-3. Structure of human FEN1. All diagrams of FEN1 molecules are viewed from the same view point as in **A**.

(**A**) Ribbon model of the molecular structure of human FEN1 (molecule Z). The α -helices and β -strands are shown in green and red, respectively. Two metal ions (M1 and M2) in the active site of molecule X are superimposed and depicted in magenta. Secondary structures of the core domain are labeled in black, and those in the C-terminal tail interacting with PCNA are labeled in blue.

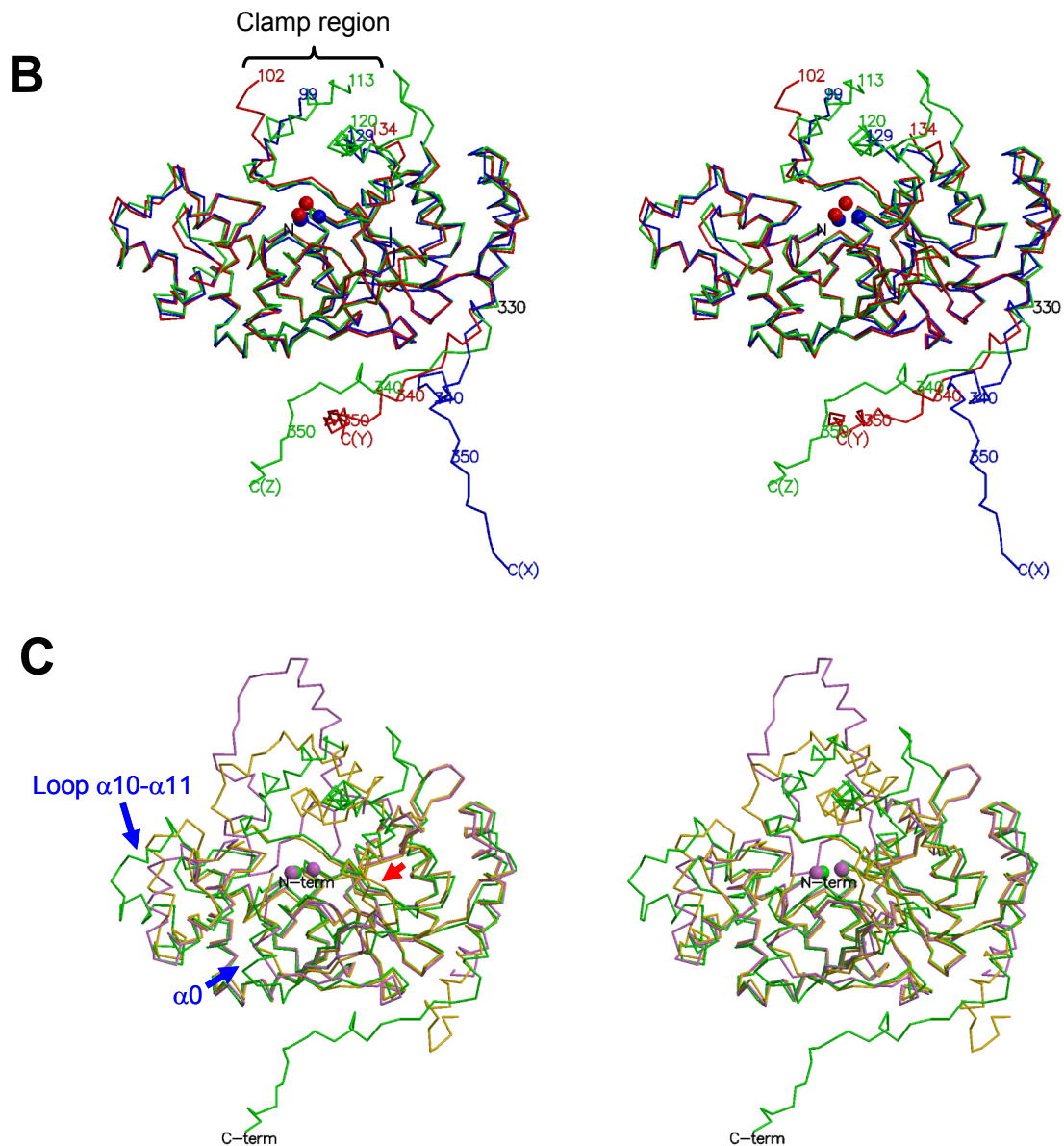


Figure 3-3. (B) A stereo diagram showing superimposition of three human FEN1 molecules (X, Y and Z) including metal ions. (C) Each molecule is shown in the same color as in Figure 3-1. A stereo diagram showing superimposition of human FEN1 (green), *M. jannachii* FEN1 (Hwang *et al.*, 1998) (violet) and *P. furiosus* FEN1 (Hosfield *et al.*, 1998) (gold). Two major insertion sites ($\alpha 0$ and loop $\alpha 10-11$) and deletion sites are indicated by blue and red arrows, respectively.

D

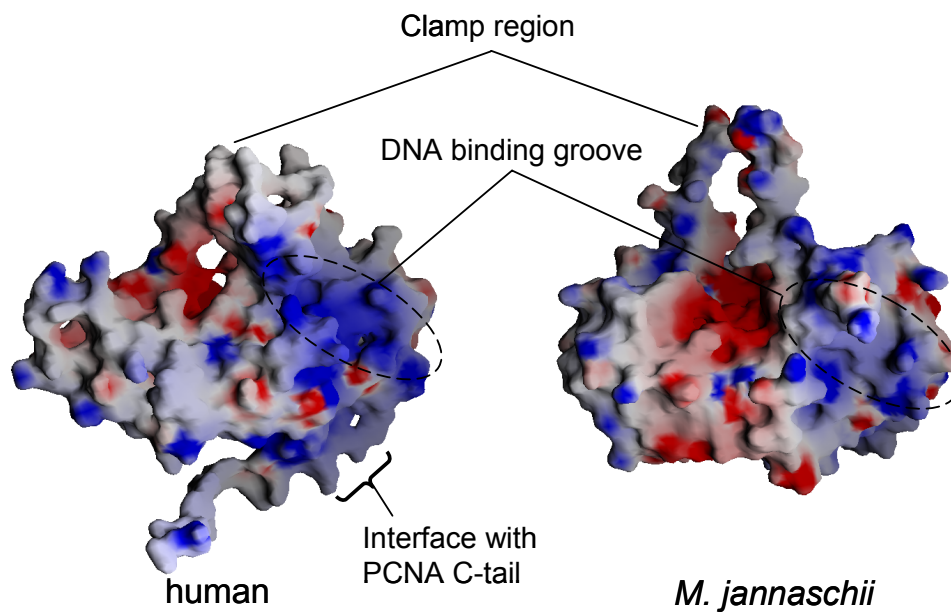


Figure 3-3. (D) Electrostatic molecular surfaces of human and *M. jannaschii* FEN1s. Each surface was calculated using GRASP, and colored in the range <-10 to $>+10 k_B T$, where k_B is the Boltzman constant and T is the temperature. The positive potential is shown in blue and the negative potential is in red.

E

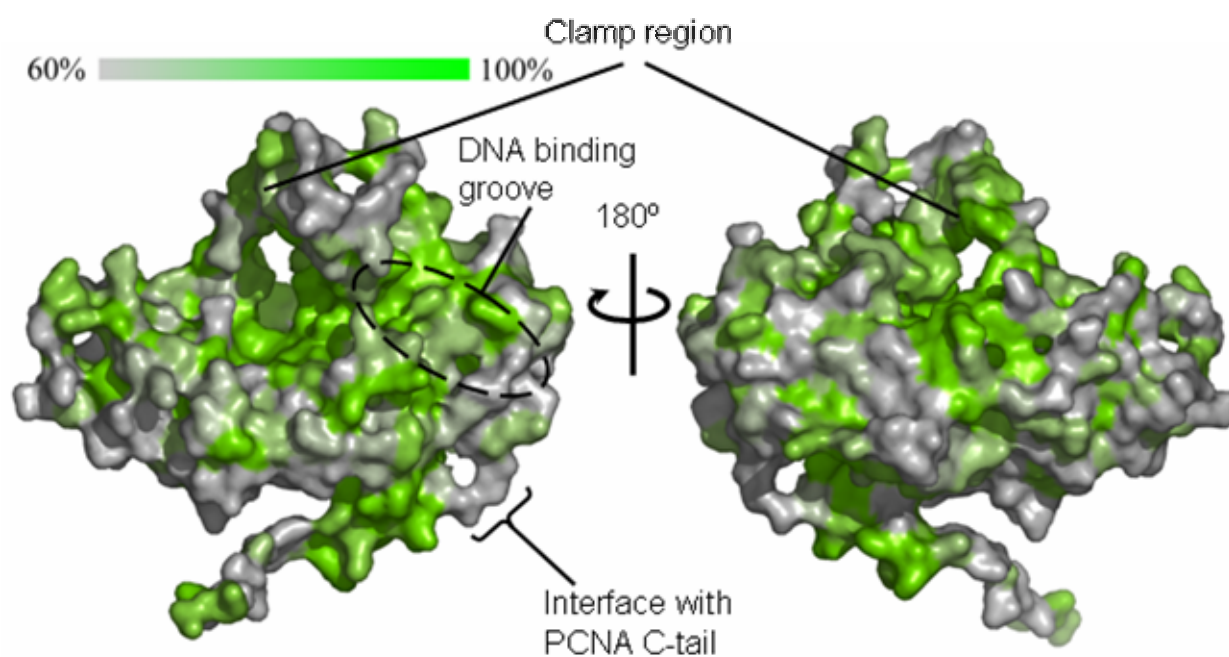


Figure 3-3. (E) Conserved residues mapped on the molecular surface of human FEN1 (Z molecule), according to color-bar on the top. Conservation of surface residues was calculated using multiple-alignments of eukaryotic FEN1s. The orientation of left image is the same as in (A). The right image is rotated by 180° around the vertical axis.

F

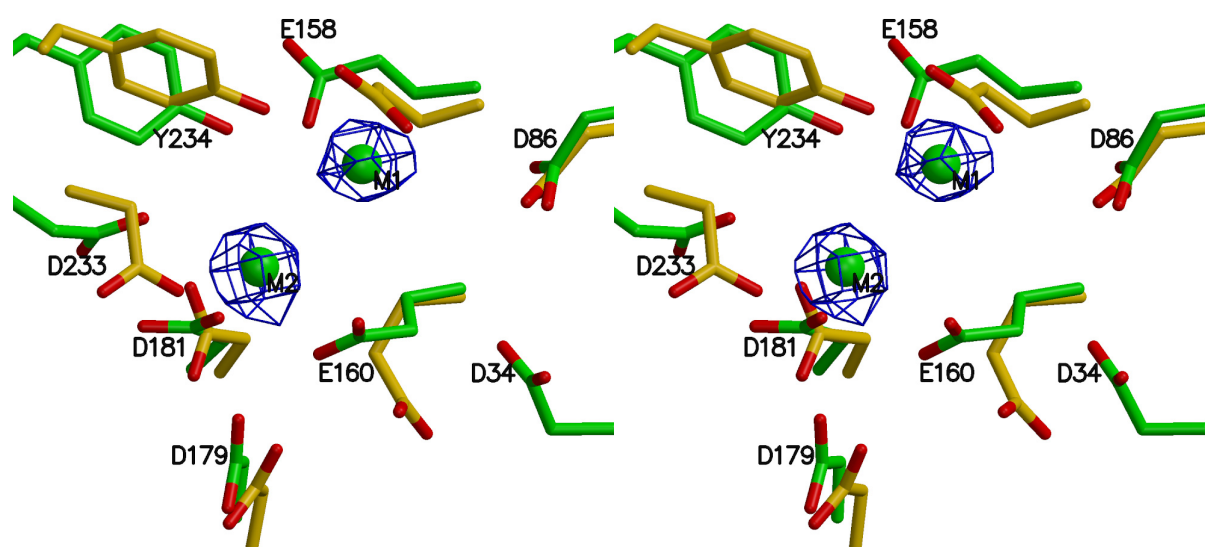


Figure 3-3. (F) A closed-up stereo view of the active sites of human (green, molecule Y) and *P. furiosus* (gold) FEN1s. The orientation viewed is approximately that of a 90° rotation from *a* along the horizontal axis. The omit $F_o - F_c$ electron density map for the two metals (M1 and M2) are shown in blue (contoured at 5σ). The metal ions and side chain of Asp34 in *P. furiosus* FEN1 have not been deposited in PDB.

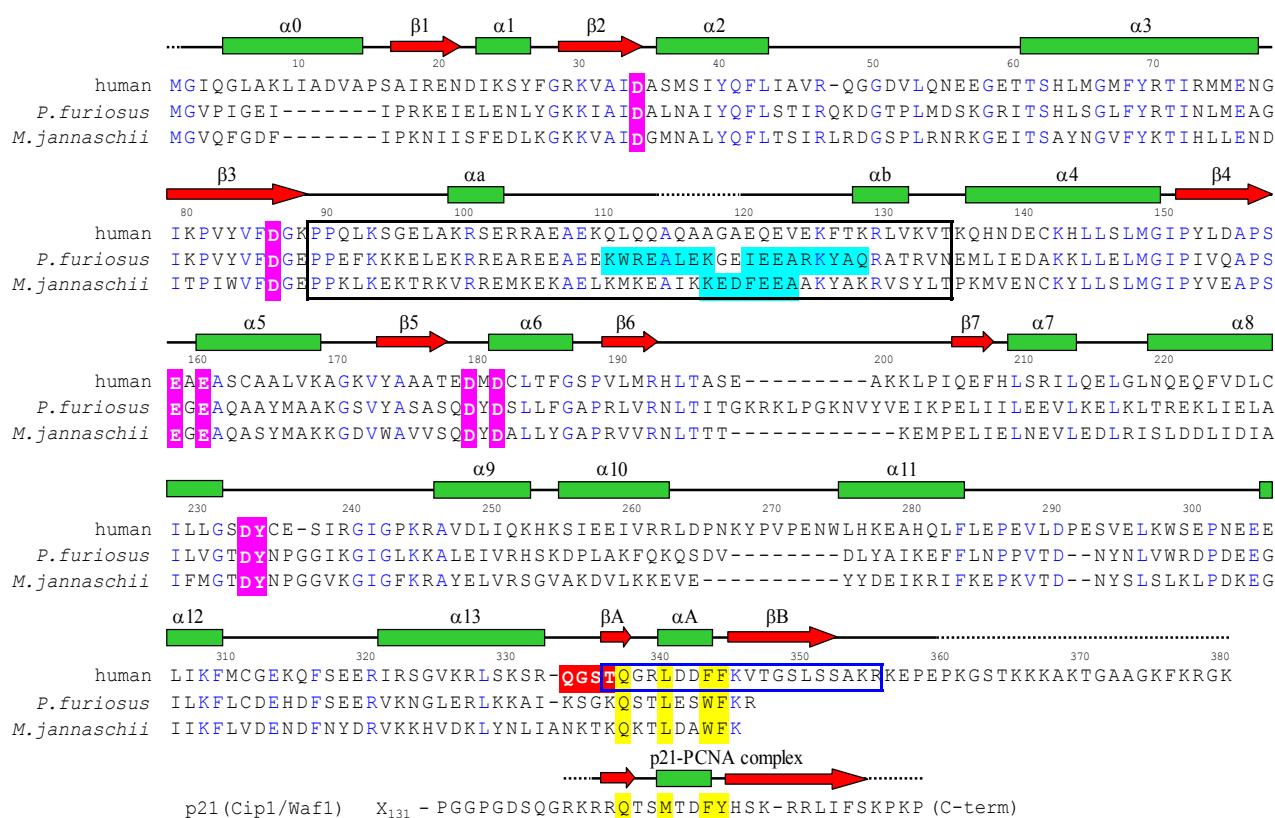


Figure 3-4. Aligned amino acid sequences of the possible flexible hinge regions and the consensus motifs for PCNA binding from FEN1s and the related human enzymes. Residues of the possible flexible hinge regions are boxed and small amino acid residues in the region are in bold and highlighted in red. Residues in the consensus motif for PCNA binding are in bold and highlighted in yellow. The number of amino acids to the N- and the C-terminus is indicated.

A

10Å 160Å

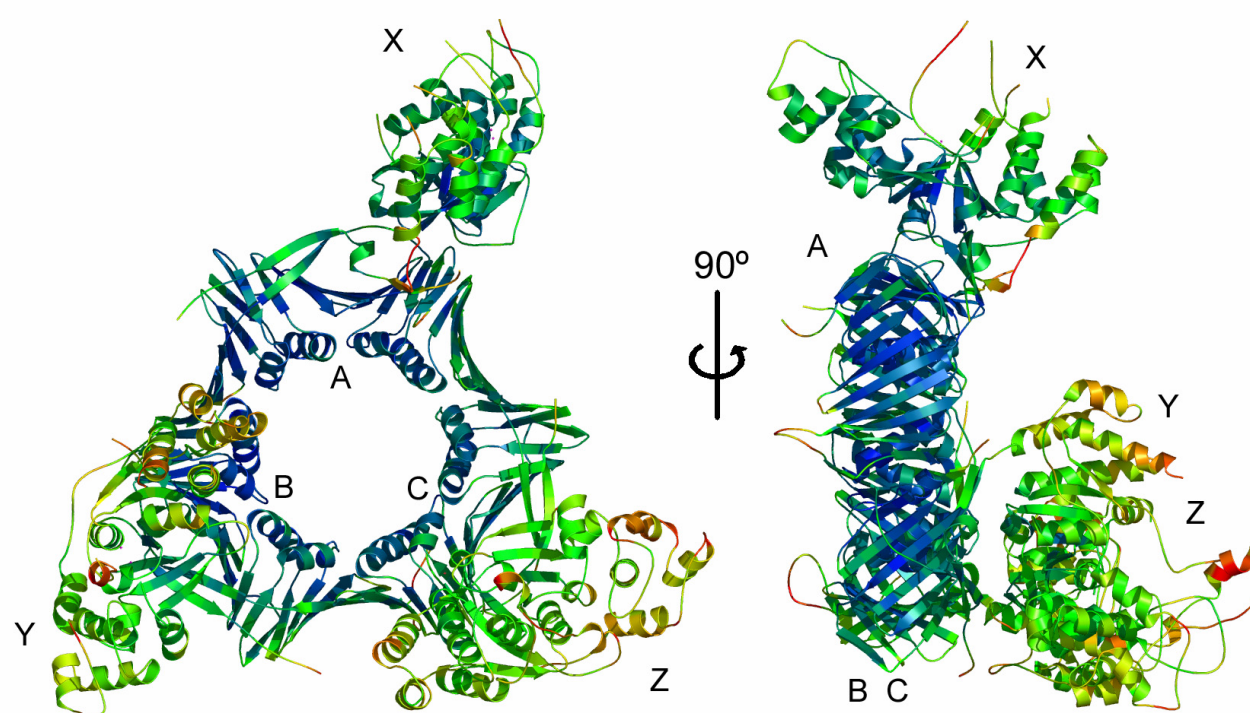


Figure 3-5. Temperature factors of FEN1 and PCNA.

(A) Temperature factors are mapped on the ribbon model of the FEN1-PCNA complex, according to the color-bar on the top. The right image is rotated by 90° around the vertical axis.

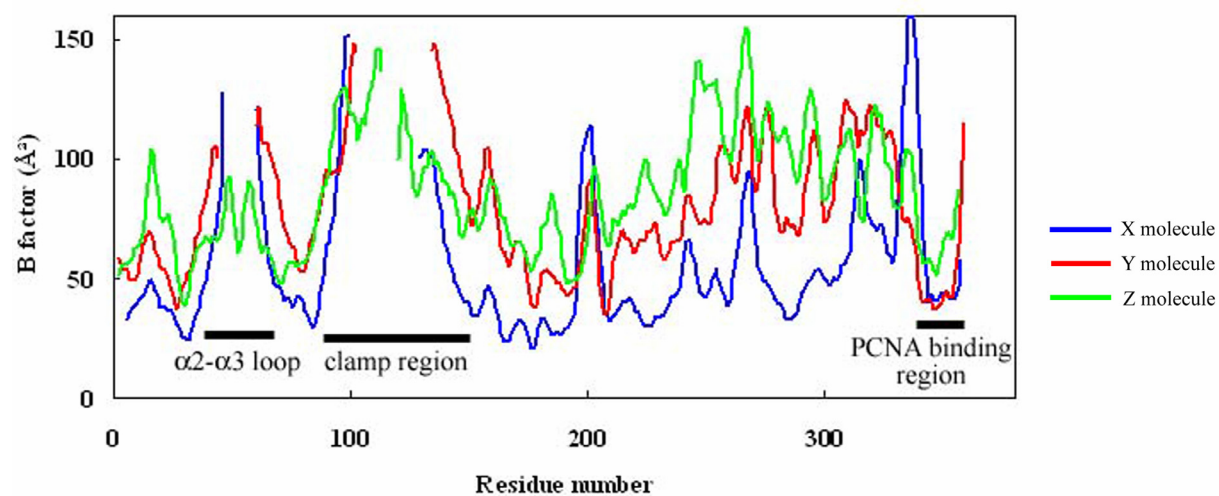
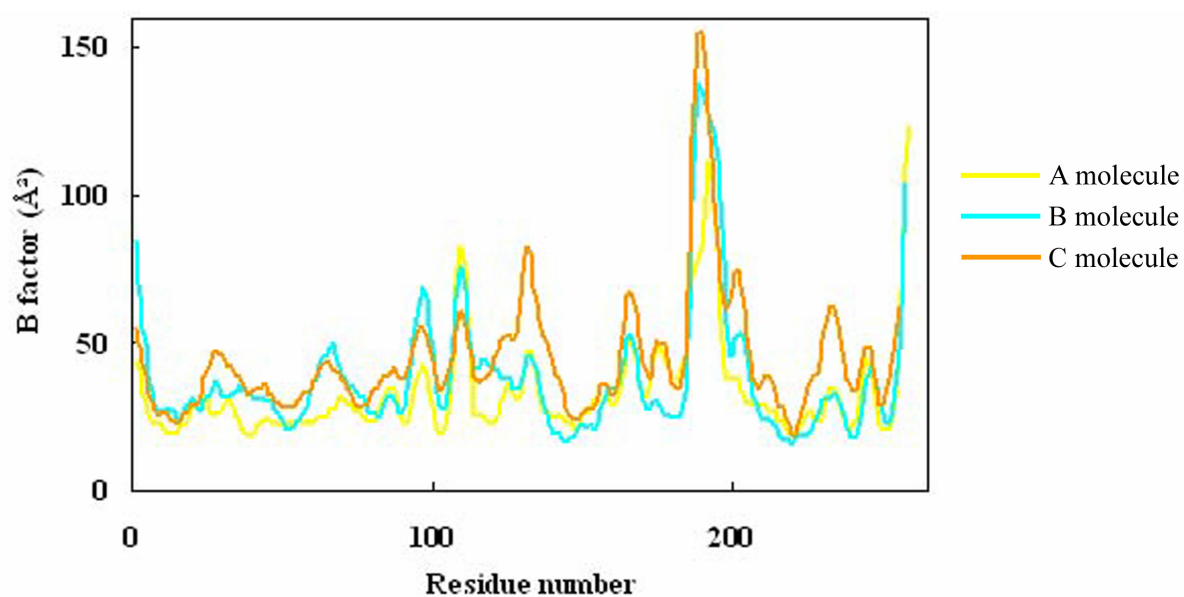
B**C**

Figure 3-5. (B) Temperature factors of FEN1 and PCNA are plotted versus the residue number. Three FEN1 molecules are in blue (X), red (Y) and green (Z). **(C)** The three subunits of PCNA are in yellow (A), cyan (B) and orange (C).

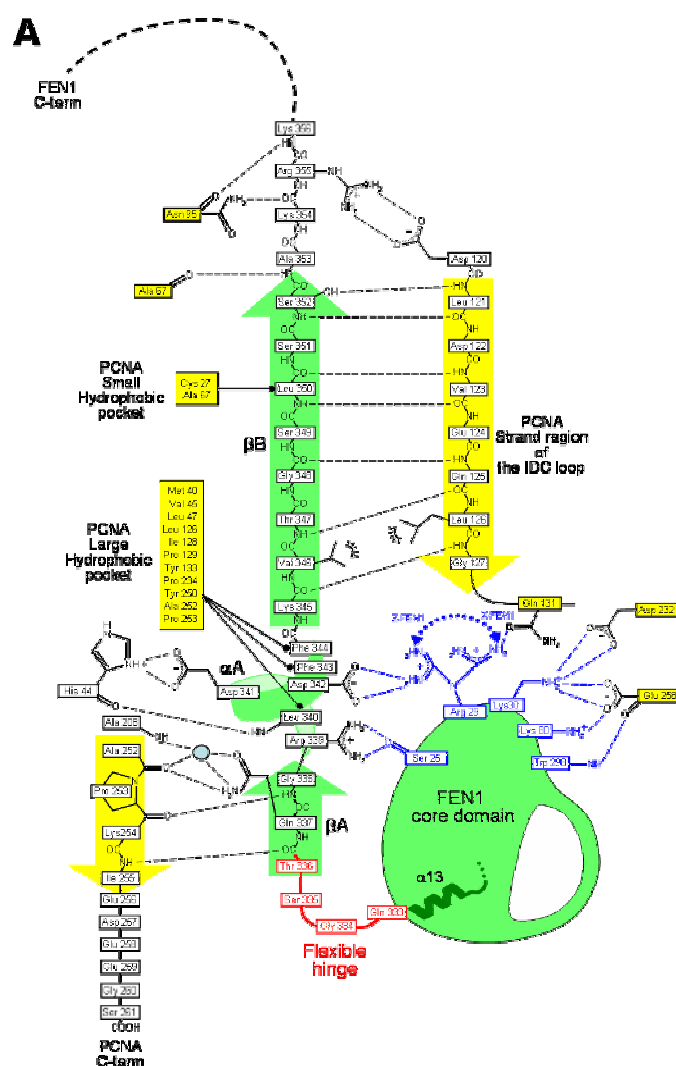


Figure 3-6. Interactions between FEN1 and PCNA. Schematic depiction of interactions between FEN1 and PCNA.

PCNA and FEN1 residues are shaded yellow and green, respectively. The flexible hinge region is colored in red. Residues shown in blue participate in protein-protein interactions in one or two FEN1 molecules out of three: Ser25 in Y and Z, Arg29 in Z and X, and Lys30, Lys80 and Trp298 in X (see text). A water molecule observed between molecules Z and C is indicated by a blue circle. In the β A-C-term interactions between molecules Z and C, Gln337 forms water-mediated hydrogen bonds to PCNA main chains (Ala208 and Ala252) in an identical interaction that was observed in the PCNA-p21 complex.

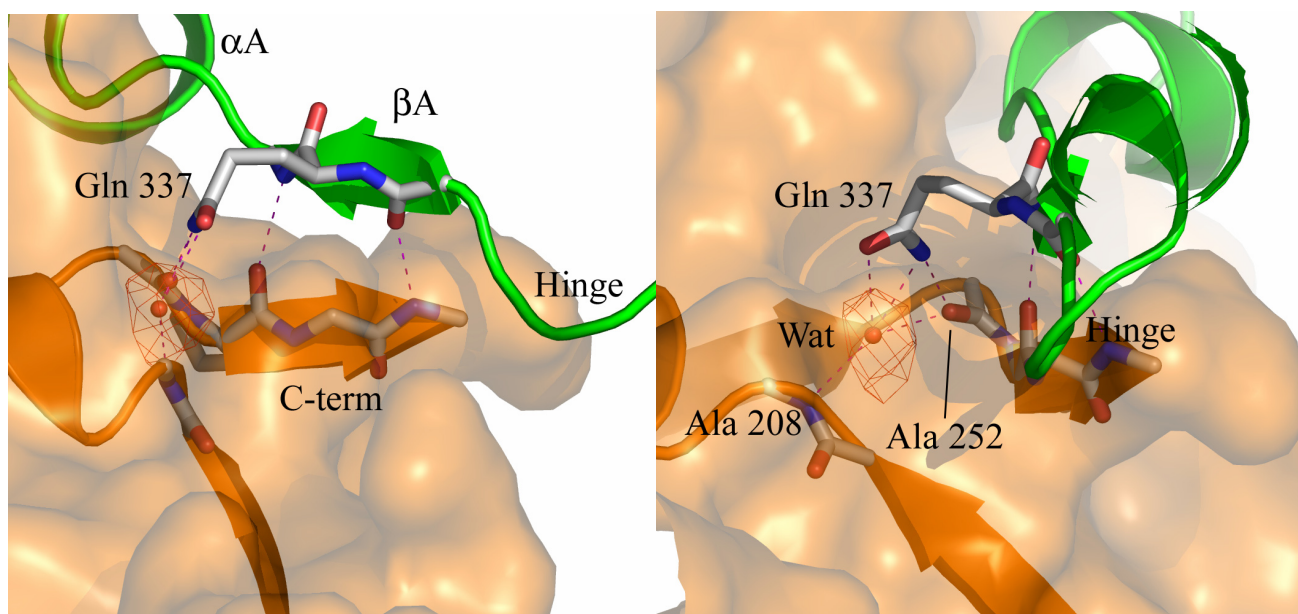


Figure 3-7. Close-up views of the β A-C-term interactions (C and Z molecules). The β A strand of FEN1 and the C-terminal residues of PCNA is shown by ribbon and stick models with part of the PCNA surface colored in semi-transparent orange (molecule C). Hydrogen bonds are shown in magenta dot lines. The omit $F_o - F_c$ electron density for water molecules are shown in red (contoured at 5σ).

A

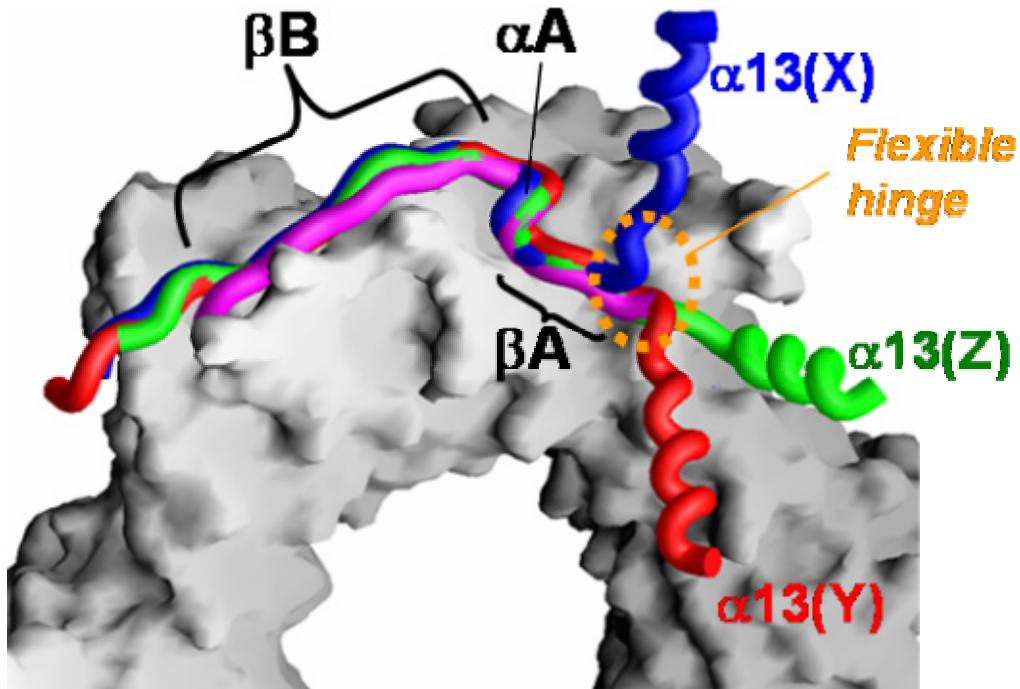
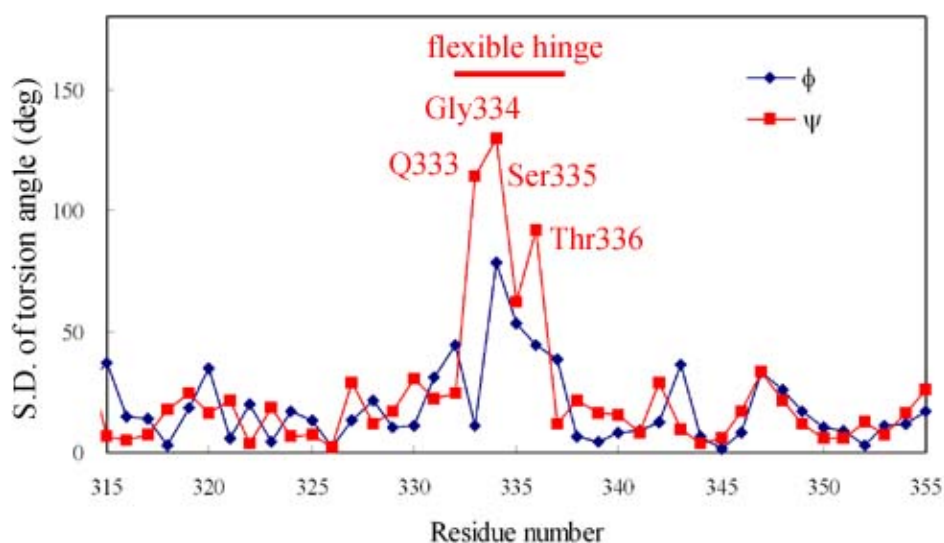


Figure 3-8. The hinge region of human FEN1.

(A) Superposition of the C-terminal tails from three human FEN1 molecules. The C-terminal tail plus helix $\alpha 13$ from three FEN1 molecules (in the same colors as in Figure 3-1) and the p21 peptide (magenta) (Gulbis *et al.*, 1996) illustrated as backbone-worm models are superimposed on part of the PCNA ring illustrated as a surface model. The orientation viewed is the same as in Figure 3-1 (PCNA-subunit A at the top). The hinge regions are marked with an orange circle with a dashed line.

B



C

FEN-1, human	G	V	K	R	L	S	K	S	R	Q	G	S	T	Q	G	R	L	D	D	F	F	K	V	T	G	S	L	S	S	A	K	R	K	E	-X ₂₃
FEN-1, <i>Rattus norvegicus</i>	G	V	K	R	L	N	K	S	R	Q	G	S	T	Q	G	R	L	D	D	F	F	K	V	T	G	S	L	S	S	A	K	R	K	E	-X ₂₃
FEN-1, <i>Mus musculus</i>	G	V	K	R	L	S	K	S	R	Q	G	S	T	Q	G	R	L	D	D	F	F	K	V	T	G	S	L	S	S	A	K	R	K	E	-X ₂₃
FEN-1, <i>Xenopus laevis</i>	G	A	K	K	L	A	K	N	R	Q	G	S	T	Q	G	R	L	D	D	F	F	K	V	T	G	S	V	S	S	T	K	R	K	E	-X ₂₅
FEN-1, <i>Caenorhabditis elegans</i>	A	L	A	K	L	K	T	S	R	K	S	G	T	Q	G	R	I	D	S	F	F	G	N	S	T	K	V	T	C	V	T	A	A	T	-X ₂₅
Rad27, <i>Saccharomyces cerevisiae</i>	G	I	S	R	L	K	K	G	L	K	S	G	I	Q	G	R	L	D	G	F	F	Q	V	V	P	K	T	K	E	Q	L	A	A	A	-X ₂₃
FEN-1, <i>Pyrococcus furiosus</i>	G	L	E	R	L	K	K	A	I	K	S	G	K	Q	S	T	L	E	S	W	F	K	R												

Figure 3-8. (B) Standard deviation of torsion angle among three FEN1 molecules is plotted versus residue number around flexible hinge. **(C)** Aligned amino acid sequences of the putative hinge regions and the consensus motifs for PCNA binding derived from eukaryotic FEN1s. Residues within putative hinge regions are boxed and small amino acid residues in the region are in bold and highlighted in red. Residues within the consensus motif for PCNA binding are in bold and highlighted in yellow. The number of amino acids to the N- and the C-terminus is indicated.

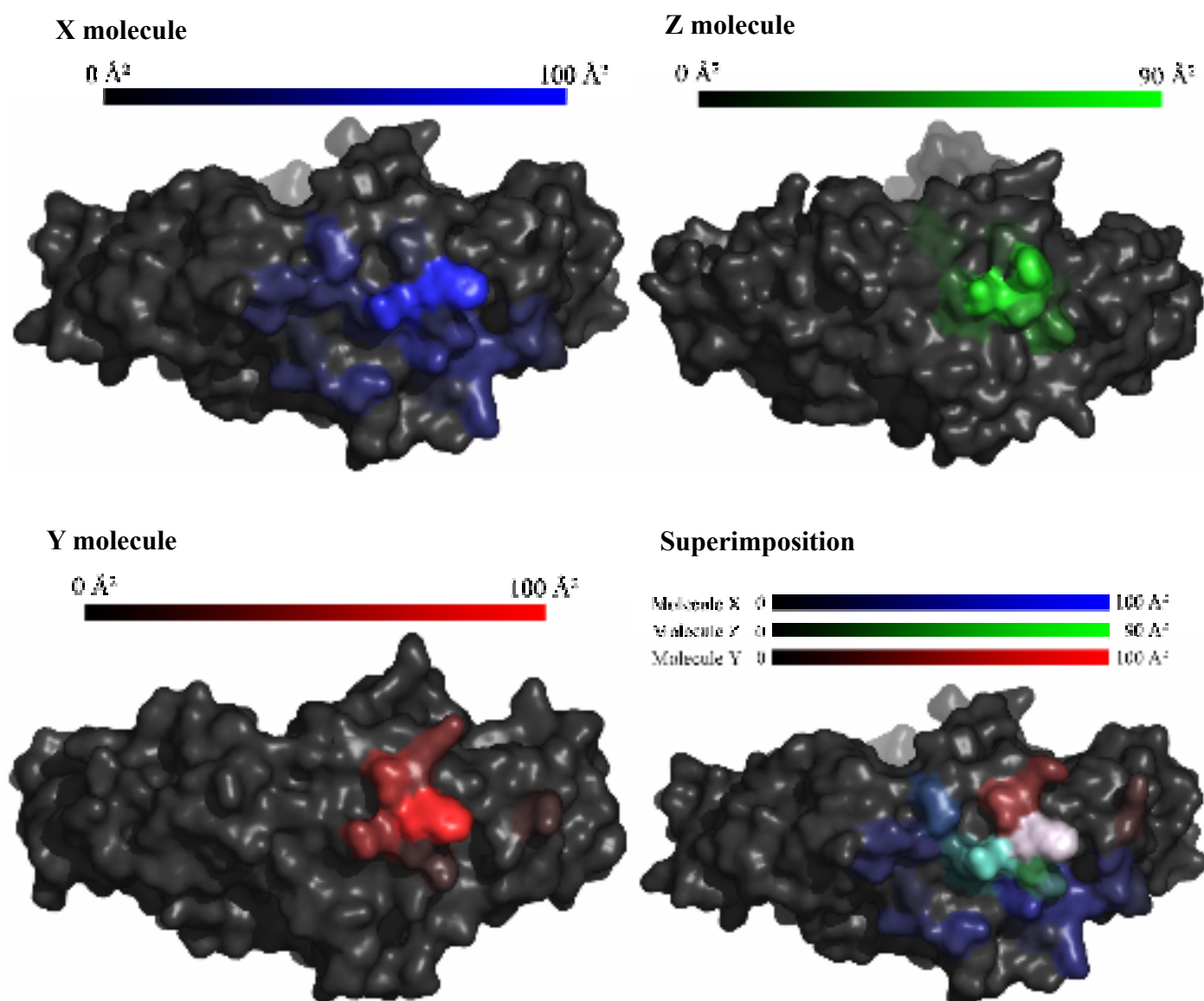


Figure 3-9. Contact residues mapped on the molecular surface of the FEN1 core domain. Residues of the FEN1 core domain (residue 2-332) contacted with PCNA residues and the C-terminal tail residues of FEN1 are mapped. Contact areas of each residue are calculated by the program AREAIMOL (Collaborative Computational Project, 1994), and colored in blue (X), red (Y) and green (Z) on the core domain. Three colors are superimposed in X molecule (the bottom right). Phe27 is colored in white as a result of superimposing three colors. The orientation of the core domain is rotated by 90° around the horizontal axis from Figure 3-3A, shown bottom side of core domain.

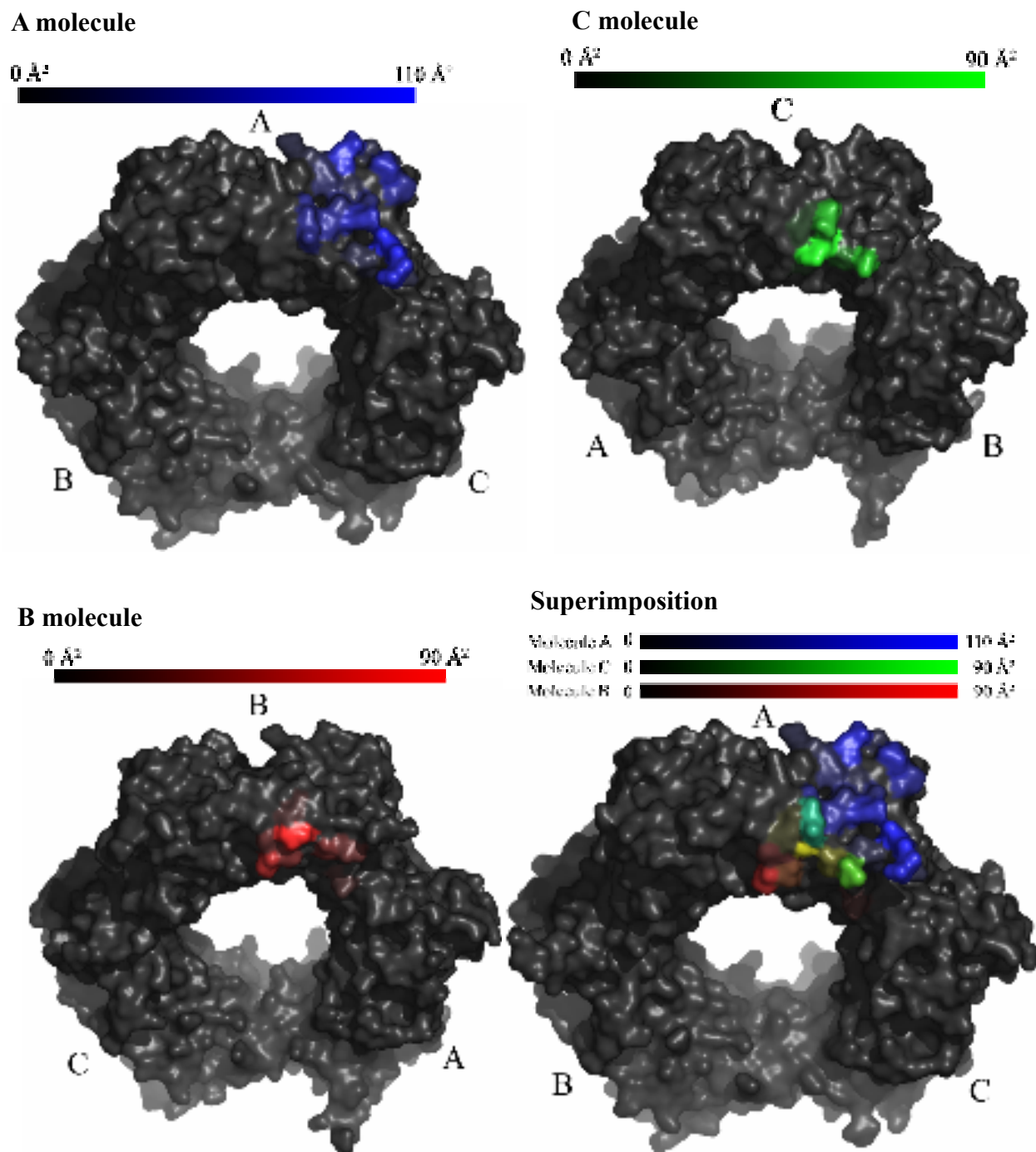


Figure 3-10. Contact residues mapped on the molecular surface of PCNA.

PCNA residues and the C-terminal tail residues of FEN1 contacted with residues of the FEN1 core domain (residue 2-332) are mapped. Contact areas of each residue are colored in blue (A:X), red (B:Y) and green (C:Z). Three colors are superimposed in A molecule (the bottom right image).

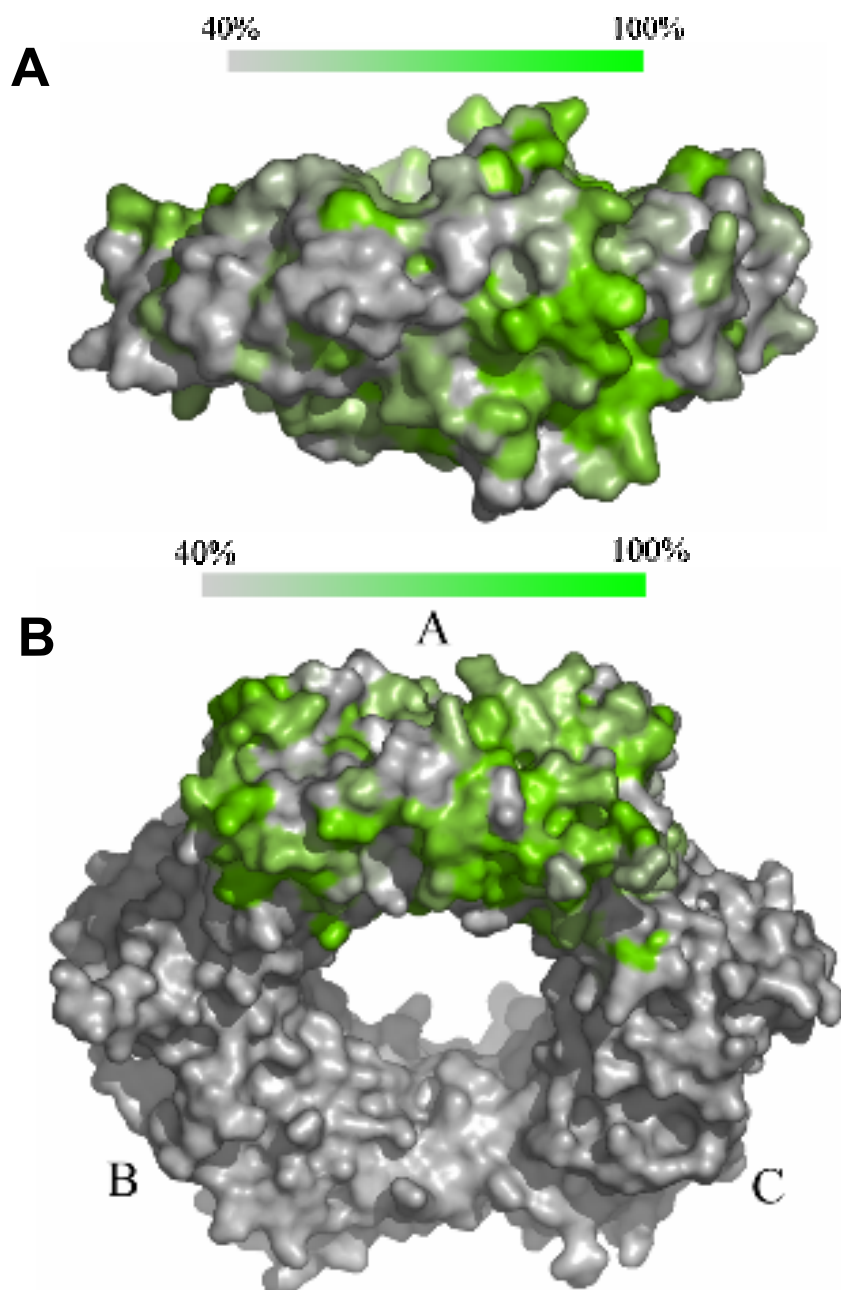


Figure 3-11. Conserved residues mapped on the molecular surfaces.

(A) Conserved residues of the human FEN1 core domain (X molecule) and (B) conserved residues of PCNA and the bound C-terminal tail from FEN1 (A molecule and residue 356-358 of X molecule) are shown according to color-bar on the top. Conservation of surface residues was calculated using alignments of eukaryotic FEN1s and PCNAs respectively.

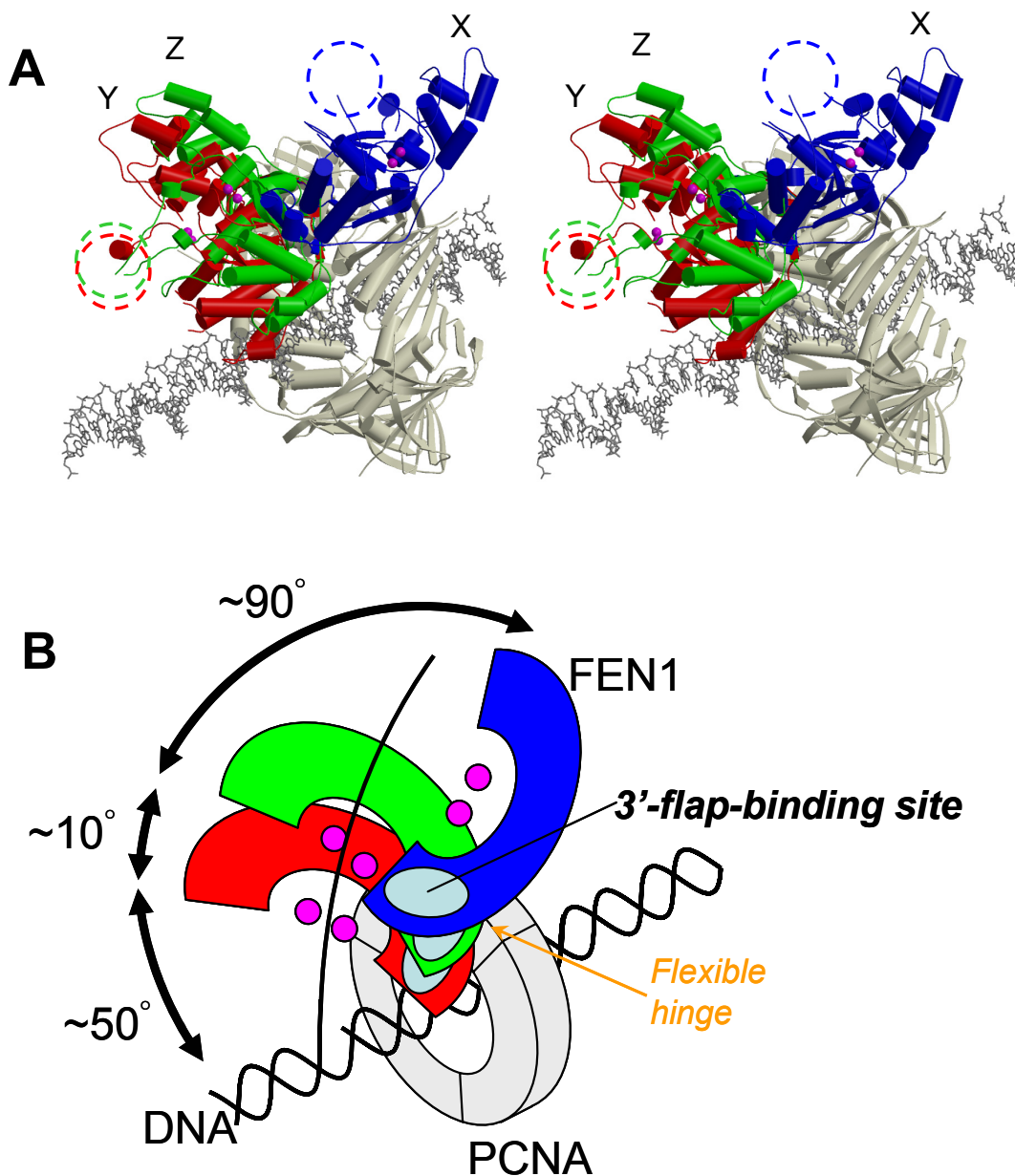


Figure 3-12. The hinge flexibility of human FEN1.

(A) A stereo diagram showing three FEN1 molecules superimposed on PCNA (a grey model) as in Figure 3-8A with a linear dsDNA in a wire model. The clamp regions are marked as dashed circles. The conceivable DNA model is superimposed at the center of PCNA.

(B) Schematic model showing the orientation of the FEN1 core domains in A.

Discussion

Our structure of the FEN1-PCNA complex has several implications concerning the molecular mechanism of action of FEN1 on PCNA. This represents the first reported structure involving a full-length DNA-editing enzyme bound to the DNA clamp and has revealed the interfaces involving the core domain of FEN1. The interactions at the interfaces maintain the enzyme core domain in an inactive orientation, whereby the FEN1 molecule would have no access to a DNA duplex running perpendicularly through the centre of PCNA. Our FEN1-PCNA complex possessing protein-protein interfaces involving the FEN1 core domain with no access to DNA may correspond to the inactive “locked-down” complex seen in the LF domain of pol IV bound to β -clamp (Bunting *et al.*, 2003). As suggested for pol IV, FEN1 could exist in equilibrium between two states: a “locked-down” and an active “tethered” complex capable of productive interaction with DNA.

In solution, DNA-clamped PCNA is known to freely slide on double-stranded (ds) DNA (Kelman, 1997). Our structure suggests that FEN1 can bind PCNA with the core domains that do not critically disturb the sliding-clamp function of PCNA (Figure 4-1). The binding models relating to the FEN1 core domains in our crystal might be utilized in rapid DNA-tracking by preserving the central PCNA hole for the purposes of sliding along the DNA. The FEN1-PCNA complex held onto the DNA by PCNA results in an effective increase in the local concentration of FEN1 on the DNA. Both the tracking and binding to the cleavage site could then be enhanced. This mechanism is consistent with *in vitro* kinetic investigations of FEN1 catalysis showing a large decrease (11-12 fold) in the Michaelis constant, K_m , following the addition of PCNA (Tom *et al.*, 2000).

A productive orientation allowing access to DNA would require disruption of the protein-protein interface. In a proposed active complex, FEN1 could swing-in the PCNA ring

and induce a kink in the substrate DNA (Chapados *et al.*, 2004). This can be achieved by hinge-like movements at the short linker that connects the core domain and the C-terminal PCNA-binding tail, while still maintaining the interaction between PCNA and the C-terminal tail. The flexible nature of the FEN1 core domains of molecules Y and Z against the bound PCNA trimer is suggested by their higher B-factors (79.0, 84.2 Å², respectively) compared to those of the PCNA subunits (Table 3-1). The βA-C-term interactions are important in providing the basis for the hinge flexibility of FEN1. Our mutation experiments consistently suggest the importance of this interaction in stimulating FEN1 activity. In the present structure, Ile255 forms a main chain-main chain hydrogen bond with Thr336 of FEN1, which provides a boundary to the hinge region (Figures 3-6 and 3-7). Thus, the βA-C-term interactions might play a role in the form of a rigid joint that can provide effective swing displacement of the core domain toward the DNA. The controlled swing of the core domain may contribute to the observed moderate increase in V_{MAX} (Tom *et al.*, 2000) and also to a proposed tracking of flap DNA through the clamp region (Hosfield *et al.*, 1998; Hwang *et al.*, 1998; Murante *et al.*, 1995; Tom *et al.*, 2000).

The βA-C-term interactions in our crystal do not involve residues 256-261 at the extreme C-terminus. Consistent with this finding, we showed that deletion of Δ5 (257-261) did not affect the binding affinity or nuclease activity of FEN1. The experimental result is in sharp contrast with the proposal that β-zipper formation between aFEN1 peptide and the PCNA C-terminus may assist in the positioning of FEN1 on the PCNA surface to facilitate flap endonuclease action (Chapados *et al.*, 2004). The proposal has been based on crystal structures of *Archaeoglobus fulgidus* PCNA in the short peptide-bound forms. β-zipper formation might be an artifact, probably due to using very short peptides in the crystallization procedure. Yet, in apparent conflict with our experimental data, the archaeal system may be

somewhat different from the eukaryotic system because of the poor conservation of the hinge sequences and lack of the interaction with the IDC loop of PCNA.

It should be noted that location of the hinge region is directly adjacent to the 3'-flap binding site. Mechanically, the hinge motion would initially position the 3'-flap binding site onto the upstream duplex containing the 3'-flap. This initial binding to the 3'-flap and the upstream duplex may assist in positioning the FEN1 active site precisely on the cleavage site of the DNA. Recently, analysis of mutations in the 3'-flap binding site of human FEN1 demonstrated a less stringent cleavage pattern (Friedrich-Heineken and Hubscher, 2004).

In the crystal structure of the *A. fulgidus* FEN1-DNA complex (Chapados *et al.*, 2004), the upstream DNA fragment has been found to bind to the right side of the helical region which contains conserved basic residues in human FEN1 (Figure 3-3D). By superimposing this *A. fulgidus* FEN1 bound to DNA onto our human FEN1, we built a model for human FEN1 complexed with an upstream DNA fragment (Figure 4-2A). It has proposed from model building and mutational studies that the phosphodiester bonds of the template DNA the downstream DNA duplex could interact to residues G244, G246, K246 and K249 of in *P. furiosus* FEN1 (G240, G242, K244 and R245 in human) (Allawi *et al.*, 2003). Based on this model, we also built a downstream DNA fragment bound to human FEN1 (Figure 4-2B). Taken together, we found that the DNA substrate bound to FEN1 should be kinked $\sim 90^\circ$, as proposed previously (Chapados *et al.*, 2004) (Figure 4-2C). During the swing-in movement of FEN1 from the inactive orientation (molecule X) to the proposed active orientation, the active site traces approximately $\sim 100\text{\AA}$ on a spherical surface (Figure 4-4). This corresponds to the length of the DNA primer (15-20 nucleotides) in DNA replication. Thus, the proposed swing motion of the core domain seems to be large enough for tracking of a flap DNA produced in DNA replication through the clamp region.

DNA replication and base excision repair are the sequential processes involving other PCNA-binding enzymes such as DNA polymerase δ/ϵ , FEN1 and DNA ligase I. PCNA is thought to equally stimulate these enzymes on the DNA by binding to their consensus PCNA-binding motif. In both processes, once the excision of flap DNA has been completed by FEN1, the enzyme needs to release its core domain away from the excision point to provide access for incoming proteins, such as ligase I. The hinge region of FEN1 might prevent steric conflicts of the core domains in the exchange process when ligase I replaces FEN1 on PCNA. Otherwise, the FEN1-PCNA complex may slide away followed by the arrival of another PCNA with ligase I. Should only one FEN1 molecule bind PCNA as in Figure 3-12, it might be possible that ligase I binds one of the two unoccupied binding sites on PCNA, thus forming a FEN1-ligase I-PCNA (and DNA) complex. Our structure demonstrated that the hinge region existing between the core domain and the tail can switch FEN1 and other enzymes bound to the same PCNA ring: FEN1 swings-out its core domain (typically as in molecule X) to allow for a space probably large enough for ligase I (and also polymerase δ/ϵ) to interact with the same PCNA and DNA (Figure 4-5). In fact, the simultaneous binding of FEN1, ligase and/or polymerase to the same PCNA has been reported for the archaeal proteins from *Sulfolobus solfataricus* (Dionne *et al.*, 2003). Considering the structure of bacteriophage RB69 polymerase, which binds the PCNA-homologous sliding clamp (gp45), a similar hinge region should be found between its core domain and the RB69-binding region (Shamoo and Steitz, 1999). Flexibility in the tail region might represent a common feature of some PCNA-binding enzymes (Hingorani and O'Donnell, 2000). The swift high performance characteristic of such a multi-protein complex has been noted both in terms of replication and repair pathways (Hingorani and O'Donnell, 2000; Hosfield *et al.*, 1998; Matsumoto, 2001).

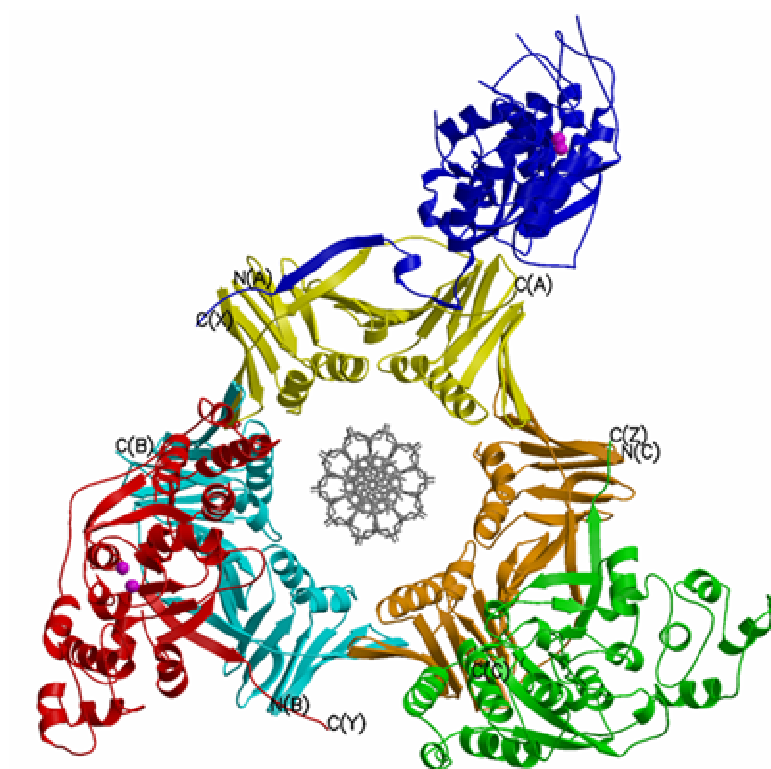


Figure 4-1

The conceivable B-DNA model (a wire model) is superimposed at the center of the PCNA ring in Figure 3-1.

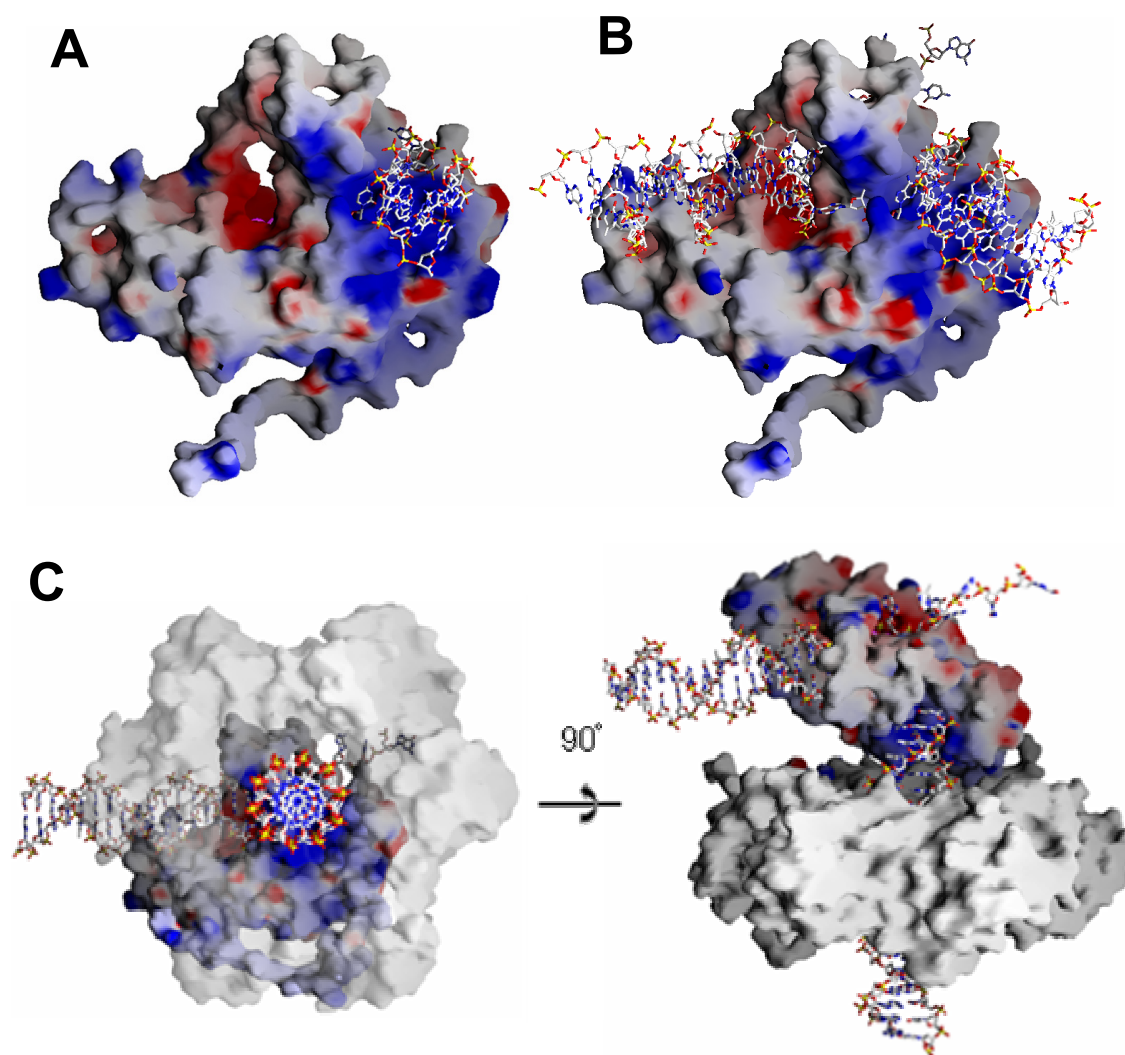


Figure 4-2 Modeling of flap DNA:FEN1 and ternary complex

- (A)** A model for human FEN1 bound to an upstream DNA fragment (a stick model). The model was built based on the crystal structure of the *A. fulgidus* FEN1-DNA complex, using human FEN1 (molecule Z). Electrostatic molecular surface is the same as Figure 3-3D left.
- (B)** A model for human FEN1 bound to both upstream and downstream DNA fragments (stick models). The model was built based on the proposed model by Allawi *et al.* Seventh and eighth phosphodiester bonds of the template DNA from the flap site were put near G240, G242, K244 and R245.
- (C)** A model for a kinked flap DNA fragment bound to an active FEN1-PCNA complex.

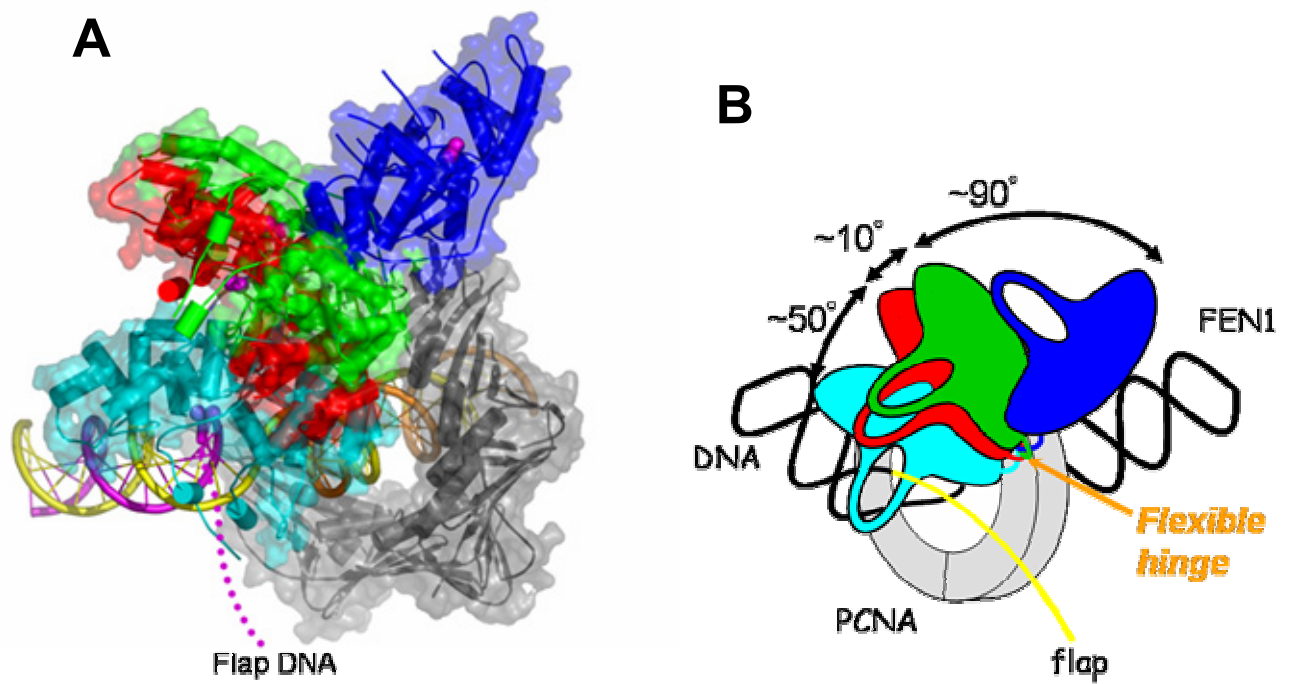


Figure 4-4. A model for the human FEN1-PCNA complex bound to a kinked flap DNA showing the hinge flexibility of human FEN1.

(A) Superimposed FEN1 molecules (molecules X, Y, Z and an active model on the cleavage site) on a kinked DNA substrate.

(B) Schematic representation of the model showing the orientation of the FEN1 core domains in A.

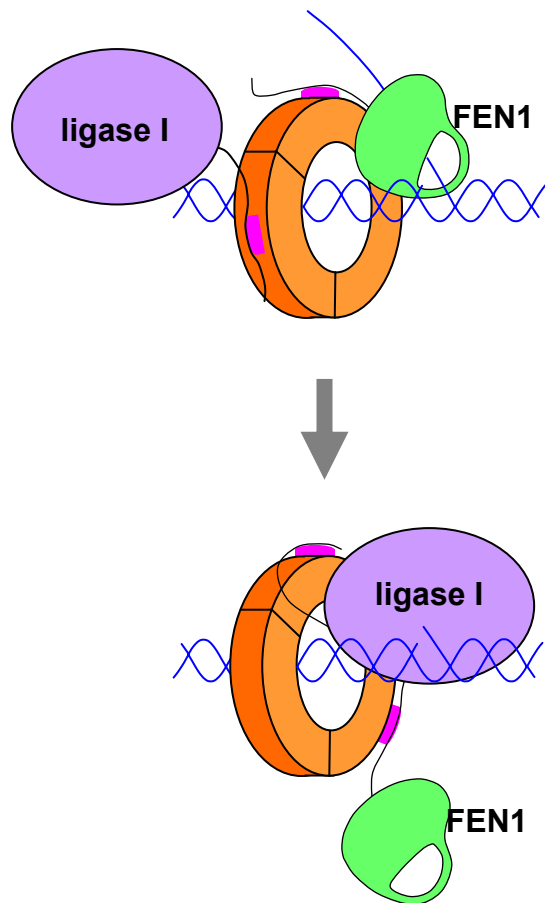


Figure 4-5. A schematic model showing the swift high performance of a multi-protein complex on PCNA.

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Publication list

I. Academic journals

1. Shigeru Sakurai, Ken Kitano, Kengo Okada, Keisuke Hamada, Hiroshi Morioka and Toshio Hakoshima, "Preparation and crystallization of human flap endonuclease FEN-1 in complex with proliferating-cell nuclear antigen, PCNA," *Acta Crystallogr. D* **59**, 933-935, 2003.
2. Shigeru Sakurai, Ken Kitano, Hiroto Yamaguchi, Keisuke Hamada, Kengo Okada, Kotaro Fukuda, Makiyo Uchida, Eiko Ohtsuka, Hiroshi Morioka and Toshio Hakoshima, "Structural basis for recruitment of human flap endonuclease 1 to PCNA," *EMBO J.*, Accepted, 23 November, 2004.

II. International conference proceedings

1. Shigeru Sakurai, Ken Kitano, Hiroto Yamaguchi, Kengo Okada, Keisuke Hamada, Eiko Ohtsuka, Hiroshi Morioka, and Toshio Hakoshima, "Crystal structure of the human FEN1-PCNA complex and its implications for replication and repair," *the 8th Conference of Biology and Synchrotron Radiation (BSR2004)*, 748, September 2004.

III. Internal

1. Shigeru Sakurai, Hiroto Yamaguchi, Ken Kitano, Kengo Okada, Keisuke Hamada, Hiroshi Morioka and Toshio Hakoshima, "Crystallization and structure determination of human FEN1-PCNA complex," *The Crystallographic Society of Japan*, P3-II-05, December 2002.
2. Shigeru Sakurai, Ken Kitano, Hiroto Yamaguchi, Kengo Okada, Keisuke Hamada, Hiroshi Morioka and Toshio Hakoshima, "Crystal structure of the human FEN1-PCNA complex: implication of a swing motion of FEN1," *The 2nd Protein Science Society of Japan*, W13-4, June 2003.
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