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ORIGINAL PAPER

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# Mutational analysis of *Pyrococcus furiosus* replication factor C based on the three-dimensional structure

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**Abstract** In eukaryotic DNA replication, replication factor C (RFC) acts as a "clamp loader" that loads PCNA onto a primed DNA template in an ATP-dependent manner. Proteins with functions essentially identical to that of RFC exist in Archaea. We have determined the crystal structure of the small subunit (RFCS) of *Pyrococcus furiosus* RFC at 2.8-Å resolution. Using the information from the determined tertiary structure, we prepared several mutations in RFCS and biochemically characterized them. Truncation of the C-terminal α-helix (α16) causes a failure in RFCS oligomerization and a loss of the stimulating activity for the PCNA-dependent DNA synthesis by DNA polymerases. The site-directed reduction of the negative charges at the center part of the RFCS complex affected the stability of the RFC-PCNA interaction and reduced the clamp-loading activity. These results contribute to our general understanding of the structure-function relationship of the RFC molecule for the clamp-loading event.

**Keywords** Archaea · DNA replication · Clamp loader · Hyperthermophile

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## Introduction

DNA replication is a fundamental process for living organisms to maintain their genetic integrity; therefore, it is very important to understand the detailed molecular mechanisms of the DNA-replication process. DNA polymerase synthesizes the nascent DNA strand along the template DNA strand. For processive DNA synthesis, the DNA polymerase has to stick onto the DNA, and a clamp molecule accomplishes this task. The bacterial DNA polymerase III β-subunit, the gp43 of bacteriophages, and the eukaryotic proliferating cell nuclear antigen (PCNA) directly interact with the replicative DNA polymerases and work as their clamps (reviewed in Hingorani and O'Donnell 2000). The active form of the clamp molecules is doughnut-shaped, and the DNA strand is encircled into the central cavity without steric hindrance. To load the clamp molecules onto the DNA strand, the ring structure must open once, and other protein factors, called "clamp-loaders," function to open the ring of the clamp molecules. The bacterial Pol III γ complex, the phage gp44–64, and the eukaryotic replication factor C (RFC) have structures and functions similar to those of clamp loaders (reviewed in Mossi and Hübscher 1998).

Archaea, the third domain of life (Woese and Fox 1977), has DNA-replication proteins with sequences similar to those of eukaryotes, although the prokaryotic unicellular shapes of the archaeal organisms look very primitive (reviewed in Olsen and Woese 1997; Edgell and Doolittle 1997; Ishino and Cann 1998; Cann and Ishino 1999; Leipe et al. 1999). Therefore, the archaeal replicational machinery or at least some of the replisome is most likely to be a simplified version of the eukaryotic one. We have been studying the molecular recognition mechanisms in the DNA-replication process in Archaea using hyperthermophilic organisms and have characterized the related proteins: Cdc6/Orc1 and MCM (Matsunaga et al. 2001); Primase (Bocquier et al. 2001; Liu et al. 2001); replication protein A (Komori and

Ishino 2001); Pol I and Pol II (Uemori et al. 1993, 1997; Imamura et al. 1995; Cann et al. 1998; Ishino et al. 1998; Komori and Ishino 2000; Ishino and Ishino 2001); PCNA (Cann et al. 1999); and RFC (Cann et al. 2001). We solved the crystal structure of the *Pyrococcus furiosus* PCNA protein and found that the ring-shaped structure with the pseudo-sixfold symmetry of the sliding clamps was clearly conserved in living organisms (Matsumiya et al. 2001). The archaeal and eukaryotic PCNAs are quite similar to each other. Furthermore, we demonstrated that the *P. furiosus* PCNA functionally interacts with mammalian replication proteins and stimulates calf thymus DNA polymerase $\delta$ activity. Moreover, in the case of a circular DNA template, human RFC enhanced the PCNA-dependent activity of Pol $\delta$ (Ishino et al. 2001). The functional interaction of *Thermococcus fumicolans* PCNA with calf thymus Pol $\delta$ has also been reported (Henneke et al. 2000). These findings further support the idea that structure-function analyses of the archaeal replication proteins will strongly contribute to our understanding of the molecular recognition mechanisms employed within human cells.

Using information from our crystal structure analysis, we have made several mutant proteins of the small subunit (RFCS) of *Pfu*RFC. We have characterized the mutant *Pfu*RFC complexes containing these RFCS for a further investigation of the molecular recognition mode of the clamp-clamp loader interaction in the processive replication machinery.

## Materials and methods

### Site-specific mutagenesis

The plasmid pTRFS (Cann et al. 2001) was used as the template for mutagenesis. A polymerase chain-reaction-mediated mutagenesis was performed, using the QuickChange site-directed mutagenesis kit (Stratagene, Calif., USA). The forward and reverse primers were complementary and contained the desired nucleotide changes (shown here as lowercase letters): m5 E260A-E270A-D271A, 5′-GAAAAGCTTA-GGGcGATACTTCTCAAGCAAGGACTT-AGTGGAGcAGcTGTACTAGTTCAG-3′; E306A-E310A, 5′-AC TTCAGACTCGTTGcAGGGGCTAATGcAATAATTCAGCT-TGA-3′, m2 E270A, 5′-AAGGACTTAGTGGAGcAGATGTAC-TAGTTC-3′; E310A 5′-TTGAAGGGGCTAATGcAATAATTC-AGCTTG-3′. The reactions were carried out essentially according to the manufacturer's instructions. The designed mutations were confirmed by nucleotide sequencing. These constructs were designated as pTRFSm5 and pTRFSm2, respectively. For the deletion mutant, a truncated gene was amplified by PCR from pTRFS. The primers were 5′-GGCATATGAGCGAAGAGA-TTAGAGAA-GTTA-3′and 5′- GGGTCGACTTAAACGAGTCTGAAGTTA-TAC-3′, which have an *Nde*I site and a *Sal*I site (underlined), respectively. The PCR product was cloned into the pGEM-T Easy vector (Promega, Wisconsin, USA) and the nucleotide sequence was confirmed. The truncated *rfcS* gene fragment was digested with *Nde*I and *Sal*I and was cloned into the corresponding site of the pET21a vector (Novagen, Wisconsin, USA). This construct was designated as pTRFSΔ.

### Purification of wild-type and mutant RFCS proteins

The RFCS, its mutant proteins, and *Pfu*RFCL were purified to homogeneity as described previously (Cann et al. 2001). Hydroxyapatite column chromatography was carried out after the removal of the nucleic acids by a polyethyleneimine treatment. Purified RFCS proteins were mixed with His-tagged RFCL, and the RFC complexes were further purified by chromatography on anion exchange and heparin affinity columns.

### Gel retardation assay

Various concentrations (0.15, 0.3, 0.6, 1.2, and 2.4 μM) of the wild-type and mutant RFCS proteins were incubated with 50 nM of a $^{32}$P-labeled, single-stranded 49mer DNA in buffer (20 mM Tris acetate, pH 8.0; 5.5 mM $MgCl_2$; 1 mM DTT; 0.1 mg/ml BSA; 0.5% glycerol; and 100 μM ATP) at 60 °C for 10 min. The reaction products were analyzed by 1% agarose gel electrophoresis in 0.1 × TAE buffer (4 mM Tris acetate and 0.1 mM EDTA, pH 8.4) and the bands were detected by autoradiography.

### ATPase assay

The ATPase activities of RFCS and the RFC complex were measured essentially as described previously (Cann et al. 2001) using [α-$^{32}$P]ATP (0.1 mCi/ml). The RFCS protein (at 0.8 μM as a monomer) or the RFCS and RFCL proteins (at 0.8 μM and 0.2 μM, respectively) were incubated with or without 20 ng/μl single-stranded M13mp18ssDNA and 0.2 μM *Pfu*PCNA in 20 μl of ATPase buffer (25 mM Tris-HCl, pH 8.0; 6 mM $MgCl_2$; 0.1 mM DTT; 0.05 mg/ml BSA) at 65 °C for the indicated times, and aliquots of the reaction were analyzed by thin-layer chromatography. The amounts of ATP and ADP were quantified from the autoradiograms using a laser-excited image analyzer (BAS5000, Fuji Film, Tokyo, Japan).

### Immunoprecipitation and Western blot analysis

Rabbit polyclonal antibodies were raised against homogenous *Pfu*PCNA and RFCS as previously reported (Cann et al. 1999, 2001). All subsequent steps were carried out at room temperature. The *Pfu*PCNA and RFCS proteins (500 ng each) were mixed in 100 μl of buffer A (20 mM Tris-HCl, pH 8.5; 10 mM $MgCl_2$; 1 mM ATP) and were incubated at 70 °C for 20 min. Anti-RFCS antiserum was mixed with protein A-Sepharose (Amersham), and the mixture was incubated for 1 h with gentle shaking. The mixtures of *Pfu*PCNA and RFCS were added to the protein A-Sepharose-bound antiserum and were further incubated in buffer B (20 mM Tris-HCl, pH 8.5; 50 mM NaCl; 0.1% BSA) for 1 h with gentle shaking. Protein A-Sepharose-bound antigen-antibody complexes were separated from the free proteins by centrifugation followed by three washes in buffer B. The precipitates were analyzed by Western blotting.

### Analytical gel filtration

Wild-type and mutated RFCS proteins with or without the *Pfu*RFCL protein were subjected to a gel filtration analysis using Superdex 200 PC3.2/30 with the SMART system (Amersham Biosciences) to investigate the complex formation. Gel filtration standards (Bio-Rad, USA) were used in a different run under the same conditions.

### DNA polymerase assay

*Pyrococcus furiosus* Pol I was prepared as described previously (Komori and Ishino 2000). The *Pfu*PCNA-dependent DNA synthesis of Pol I was measured essentially as described previously (Cann et al. 2001; Mayanagi et al. 2001), with wild-type and mutant RFCS proteins in the presence of *Pfu*RFCL.

## Results

### Design of mutant RFCS proteins based on the crystal structure

The crystal structure of the RFCS hexamer provided an interaction mode of each subunit (Oyama et al. 2001). There are two interfaces in the subunit interaction. Interface I is formed mainly by domain 1 of one subunit and domain 3 of the adjacent subunit in a head-to-tail configuration. Interface II consists of only domain 3 of the neighboring two subunits. An analysis of the interaction mode suggested that interface II is tighter and more specific than interface I for the RFCS complex formation. At interface II, the α16 helix of one subunit is closely packed with the α15 helix of the other subunit (Fig. 1A). Therefore, we designed a deletion mutation of the α16 helix (306–327) at the C-terminal region of the RFCS protein (RFCS-Δ). One more interesting feature of the crystal structure of the RFCS hexamer is the localization of the negatively charged residues of each subunit within the central region of the hexamer ring. The negative charge in this region may contribute to the interaction of RFC with PCNA, because some positive charges are localized at the center of the PCNA ring. To address this question, the mutation of the negatively charged residues to alanine is necessary. We designed a site-specific mutagenesis to change five acidic residues (E260, E270, D271, E306, and E310) to alanine (RFCSm5). To compare the effect of the negative charge strength, we created another mutant RFCS, in which two of the five acidic residues (E270 and E306) are replaced with alanine (RFCSm2). A calculated electrostatic potential map of the molecular surfaces showed that the negatively charged area observed in the wild-type RFCS was absent from the mutant RFCSm5 and RFCSm2 surfaces (Fig. 1B).

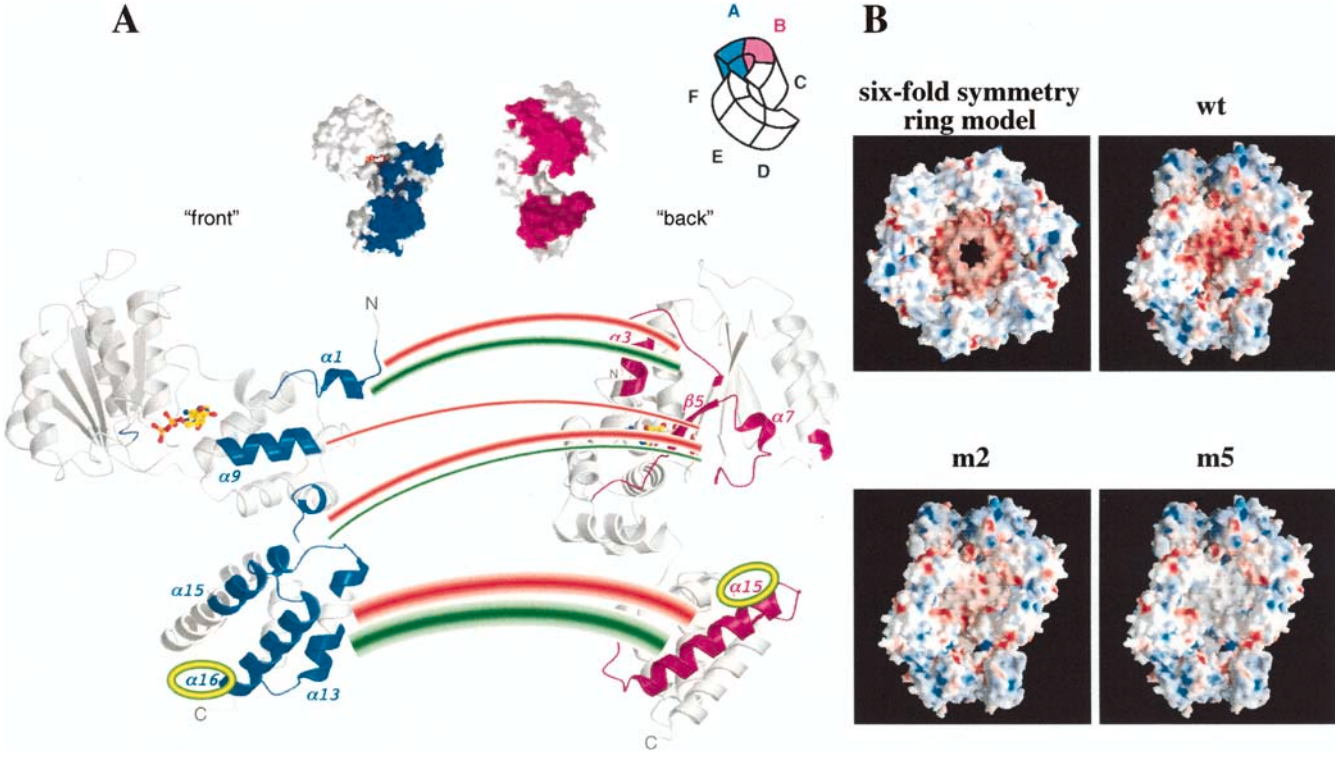


**Fig. 1** **A** Interaction mode of RFCS subunits. Direct interactions observed in more than two out of the four subunit-subunit interfaces in the hexameric crystal are shown. The red and green lines indicate hydrogen bonds and hydrophobic interactions, respectively. **B** Electrostatic potential on the surface of the RFCS hexamer, calculated by using the program GRASP with NaCl at 0.15 M (Nicholls et al. 1991). The 6-fold symmetry ring model was built from a semicircular trimer of RFCS in the crystal. Positively charged surfaces and negatively charged surfaces are colored blue and red, respectively

### Preparation of the mutant RFC

The genes for the mutant RFCS proteins, RFCS-Δ, RFCSm5, and RFCSm2 (schematically shown in Fig. 2A), in the pET21a expression vector were expressed in *Escherichia coli*, and the gene products were purified by basically the same procedure as for the wild-type RFC, as described in Materials and methods. The purified proteins were analyzed by SDS-PAGE, and each protein was obtained as a single band on the gel (Fig. 2B). The native PAGE analysis (without SDS) showed that the RFCS migrated more slowly with the acidic residues, indicating the reduction of negative charges on the molecular surfaces of the mutant proteins (Fig. 2C). The elution profiles of the ion-exchange and heparin affinity chromatographies of the *Pfu*RFC complex containing mutant RFCSs were not very different

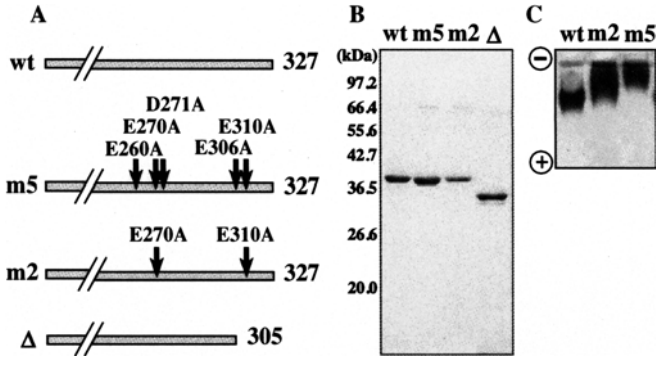


**Fig. 2** **A** Series of the mutant RFCS proteins. **B** Purification of the mutant RFCS proteins. Wild-type and mutant RFCS proteins (each 1 μg) were separated by electrophoresis on a 12% acrylamide gel containing SDS with Tris-glycine buffer (**B**) or a 6% acrylamide gel with Tris-acetate buffer (pH 8.0) under native conditions (**C**). After separation, the proteins were visualized by staining with Coomassie brilliant blue and silver, respectively

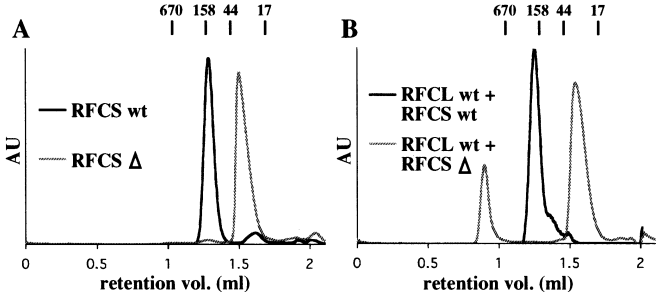


**Fig. 3A, B** Investigation of the RFCS complex by gel filtration analysis. **A** The wild-type and mutant RFCS proteins were fractionated on a gel filtration column. Elution of the proteins was monitored by the absorbance at 280 nm. **B** The wild-type and mutant RFCS proteins were mixed with the RFCL proteins, and the RFC complexes were fractionated on the same column as in **A**

from those of the wild-type *Pfu*RFC complex, indicating that critical conformational changes in the overall structure did not occur by the amino acid deletions or substitutions presented in this study.

## Oligomer formation

Our previous report showed homo-oligomer formation of the RFCS protein by a gel filtration analysis (Cann et al. 2001). The present analysis using Superdex 200 showed that the RFCS protein eluted at the position corresponding to a molecular mass of about 150 kDa (Fig. 3A). The mutant RFCS proteins m2 and m5 eluted at the same position as that of the wild-type RFCS in the gel filtration chromatography (data not shown). On the other hand, the C-terminal deletion mutant protein, RFCS-Δ, elevated at the position corresponding to the monomer (38 kDa), as shown in Fig. 3A. Furthermore, as expected, RFCS-Δ could not form the heterocomplex with RFCL, while in contrast, the wild-type RFCS and

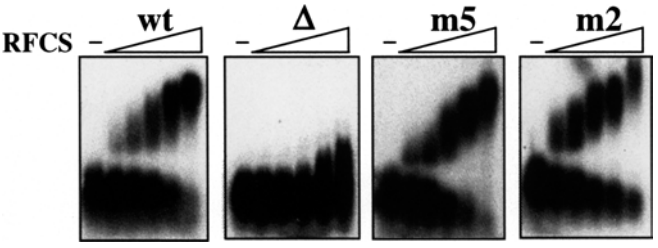


**Fig. 4** DNA-binding activities of mutant RFCS. Various concentrations (described in the text) of proteins were incubated with a $^{32}$P-labeled DNA (49mer ) at 60 °C for 5 min. The reaction products were analyzed by 1% agarose gel electrophoresis followed by autoradiography. The left lane in each panel shows the reaction without protein

RFCL eluted together as a complex at about 200 kDa (Fig. 3B). The RFCL protein tends to self-aggregate in the absence of RFCS proteins and was usually eluted at a very early position.

## DNA-binding ability

The negative charges on the surfaces of RFC may affect its DNA-binding ability. Therefore, a gel retardation assay was performed using the purified RFCS proteins to compare their DNA affinities. As shown in Fig. 4, the DNA band was shifted with increasing amounts of the wild-type, RFCSm5, and RFCSm2 proteins. No difference was observed in the efficiency of the band shift from the three RFCSs, suggesting that the affinity of RFCS to DNA was not changed drastically by the removal of the negatively charged area. However, almost no band shift was observed by RFCSΔ under the same conditions. This result indicates that RFCS oligomerization may be necessary for its distinct DNA-binding ability. ATP did not affect the affinity of the RFCS proteins for DNA (data not shown).

## ATPase activity and stimulation of PCNA-dependent DNA synthesis

The most prominent function of RFC is to stimulate the DNA synthesis activity of replicative DNA polymerases by loading PCNA onto DNA. *Pfu*RFC clearly stimulates the *Pfu*PCNA-dependent DNA synthesis of Pol I and Pol II in vitro (Cann et al. 2001). Primer extension reactions of *Pyrococcus furiosus* Pol I on M13 single-stranded DNA were performed using mutant *Pfu*RFCs made with RFCS-Δ, RFCSm2, and RFCSm5. As shown in Fig. 5, the mixture of RFCL and RFCS-Δ could not stimulate the DNA synthesis reaction (lane 4). Although RFCSm5 can form a complex with RFCL, as shown above, the complex obviously reduced the efficiency of strand synthesis (41% of the wild-type RFC activity) by the *Pfu*PCNA-dependent Pol I reaction (lane 5). The RFC complex containing RFCSm2 showed slightly

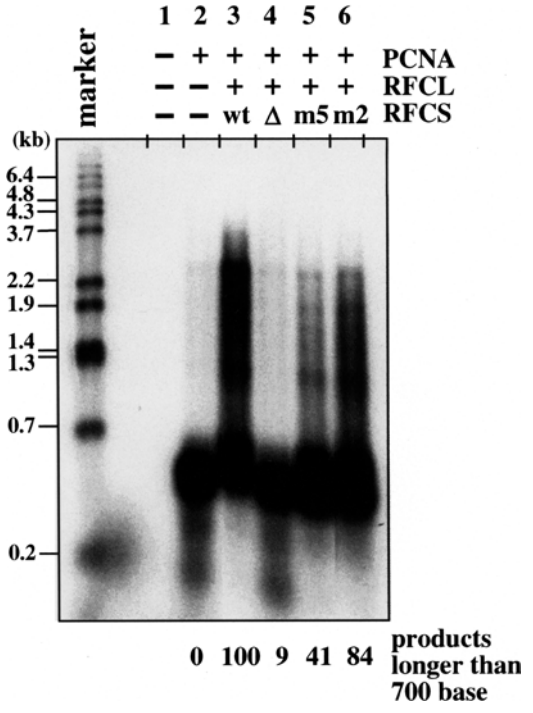


**Fig. 5** Effects of the mutations in RFCS on the *Pfu*PCNA-dependent DNA synthesis of *Pyrococcus furiosus* Pol I. The reaction mixtures with the indicated proteins in each lane were analyzed by 1% alkaline agarose gel electrophoresis, and the products were visualized by autoradiography. The sizes indicated on the left were from *Bst*I-digested $\lambda$ phage DNA labeled by $^{32}$P at each 5′ end. Amounts of DNA products longer than 700 bases by each RFCS are shown as the %percent relative to the product by *Pfu*RFC containing the wild-type RFCS

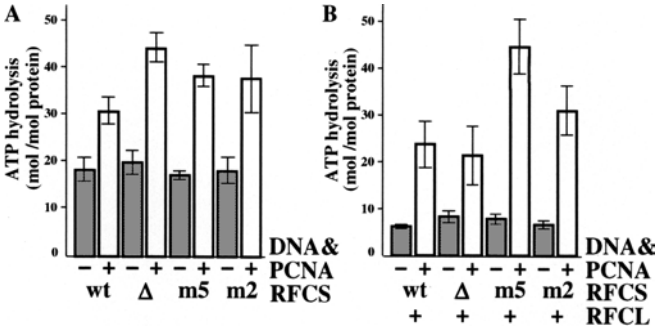


**Fig. 6A, B** ATPase activity of RFCS and the RFCS-RFCL complex. **A** The RFCS protein (at 0.8 μM as a monomer), [α-$^{32}$P]ATP(0.1 mCi/ml), and 100 μM of ATP were incubated with or without DNA and *Pfu*PCNA at 65 °C for 30 min, and aliquots of the reactions were analyzed by thin-layer chromatography. **B** The RFCS and RFCL proteins (at 0.8 μM and 0.2 μM, respectively) were incubated with or without DNA and *Pfu*PCNA at 65 °C for 10 min, and the reactions were analyzed as described above. The graphs show the values after subtraction of the background (radioactivities from the reaction without protein at each time point)

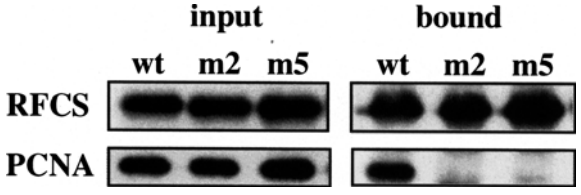


**Fig. 7** Immunoprecipitation of the purified *Pfu*PCNA and RFCS proteins by the anti-RFCS antibody in vitro. *Pfu*PCNA and the wild-type or mutant RFCS proteins were mixed and immunoprecipitated by the anti-RFCS antiserum. The precipitates were analyzed by Western blotting using each specific antibody indicated on the left side

reduced (84%) synthesis efficiency (lane 6). To anatomize the function of the RFC molecule, the ATPase activity of each mutant RFCS and their complexes were compared. As shown in Fig. 6, the ATPase activities of the RFCS mutant proteins prepared in this study were not different from that of the wild-type RFCS, in both the presence and absence of DNA and PCNA. When the mutant RFCS proteins were complexed with RFCL, all of the RFC complexes displayed almost the same ATPase activity. The ATPase activity and the stimulation of primer extension in the RFC complex were not correlated in the assay system in this study.

### Physical interaction of RFC-PCNA

To investigate the effect of the gathered negative charges of RFCS complex on the direct interaction with the PCNA molecule, an immunoprecipitation experiment was performed. The purified PCNA and RFCS proteins were mixed, and the fraction that precipitated with anti-*Pfu*RFCS was subjected to Western blot analyses using anti-PCNA and anti-RFCS antisera, respectively. The PCNA band was detected with the wild-type RFCS by the anti-RFCS-bound fraction. Under the same conditions, when RFCSm5 or RFCSm2 was used instead of wild-type RFCS, the PCNA band was not detected (Fig. 7). This experiment shows that the strength of the direct interaction between the PCNA and RFCS molecules was reduced to some extent by these charge-substituting mutations. Conversely, when the anti-PCNA-bound fraction was used for the Western blot analysis, RFCS nonspecifically bound to the anti-PCNA bound protein A-Sepharose, and the specific PCNA-RFCS interaction could not be detected (data not shown).

## Discussion

We have presented the effects of mutations in the small subunit of *Pyrococcus furiosus* RFC on the complex formation within the RFC complex and also with *P. furiosus* PCNA. The deletion of the C-terminal α-helix clearly reduced the oligomer formation, as shown by gel filtration (Fig. 3). This is consistent with the deletion analysis of the human RFC proteins. The C-terminal regions of each subunit are essential for RFC complex

formation (Uhlmann et al. 1997a, 1997b). The deleted regions in the studies of human RFC proteins are somewhat large (Δ166 amino acids for p140, Δ14 for p40, Δ63 for p38, Δ29 for p37, and Δ17 for p36). Our study defined the critical region for the complex formation, which exists within the C-terminal α-helix, with 22 amino acids. We also discovered that the affinity of the RFC small subunit for DNA is strongly enhanced by oligomerization (Fig. 4).

The substitution mutant protein, RFCSm5, reduced the stimulation efficiency of the *Pfu*PCNA-dependent DNA synthesis activity of Pol I (Fig. 5). This result indicates that the central, negatively charged region has some effect on the RFC function in the replisome. Our immunoprecipitation analysis showed that the stability of the PCNA-RFC complex was decreased by the substitution of the negatively charged residues (Fig. 7). This decreased stability of the PCNA-RFC complex may have reduced the efficiency of the PCNA-dependent reaction. Unexpectedly, the ATPase activity of the RFCS proteins was not affected by the mutations in this study. Although RFCSΔ does not form a stable oligomeric complex, its ATPase activity is not different from that of the wild-type RFCS, in both the presence and absence of RFCL (Fig. 6). This result is in contrast with the case of human RFC, in which the p40 subunit has DNA-dependent ATPase activity by itself and its specific activity is increased by complex formation with the subunits p36, p37, p38, and p140 (Podust et al. 1998). In addition, the reason that the ATPase activity of the RFC complex containing RFCSm5 or RFCSm2 is enhanced to a greater extent than that of the wild-type RFC is presently not clear. Further investigations are required to explain the relationship between the ATPase and clamp-loading activities in the archaeal RFC molecules.

To elucidate the molecular mechanism of the clamp-loading event, an understanding of the detailed interaction mode between RFC and PCNA is necessary. The crystal structure of the complex between the δ-subunit of the γ-complex (clamp loader) and the β-subunit (clamp) of *Escherichia coli* Pol III has recently been solved (Jeruzalmi et al. 2001). In this crystal, only the N-terminal domain 1 of the three δ-subunit domains was found to interact with the β-subunit through a protruding helical hydrophobic region. According to the fact that the interaction between the δ- and β-subunits does not involve the dimer interface directly, a spring-loaded mechanism, in which δ-subunit binding induces a conformational change in the β-subunit, has been proposed for the clamp opening (Jeruzalmi et al. 2001). In this β-δ complex structure, domains 2 and 3 of the δ-subunit are extended over the face of the β-ring. However, mutational analyses of the human RFC subunits showed that the DNA-binding and PCNA-binding abilities of the RFC molecule are retained in the mutant RFC complexes lacking some N-terminal regions of one subunit from p140, p40, p37, and p36 (Uhlmann et al. 1997a, 1997b). Complex formation by the RFC subunit may be essential to sufficient binding to PCNA as well as to DNA. The gathered negative charges of the RFC complex from each domain 3 of the RFC subunits may contribute toward attracting PCNA for their stable binding. An amino acid sequence alignment showed that the acidic residues targeted in this study are conserved in two of the four human RFC subunits in each position (Oyama et al. 2001).

Exciting progress has been made in molecular analyses of the clamp-loading process using various biological materials. The structures of the archaeal RFC and PCNA molecules strikingly resemble their eukaryotic counterparts. Further detailed structural and biochemical analyses of the stable archaeal proteins will greatly contribute to a complete understanding of the molecular recognition mechanisms of the proteins involved in the clamp-loading and -unloading processes.

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