PCNA stimulates catalysis by structure-specific nucleases using two distinct mechanisms: substrate targeting and catalytic step

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ABSTRACT
The sliding clamp Proliferating Cell Nuclear Antigen (PCNA) functions as a recruiter and organizer of a wide variety of DNA modifying enzymes including nucleases, helicases, polymerases and glycosylases. The 5’-flap endonuclease Fen-1 is essential for Okazaki fragment processing in eukaryotes and archaea, and is targeted to the replication fork by PCNA. Crenarchaeal XPF, a 3’-flap endonuclease, is also stimulated by PCNA in vitro. Using a novel continuous fluorimetric assay, we demonstrate that PCNA activates these two nucleases by fundamentally different mechanisms. PCNA stimulates Fen-1 by increasing the enzyme’s binding affinity for substrates, as suggested previously. However, PCNA activates XPF by increasing the catalytic rate constant by four orders of magnitude without affecting the $K_m$. PCNA may function as a platform upon which XPF exerts force to distort DNA substrates, destabilizing the substrate and/or stabilizing the transition state structure. This suggests that PCNA can function directly in supporting catalysis as an essential cofactor in some circumstances, a new role for a protein that is generally assumed to perform a passive targeting and organizing function in molecular biology. This could provide a mechanism for the exquisite control of nuclease activity targeted to specific circumstances, such as replication forks or damaged DNA with pre-loaded PCNA.

INTRODUCTION
The Proliferating Cell Nuclear Antigen (PCNA) is a ring-shaped protein that encircles DNA, acting as a sliding clamp or platform. PCNA is conserved in eukarya and archaea, and in bacteria the $\beta$ subunit of DNA polymerase III plays an analogous role. PCNA is an essential component of the core processes of DNA replication, recombination and repair and cell-cycle control (1). PCNA is loaded onto DNA at the replication fork by the clamp loader Replication Factor C (RFC), and subsequently recruits a variety of proteins to the fork. The interaction between the 5’-flap endonuclease Fen-1 and PCNA is essential for the recruitment of Fen-1 to replication forks, where it catalyses Okazaki fragment processing. Photobleaching experiments have shown that PCNA persists for long periods at replication forks, whilst Fen-1 and DNA ligase associate and dissociate rapidly—consistent with the view that PCNA functions as a stable loading platform for DNA modification enzymes (2). Although Fen-1 is quite active in the absence of PCNA in vitro, disruption of the Fen-1:PCNA interaction leads to DNA replication defects and newborn lethality in mice (3). Disruption of the interaction between PCNA and mismatch repair (MMR) proteins using an oligopeptide abolishes MMR in an in vitro system (4). Similarly, disruption of PCNA:MMR protein interactions in Saccharomyces cerevisiae abolishes MMR in meiotic recombination in vivo (5). These observations emphasize the important role of PCNA in vivo.

PCNA is generally considered to function as a DNA-targeting factor, allowing non-sequence-specific enzymes, such as DNA polymerases, endonucleases, ligases, helicases, mismatch repair proteins and glycosylases to associate more closely with their DNA substrates [reviewed in (6,7)]. The trimeric structure of PCNA yields three potential binding sites for proteins, leading to the suggestion that PCNA may coordinate cellular processes by bringing consecutive enzymes in a DNA processing pathway together, for example, in the Okazaki fragment processing pathway where the nuclease Fen-1, DNA ligase and DNA polymerase can bind PCNA simultaneously (8). Covalent modification of PCNA by ubiquitination and sumoylation has been shown to play an important role in damage bypass pathways during replication in S. cerevisiae.

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probably by changing the affinity of different DNA polymers and repair factors for the clamp (9,10).

Whilst the eukaryotic PCNA protein is a homotrimer, in *Sulfolobus solfataricus* (and probably other crenarchaeal) PCNA is heterotrimeric, with a tight association between subunits 1 and 2 and a much weaker interaction of the 1–2 heterodimer with subunit 3 that may facilitate DNA loading (8). The structure of the PCNA heterotrimer has been solved both on its own (11) and as a complex with Fen-1 (12,13) and DNA ligase (13), and closely resembles the euryarchaeal and eukaryotic homotrimeric protein structures. In addition to interactions with ligase, Fen-1, DNA polymerases and glycosylases, *S. solfataricus* PCNA stimulates the activities of the Holliday junction endonuclease Hjc (14) and the 3’-flap endonuclease XPF (15). We showed previously that PCNA stimulates the cleavage activity of XPF for a wide variety of DNA substrates to a remarkable degree (16,17). Here, we utilize a novel fluorescence resonance energy transfer (FRET) assay to demonstrate that PCNA stimulates Fen-1 and XPF in quite distinct ways. The activity of Fen-1 is increased primarily by lowering the *K*<sub>M</sub> for substrates, as was shown previously for the human proteins (18). This represents the widely accepted DNA targeting model for PCNA activation. In sharp contrast, PCNA is an essential cofactor for the XPF nuclease, stimulating catalytic rates by almost 10,000-fold. This is achieved by increasing the maximal velocity of the DNA cleavage reaction, whilst *K*<sub>M</sub>’s for substrates are not affected. These data suggest that PCNA increases the catalytic rate constant by reducing the activation barrier of the cleavage reaction. This represents a novel role for the PCNA sliding clamp that may be relevant for other PCNA-dependent enzymes.

**MATERIALS AND METHODS**

**Protein expression and purification**

*Sulfolobus solfataricus* XPF and PCNA heterotrimer were expressed and purified as described previously (8,16). The *S. solfataricus* fen-1 gene was amplified from *S. solfataricus* strain P2 genomic DNA using the following primers:

5’-primer: 3’-CGTCCGGATCCCCATGGATTTAGCAGA-TTTA GTAAAG

3’-primer: 5’-CCGGGGATCCCTCGACTCTAAACCACTTGG CCAATCCTGGTTGTC

The PCR product was cloned into the Ncol/BamHI sites of the vector pET28c (Novagen, Darmstadt, Germany) for native protein expression. Protein expression was carried out in BL21 Rosetta (DE3) cells induced by adding 0.2 mM IPTG when cultures reached A<sub>600</sub> 0.7, grown for further 3 h and the cells pelleted. The bacterial pellet was resuspended in ~35 ml lysis buffer (20 mM MES pH 6.0, 1 mM EDTA, 0.5 mM DTT, 100 mM NaCl, 1 mM benzamidine) and sonicated for 4 × 2 min with cooling. The lysate was centrifuged at 48 000g for 20 min, 4°C and the supernatant heated to 70°C to precipitate *Escherichia coli* proteins before centrifugation for a further 20 min. The supernatant was filtered (Acrodisc 0.1 μm syringe filter, Pall Corporation, East Hills, NY, USA) and diluted 3-fold with buffer A (20 mM MES pH 6.0, 1 mM EDTA, 0.5 mM DTT). This was applied to a 5 ml Hitrap heparin column (GE Healthcare, Chalfont St Giles, UK) equilibrated with buffer A and the bound cationic proteins eluted over a 120 ml linear gradient of 0–1000 mM NaCl. The fractions containing Fen-1 were identified by SDS–PAGE, pooled and concentrated to ~7 ml and loaded onto a HiLoad® 26/60 Superdex® 200 gel filtration column (GE Healthcare) equilibrated with buffer (20 mM MES pH 6.0, 1 mM EDTA, 0.5 mM DTT, 150 mM NaCl). Fractions corresponding to the peak(s) were concentrated as before and the protein concentration calculated from the extinction coefficient at 280 nm (15). Protein was stored at −80°C in 15% glycerol until required.

**Substrate formation**

Unlabelled oligonucleotides used to make the DNA structures were purchased from Operon Biotechnologies GmbH (Cologne, Germany), the fluorescent oligonucleotides used were purchased from Integrated DNA Technologies (Coralville, USA). The 3’-flap and 5’-double-flap substrates were assembled using 0.1 OD of each strand (Table 1) and mixed with hybridization buffer (20 mM Tris–HCl pH 7.8, 25 mM NaCl). The sample was then heated at 93°C for 2 min followed by slow overnight cooling to 4°C. The substrate was purified on a 10% non-denaturing acrylamide gel at 110 V for 4 h at 4°C. Bands were visualized and cut by UV shadowing and then extracted from the gel using a overnight crush and soak protocol at 4°C (CSH Protocols; 2006; doi:10.1101/pdb.prot2936), followed by ethanol precipitation. The absorption spectrum from 650 nm to 220 nm was used to determine DNA concentration and labelling efficiency of the fluorescent dyes. In addition, the sequences of all oligonucleotides were selected to have predicted melting temperatures >55°C such that under reaction conditions the duplex structure was favoured.

**Endonuclease assays**

Multiple turnover nuclease reactions were assembled in 30 mM HEPES, pH 7.6, 40 mM KCl, 5% glycerol, 0.1 mg/ml bovine serum albumin with 25 nM labelled DNA substrate and 1 nM XPF dimer or FEN-1 monomer.

**Table 1. Oligonucleotides used for DNA substrates**

| Oligo   | Sequence (5’ to 3’) |
|---------|---------------------|
| XPF A   | ACCGTCGCCdIT-FluoCCTAGCAACGACTT[Cy3] |
| XPF B   | TCTGACTGAGCTGCGGCT |
| XPF C   | AGCGCCAAGCAGTCAAGCGT |
| FEN-1A  | ACCTAGGTCGCTCGCTAGCAAGCC |
| FEN-1B  | [Cy3]TTATCTGACTGAGCAGT[CdIT-Fluo] AGCTACTG |
| FEN-1C  | CAGTAGGACTGACTGAGCGAGTT GCTAGGAGCACCTAGG |
Unlabelled DNA substrate and PCNA heterotrimer were added to the required concentration. When using short synthetic DNA substrates, PCNA can diffuse readily onto the DNA and there is no need for the addition of the clamp loader RFC. Reactions were equilibrated at 55°C for 10 min before the reaction was initiated, readings were then taken for 5 min so that a stable baseline was achieved. The reaction was initiated by the addition of MgCl₂ in 5% glycerol to a final concentration of 10 mM, and monitored to completion. To minimize evaporation a layer of mineral oil was added to prevent evaporation within the cell. Cleavage rates were calculated by fitting the raw data to a single exponential equation generating reaction start and end point values. The Y-axis was rescaled to reflect fmol product, and the reaction rate was obtained by linear regression (fmol product/min/fmol enzyme). Data were obtained in triplicate and fitted to the Michaelis–Menten equation by non-linear regression.

Experiments were performed on a Cary Eclipse spectrofluorimeter (Varian Inc., Palo Alto, USA) under magic angle conditions to avoid anisotropy artefacts on the fluorimeter (Varian Inc., Palo Alto, USA) under magic angle conditions to avoid anisotropy artefacts on the fluorimeter (Varian Inc., Palo Alto, USA). Data were obtained in triplicate and fitted to the Michaelis–Menten equation by non-linear regression.

For diffusion of ligand/receptor across the needle tip during the equilibration period. Heats of dilution experiments were measured independently and subtracted from the integrated data before curve-fitting in Origin 7.0 with the standard one site model supplied by MicroCal.

**RESULTS**

Quantification of XPF and Fen-1 binding to PCNA

Both *S. solfataricus* XPF and Fen-1 have been shown previously to interact with the heterotrimeric PCNA molecule in the absence of DNA, but binding affinities have not been measured. We used ITC to determine the dissociation constants of both proteins for PCNA in solution. Fen-1 bound to PCNA with a $K_D$ of $210 \pm 13$ nM and a stoichiometry of 0.998 ± 0.002 Fen-1 monomer per PCNA trimer (Figure 1A). XPF bound to PCNA with a stoichiometry of 1.1 ± 0.2 XPF dimer per PCNA trimer and a $K_D$ of 3.8 ± 0.6 μM (Figure 1B). By comparison, human PCNA interacts with the mismatch binding protein MutSβ in solution with a 1:1 stoichiometry and a $K_D$ of 0.7 μM (19).

Development of a continuous, fluorescence-based assay for XPF and Fen-1

Previous experiments on *S. solfataricus* XPF have utilized a discontinuous, single-turnover assay with a radiolabelled substrate (16). To facilitate analysis of multi-turnover kinetics with a continuous assay, we developed a FRET-based assay where the DNA strand targeted for cleavage has two dyes: a donor dye (fluorescein) located 5′ to the cleavage point and an acceptor dye (Cy3) located on the terminus of the 3′-flap (Table 1, Figure 2). The close proximity of the donor and acceptor groups in the same DNA strand facilitated efficient energy transfer thereby reducing fluorescence emission intensity from the donor moiety at 520 nm upon excitation at 490 nm (Figure 2B). Cleavage of the strand resulted in alleviation of this quenching and an increase in fluorescence emission intensity at 520 nm. The assay was based on a previously reported method (20). The expected cleavage products of the fluorescent construct were observed by separating the reaction products by denaturing gel electrophoresis and visualization by phosphorimaging (Fuji FLA5100, Fujifilm, Tokyo, Japan) using a SHG green laser (532 nm) to observe Cy3 fluorescence, confirming that substrate strand cleavage had taken place (Figure 2C).

A similar strategy was used to design a fluorescent substrate for Fen-1. The optimal double-flap substrate was chosen, which consists of an unpaired 3′-nucleotide and a 5′-ssDNA flap (21). The donor dye (fluorescein) was located 3′ of the cleavage point and the acceptor dye (Cy3) was located on the terminus of the 5′-flap on the same strand (Table 1, Figure 2A). Cleavage of the flap by Fen-1 resulted in an increase in fluorescence emission of the fluorescein reporter at 520 nM that could be followed in a continuous assay format as for XPF.

**Isothermal titration calorimetry**

Binding of XPF or Fen-1 to PCNA was assessed by isothermal titration calorimetry (ITC) using a VP-ITC unit operating at 328 K for XPF and 293 K for Fen-1 (Microcal, GE Healthcare, Chalfont St Giles, UK). Before use, proteins were dialysed against binding buffer and degassed in a vacuum. All concentrations were measured by UV absorption immediately before titrations were started. For Fen-1, binding buffer was 30 mM HEPES pH 7.6, 40 mM KCl and titrations comprised 50 injections of Fen-1, one 2-μl injection followed by 49 5-μl injections. For XPF binding buffer was 50 mM Tris–HCl pH 7.4, 200 mM NaCl and titrations comprised 26 injections of XPF, one 2-μl injection followed by 25 10-μl injections. The initial data point was routinely deleted to allow
Figure 1. Interaction of PCNA with XPF and Fen-1 quantified by ITC. (A) Quantification of Fen-1 and PCNA interaction by ITC. Fen-1 (268 μM in the syringe) was injected into a solution of 13 μM PCNA heterotrimer at 293 K and heats of dilution monitored. The data were fitted with a simple one site binding model, yielding a $K_D$ of 210 nM and a binding stoichiometry of 1:1. (B) Quantification of XPF and PCNA interaction by ITC. XPF (170 μM dimer in syringe) was injected into a solution of 4 μM PCNA heterotrimer at 328 K and heats of dilution monitored. The data were fitted with a simple one site binding model, yielding a $K_D$ of 3.8 μM and a binding stoichiometry of 1:1.

Figure 2. A continuous fluorescence assay for XPF and Fen-1. (A) Schematic showing design of steady-state FRET-based cleavage assays for XPF and Fen-1. Refer to Table 1 for the sequences. (B) Progress of the XPF substrate cleavage by the XPF–PCNA holoenzyme can be monitored by the increase in the donor (fluorescein) emission at 520 nm. The solid line represents the fitting of the experimental data to a single exponential model. Inset: change in the fluorescence emission spectrum ($\lambda_{ex}$ 490 nm) observed upon substrate cleavage by XPF–PCNA. The emission spectrum before initiation of the reaction by addition of magnesium (pink), and (yellow) are shown. (C) Denaturing gel electrophoresis confirms that the fluorescent XPF substrate is cleaved by XPF–PCNA.
PCNA activates the catalytic step of XPF but not Fen-1

Single-turnover experiments were performed over a temperature range from 25°C to 55°C. The XPF concentration was 1 μM and DNA substrate concentration 80 nM. Data are shown graphically in Figure 3A and summarized in Table 2. Experiments performed at 55°C in the presence of 1 μM PCNA yielded a $k_c$ value of 9.1 min$^{-1}$. This compares well with the rate of 6.8 min$^{-1}$ estimated using a discontinuous radiation-based assay (16), giving confidence that the fluorescent labels did not interfere with catalysis by XPF. Experiments performed under identical conditions but without added PCNA yielded a $k_c$ value of $1 \times 10^{-3}$ min$^{-1}$, ~7000-fold less active than in the presence of PCNA (Figure 3B, Table 2). This is clear evidence that PCNA activates the catalytic cleavage activity of XPF, presumably by stabilizing the transition state or destabilizing the substrate. In marked contrast, single-turnover rate constants for Fen-1 in the presence and absence of 1 μM PCNA yielded highly similar $k_c$ values of 10.3 min$^{-1}$ and 10.6 min$^{-1}$, respectively, at 55°C, suggesting there is no intrinsic stimulation of the catalytic step of Fen-1 by PCNA (Figure 3B, Table 2). Both enzymes showed temperature-dependent kinetics as expected, with a 2- and 3-fold increase in catalytic rate for every 10°C increase in temperature for Fen-1 and XPF, respectively.

Steady-state kinetic analyses of XPF and Fen-1

The pre-steady-state rate constants measured for XPF highlighted the crucial role of PCNA in the activation of catalysis by this endonuclease, and confirmed previous measurements using discontinuous assays. The fluorescence-based assay allowed measurement of steady-state kinetics for XPF, which yield complementary information on the cycle of binding, catalysis and product release. Steady-state kinetic measurements of XPF were carried out by assaying 1 nM XPF and a DNA substrate concentration range of 25–650 nM at 55°C. Initial velocities at each substrate concentration (Figure 4A) were fitted to the Michaelis–Menten equation (Figure 4B) to yield kinetic constants $k_{cat}$ and $K_M$. Steady-state experiments in the absence of PCNA showed no activity for XPF over a time period of 72 h. Inclusion of PCNA at concentrations from 50 nM to 20 μM increased the $k_{cat}$ from 0.37 min$^{-1}$ to 5.5 min$^{-1}$ (Figure 4C), without changing the $K_M$ which remained constant at 85 ± 10 nM at all concentrations of PCNA tested (Figure 4D). The close agreement between the catalytic rate constant $k_c$ and the turnover number $k_{cat}$, 9.1 min$^{-1}$ and 5.5 min$^{-1}$, respectively, suggests that the catalytic step, rather than substrate binding or product release by XPF, is rate-limiting. The variation of $k_{cat}$ with PCNA concentration shown in Figure 4C yielded an apparent $K_M$ of 4 ± 0.7 μM for XPF and PCNA. This was in good agreement with the $K_D$ of 3.8 μM determined from the ITC experiment, reinforcing the conclusion that the PCNA–XPF complex is essential for nuclease activity.

Steady-state kinetic analysis was also carried out for Fen-1. As observed previously, Fen-1 activity was not strictly dependent on the presence of PCNA. An ~3-fold increase in $k_{cat}$ from 2.6 min$^{-1}$ to 7.3 min$^{-1}$ was observed

![Figure 3](image-url) DNA substrate cleavage by XPF and Fen-1 in the presence and absence of PCNA. (A) Representative traces showing the continuous monitoring by FRET of DNA substrate cleavage and product formation by the XPF–PCNA complex at different temperatures under single-turnover conditions. (B) Variation in the catalytic rate constants for DNA substrate cleavage by 1 μM XPF or Fen-1 in the presence and absence of 10 μM PCNA as a function of temperature. Fen-1 is not significantly stimulated by PCNA under single-turnover conditions, whilst XPF is only marginally active in the absence of PCNA. Experiments were carried out in triplicate and means with standard errors are shown.

|          | $k_c$ (min$^{-1}$) | $k_{cat}$ (min$^{-1}$) | $K_M$ (M)         | $k_{cat}/K_M$ (M$^{-1}$min$^{-1}$) |
|----------|------------------|-----------------------|------------------|-----------------------------------|
| XPF      | (1.0 ± 0.2) × 10$^{-3}$ | –                     | –                | –                                 |
| XPF + PCNA | 9.1 ± 0.3         | 5.5 ± 0.2             | (93 ± 8) × 10$^{-6}$ | (59 ± 7) × 10$^6$               |
| Fen-1    | 10.6 ± 0.9        | 2.6 ± 0.2             | (290 ± 40) × 10$^{-6}$ | (9 ± 2) × 10$^6$               |
| Fen-1 + PCNA | 10.3 ± 0.3       | 7.3 ± 0.3             | (67 ± 10) × 10$^{-6}$ | (110 ± 16) × 10$^6$             |
as the PCNA concentration increased from 0 μM to 20 μM (Figure 4C). In contrast to XPF, the \( K_M \) for Fen-1 was strongly dependent on PCNA concentration, dropping from 290 nM to 60 nM as PCNA increased from 0 μM to 5 μM (Figure 4D). Together with the single-turnover experiments, this suggests that the stimulatory effect of PCNA on Fen-1 is largely related to substrate binding.

**DISCUSSION**

**Stimulation of Fen-1 by PCNA—substrate targeting**

The sliding clamp PCNA binds to DNA and interacts with a wide variety of proteins. It is generally accepted that PCNA’s function is to recruit a variety of DNA modification proteins to relevant DNA structures. This implies a rather passive role in catalysis, as a moderately stimulatory factor whose main function is in targeting and organization of components of quite complex molecular machines catalysing processes, such as DNA replication or MMR. Virtually all previously described PCNA-interacting proteins are reasonably active in the absence of PCNA. A good example is the 5’-flap endonuclease Fen-1, whose activity is only moderately stimulated by PCNA *in vitro*. Nevertheless, interactions with PCNA have been shown to be crucial for cell survival *in vivo* (3), emphasizing the role of PCNA as a molecular organizer or mediator.

Previously published kinetic studies of human and archaeal Fen-1’s have reported widely divergent kinetic constants. In several instances, this has been complicated by reporting of multiple turnover rate constants and \( K_M \)’s derived from assays where Fen-1 was equimolar to or in excess of the substrate concentration. Some Fen-1’s seem to be highly sensitive to the presence of DTT for full...
activity (22), and the pH used by different investigators has varied widely. The continuous fluorescent assay system we have reported here has clear advantages over discontinuous assays used previously. The single-turnover rate \( k_{\text{cat}} \) for \textit{S. solfataricus} Fen-1 of 10.3 min \(^{-1} \) was close to the multiple turnover \( k_{\text{cat}} \) of 7.3 min \(^{-1} \) suggesting that, as with XPF, the catalytic step is rate limiting under the multiple turnover conditions used in these experiments. This contrasts with the phage T5-flap endonuclease, where turnover rates are limited by product release rather than the catalytic step (22).

The \( k_{\text{cat}} \) for human Fen-1 in the absence of PCNA, with a double-flap substrate at pH 8.0, 37°C, is reported as 11 min \(^{-1} \) (23). The turnover number for \textit{Archaeoglobus fulgidus} Fen-1 is reported to be considerably higher, about 100 min \(^{-1} \) with a similar substrate at pH 9.3, 55°C and a large increase in reaction rate was observed when 1 mM DTT was present in the reaction buffer (22). We observed no influence of DTT on the cleavage rate of \textit{S. solfataricus} Fen-1, and note that whilst \textit{A. fulgidus} Fen-1 has a single cysteine residue that may conceivably influence catalysis, \textit{S. solfataricus} Fen-1 has no cysteines in its primary sequence. However, we did observe an ~10-fold increase in both the single- and multiple-turnover rate constants for \textit{S. solfataricus} Fen-1 assayed at pH 9.3, bringing the catalytic constants close to those reported for \textit{A. fulgidus} Fen-1 (22) (data not shown). In common with studies of other Fen-1 enzymes, we have shown that \textit{S. solfataricus} Fen-1 is only moderately stimulated by PCNA and that this effect is mostly at the level of DNA binding. Under single-turnover conditions, PCNA has no effect on Fen-1 activity. Under steady-state conditions, the \( K_M \) of \textit{S. solfataricus} Fen-1 for a double-flap substrate was reduced from 290 nM in the absence of PCNA to 60 nM at saturating concentrations of PCNA. By comparison, the \( K_M \) of \textit{A. fulgidus} Fen-1 for a double-flap substrate was estimated at 1.4 \( \mu \)M in the absence of PCNA (22). In reactions carried out at pH 9.3, the effect of PCNA (20 \( \mu \)M) on \textit{S. solfataricus} Fen-1 activity was qualitatively similar to that observed at pH 7.6, with a modest increase in \( V_{\text{max}} \) and a 4-fold decrease in \( K_M \). This suggests that the mechanism by which PCNA stimulates Fen-1 is similar at both the high and low pH’s, and is likely to be a general phenomenon for Fen-1’s from different organisms.

**Stimulation of XPF by PCNA—an essential cofactor for catalysis**

In sharp contrast to the situation for Fen-1, the 3’-flap endonuclease XPF has a 7000-fold lower catalytic rate constant in the absence of PCNA. Under steady-state conditions, activity was undetectable in the absence of PCNA, and increasing concentrations of PCNA resulted in an increase \( k_{\text{cat}} \) but had no effect on \( K_M \). These observations suggest strongly that the role of the sliding clamp in the XPF-mediated reaction is fundamentally different from that observed with Fen-1 and other DNA modification enzymes. Here, PCNA must be regarded as an essential cofactor or protein subunit of the XPF holoenzyme. In other words, the XPF:PCNA complex is the active entity. Consistent with this hypothesis, the variation of XPF \( k_{\text{cat}} \) with increasing PCNA concentration yields an apparent \( K_M \) for PCNA of 4 \( \mu \)M—in close agreement with the dissociation constant of the two proteins in solution measured by ITC at 3.8 \( \mu \)M.

Why is PCNA absolutely essential for catalysis by cre-narcarchaeal XPF when its role in other situations is predominantly in substrate targeting and processivity? The XPF endonuclease is unusual in being organized with two dimeric domains joined by a flexible linker (24) (Figure 5). The C-terminal dimeric HhH \(_2\) domain has a DNA-binding role, and is thought to distort DNA substrates, allowing the nuclease domain to remove the 3’-flap, or extend the gap upstream of a nicked DNA duplex. It is possible that PCNA functions as a molecular fulcrum in this situation; acting as a platform against which XPF can exert force to distort the DNA towards a productive structure for catalysis.

![Figure 5](image)

**Figure 5.** Scale cartoon showing a potential mechanism for PCNA activation of XPF endonuclease activity. (A) PCNA loaded onto duplex DNA can associate reversibly with the XPF endonuclease, which is composed of two dimeric domain joined by a flexible linker and binds to PCNA via a C-terminal PIP motif. (B) On encountering a suitable substrate for XPF, such as a 3’-flap, PCNA acts as a molecular fulcrum, providing a platform against which XPF can exert force to distort the DNA towards a productive structure for catalysis.
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