Investigation of Sliding DNA Clamp Dynamics by Single-Molecule Fluorescence, Mass Spectrometry, and Structure-Based Modeling

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Abstract

Proliferating cell nuclear antigen (PCNA) is a trimeric ring-shaped clamp protein that encircles DNA and interacts with many proteins involved in DNA replication and repair. Despite extensive structural work to characterize the monomeric, dimeric, and trimeric forms of PCNA alone and in complex with interacting proteins, no structure of PCNA in a ring-open conformation has been published. Here, we use a multidisciplinary approach, including single-molecule Förster resonance energy transfer (smFRET), native ion mobility-mass spectrometry (IM-MS), and structure-based computational modeling, to explore the conformational dynamics of a model PCNA from Sulfolobus solfataricus (Sso), an archaeon. We found that Sso PCNA samples ringopen and ring-closed conformations even in the absence of its clamp loader complex, replication factor C, and transition to the ring-open conformation is modulated by the ionic strength of the solution. The IM-MS results corroborate the smFRET findings suggesting that PCNA dynamics are maintained in the gas phase and further establishing IM-MS as a reliable strategy to investigate macromolecular motions. Our molecular dynamic simulations agree with the experimental data and reveal that ring-open PCNA often adopts an out-of-plane left-hand geometry. Collectively, these results implore future studies to define the roles of PCNA dynamics in DNA loading and other PCNA-mediated interactions.

Introduction

In all three domains of life, DNA replication is an intricate, and stringently coordinated process that ensures faithful and efficient copying of genetic material. Such a complicated process is accomplished by a variety of enzymes including DNA polymerases (1-8), glycosylases (9-11), ligases (12,13), and nucleases (14-16), which often interact with an evolutionarily conserved sliding DNA clamp. While encircling duplexed DNA, the sliding DNA clamp functions as a scaffold for DNA replication and repair machinery (17-19). Moreover, sliding DNA clamps have been identified and studied in all domains of life, *e.g.* the β clamp of *Escherichia coli*, the gene 45 protein (gp45) of T4 bacteriophage, and proliferating cell nuclear antigen (PCNA) in yeast, humans, and the archaeon *Sulfolobus solfataricus* (*Sso*) (20,21).

Previous studies have shown that despite low sequence similarity, all sliding DNA clamps share a similar toroidal structure with a central hole to accommodate a DNA duplex (20). Despite the highly conserved ring-shaped structure of DNA sliding clamps, the complexity of their subunit composition varies. For instance, the β clamp of *E. coli* is homodimeric, while PCNA clamps in archaeal and eukaryotic organisms are trimeric (20). Although many PCNA clamps are homotrimeric, *Sso* PCNA is heterotrimeric, consisting of three distinct PCNA monomer subunits: PCNA1, 2, and 3 (21). As a result, proper subunit arrangement, subunit interfaces (22-24), and exclusive subunit binding partners have been identified for *Sso* PCNA1, PCNA2, and PCNA3 (21,24-26). Previous studies have established that formation of the *Sso* PCNA trimer follows a sequential process, which begins with dimerization of PCNA1 and PCNA2, followed by recruitment of PCNA3 to form the heterotrimer (21-24).

While crystal structures exist for each *Sso* PCNA monomer (23), the PCNA1:PCNA2 dimer (23), and the ring-closed PCNA heterotrimer (22-24), no crystal structure is available for

the *Sso* PCNA heterotrimer in a ring-open conformation. However, biochemical experiments have shown that the *Sso* PCNA heterotrimer opens at the interface between PCNA1 and PCNA3 in order to be loaded onto DNA by a clamp loading accessory protein, replication factor C (RFC) (27). To date, only the gp45 homotrimer of T4 bacteriophage has been crystallized in a ring-open conformation, but only while in complex with its clamp loader assembly (gp62/gp44 complex) (28). In this structure, the clamp is open out-of-plane and adopts a right-hand geometry, consistent with the right-handed helix of double-stranded DNA (28). Paradoxically, fluorescence studies report that the gp45 DNA clamp exists primarily in a ring-open form while in solution (29), yet only the ring-closed form has been crystallized in the absence of the clamp loader (30). Accordingly, sliding DNA clamps likely undergo conformational dynamics while free in solution, as well as in complex with clamp loaders, DNA, or both.

As sliding DNA clamps serve significant roles in numerous fundamental aspects of DNA metabolism, it is crucial to understand if their conformational dynamics may influence biochemical events. Here, we use a state-of-the-art multidisciplinary approach, including single-molecule Förster resonance energy transfer (smFRET), native ion mobility-mass spectrometry (IM-MS), and structure-based computational modeling, to characterize the dynamics of *Sso* PCNA. Our smFRET results suggest that PCNA exists in dynamic equilibrium between ring-open and ring-closed conformations at the PCNA1:PCNA3 interface, and that ionic strength can modulate the transition kinetics between these states. Moreover, this dynamic open-closed equilibrium of *Sso* PCNA can also be monitored in the gas-phase using IM-MS as demonstrated by two populations of distinct collisional cross sections, which were also sensitive to changes in ionic strength. Finally, structure-based computational modeling of *Sso* PCNA agreed with the experimentally observed two-state system and its dependence on ionic strength. However, in contrast to the ring-

open structure of gp45 in complex with its clamp loader, our computational results suggest an unusual out-of-plane ring-opening of PCNA at the PCNA1:PCNA3 interface to mainly adopt left-hand geometry. This result may have general implications for loading and unloading of PCNA to DNA. Taken together, our results demonstrate the dynamics of an evolutionarily conserved sliding DNA clamp, and implore future efforts to understand how PCNA motions may influence protein-protein and protein-DNA interactions.

Materials & Methods

Expression and Purifications of Proteins

Sso PCNA plasmids were obtained as generous gifts from the lab of Dr. Stephen D. Bell at Indiana University in Bloomington, IN. Genes coding for Sso PCNA1, PCNA2, PCNA3 were expressed by three separate plasmids, while the covalently-linked PCNA heterotrimer (PCNA1-2-3) was expressed as a fusion from a single gene encoding PCNA1, PCNA2, and PCNA3 covalently joined at the PCNA1:PCNA2 and PCNA2:PCNA3 interfaces by amino acid linkers (ASGAGGSEGGGSEGGTSGAT) as described previously (27). To this construct, site-specific S→C mutations were engineered at residues S64 of PCNA1 and S189 of PCNA3 for fluorophore labeling. Additionally, a gene sequence encoding an AviTag peptide (GLNDIFEAQKIEWHE) to allow site-specific biotinylation by BirA (31) and subsequent surface immobilization for smFRET studies was added to the C-terminus of PCNA1-2-3 before the 6xHis tag.

All expression plasmids were individually transformed into *E. coli* strain Rosetta (DE3) and expressed separately using autoinduction in ZYP-5052 medium (32). After growth, cells were harvested (4,000 rpm for 20 min), resuspended in Buffer A (50 mM HEPES (pH 8 at 4°C), 100 mM NaCl, and 1 mM DTT), and lysed by French press at 20,000 PSI before clarifying the lysate

by ultracentrifugation (40,000 rpm for 40 min). As *Sso* PCNA is thermostable, the clarified lysate was subject to heat shock by incubation at 60°C for 10 min to precipitate contaminating proteins which were subsequently removed by ultracentrifugation (40,000 rpm for 40 min).

Sso PCNA1, PCNA2, and PCNA3 subunits were expressed and purified independently. Each subunit was purified by anion exchange, heparin, and size exclusion chromatography. Briefly, a PCNA subunit was bound to a 5 mL HiTrap Q column (GE Healthcare) and eluted through a linear salt gradient (up to 1 M NaCl). Fractions containing the PCNA monomer were dialyzed against Buffer A, loaded onto a 5 mL HiTrap heparin column (GE Healthcare), and eluted through a linear salt gradient (up to 1 M NaCl). Fractions with negligible nucleic acid contamination (260/280 ratio < 0.6) were dialyzed against Buffer A, concentrated to less than 1 mL, loaded and eluted from a HiPrep 26/60 Sephacryl S-100 column (GE Healthcare). The PCNA heterotrimer was formed by incubating equimolar amounts of purified PCNA1, PCNA2, and PCNA3 at 4°C for one hour in Buffer A, before purification by size-exclusion chromatography, as described above.

Following heat shock, the covalently-linked PCNA1-2-3 protein was bound to Ni²⁺-NTA resin (Qiagen) and eluted through a linear imidazole gradient (up to 1 M imidazole) in Buffer A. Following dialysis against Buffer A, the protein was purified by HiTrap heparin and size-exclusion chromatography as described above for the PCNA monomers. The purified PCNA1-2-3 was then biotinylated by following a previously published protocol (31,33) before fluorophore labeling with a 15-fold molar excess of thiol-reactive Cy3- and Cy5-maleimide (Lumiprobe) overnight at 4°C in a buffer containing 50 mM Tris (pH 7.2), 150 mM NaCl, 0.5 mM TCEP, and 10% glycerol. Free dye was removed by size-exclusion chromatography. The extent of fluorophore labeling was verified by measuring absorbance at 280, 552, and 650 nm for PCNA, Cy3, and Cy5, respectively.

Single Molecule Measurements

All single-molecule measurements were conducted on a custom built, prism-type total internal reflection microscope, as described previously (34-36). Imaging chambers were assembled from quartz slides and coverslips that were cleaned, passivated, and biotinylated by following a published protocol (34,37). Biotinylated, Cy3-Cy5 labeled PCNA1-2-3 was then surface immobilized following the addition of neutravidin (0.2 mg/mL). After rinsing the imaging chamber with T50 buffer (10 mM Tris HCl, pH 8.0, 50 mM NaCl) and addition of imaging buffer (50 mM HEPES [pH 7.6], 0.8% w/v D-Glucose, 2 mM Trolox, 0.1 mg/ml BSA, 1 mg/ml glucose oxidase, 0.04 mg/ml catalase) containing 0, 250, 500, 750, or 1,000 mM NaCl, single-molecule movies were recorded at 25°C using an Andor iXon 897 EM-CCD. Notably, the inclusion of Trolox in the imaging buffer limited acceptor blinking (38). The movies were recorded over several minutes at a reduced laser power and a slow frame rate (2 frames per second) to limit observed rapid photobleaching of Cy3 and Cy5 conjugated to PCNA1-2-3 at higher laser powers.

Single Molecule Data Analysis

Single-molecule movies were processed using IDL (ITT Visual Information Solutions) and custom MATLAB scripts (Center for the Physics of Living Cells, University of Illinois at Urbana-Champaign) to generate traces of donor and acceptor fluorescence intensities for each detected molecule, as previously described (35,36). The traces were subsequently processed and background corrected in MATLAB. Traces showing clear anti-correlated donor and acceptor signals, as well as exhibiting single donor photobleaching events, were selected. FRET efficiency values were calculated as apparent FRET (E_{app}) using **Equation 1**, where I_D , and I_A are the donor

and acceptor fluorescence intensities, respectively.

$$E_{app} = \frac{I_{A}}{I_{D} + I_{A}} \text{ (Eq. 1)}$$

For every experiment, FRET efficiency values from >200 FRET trajectories were collected and binned to generate histograms depicting the relative population distributions of FRET events. These data were fit to a sum of Gaussian functions using MATLAB, and percent occupancy of FRET states was calculated as the total area under the individual Gaussian fits. Dwell time analysis was performed as previously described (35,36). Briefly, the duration of each FRET event for the selected molecules was quantified using a thresholding analysis. The "Low FRET" state was limited by thresholds at FRET efficiencies of 0.2 and 0.65, and the "High FRET" state was limited by thresholds at FRET efficiencies of 0.65 and 1. The resulting dwell times were compiled to generate survivor functions as previously described (35,36) which were fit to a single exponential decay equation (**Equation 2**), where f(t) is the fraction of molecules in the designated FRET state after time t, A is the amplitude of the function, and k is the decay rate constant associated with the designated FRET state.

$$f(t) = Ae^{-kt} \text{ (Eq. 2)}$$

Mass Spectrometry

All mass spectrometry and ion mobility-mass spectrometry studies were performed on an in-house modified Synapt G2 (Waters, Milford, UK) (39). Typical experimental parameters were as follows; capillary voltage of 1.2-1.4 kV, a sampling cone of 20 V, source temperature of 20°C and trap collision energy of 5 V. Gas flows in the trap, helium cell and IM cell were 4, 120, and 60 mL/min respectively. Experimental collisional cross sections were determined using a calibration procedure, with β-lactoglobulin, avidin, concanavilin A, transthyretin, and serum

amyloid P component as standards.(40) In all cases, a trap wave velocity of 250 m/s and height of 3.5 V, ion mobility wave velocity of 300 m/s and height of 17 V, and a transfer wave velocity of 100 m/s and a height of 2 V were used.

Structure-based Model

An initial coarse-grained $C\alpha$ structure-based model (SBM) was generated from the crystal structure of *Sso* heterotrimeric PCNA (PDB 2HII) (Chains 1, 2, and 3 contain 249, 243, and 243 amino acids, respectively)(24) using the SMOG on-line toolkit, which included one bead on the $C\alpha$ atom of each residue of the complex (41-43). The native contact map was built by the Shadow Algorithm (43). Aiming to sample the ring-open conformation of heterotrimeric PCNA, the native contacts between chain 1 and chain 3 were not included in this model. The potential energy function consisted of both bonded and nonbonded terms. Additionally, we introduced charge characterization into our SBM to study the electrostatic interactions in the system. As a result, the potential energy form used in this study is given by the following equation:

$$V = \sum_{bonds} \varepsilon_r (r - r_0)^2 + \sum_{angles} \varepsilon_\theta (\theta - \theta_0)^2$$

$$+ \sum_{dihedrals} K_{\phi}^{(n)} (1 - \cos(n \times (\phi - \phi_0)))$$

$$+ \sum_{contacts} \varepsilon_{ij} \left(5 \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - 6 \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{10} \right)$$

$$+ \sum_{non-contacts} \varepsilon_{NC} \left(\frac{\sigma_{NC}}{r_{ij}} \right)^{12} + V_{Debye-Hückel}$$
(Eq. 3)

In Eq. 3, $\varepsilon_r = 100\varepsilon$, $\varepsilon_\theta = 20\varepsilon$, $K_\phi^{(1)} = \varepsilon$ and $K_\phi^{(3)} = 0.5\varepsilon$. The interaction strength of the Lennard-Jones type potential is proportional to the statistical potential reported for the residue types of i and j by Miyazawa and Jernigan (MJ) (44) to generate the "flavored model" (45). Therefore, the

coefficient of nonbonded contacts, ε_{ij} , is set as follows:

$$\varepsilon_{ij} = \left(\gamma \left(\frac{\varepsilon_{ij}^{MJ}}{\overline{\varepsilon}^{MJ}} - 1 \right) + 1 \right)$$
 (Eq. 4)

where \mathcal{E}_{ij}^{MJ} is the original MJ potential, $\overline{\mathcal{E}}^{MJ}$ is the mean value of the entire set of MJ weights in the complex system, and γ is set to 1.0 corresponding to the "flavored model" (45).

The electrostatic interaction is calculated by the Debye-Hückel model, which can quantify the strength of charge-charge attractions and repulsions at various salt concentrations:

$$V_{Debye-Hückel} = \Gamma_{DH} \times K_{coulomb} B(\kappa) \sum_{ij} \frac{q_i q_j \exp(-\kappa r_{ij})}{\varepsilon r_{ij}}$$
 (Eq. 5)

In Eq. 5, $K_{coulomb} = 4\pi\epsilon_0 = 138.94 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{nm} \cdot \text{e}^{-2}$ is the electric conversion factor; $B(\kappa)$ is the salt-dependent coefficient; κ^{-1} is the Debye screening length, which is directly influenced by the salt concentration C_{salt} ($\kappa \approx 3.2 \sqrt{C_{\text{salt}}}$); ε is dielectric constant, which is set to 80 during the simulations; and Γ_{DH} is the energy scaled coefficient, which aims to make the total energy balanceable. In our model, Lys and Arg residues have a positive point charge (+e) while Asp and Glu have a negative point charge (-e). All charges are placed on the $C\alpha$ atoms. Besides the systems of variable ionic strengths, there is also a system with no electrostatic interactions. Under physiological ionic strengths ($C_{\text{salt}} \sim 0.15 \text{M}$), κ is 1.24 nm⁻¹. In our simulations, $\Gamma_{DH} = 0.535$ such that V_{DH} for two oppositely charged atoms located at a distance of 0.5 nm matches the native contact energy. For more details regarding the Debye-Hückel model, the authors cite several helpful publications (46-49).

Molecular Dynamics Simulations

All molecular dynamic simulations (MD) were performed with Gromacs 4.5.5 (50). In the SBM, reduced units were used in the potentials. Accordingly, the value of simulation temperature is not the same as the "normal" temperature. To obtain an appropriate simulation temperature, replica-exchange molecular dynamic (REMD) simulations with 24 replicas ranging from about 38 K to 150 K were performed. Each replica was performed for 100 ns. The exchanges were attempted every 5000 steps. As a result, the simulation temperature was set to 85 K. The MD used the Langevin equation with a constant friction coefficient of $\gamma = 1.0$. The cutoff for nonbonded interactions was set to 3.0 nm, and all bonds were constrained using the LINCS algorithm (51). The MD time step was set to 2.0 fs. For thermodynamical simulations, a 1 μ s MD simulation was performed on each salt concentration (including 10, 50, 100, 150, 200, 250, 300, and 500 mM, as well as a system without charge interactions). We define that a native contact is formed if the C α -C α distance between any given native atom pair is within 1.2x of its native distance. The native distance is calculated from the initial structure model. Therefore, Q_{inter} is defined as the fraction of the interchain native contact number.

Theoretical CCS determination

Theoretical CCS were determined for the C alpha models using the projection approximation (PA) and trajectory method (TJM) models in IMPACT (52), for 50,000 frames in each trajectory. Results were filtered and plotted based on the fraction of native contacts between chain 1 and chain 3 ($Q_{inter13}$) and the ion strength, where $Q_{inter13} = 0$ represents the open state, $Q_{inter13} = 1$ represents the closed state.

Results

Design of Covalently-linked Sso PCNA for smFRET

To understand how sliding DNA clamp conformational dynamics may influence biological processes, we sought to first characterize the dynamics of heterotrimeric PCNA from the model organism Sulfolobus solfataricus. This well-studied protein consists of three distinct subunits, PCNA1, PCNA2, and PCNA3, serves as a processivity factor during DNA replication, and is implicated as a key component of polymerase switching during translesion DNA synthesis (53-55). Previous studies have revealed that Sso PCNA opens at the PCNA1:PCNA3 interface to be loaded onto DNA by RFC (27). However, no crystal structure of PCNA in the ring-open conformation is currently available despite structures of the ring-closed conformation alone (22-24) and in complex with protein binding partners (24-26). Accordingly, we wondered if PCNA maintains an equilibrium between ring-open and ring-closed conformations even in the absence of DNA or RFC. To directly probe for this conformational equilibrium, we designed an inter-subunit FRET construct wherein PCNA1 and PCNA3 were modified with site-specific Cys mutations at S64 and S189, respectively, and were simultaneously labeled with Cy3- and Cy5-maleimide (Fig. **1A** and **B**). Based on the crystal structure of Sso PCNA, the inter-fluorophore distance in the ringclosed conformation is 45 Å which corresponds to a FRET efficiency of ~ 0.80 (Cy3-Cy5 R₀ = ~ 54 Å) (56,57). Consequently, conformational transition of Sso PCNA from the ring-closed to the ringopen conformation should cause a decrease in FRET efficiency (Fig. 1A and 1B). Notably, while our labeling strategy can result in a heterogeneous population of labeled species (i.e. Cy3 at both S64 and S189, Cy5 at both S64 and S189, and single-label species), these molecules do not demonstrate anti-correlated donor and acceptor fluorescence signals during smFRET experiments, and thus were either not observed during smFRET imaging or easily identified for exclusion during data processing. Moreover, the two possible dual-labeled PCNA molecules (i.e. C64-Cy3/C189-Cy5 PCNA and C64-Cy5/C189-Cy3 PCNA) from Cy3-Cy5 labeling are expected to yield

equivalent FRET signals during smFRET experiments as each labeling site was carefully selected (*i.e.* based on the *Sso* PCNA heterotrimer crystal structure, PDB code: 2HII) to avoid secondary structural elements of PCNA while maintaining a high degree of flexibility and solvent accessibility for the conjugated fluorophores. Thus, we assume that the dipole orientation factors (κ^2) (58) for the dual-labeled PCNA molecules with fluorophores positioned at either site should be similar and therefore not affect apparent FRET efficiencies and relative distance changes during our smFRET experiments.

To simplify surface-immobilization for smFRET experiments, PCNA1, PCNA2, and PCNA3 were covalently fused (PCNA1-2-3) through peptide linker (ASGAGGSEGGSEGGTSGAT) between PCNA1 and 2 as well as PCNA2 and 3 (Fig. 1C). This same strategy was previously applied to define the single PCNA subunit interface (PCNA1:PCNA3) for ring-opening, and this linked construct enhanced DNA polymerase processivity in primer extension assays just as wild-type heterotrimeric PCNA (27). Thus, this covalently-linked PCNA heterotrimer offered the advantage of simplifying the interpretation of smFRET results (i.e. by limiting complicating signals from in situ PCNA heterotrimer association and dissociation) to exclusively investigate ring-opening and ring-closing, as well as permitted direct comparison with the previous study (27). To permit wide-field smFRET imaging, we achieved surface immobilization of Sso PCNA by engineering a short AviTag (15 amino acid residues) to the C-terminus of the protein to be biotinylated at a specific Lys residue by BirA (31,33). The biotinylated PCNA was then bound by NeutrAvidin which was immobilized to the surface of a quartz microscope slide passivated with biotinylated-PEG molecules. Following surface-immobilization, fluorescence intensity from both donor and acceptor channels was simultaneously recorded during Cy3 excitation with a 532 nm laser. Initial experiments yielded

single-molecule trajectories with anti-correlated donor and acceptor signals representing two, nonzero FRET efficiency values near 0.80 (high FRET) and 0.55 (low FRET), which we have attributed to the ring-closed (Fig. 1D) and ring-open (Fig. 1E) conformations of PCNA, respectively. PCNA was observed to interconvert between the ring-closed and ring-open conformations as demonstrated by FRET fluctuations between the high and low efficiency values (Fig. 2) in some single-molecule trajectories. The inclusion of Trolox (2 mM) as an anti-blinking agent in the single-molecule imaging buffer, as well as the anti-correlated signal changes and fluctuations to non-zero FRET efficiency values suggests that the observed FRET transitions do not occur as a result of acceptor blinking (38). However, many single-molecule trajectories demonstrated only high FRET (Fig. 1D) or low FRET (Fig. 1E) efficiencies until irreversible photobleaching, suggesting that PCNA ring-opening and ring-closing transition kinetics are slow. Accordingly, reversible fluctuations between FRET efficiency states were only occasionally observed (data not shown) before photobleaching during the imaging period of our experiments (~120 s). These results verify the utility of our FRET construct and immobilization strategy, and support the hypothesis that Sso PCNA is conformationally dynamic even in the absence of DNA or protein partners.

Investigation of Sso PCNA by smFRET

Previous studies have established that the interfaces between each PCNA monomer are partly stabilized by ionic interactions (22-24). Moreover, the interacting regions of PCNA1 and PCNA3 at the PCNA1:PCNA3 interface have clear positive and negative surfaces, respectively (**Fig. S1**). We thus wondered if PCNA ring-opening and ring-closing detected through our smFRET methodology (**Figs. 1** and **2**) would be sensitive to the ionic strength of the imaging

buffer, which would be observed as changes in the relative distribution of FRET efficiency values and ring-opening/ring-closing transition kinetics upon titration with increasing amounts of NaCl. Accordingly, we performed single-molecule imaging of surface-immobilized, Cy3/Cy5-labeled PCNA1-2-3 in imaging buffer containing different NaCl concentrations (0-1,000 mM). Many single-molecule trajectories (>200) at each NaCl concentration were collected and observed FRET efficiencies were binned to generate FRET population distribution histograms (Fig. 3). Two FRET populations, corresponding to a high FRET, ring-closed PCNA conformation ($E_{app} = 0.7$ -0.9) and a low FRET, ring-open PCNA conformation ($E_{app} = 0.5-0.6$) were readily apparent within the FRET distribution histograms (Fig. 3A - E). The bimodal FRET distributions were fit to a sum of Gaussian functions in MATLAB to quantify the effect of solution ionic strength on the open-close equilibrium of PCNA. The low- and high-FRET populations (Fig. 3A – E, dashed red lines) showed a strong dependence on solution ionic strength as low NaCl concentrations favored the ring-closed PCNA conformation (peak center = 0.80) and high NaCl concentrations favored the ring-open PCNA conformation (peak center = 0.55). In fact, by plotting the relative fraction of low-FRET events against NaCl concentration, a clear linear correlation is apparent as increasing ionic strength presumably disfavors the ring-closed PCNA conformation but favors the ring-open PCNA conformation (Fig. 3F). With no NaCl added to the imaging buffer (Fig. 3A), a substantial fraction (~25%) of low-FRET events are still observed in the FRET distribution, suggesting that PCNA ring-opening can still spontaneously occur despite weak shielding of electrostatic interactions. Similarly, at high ionic strength (Fig. 3E, 1,000 mM NaCl), PCNA ring-closing can still spontaneously occur as ~25% of molecules remain in the high-FRET, ring-closed conformation despite significant shielding of electrostatic interactions.

To further investigate the conformational dynamics of Sso PCNA, we measured the transition kinetics of PCNA ring-opening and ring-closing through a dwell time analysis of our smFRET data at each NaCl concentration. Briefly, we measured the duration of time each molecule spent at a particular FRET efficiency level before transitioning to a different level or photobleaching. Dwell times of PCNA in the ring-closed (i.e. high-FRET) and ring-open (i.e. low-FRET) conformations were binned into histograms which were subsequently integrated, normalized, and inverted to generate survivor functions (Fig. 4A, B). Single exponential decay fits to the survivor functions of the high-FRET and low-FRET efficiencies yielded rate constants for PCNA ring-opening (k_{open}) and PCNA ring-closing (k_{close}) at each tested NaCl concentration (**Table 1**). The value for k_{open} was affected by solution ionic strength. Consistent with the decrease in population of the ring-closed PCNA conformer at increasing ionic strength (Fig. 3), kopen became more rapid (0.0258-0.130 s⁻¹) as the NaCl concentration was raised from 0 to 1,000 mM (Fig. 4C and **Table 1**). In contrast, k_{close} (0.038-0.051 s⁻¹) was not significantly affected by increasing solution ionic strength, suggesting that PCNA ring-closing may occur in two distinct steps, one of which (i.e. the protein conformational change associated with ring-closing) is unaffected by solution ionic strength. Consistently, as the solution ionic strength increased, the equilibrium constant (K_{eq}) , calculated as k_{open}/k_{close} , also increased thereby reflecting the shift in equilibrium towards the ring-open conformation of PCNA (Table 1). In agreement with the lack of reversible fluctuations between FRET efficiency states before photobleaching in smFRET trajectories, the transition rates measured for ring-opening and ring-closing were indeed small (Table 1). In fact, the time required for all molecules to complete the reaction $(7*t_{1/2} = 7*(\ln 2/k), t_{1/2} = \text{reaction half-}$ life) was 75 s for PCNA opening (i.e. calculated based on average kopen) and 109 s for PCNA closing (i.e. calculated based on average k_{close}), while photobleaching typically occurred within

120 s during smFRET experiments. As a result, the likelihood of observing both slow ring-opening and slow ring-closing within an individual single-molecule trajectory before donor or acceptor photobleaching was minimal.

Ion Mobility-Mass Spectrometry of Sso PCNA

To further probe the conformational dynamics of *Sso* PCNA and support the conclusions of our smFRET analysis, we studied the structure and conformations of *Sso* PCNA by native mass spectrometry (MS). This unique methodology has been applied to investigate protein-protein interactions in the gas phase as well as to provide low-resolution structural information for proteins and protein complexes, without the need for complicated or potentially disruptive protein labeling (59,60). Coupling nano-electrospray ionization (nESI), a soft ionization technique that preserves noncovalent interactions for transmission into the mass spectrometer (61-63), with ion mobility-mass spectrometry (IM-MS), we can probe protein shape (64,65) and can even observe differences in molecular conformations (66,67).

To verify that subunit fusion did not alter the conformational dynamics of covalently-linked *Sso* PCNA1-2-3 utilized in the smFRET experiments relative to wild-type *Sso* PCNA heterotrimer, we performed native MS on both constructs. For gas phase analysis, each protein was buffer exchanged into a solution consisting of increasing concentrations of 20% triethylammonium acetate (TEAA), and 80% ammonium acetate (AmAc) (100-500 mM mixed acetate salts) to increase ionic strength. We chose to use a combination of AmAc and TEAA as previous studies have shown that TEAA reduces the charge states of the protein ions and lower charge states are attributed to a more compact and native-like form of the complex (68,69). While the wild-type PCNA heterotrimer is the predominant species at 100 and 200 mM mixed acetate

salts (Fig. 5A, B), the mass spectra revealed sample heterogeneity as peaks attributed to monomer and dimer were apparent. The experimental mass for the wild-type PCNA heterotrimer (Fig. 5A) was determined to be 82982 ± 41 Da, which is in good agreement with the theoretical mass (82395) Da), when adducting of salt and solvent are considered (common in native MS when attempting to keep structures native). Moreover, masses of monomer and dimer were determined to be 27675 \pm 94 Da and 55393 \pm 145 Da, respectively, which are consistent with the theoretical masses (PCNA1: 27536 Da, PCNA2: 27436 Da, PCNA3: 27459 Da, PCNA1+2: 54972 Da, PCNA2+3: 54895 Da, PCNA1+3: 54995 Da). The differences in theoretical masses for the monomer and dimer species are relatively small $(\Delta MW_{(PCNA1-PCNA2)}) = 100 \text{ Da}; \Delta MW_{(PCNA1-PCNA3)} = 77 \text{ Da};$ $\Delta MW_{(|PCNA2-PCNA3|)} = 23 \text{ Da}; \Delta MW_{(|(PCNA1+2)-(PCNA2+3)|)} = 77 \text{ Da}; \Delta MW_{(|(PCNA1+2)-(PCNA1+3)|)} = 23 \text{ Da};$ $\Delta MW_{(|(PCNA1+3)-(PCNA2+3)|)} = 100 \text{ Da}$), corresponding to a theoretical $\Delta m/z$ of 3-14 for the 7+ charge state of the monomer and a theoretical $\Delta m/z$ of 2 – 9 for the 11+ charge state of the dimer. Accordingly, we cannot confidently assign the identities of the monomer and dimer species present in Fig. 5A – 5C which were acquired using a time-of-flight mass spectrometer and have peak widths (126 and 122, respectively) greater than these theoretical $\Delta m/z$ values. Indeed, given such subtle mass differences, the reported enzyme-catalyzed removal of the N-terminal methionine residue during protein expression in E. coli, which occurs with varying efficiency based on protein sequence (70), further complicates assignment of the monomer and dimer species by mass. The higher observed molecular weights in comparison to the theoretical sequences are attributed to the fact that the protein ions will retain salt, solvent, and other adducts during the gentle ionization and desolvation conditions employed here, which were critical for maintaining the native state of the wild-type PCNA heterotrimer (71). However, previous biochemical studies of Sso PCNA have reported that only PCNA1 and PCNA2 form a dimer and no homodimerzation of any of the PCNA

subunits occurs (21). Hence, the dimer species in our experiments is likely PCNA1+2, while the monomer is PCNA3. The relative intensities of the monomer and dimer species increased at higher ionic strength (Fig. 5C, 500 mM) suggesting heterotrimer dissociation as stabilizing electrostatic interactions at the subunit interfaces were presumably shielded. As 500 mM mixed acetate salts resulted in significant decomposition of the heterotrimeric PCNA to monomer or dimer constituents, the wild-type *Sso* PCNA was not analyzed at higher ionic strengths.

As depicted for the dominant charge state (14+), two populations of distinct rotationally averaged collisional cross sections (CCS) of 49 and 52 nm² were observed suggesting conformational heterogeneity of heterotrimeric PCNA (**Fig. 5D – F**). As the concentration of mixed acetate salts increased from 100 to 500 mM, the more compact population of ions (*i.e.* possessing a smaller CCS of ~49 nm²) began to diminish while the more extended population of ions (*i.e.* possessing a larger CCS of ~52 nm²) began to increase (**Table 2** and **Fig. 5D – F**). These results are consistent with the smFRET data (**Fig. 3**) and suggest that ions with smaller CCS represent the ring-closed conformer of PCNA (discussed in more detail in following sections), which is stabilized at low ionic strength, while ions with larger CCS represent the ring-open conformer of PCNA, which is favored at high ionic strength.

We next examined biotinylated *Sso* PCNA1-2-3 by native IM-MS to better simulate conditions of the smFRET analysis. As expected, the experimental mass of the biotinylated PCNA1-2-3 (**Fig. 6A**) is larger (89667 ± 103 Da) than wild-type PCNA heterotrimer (82982 ± 41 Da) which is consistent with the 20 amino acid linkers between PCNA1 and 2 and PCNA2 and 3, as well as the 15 amino acid AviTag conjugated to biotin (total theoretical MW: 88894 Da). The higher measured molecular weight in comparison to the theoretical is likely due to adducts (such as solvent or salt molecules) remaining on the protein during the ionization and desolvation

processes. As with the wild-type PCNA heterotrimer, we systematically increased ionic strength by buffer exchanging aliquots of the biotinylated PCNA1-2-3 into increasing amounts of mixed acetate salts (100-1,000 mM) before IM-MS analysis. As expected for a covalent peptide-linked construct, the mass spectra (Fig. 6 A - E) obtained at each concentration of mixed acetate salts revealed a homogenous sample of biotinylated PCNA1-2-3 that did not decompose to smaller subunit constituents as ionic strength was increased. The collisional cross section distributions of the two dominant charge states (14+ and 15+) of biotinlylated PCNA1-2-3 (Fig. 6 $\mathbf{F} - \mathbf{O}$) were similar to the wild-type PCNA heterotrimer and revealed two populations of distinct CCS: a compact species at ~53 nm² and an extended species at ~55 nm² (Table 2). Notably, the CCS values for compact and extended biotinylated PCNA1-2-3 were noticeably larger than for wildtype PCNA heterotrimer (~49 vs. ~53 nm² and ~52 vs. ~55 nm², respectively) (**Table 2**). We attribute these differences in CCS to the 20 amino acid long linkers between PCNA1 and 2 and PCNA2 and 3, as well as the 15 amino acid long AviTag at the protein C-terminus. As with the wild-type PCNA heterotrimer, increasing ionic strength resulted in reduction of the intensity of the compact conformational family and concomitant increase in the intensity of the extended conformational family (Fig. 6), which is consistent with the IM-MS data collected for the wildtype PCNA heterotrimer (Fig. 5) and indicates that, relative to the wild-type PCNA, neither subunit fusion nor biotinylation of PCNA1-2-3 significantly diminished the ability of the trimer to adopt two distinct conformations. This experimental consistency between the wild-type and covalentlylinked constructs verifies the biological relevance of smFRET experiments completed with the covalently-linked PCNA heterotrimer (Figs. 1 and 2). We attribute the compact conformational family (Fig. 6F – O), at lower CCS, to PCNA1-2-3 in a ring-closed conformation which transitions to an extended ring-open conformation as ionic strength increases. The collisional cross section

distributions between the 15+ and 14+ charge states are marginally different (Fig. 6). For example, even at lower concentrations of mixed acetate salts (Fig. 6F, 100 mM), the majority of ions at the 15+ charge state adopt the extended ring-open conformation (~55 nm²), but the minority ringclosed population continues to diminish as ionic strength increases (Fig. 6 G, H, I, J). This is in contrast to ions of the 14+ charge state which show the majority of ions in the compact ring-closed conformation (52.81 \pm 0.15 nm²) (Fig. 6K, 100 mM) prior to increasing ionic strength (Fig. 6 L, M, N, O). This difference with charge state is not unexpected as the more compact population would be expected, on average, to accept fewer charges (i.e. smaller charge carrying capacity) during the nano-electrospray ionization process than the more extended population (i.e. larger charge carrying capacity). Hence, we would expect to see a higher population of the compact conformational family in the lower charge states (i.e. 14+) and a higher population of the extended conformational family in the higher charge states (i.e. 15+). Furthermore, while the overall trend observed for the conformational dynamics of biotinylated PCNA1-2-3 at increasing ionic strengths is consistent between the smFRET and IM-MS data, the distributions of PCNA1-2-3 in the ringopen and ring-closed conformations at each salt concentration were slightly different between the two methods. For instance, at concentrations greater than 500 mM NaCl (Fig. 3) in the smFRET experiments, the majority of PCNA1-2-3 molecules adopt the ring-open conformation. However, for the 15+ and 14+ charge states of the IM-MS experiments (Fig. 6), concentrations of mixed acetate salts as low as 100 (Fig. 6F) or 250 mM (Fig. 6L), respectively, are sufficient to favor the extended ring-open conformation. Accordingly, as choice of monovalent salt (i.e. ammonium acetate versus sodium chloride) did not appreciably influence the smFRET results (Fig. S2), the observed conformational equilibrium of PCNA may be moderately sensitive to IM-MS processes, such as desolvation and ionization, which may positively contribute to PCNA transitions to the

extended, ring-open conformation. Nevertheless, these IM-MS results demonstrate a strong correlation between ionic strength and an extended (ring-open) conformation of PCNA1-2-3, and therefore strongly support the in-solution smFRET data of favorable transitions to a low-FRET, ring-open conformation as solution ionic strength increases (**Fig. 3**). As a result of the wild-type (**Fig. 5**) and covalently-linked (**Fig. 6**) PCNA heterotrimer demonstrating similar dynamic properties during IM-MS analysis, the conformational dynamics of PCNA1-2-3 observed by smFRET (**Fig. 3**) likely emulate those of the wild-type PCNA heterotrimer as well.

Molecular Dynamics Simulation of Sso PCNA

A powerful tool for understanding the mechanisms of protein folding, binding, as well as conformational changes associated with a biological process (72-76) is generation of a structurebased model (SBM). Accordingly, we used the crystal structure of Sso PCNA along with our smFRET and IM-MS experimental findings to develop a SBM of PCNA to simulate ring- opening and ring-closing. A coarse-grained SBM consisting of Cα atoms from the wild-type Sso PCNA heterotrimer was generated from PDB code 2HII (24). The native contacts between PCNA1 and PCNA3 were omitted from the starting model to allow sampling to the ring-open conformer. Using the Debye-Hückel model, a series of long-time (1 µs) molecular dynamic simulations were performed at different ionic strengths (10-500 mM), and the ring-open conformation of the wildtype PCNA heterotrimer was observed at higher ionic strengths. Consistent with the collisional cross section distributions of the wild-type PCNA heterotrimer (Fig. 5), our molecular dynamic simulations suggest that the ring-closed conformation of Sso PCNA is more stable at lower salt concentrations based on the root-mean-square deviation (RMSD) (Fig. 7A) of our SBM with respect to the crystal structure of PCNA in the ring-closed conformation (2HII). Furthermore, the RMSD is shown to increase as the salt concentration is raised from 10 to 500 mM, indicating conformational heterogeneity driven by increased ionic strength (Fig. 7A). Our simulation tracked the fraction of native contacts at the PCNA1:PCNA3 interface ($Q_{inter13}$) as ionic strength was increased from 10 to 500 mM (Fig. 7B). With Sso PCNA in the fully ring-open conformation, $Q_{inter13}$ equals 0, while in the fully ring-closed conformation, $Q_{inter13}$ equals 1, indicating that all native contacts are made. Clearly, the fraction of ring-open PCNA conformers is much lower (i.e. low probability, low $Q_{inter13}$) at low ionic strength, but increases as ionic strength is raised (**Fig.** 7B). Notably, a simulation removing all charge from the system reveals Sso PCNA to be in the ring-open conformation as all electrostatic contacts at the PCNA1:PCNA3 interface are eliminated (no charge, Fig. 7B). By calculating the distance between the center of mass of residues involved in native contacts at the PCNA1:PCNA3 interface at varying ionic strengths, we observed that the distance of the PCNA1:PCNA3 interface lengthens as salt concentration increases (Fig. 7C) and is greatest when all charges are eliminated (i.e. no attractive electrostatic forces) (no charge, Fig. 7C). Our simulation also reveals that as ionic strength is increased from 10 to 500 mM, the mean number of salt bridges in the SBM decrease as interacting charges become shielded (Fig. 7D), which provides a basis for the experimentally observed increases in k_{open} (Table 1) and the population of ring-open PCNA conformer as salt concentration is increased (Figs. 3, 5, and 7). Taken together, our molecular dynamic simulation suggests that the proportion of ring-open PCNA conformer is dependent on ionic strength. Notably, most configurations of Sso PCNA from our simulation open out-of-plane with an unexpected left-hand geometry (Fig. 7E).

Discussion

Replication and repair of DNA are critical for cell functioning, survival, and proliferation.

Accordingly, these complex processes are executed and tightly regulated *in vivo* by a host of

enzymes and protein cofactors, including the sliding DNA clamp, PCNA. This ring-shaped molecule encircles DNA to serve as a processivity factor for replicative DNA polymerases, but has also been implicated as a protein scaffold upon which DNA replication and repair components assemble. As PCNA is often localized at the replication fork, any complications that arise during DNA replication, such as DNA damage or replication fork stalling, can be efficiently resolved by the applicable enzymes recruited to PCNA. Structures of sliding DNA clamps from all three domains of life show a ring-closed conformation, suggesting that a ring-closed-ring-open conformational change is necessary for PCNA loading onto DNA. Indeed, RFC is known to pry open the closed PCNA ring for efficient loading onto DNA at primer/template junctions. Consistently, a structure of gp45 clamp from T4 bacteriophage was solved in a ring-open conformation only when in complex with the gp62/gp44 clamp loader and DNA (27,28,77). Similarly, a low resolution (12 Å) cryo-electron microscopy structure of archaeal PCNA in complex with RFC and DNA from *Pyrococcus furiosus* also reveals out-of-plane ring-opening (78). However, biochemical and computational evidence suggest that sliding DNA clamps may sample ring-open and ring-closed conformations (29,79), even in the absence of their respective clamp loaders, despite the aforementioned structural data. In fact, the ring-open conformation of gp45 was shown to dominate in solution by fluorescence spectroscopy (29), while only the ringclosed structure (in the presence of clamp loader) could be solved by X-ray crystallography (30). For this reason, it was previously hypothesized that crystallization conditions may in fact artificially select for the ring-closed conformation of sliding DNA clamps (79). Here, using smFRET, IM-MS, and SBM molecular dynamic simulations, we show that Sso PCNA exists in equilibrium between ring-open and ring-closed conformations that are sensitive to ionic strength. To our knowledge, these data are the first to demonstrate passive ring-opening and ring-closing of a sliding DNA clamp in real-time. Importantly, these dynamic conformational transitions may be significant for regulating protein-protein and protein-DNA interactions mediated by PCNA during DNA replication and repair.

The opening of sliding DNA clamps by clamp loader assemblies (i.e. gp62/gp44 in T4 bacteriophage, γ-complex in E. coli, RFC in archaea and eukaryotes) has been structurally and biochemically evaluated to reveal a largely conserved mechanism (80). While in complex with ATP, clamp loaders first bind and then open sliding DNA clamps. Next, this complex binds to the primer-template junction of DNA which activates ATP hydrolysis by the clamp loader. ATP hydrolysis results in the closure of the sliding DNA clamp and dissociation of the clamp loader from the complex. While it is suspected that ATP binding to the clamp loader permits the forcible opening of the DNA clamp, an alternative hypothesis suggests that an ATP-stabilized clamp loader conformation preferentially associates with the ring-open conformation of the sliding DNA clamp (28). For the first time, our smFRET analysis has provided direct evidence for dynamic interconversion of PCNA between ring-open and ring-closed conformations (Fig. 2) which supports the latter hypothesis for RFC-catalyzed clamp opening (the ATP-stabilized clamp loader associates with the ring-open PCNA). While ensemble FRET has been previously utilized to investigate the conformations of sliding DNA clamps (29,81-84), these studies could only report on the average behavior of clamp molecules. In contrast, smFRET is not constrained by ensemble avaraging to permit dynamic visualization of individual molecules. Here, we surface-immobilized PCNA that was simultaenously labeled with Cy3 and Cy5 at positions sensitive to ring-opening and ring-closing at the PCNA1:PCNA3 interface for wide-field smFRET imaging (Fig. 1). Based on the observed, apparent FRET efficiencies for the high- and low-FRET states (~0.80 and 0.55, respectively) (Figs. 2 and 3), as well as the Förster radius for Cy3-Cy5 (~54 Å) (56,57), the

distance change between the ring-closed and ring-open PCNA conformers is roughly 10 Å. This distance is consistent with in-plane ring-opening of ~5-9 Å observed structurally for gp45 and archaeal PCNA in complex with their respective clamp loaders and DNA (28,78). This supports the notion that single-stranded DNA is first passed through the open ring, followed by PCNA ringclosing and subsequent sliding to the double-stranded primer-template junction, rather than additional ring-opening to permit passage of the wider double-stranded DNA (~20 Å) (28,78). Notably, while proper data corrections are necessary to measure the absolute inter-fluorophore distance of the low- and high-FRET states, the ~10 Å difference estimated above is a reasonable approximation as both the low-(0.40 - 0.60, center = 0.55) and high-(0.60 - 0.90, center = 0.80) FRET distributions fall squarely in the linear region of the distance-dependency of FRET efficiency for the Cy3-Cy5 pair (Fig. S3). Our molecular dynamic simulations revealed that most ring-open configurations sampled by PCNA were out-of-plane with left-hand geometry (Fig. 7E). This result is consistent with previous computational reports (79,85,86) which revealed that PCNA (including yeast and human PCNA (86)) is not biased towards right-handed spiral configurations, which is in contrast to structures of DNA clamps in complex with clamp loaders (28,78,80). Accordingly, passive loading of PCNA may occur at the single-stranded region of primer-template junctions via left-hand helical geometry. Alternatively, PCNA may be stabilized in the right-hand helical geometry for DNA loading by its binding to RFC.

The novel results of our smFRET investigation were supported and cross-validated by native mass spectrometry of the wild-type *Sso* PCNA (**Fig. 5**) and the biotinylated PCNA1-2-3 construct (**Fig. 6**). IM-MS revealed that two conformational families existed for each construct with distinct collisional cross sections (**Figs. 5** and **6**, **Table 2**). These populations are attributed to PCNA heterotrimer in either compact (ring-closed) or extended (ring-open) conformational

families and are readily separated by their difference in drift time within the ion mobility cell. In order to further validate these findings we compared the experimental CCS to theoretical CCS determined from the molecular dynamics simulations performed in Fig. 7. Our MD simulations were performed using coarse grained (C alpha only) structures and to the best of our knowledge, no CCS algorithm exists for CCS determination of coarse grained structures of this type. We therefore could not directly compare the values generated from the theoretical calculations to the experimental values, but could compare the trends observed. Theoretical CCS were determined using both the projection approximation (PA) and the trajectory method (TJM) algorithms in IMPACT and although the values vary significantly for these coarse grained models, the trends remain the same. Fig S4 and Table S1 summarize the theoretical CCS results for the wild-type PCNA. As expected, the ring-closed form is more compact than the ring-open form with the average percentage difference in CCS between the two forms being 2.1%. This is in line with, although smaller than, our experimental observations of the wild-type PCNA heterotrimer in which an average 4.7% difference between the two conformational families was observed. It has been previously reported that ring complexes can undergo compaction on transfer into the gas-phase (87), which could in part explain the larger percentage difference between the two conformational families observed experimentally. Interestingly, although the absolute values are significantly different for PA and TJM, the percentage difference calculated between the two conformational families is very similar, 2.1% and 2.2% respectively.

Furthermore, the intensity of each conformational ensemble was shown to be sensitive to the concentration of mixed acetate salts (**Fig. 6**), with higher concentrations favoring the extended conformer. The IM-MS data at different mixed acetate salt concentrations agreed with the observed influence of increasing NaCl concentrations on the proportions of FRET events at high

and low efficiencies (Fig. 3) and our molecular dynamic simulations (Fig. 7), with low ionic strength favoring the closed PCNA conformation and high ionic strength favoring the ring-open PCNA conformation. Moreover, dwell time analysis (Fig. 4) of the high- and low-FRET events yielded transitions rates for ring-closed→ring-open and ring-open→ring-closed conformational changes, respectively (Table 1). As the transition kinetics (Fig. 4 and Table 1) from the ringclosed conformer (PCNA^C, Scheme 1) to the ring-open conformer (PCNA^O, Scheme 1) and occupancy of the low-FRET state (Fig. 3F) showed an increasing linear dependence on ionic strength, but the reverse transition (i.e. $PCNA^O \rightarrow PCNA^C$) was not sensitive to increasing ionic strength, we expanded the simple one-step mechanism for PCNA ring-opening/ring-closing (Scheme 1A) to include an additional step (Scheme 1B). Supported by our molecular dynamic simulations (Fig. 7B, C, D), as native electrostatic interactions at the PCNA1:PCNA3 interface become sufficiently shielded by electrolyte ions, PCNA^C is first converted to PCNA^Z which maintains the closed ring conformation but lacks all associated stabilizing contacts. Conversion to PCNA^Z (k_I) becomes more rapid as ionic strength increases as illustrated by the slope of 1.0 x 10⁻¹ ⁴ mM⁻¹s⁻¹ for the best fit line to the data in **Fig. 4C** which quantitatively describes how the ringclosed—ring-open transition rate scales with increasing NaCl concentrations. Following PCNAZ formation, conformational change to the ring-open state (PCNA O , **Scheme1B**) occurs rapidly (k_2) and is independent of salt concentrations. Thus, k_{open} in Table 1 is limited by k_1 . During the reverse transition from PCNA^O to PCNA^O, k-2 (0.038-0.051 s⁻¹, **Table 1**) describes the rate of the reverse conformational change to the closed ring conformation wherein native electrostatic contacts have not yet re-formed (PCNA^Z), and is independent of salt concentrations. Moreover, k_{-1} describes the reinstatement of native electrostatic contacts at the PCNA1:PCNA3 interface which presumably does not lead to a measurable FRET change and was not determined in our studies. Accordingly,

 k_{close} in Table 1 equals k_{-2} . Notably, the remaining interfaces of the PCNA heterotrimer (i.e. PCNA1:PCNA2 and PCNA2:PCNA3) have been shown to be much more charge-charge compatible, as well as occupy more buried surface area, than the PCNA1:PCNA3 interface, suggesting that disruption through electrostatic shielding of native interactions at these interfaces would occur at higher ionic strengths than for the PCNA1:PCNA3 interface, as supported by our native mass spectra of the wild-type PCNA heterotrimer (Fig. 5) which shows dissociation of the trimer at higher ionic strengths (23). The observed instability of the wild-type PCNA heterotrimer as buffer ionic strength is increased beyond the physiological salt concentrations which are ≤500 mM (88,89) may suggest a mechanism for PCNA subunit dissociation. Indeed, it may be that disassembly of the PCNA heterotrimer favorably occurs from the ring-open state as a result of the loss in electrostatic contacts between PCNA1 and PCNA3, which agrees with the positive correlation between the ring-open state of the PCNA heterotrimer (Fig. 5 D-F) and observed subunit dissociation (Fig. 5 A-C) at higher buffer ionic strengths. Notably, the low FRET state assigned as the ring-open conformer of covalently fused PCNA during smFRET experiments (Figs.1 – 3) cannot be attributed to the observed subunit dissociation of the PCNA heterotrimer at higher ionic strengths as such a dissociation event would most likely result in FRET efficiencies of less than 0.2 as a result of the 20 amino acid linkers between PCNA1 and PCNA2, as well as PCNA2 and PCNA3. The length of the fully-extended linker is approximately 70 Å (i.e. assuming 3.5 Å per amino acid) which would result in a FRET efficiency of ≤ 0.17 (assuming Cy3-Cy5 R₀ \approx 54 Å and not including the length of a typical PCNA subunit which would result in an even lower expected FRET value, near zero) upon PCNA subunit dissociation. As a low FRET efficiency threshold of 0.2 was applied during smFRET data analysis (see Materials & Methods),

such near-zero FRET efficiencies, occurring from PCNA subunit dissociation at high ionic strengths, would have been removed from the overall analysis.

The observed conformational dynamics of PCNA are supported by structural data collected for Sso monomeric, dimeric, and trimeric PCNA (22-24). Although the angle between each monomer in the crystal structure of the heterotrimer was determined to be 120°, the angle between PCNA1 and PCNA2 in the crystal structure of the heterodimer was determined to be 130°. Accordingly, during formation of the Sso heterotrimer (i.e. binding of PCNA3 to the PCNA1/PCNA2 heterodimer), a significant spring tension is introduced as PCNA1 and PCNA2 are forced to bend ~10° to accommodate PCNA3. It is thus hypothesized that PCNA ring-opening at the PCNA1:PCNA3 interface occurs to relieve the spring tension induced by PCNA3 (23). Indeed, a structural model of PCNA3 bound to the PCNA1/PCNA2 heterodimer reveals a ~7 Å gap between PCNA1 and PCNA3 which is consistent with the ~10 Å distance change roughly estimated through our smFRET experiments (23). As our data reveal that PCNA can open and close even in the absence of RFC, the role of the clamp loader becomes less defined. Consistently, DNA polymerase processivity assays revealed that while Sso PCNA was necessary to synthesize full-length DNA product, Sso RFC was dispensable, suggesting that Sso PCNA is capable of loading to a primed M13 template in the absence of RFC (77). However, the fastest rate of DNA synthesis and greatest amount of DNA product formation was achieved in the presence of both PCNA and RFC (77). Accordingly, it seems that RFC may bind to the ring-open conformation of PCNA and guide the sliding DNA clamp to primer-template junctions of DNA substrates. Moreover, Sso RFC may be required to accelerate the rate of Sso PCNA ring-opening as the passive rate of PCNA ring-opening (k_{open}), even at the highest NaCl concentration, was only 0.13 s⁻¹ (**Table 1**) which is considerably slower than the estimated association rate of the binary

complex of RFC/PCNA to DNA (100 s⁻¹) (77). At a more physiologically relevant salt concentration (250 mM), k_{open} was determined to be even slower (0.0374 s⁻¹, **Table 1**) and the majority of PCNA molecules were observed in the ring-closed conformation (**Fig. 3B**), further underlining the need for RFC to accelerate and stabilize the ring-open conformation of *Sso* PCNA for loading onto DNA. As the rate of replication fork progression in a hyperthermophilic organism such as *Sulfolobus solfataricus* is likely very fast, it is crucial that *Sso* PCNA be quickly and efficiently directed to priming sites for Okazaki fragment synthesis during lagging strand replication. Furthermore, MS data demonstrating that the wild-type *Sso* PCNA heterotrimer significantly dissociates into monomer and dimer constituents when the ionic strength is increased to a 500 mM mixed acetate salt threshold (**Fig. 5**) underlines the need for RFC to stabilize the PCNA heterotrimer for clamp loading.

Collectively, our interdisciplinary approach has revealed a model PCNA to be a conformationally dynamic protein. The trends in protein conformational dynamics observed in solution during our smFRET experiments are conserved in the gas phase measurements of the solution structures during our IM-MS experiments. Thus, our work further validates native mass spectrometry as an effective tool to study the dynamic motions of proteins. We are eager to apply this methodology to investigate how the observed dynamics exhibited by PCNA may contribute to clamp loading onto DNA as well as key protein-protein and protein-DNA interactions mediated by PCNA during DNA replication, DNA damage response, and DNA repair.

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Tables

Table 1: Kinetic rates of PCNA ring-opening and ring-closing

NaCl (mM)	k _{open} (s ⁻¹) ^a	k_{close} (s ⁻¹) ^b	Keq (kopen/kclose)
0	0.0258 ± 0.0004	0.044 ± 0.004	0.59
250	0.0374 ± 0.0007	0.050 ± 0.002	0.75
500	0.0476 ± 0.0008	0.0405 ± 0.0009	1.18
750	0.081 ± 0.002	0.051 ± 0.002	1.59
1,000	0.130 ± 0.002	0.038 ± 0.002	3.42

[&]quot;Dwell time survivor functions to calculate k_{open} (Fig. 4A) were generated from smFRET trajectories exhibiting ring-closed \rightarrow ring-open FRET transitions at 0 (n = 39), 250 (n = 50), 500 (n = 84), 750 (n = 80), and 1000 (n = 64) mM NaCl

^bDwell time survivor functions to calculate k_{close} (Fig. 4B) were generated from smFRET trajectories exhibiting ring-open→ring-closed FRET transitions at 0 (n = 20), 250 (n = 23), 500 (n = 77), 750 (n = 68), and 1000 (n = 40) mM NaCl

Table 2: Collisional cross sections (CCS) in nm² for PCNA

14+ charge state wild-type PCNA heterotrimer						
ionic strength ^{a,b}	100 mM	200 mM	500 mM			
ring-closed PCNA	49.7 ± 0.31	49.76 ± 0.46	49.67 ± 0.43			
ring-open PCNA	51.86 ± 0.44	52.32 ± 1.12	51.96 ± 0.45			
14+ charge state PCNA1-2-3						
ionic strength ^{a,b}	100 mM	250 mM	500 mM	750 mM	1000 mM	
ring-closed PCNA	52.81 ± 0.15	53.56 ± 0.91	52.77 ± 0.15	52.70 ± 0.39	52.86 ± 0.45	
ring-open PCNA	54.45 ± 0.27	54.59 ± 0.25	54.43 ± 0.09	54.44 ± 0.42	54.50 ± 0.45	
15+ charge state PCNA1-2-3						
ionic strength ^{a,b}	100 mM	250 mM	500 mM	750 mM	1000 mM	
ring-closed PCNA	52.90 ± 0.23	52.81 ± 0.23	53 ± 0.02	53.20 ± 0.38	52.87 ± 0.38	
ring-open PCNA	54.53 ± 0.26	54.55 ± 0.22	54.59 ± 0.10	54.54 ± 0.034	54.66 ± 0.33	
"Ionic strength results from 80% ammonium acetate, 20% triethylammonium acetate mixture						

^bValues reported are the average of three different day repeats and the error represents the standard deviation between the three repeats.

Schemes

Scheme 1

$$\mathsf{B} \quad \mathsf{PCNA}^{\mathsf{C}} \xrightarrow[k_1]{k_1} \mathsf{PCNA}^{\mathsf{Z}} \xrightarrow[k_2]{k_2} \mathsf{PCNA}^{\mathsf{O}}$$

Scheme 1: (A) Simple mechanism of PCNA ring-opening and ring-closing. PCNA^C denotes the ring-closed conformation of PCNA while PCNA^O denotes the ring-open conformation. (B) Expanded mechanism of PCNA ring-opening and ring-closing which shows an intermediate conformation PCNA^Z, which represents a ring-closed form of PCNA lacking native interactions at the PCNA1:PCNA3 interface.

Figures

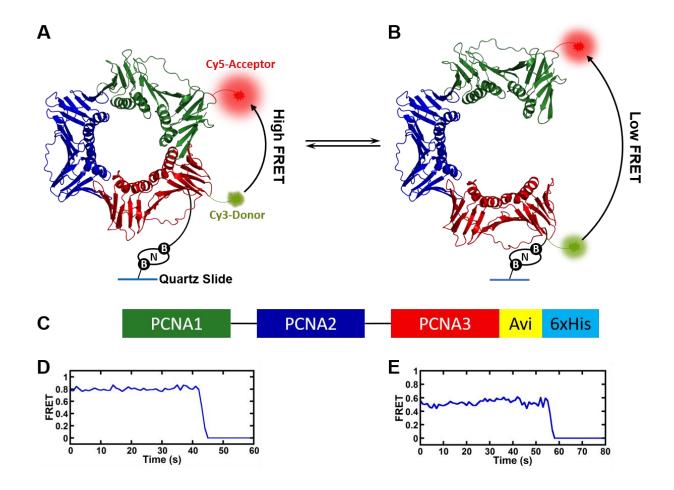


Figure 1: Single-molecule FRET system for Sso PCNA conformational dynamics. (A, B) Cy3 donor and Cy5 acceptor fluorescent dyes were introduced at the PCNA1:3 interface to probe conformational changes associated with PCNA ring-opening and ring-closing. (C) PCNA1-2-3 is covalently-linked at the PCNA1:2, and PCNA2:3 interface. (D) Ring-closed PCNA1-2-3 molecules produce high-FRET efficiency signal from 0.60-0.90 while (E) ring-open PCNA1-2-3 molecules produce low-FRET efficiency signal from 0.40-0.60.

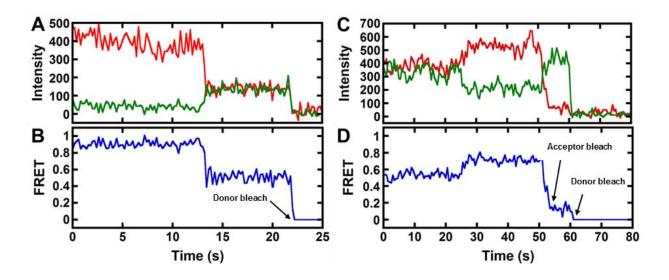


Figure 2: PCNA interconverts between its ring-open and ring-closed conformations. (A) Representative donor (green) and acceptor (red) fluorescence trajectories depicting transition from the PCNA ring-closed (high-FRET) to ring-open (low-FRET) conformation before donor photobleaching. (B) FRET efficiency trajectory (blue) calculated from the donor and acceptor intensities in (A). (C) Representative donor (green) and acceptor (red) fluorescence trajectories depicting transition from the PCNA ring-open (low-FRET) to ring-closed (high-FRET) conformation before acceptor photobleaching. (D) FRET efficiency trajectory (blue) calculated from the donor and acceptor intensities in (C).

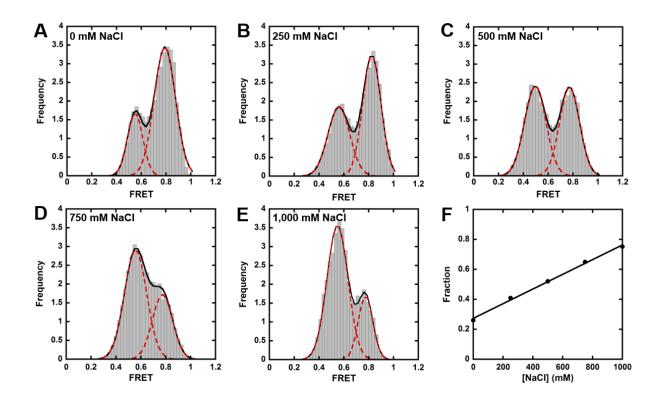


Figure 3: FRET distributions change with increasing NaCl concentrations. FRET trajectories for >200 molecules were compiled and binned to generate FRET distribution histograms for each NaCl concentration, (A) 0 mM, (B) 250 mM, (C) 500 mM, (D) 750 mM, and (E) 1,000 mM NaCl. FRET distribution histograms were fit to a sum of Gaussians function (black line) to extract the individual low-FRET (0.40-0.60) and high-FRET (0.60-0.90) populations (red dashed lines). (F) Fraction of PCNA1-2-3 molecules in the ring-open conformation (calculated in A-E) increased linearly as NaCl concentration was increased.

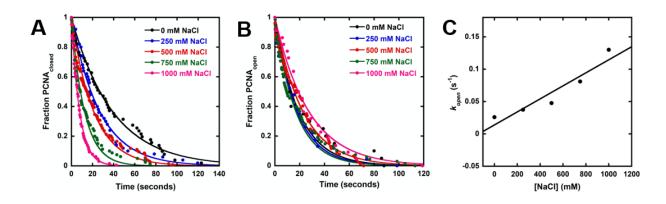


Figure 4: Dwell time analysis of PCNA states. Rates of PCNA (A) ring-opening (k_{open}) and (B) ring-closing (k_{close}) at each NaCl concentration were extracted by fitting the dwell time survivor functions of the high-FRET and low-FRET states, respectively, to a single exponential decay equation. (C) Rates of PCNA ring-opening (k_{open}) were plotted against NaCl concentrations. A linear fit with slope of 1.0 x 10⁻⁴ mM⁻¹s⁻¹ is shown (black line) to describe the dependence of k_{open} values on NaCl concentrations.

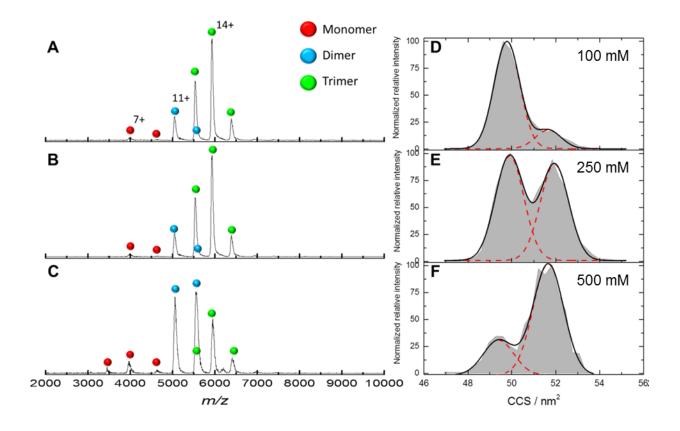


Figure 5: Ion mobility-mass spectrometry of wild-type *Sso* PCNA. Wild-type PCNA trimer was analyzed by IM-MS. Samples were exchanged into buffers containing (A) 100, (B) 200, and (C) 500 mM of 20% triethylammonium acetate (TEAA), and 80% ammonium acetate (AmAc) before introduction to the mass spectrometer by nano-electrospray ionization. Collisional cross section (CCS) distributions for the 14+ charge state of the trimer were obtained from the arrival time distributions using the previously published calibration procedure.(40) In (D) 100, (E) 200, and (F) 500 mM mixed acetate salt, the 14+ trimer showed two distributions. The lower CCS distribution is assigned to a ring-closed PCNA trimer, while the higher CCS distribution is expected to be a ring-open form of the PCNA trimer. We observe a transition from the ring-closed to the ring-open PCNA conformation as we increase ionic strength.

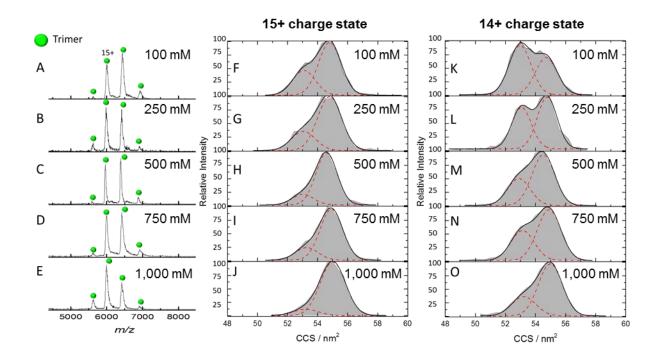


Figure 6: Mass spectrometry of covalently-linked *Sso* PCNA. Biotinylated, covalently-linked PCNA trimer (PCNA1-2-3) was analyzed by mass spectrometry. A mass spectrum is shown for each concentration of 20% triethylammonium acetate (TEAA), and 80% ammonium acetate (AmAc), (A) 100, (B) 250, (C) 500, (D) 750, and (E) 1,000 mM. At all ionic strengths, the dominant species are the 15+ and 14+ charge states of the covalently-linked PCNA trimer. Arrival time distributions were converted to collisional cross section (CCS) distributions, using a previously published calibration procedure (40). CCS distributions of the 15+ charge state are shown for increasing concentrations of mixed acetate salt, (F) 100, (G) 250, (H) 500, (I) 750, and (J) 1,000 mM. CCS distributions of the 14+ charge state are shown for increasing concentrations of mixed acetate salt, (K) 100, (L) 250, (M) 500, (N) 750, and (O) 1,000 mM.

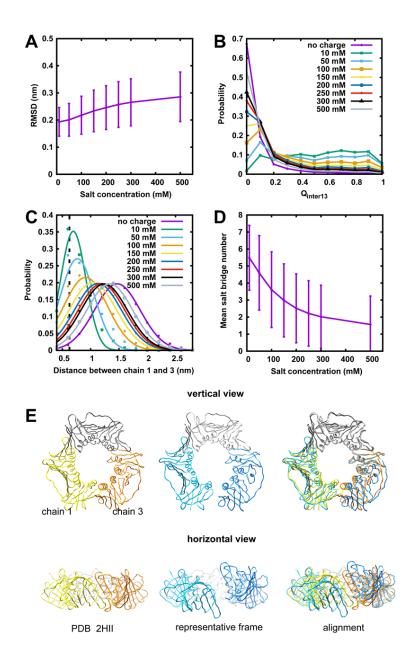


Figure 7: Molecular Dynamic Simulation of the Wild-Type Sso PCNA. (A) Average RMSD (with standard deviation) of heterotrimeric PCNA at different salt concentrations, with respect to the structure 2HII. (B) Distribution of Q_{inter13} of heterotrimeric PCNA at different salt concentrations, as well as heterotrimeric PCNA without charges. (C) Distribution of distance between PCNA1 and PCNA3 at different salt concentrations, as well as without charges. This distance is 0.625 in 2HII (shown with black dashed line). (D) Mean salt bridge number (with

standard deviation) between PCNA1 and PCNA3 at different salt concentrations. (**E**) Comparison between ring-open (middle, blue) and ring-closed (left, yellow) states of heterotrimeric PCNA. According to our simulation results, ring-open PCNA mainly adopts out-of-plane, left-hand spiral geometry.

Supplementary Data

Investigation of Sliding DNA Clamp Dynamics by Single-Molecule Fluorescence, Mass Spectrometry, and Structure-Based Modeling

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- Supplementary Figures S1-S4
- Table S1

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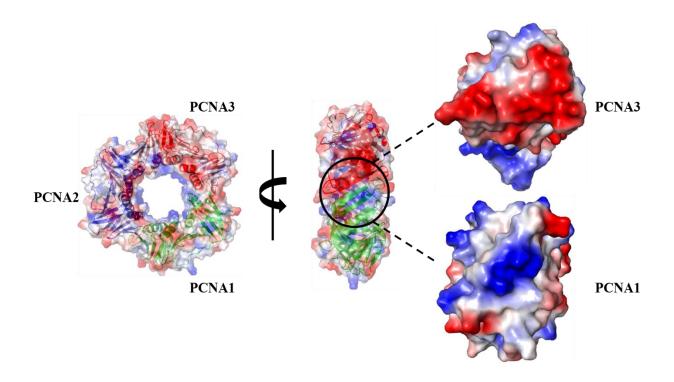


Figure S1. Electrostatic potential of the PCNA1:PCNA3 interface. Structure of

heterotrimeric PCNA from *Sulfolobus solfataricus* (PDB code: 2HII) with PCNA1, PCNA2, and PCNA3 colored green, blue, and red, respectively. Vacuum electrostatic protein contact potential for PCNA was generated in PyMOL with darker red colors indicating more negative potential and darker blue colors indicating more positive potential. Individual inspection of PCNA1 and PCNA3 interfaces reveals clear electrostatic compatibility, with PCNA1 possessing an overall positive potential and PCNA3 possessing an overall negative potential.

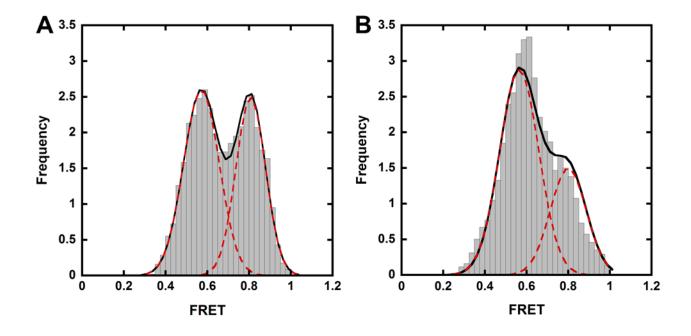
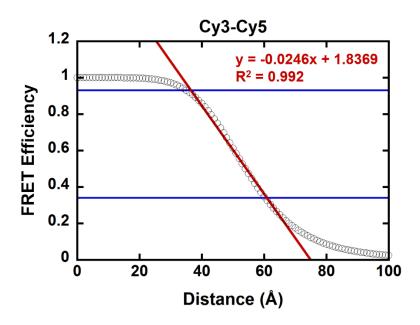


Figure S2. Single-molecule FRET experiments using ammonium acetate. smFRET experiments similar to Fig.3 were conducted using ammonium acetate, rather than NaCl, to modulate ionic strength. FRET trajectories for >200 molecules were compiled and binned to generate FRET distribution histograms at each ammonium acetate concentration, (A) 500 mM, (B) 1,000 mM. FRET distribution histograms were fit to a sum of Gaussians function (black line) to extract the individual low-FRET (0.40-0.60) and high-FRET (0.60-1.0) populations (red dashed lines).



FRET efficiency (black circles) was calculated with respect to distance using the equation $E = 1/((1+(r/R_0)^6))$, where E is FRET efficiency, r is inter-fluorophore distance (Å), and R_0 is the Cy3-Cy5 Förster radius (54 Å). The blue lines set the boundaries for the linear portion of the plot (0.34-0.93). The red line depicts the best fit line with a slope of -0.0246 FRET/Å to the linear region of the plot $(i.e.\ 0.34-0.93)$. As the apparent FRET efficiencies of the low- (0.40-0.60), center = 0.55) and high- (0.60-0.90), center = 0.80) FRET states in Fig. 3 fall squarely within the linear range, it is reasonable to estimate that the observed difference in FRET efficiency between the low- and high-FRET states (0.80-0.55=0.25) corresponds to a distance of ~10 Å

 $(FRET = -0.0246 \frac{FRET}{\text{Å}} * X\text{Å}; \ 0.25 = -0.0246 \frac{FRET}{\text{Å}} * X\text{Å}).$

Figure S3. Distance-dependency of FRET efficiency for the Cy3-Cy5 FRET pair. Cy3-Cy5

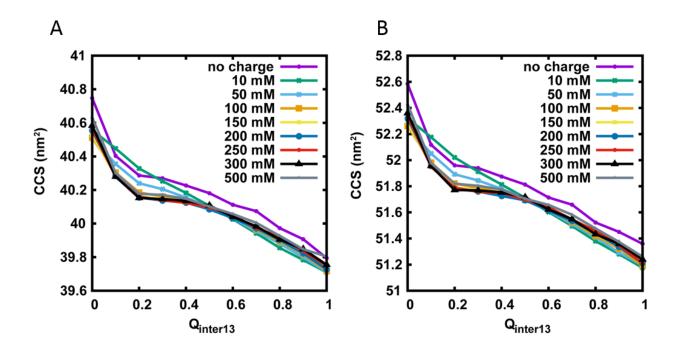


Figure S4. Theoretical CCS determined from MD simulations for the wild-type PCNA.

CCS values were determined for 50,000 frames in each trajectory, and filtered according to the fraction of native contacts between chain 1 and chain 3 ($Q_{inter13}$) and the ion strength, where $Q_{inter13} = 0$ represents the open state, $Q_{inter13} = 1$ represents the closed state. A) Determined using the projection approximation algorithm in IMPACT; and B) determined using the trajectory method in IMPACT.

Table S1. Average theoretical CCS determined in 100, 200, and 500 mM salt using both the projection approximation (PA) and trajectory method (TJM) algorithms.

	Ionic Strength				
	100 mM	200 mM	500 mM		
PA					
ring-closed PCNA	$39.72 \pm 0.22 \text{ nm}^2$	$39.73 \pm 0.22 \text{ nm}^2$	$39.80 \pm 0.24 \text{ nm}^2$		
ring-open PCNA	$40.51 \pm 0.37 \text{ nm}^2$	$40.55 \pm 0.40 \text{ nm}^2$	$40.63 \pm 0.43 \text{ nm}^2$		
TJM					
ring-closed PCNA	$51.19 \pm 0.30 \text{ nm}^2$	$51.21 \pm 0.31 \text{ nm}^2$	$51.26 \pm 0.29 \text{ nm}^2$		
ring-open PCNA	$52.26 \pm 0.51 \text{ nm}^2$	$52.32 \pm 0.55 \text{ nm}^2$	$52.43 \pm 0.59 \text{ nm}^2$		