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Kinetic analysis of PCNA clamp binding and release in the clamp loading reaction catalyzed by *Saccharomyces cerevisiae* replication factor C

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ABSTRACT

DNA polymerases require a sliding clamp to achieve processive DNA synthesis. The toroidal clamps are loaded onto DNA by clamp loaders, members of the AAA+ family of ATPases. These enzymes utilize the energy of ATP binding and hydrolysis to perform a variety of cellular functions. In this study, a clamp loader-clamp binding assay was developed to measure the rates of ATP-dependent clamp binding and ATP-hydrolysis-dependent clamp release for the *Saccharomyces cerevisiae* clamp loader (RFC) and clamp (PCNA). Pre-steady-state kinetics of PCNA binding showed that although ATP binding to RFC increases affinity for PCNA, ATP binding rates and ATP-dependent conformational changes in RFC are fast relative to PCNA binding rates. Interestingly, RFC binds PCNA faster than the *Escherichia coli* γ complex clamp loader binds the β-clamp. In the process of loading clamps on DNA, RFC maintains contact with PCNA while PCNA closes, as the observed rate of PCNA closing is faster than the rate of PCNA release, precluding the possibility of an open clamp dissociating from DNA. Rates of clamp closing and release are not dependent on the rate of the DNA binding step and are also slower than reported rates of ATP hydrolysis, showing that these rates reflect unique intramolecular reaction steps in the clamp loading pathway.

1. Introduction

Sliding clamps are toroidal proteins that encircle duplex DNA and tether the DNA polymerase to the template DNA, thereby reducing the number of binding events and limiting dissociation. Thus, the speed of DNA synthesis is limited by rates of nucleotide incorporation (reviewed in [1]). In eukaryotes, the replicative sliding clamp, proliferating cell nuclear antigen (PCNA), is a homotrimer of subunits arranged in a head-to-tail fashion [2–4]. PCNA is required not only for DNA replication but also for a variety of other cellular functions including DNA repair pathways, chromatin remodeling, and sister chromatid cohesion (reviewed in [5]). In each case, a clamp loader is required to load PCNA onto DNA. The primary clamp loader required for DNA replication, replication factor C (RFC), is a heteropentameric complex belonging to the AAA+ family of ATPases [6–10]. These molecular motors use the energy from ATP binding and hydrolysis to load clamps onto DNA (reviewed in [11]). The clamp loading reaction is complex, involving many steps and conformational changes in the clamp loader and clamp. Simplistically, RFC, in the presence of ATP, binds to the clamp and DNA, forming an intermediate ternary complex [12–18]. Formation of this complex causes conformational changes that induce ATP hydrolysis, causing RFC to release PCNA onto DNA [17,19–23]. However, many individual kinetic steps are required to complete a single clamp loading reaction cycle. In this study, we have developed a fluorescence-based assay to measure RFC–PCNA binding interactions specifically. This method was used to characterize ATP-dependent binding of RFC to PCNA and to compare the relative rates of PCNA closing around DNA to PCNA dissociation from RFC during a productive clamp loading reaction.

2. Materials and methods

2.1. Buffers and reagents

RFC assay buffer consists of 30 mM HEPES pH 7.5, 150 mM sodium chloride (NaCl), 2 mM dithiothreitol (DTT), 10 mM magnesium chloride ($MgCl_2$), and 10% glycerol. γ complex assay buffer consists of 20 mM Tris–HCl pH 7.5, 50 mM NaCl, 8 mM $MgCl_2$, and 10% glycerol. PCNA storage buffer contains 30 mM HEPES pH 7.5, 0.5 mM EDTA, 2 mM DTT, 150 mM NaCl, and 10% glycerol. RFC storage buffer is the same as for PCNA except the NaCl concentration was increased to 300 mM. Storage buffers for γ complex and β-pyrene (β-PY) are as reported previously [24].

Abbreviations: RFC, replication factor C; PCNA, proliferating cell nuclear antigen; DTT, dithiothreitol; $MgCl_2$, magnesium chloride; EDTA, ethylenediaminetetraacetic acid; MDCC, N-(2-(1-maleimidyl)ethyl)-7-(diethylamino)coumarin-3-carboxamide; PCNA-MDCC, PCNA labeled with MDCC; PY, pyrene; β-PY, β labeled with pyrene; K_d, dissociation constant; SPR, surface plasmon resonance

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Concentrations of ATP (GE Healthcare) diluted with 30 mM HEPES pH 7.5 were determined from the absorbance at 259 nm and using an extinction coefficient of 15,400 M^{-1} cm^{-1}.

2.2. Proteins

RFC and PCNA expression vectors were provided by M. O'Donnell and colleagues (Rockefeller University) [25,26]. RFC containing a truncated Rfc1 protein, lacking the first 283 amino acids, was expressed as previously described [27–29]. Site-directed mutagenesis of the PCNA coding sequence converted naturally occurring Cys residues 22, 62, and 81 to Ser, Ser-43 to Cys for labeling the binding mutant, and Ile-111 and Ile-181 to Cys for labeling the opening mutant as previously reported [30]. PCNA was expressed, purified, and labeled as previously described [2,30,31] with minor modifications. RFC was expressed and purified as previously described [25,26,32] with minor modifications. The γ complex clamp loader subunits γ [33], δ [34], δ′ [34], and χψ [35], were purified, and the γ complex ($\gamma_3\delta\delta'\chi\psi$) was reconstituted [36], as previously described. The β clamp was purified [37] and labeled with pyrene [24] as previously described.

2.3. Steady-state fluorescence assays

All steady-state measurements were made and analyzed as described previously [38]. Final concentrations were 0.5 mM ATP, 5, 10, or 20 nM PCNA-MDCC, and 0–400 nM RFC. Average values and standard deviations for three independent experiments are reported and the average relative intensities and standard deviations were plotted and fit using KaleidaGraph [38]. Dissociation constants (K_d) were calculated using Eq. (1) where R_o and P_o are the total concentrations of RFC and PCNA-MDCC, respectively, and I_{max} and I_{min} are the maximum and minimum MDCC intensities, respectively.

$$I_{obs} = \frac{K_d + R_o + P_o - \sqrt{(K_d + R_o + P_o)^2 - 4R_oP_o}}{2P_o} * (I_{max} - I_{min}) + I_{min} \quad (1)$$

Competition-binding of RFC to PCNA-MDCC versus unlabeled wild-type PCNA (wt PCNA) was done by adding reagents sequentially to a cuvette starting with assay buffer with ATP followed by PCNA-MDCC and unlabeled wt PCNA, and finally, RFC. Final concentrations were: 0.5 mM ATP, 20 nM PCNA-MDCC, 0–400 nM wt PCNA, and 20 nM RFC. Emission spectra were corrected for background by subtracting the buffer signal. Relative MDCC intensities were calculated from the ratio of emission at 467 nm for PCNA-MDCC with RFC to PCNA-MDCC without RFC, and plotted as a function of wt PCNA concentration and fit to Eq. (2) using KaleidaGraph [38]. In Eq. (2), $PCNA_{MDCC}$ is the concentration of PCNA-MDCC (20 nM), $PCNA_{wt}$ is the concentration of wt unlabeled PCNA, I_{max} is MDCC intensity in the absence of wt PCNA (set to a value of 1) and I_{min} is the signal at saturating concentrations of wt PCNA (fit as an adjustable parameter, calculated value of 0.74 ± 0.11) [30].

$$I_{obs} = \left(\frac{PCNA_{MDCC}}{PCNA_{MDCC} + PCNA_{wt}}\right) * (I_{max} - I_{min}) + I_{min} \quad (2)$$

2.4. Pre-steady-state kinetic measurements

Assays were performed using an Applied PhotoPhysics SX20MV stopped-flow apparatus at 20 °C. MDCC emission fluorescence was monitored using a 455 nm cut-on filter while exciting at 420 nm. Pyrene emission fluorescence was monitored using a 365 nm cut-on filter while exciting at 345 nm. Alexa Fluor 488 emission was monitored using a 515 nm cut-on filter while exciting at 495 nm. For clamp binding assays, experiments were performed in single mix mode, mixing equal volumes (60 μL) of two solutions containing reactants in assay buffer. For clamp closing and release assays, experiments were performed in sequential mix mode, in which a solution of RFC was mixed with a solution of PCNA and ATP prior to adding a solution of DNA and ATP. Eight or more individual kinetic traces were averaged. The time courses were corrected for background by subtracting the signal for buffer. For PCNA release and closing assays, a solution of RFC, labeled PCNA, and ATP was added to a solution of wt PCNA, DNA (when present), and ATP at concentrations indicated in Figure legends. Time courses were normalized to the intensity at the start of the reaction. The rate of change in fluorescence, k_{obs}, was fit to a single exponential decay [38]. For clamp binding reactions, a solution of clamp loader (RFC or γ complex) and ATP was added to a solution of labeled clamp (PCNA-MDCC or β-PY) and ATP at concentrations indicated in figure legends. Data were collected for a total of 4 s at 0.4 ms intervals. Time courses were divided by fluorescence of the free clamp to give relative fluorescence increases, and fit to double exponential rises (γ complex/β) or single exponential rises (RFC/PCNA) using KaleidaGraph [38] to calculate observed rate constants, k_{obs}.

3. Results

3.1. Equilibrium RFC·PCNA binding

A fluorescence intensity-based assay was developed to measure clamp binding by RFC during the clamp loading cycle. A surface residue on PCNA, Ser-43, was mutated to Cys to covalently label this site with MDCC (Fig. 1A). Three of the four naturally occurring Cys residues, 22, 62, and 81, in PCNA were converted to Ser to allow for selective labeling of Cys-43 [30]. The fourth naturally occurring Cys residue should not be solvent accessible for labeling under these conditions [3]. The predicted RFC "footprint" on PCNA upon binding is such that RFC may interact with two or more of the fluorophores [4,39–41] (Fig. 1A). When RFC binds PCNA-MDCC, MDCC fluorescence increases by about three-fold (Fig. 1B & C).

Using this assay, RFC binding to PCNA was measured under equilibrium conditions in assays with ATP. Three separate titrations were done at three different PCNA concentrations (Fig. 1C). Dissociation constants, K_d values, for RFC-PCNA-MDCC binding were calculated from these data using Eq. (1). Average K_d values from the three independent experiments were 5.5 ± 1.9 nM, 7.9 ± 0.5 nM, and 9.6 ± 2.3 nM for 5 (blue), 10 (black), and 20 (orange) nM PCNA-MDCC, respectively. These values are in agreement with one another with an average of 7.7 ± 2.3 nM for the nine measurements. This K_d is within the same order of magnitude as the value of 1.3 nM measured in a single surface plasmon resonance experiment [14] and the values of 2.6 to 4.5 nM measured in clamp closing assays [30].

To determine whether the mutations introduced into PCNA for labeling affected interactions with RFC, a competition-binding assay was performed in which unlabeled wild-type (wt) PCNA competed for RFC binding with PCNA-MDCC. In this assay, 20 nM RFC was added to solutions of 20 nM PCNA-MDCC and increasing concentrations of wt PCNA (Fig. 1D). The decrease in MDCC fluorescence with increasing unlabeled PCNA can be adequately fit to Eq. (2), which simply takes into account the fraction of total PCNA that is labeled with MDCC, demonstrating that RFC binds with equal affinity to both PCNA-MDCC and unlabeled wt PCNA. Together, these data show that RFC binds PCNA-MDCC with same affinity as wt PCNA.

3.2. Pre-steady-state PCNA binding by RFC with and without ATP preincubation

The PCNA-MDCC binding assay was used to determine the rate constant for RFC binding PCNA. In these experiments, a pre-incubated solution of RFC and 0.5 mM ATP was rapidly mixed with a solution of PCNA-MDCC and 0.5 mM ATP (Fig. 2A). The rate of the increase in MDCC fluorescence increased with increasing concentration of RFC as