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Improved solubility of replication factor C (RFC) Walker A mutants

Melissa R. Marzahn, Linda B. Bloom *

University of Florida, Department of Biochemistry & Molecular Biology, 1600 SW Archer Road, P.O. Box 100245, Gainesville, FL 32610-0245, USA

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ABSTRACT

Protein insolubility often poses a significant problem during purification protocols and in enzyme assays, especially for eukaryotic proteins expressed in a recombinant bacterial system. The limited solubility of replication factor C (RFC), the clamp loader complex from *Saccharomyces cerevisiae*, has been previously documented. We found that mutant forms of RFC harboring a single point mutation in the Walker A motif were even less soluble than the wild-type complex. The addition of maltose at 0.75 M to the storage and assay buffers greatly increases protein solubility and prevents the complex from falling apart. Our analysis of the clamp loading reaction is dependent on fluorescence-based assays, which are environmentally sensitive. Using wt RFC as a control, we show that the addition of maltose to the reaction buffers does not affect fluorophore responses in the assays or the enzyme activity, indicating that maltose can be used as a buffer additive for further downstream analysis of these mutants.

Introduction

Clamps and clamp loaders are essential components of the replisome. During replication the clamp serves to tether the polymerase to the DNA template so that the rate of DNA synthesis is limited only by the rate of nucleotide incorporation [1–3]. The clamp loader is responsible for loading the clamp onto DNA. The eukaryotic clamp, proliferating cell nuclear antigen (PCNA),[1] is a toroidal homotrimer while the eukaryotic clamp loader, replication factor C (RFC), is a heteropentamer (Fig. 1a) [4,5]. RFC belongs to a family of proteins known as AAA + ATPases, which utilize the energy from ATP hydrolysis to perform cellular functions [6–8]. At least four of the five RFC subunits are able to bind ATP (Fig. 1b) [6,9]. Proteins within this family share several structural motifs that serve to coordinate and promote ATP binding and hydrolysis. Three of these are the Walker A, Walker B, and arginine finger motifs and they are involved in ATP binding, ATP hydrolysis, and sensing bound ATP, respectively [10–12]. The clamp loading reaction is complex, involving many interactions and conformational changes. Simplistically, RFC, in the presence of ATP, binds and opens PCNA, and then binds DNA, allowing the formation of a ternary complex (cartoon schematic Fig. 1c). Formation of this complex triggers ATP hydrolysis, promoting loading of the clamp onto DNA and RFC dissociation. The functions these motifs play during the clamp loading reaction are generally only analyzed from an endpoint assay such as the number of clamps loaded onto DNA or the ability to support processive DNA synthesis. Fluorescence-based assays developed in our laboratory can be used to monitor specific steps within the clamp loading reaction and determine the precise role individual residues play. For example, rather than simply evaluating the effects of a mutation on the overall efficiency of clamp loading or DNA replication, these assays identify specific steps, such as DNA binding or clamp opening, that are affected by the mutation. Clamp binding and opening are measured in two different assays. In the clamp binding assay, the fluorescence of an environmentally sensitive fluorophore covalently attached to the surface of PCNA increases when RFC binds [13,14]. In the clamp opening assay, two fluorophores, juxtaposed on either side of the clamp monomer interfaces, are quenched when the clamp is closed, and the fluorescence increases when RFC opens the clamp and "pulls apart" the two fluorophores [13,15]. DNA binding is measured by monitoring the increase in anisotropy that occurs when proteins bind the fluorescent-labeled DNA [16–18].

To identify the role that the conserved Lys residue from the Walker A motif (consensus sequence with Lys shown in orange, Fig. 1b) plays in clamp loading, this residue was mutated to Ala. This mutation is predicted to prevent ATP binding [9,12]. Even though the Rfc5 subunit has been shown to bind ATP [5,6], there is no arginine finger motif extending from an adjacent subunit to create an active site for ATP hydrolysis (Fig. 1b). For this reason, we have chosen only to mutate residues within four subunits: Rfc1, Rfc2, Rfc3, and Rfc4. Because different

* Corresponding author. Fax: +1 352 392 6511.
E-mail address: lbloom@ufl.edu (L.B. Bloom).

[1] Abbreviations used: RFC, replication factor C; PCNA, proliferating cell nuclear antigen; DTT, dithiothreitol; $MgCl_2$, magnesium chloride; RhX, X-rhodamine; DNA-RhX, DNA labeled with X-rhodamine; RFC4GAT, RFC with mutation of the Rfc4 Walker A Lys to Ala; AF488, Alexa Fluor 488; PCNA-AF488, PCNA labeled with Alexa Fluor 488; MDCC, *N*-(2-(1-maleimidyl)ethyl)-7-(diethylamino)coumarin-3-carboxamide; PCNA-MDCC, PCNA labeled with MDCC; EDTA, ethylenediaminetetraacetic acid; K_d, dissociation constant.

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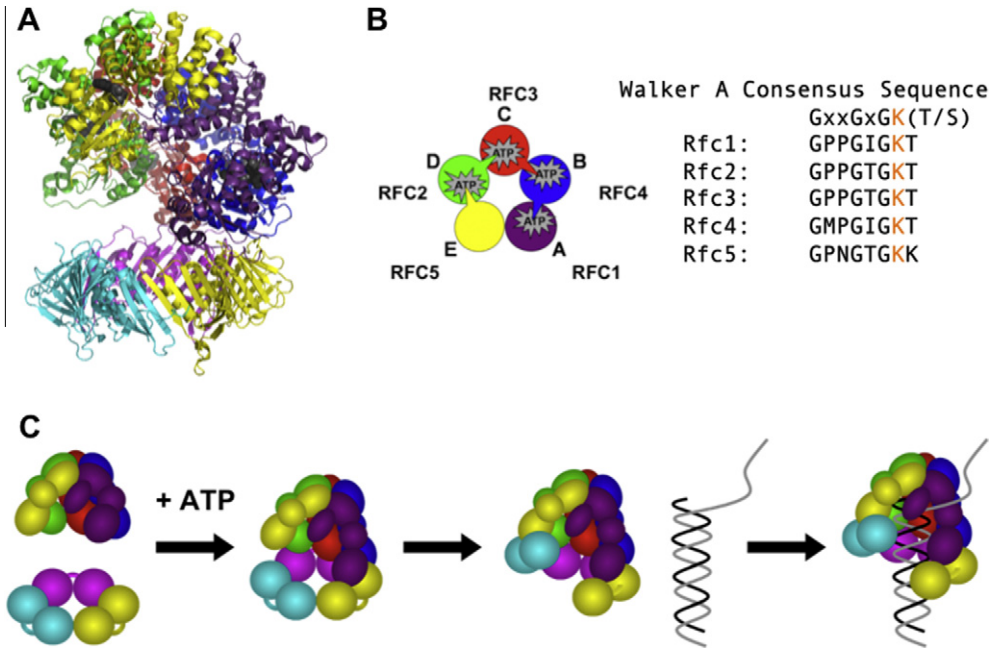

Fig. 1. RFC structure and clamp loading reaction schematic. (A) Ribbon diagram of the *Sc*RFC/PCNA complex structure (PDB ID: 1SXJ) with α-helices shown as *coils*, β-sheets as arrows, and bound nucleotide as dark gray spheres is shown. The RFC subunits are: Rfc1 (purple), Rfc2 (green), Rfc3 (red), Rfc4 (blue) and Rfc5 (yellow). Each PCNA monomer is shown as a different color. (B) A cartoon depicting the arrangement of subunits and arginine finger interactions in RFC using the same color scheme as in panel A is shown. The arginine finger motif is shown as a wedge-like protrusion extending to the ATP site of the neighboring subunit. The Walker A consensus sequence is aligned with the individual sequence for each RFC subunit. The conserved Lys residue that was mutated to Ala for these studies is shown in orange. (C) Schematic of the clamp loading reaction in the presence of ATP leading to the formation of a ternary complex (RFC•PCNA•DNA) prior to ATP hydrolysis is shown.

genes encode all four subunits, this can be done site-specifically to create four different mutant RFC complexes and determine whether individual ATP binding sites have distinct functions. As no laboratory has been able to purify the human proteins in the quantities needed for these assays, we chose *Saccharomyces cerevisiae* as our model system. Using a dual vector system, all five RFC subunits were co-expressed in *Escherichia coli* and the assembled complex was purified using ion exchange and affinity chromatography [4,11,19]. Although much of the protein is present in the insoluble fraction, at least 5 mg of mutant RFC per liter of expression media is in the soluble fraction. Solutions of purified mutant complexes at high concentrations were cloudy, and at lower concentrations in anisotropy experiments a high background due to light scattering was observed, both of which indicate that the mutant proteins precipitate. Given that protein precipitation was likely occurring under our assay conditions, quantitative measurements of the activities of mutant complexes could not be made reliably. Therefore, different buffer conditions were evaluated to identify additives that would increase protein solubility and limit precipitation while not having an adverse effect on our fluorescence-based assays under equilibrium or pre-steady state analysis.

Materials and methods

Buffers and reagents

Assay buffer consists of 30 mM HEPES pH 7.5, 150 mM sodium chloride (NaCl), 2 mM dithiothreitol (DTT), 8 mM magnesium chloride ($MgCl_2$), 0.5% or 4% glycerol where indicated, and 750 mM maltose (when present). Storage buffer for PCNA contains 30 mM HEPES pH 7.5, 0.5 mM EDTA, 2 mM DTT, 150 mM NaCl, and 10% glycerol. Storage buffer for wild-type RFC (wt RFC) contains 30 mM HEPES pH 7.5, 0.5 mM EDTA, 2 mM DTT, 300 mM NaCl, and 10% glycerol. Storage buffer for RFC4GAT is the same as for RFC except for the addition of 750 mM maltose.

Nucleotides and oligonucleotides

Concentrations of ATP (GE Healthcare) diluted with 30 mM HEPES pH 7.5 and ATPγS diluted with water (Roche Diagnostics) were determined by measuring the absorbance at 259 nm and using an extinction coefficient of 15,400 M^{-1} cm^{-1}. Synthetic oligonucleotides were obtained from Integrated DNA Technologies and purified by 12% denaturing polyacrylamide gel electrophoresis. The sequences of the 60-nucleotide template and the complementary 30-nucleotide primer are as follows: 5′-(5AmMC6)TTC AGG TCA GAA GGG TTC TAT CTC TGT TGG CCA GAA TGT CCC TTT TAT TAC TGG TCG TGT-3′ and 5′-ACA CGA CCA GTA ATA AAA GGG ACA TTC TGG-3′, where 5AmMC6 is a T with a C6 amino linker that was covalently labeled with X-Rhodamine (RhX) (Invitrogen) as described [16–18]. Primed templates were annealed by incubating the 30-nucleotide primer with the 60-nucleotide template at 85 °C for 5 min and then allowing the solution to slowly cool to room temperature. For all assays, the molar ratios of primer to template were 1.2:1 in annealing reactions.

Proteins

The DNA constructs for expressing wt RFC, each of the mutants, and PCNA were obtained from M. O'Donnell (Rockefeller University) [19,11]. PCNA was expressed, purified, and labeled as previously described [20] with minor modifications [13]. Fluorophores for labeling PCNA were purchased from Molecular Probes (Invitrogen). RFC Walker A mutants are named based upon the subunit containing the mutation (for example, if the RFC4 subunit has the Lys to Ala substitution, the complex would be termed RFC4GAT). RFC and RFC4GAT (both with 283 amino acid truncation of the N terminus of RFC1) were expressed and purified as