Biochemical Characterization of a CDC6-like Protein from the Crenarchaeon Sulfolobus solfataricus*

Received for publication, June 10, 2003, and in revised form, August 1, 2003
Published, JBC Papers in Press, September 8, 2003, DOI 10.1074/jbc.M306075200

Mariarita De Felice, Luca Esposito, Biagio Pucci, Floriana Carpentieri, Mariarosaria De Falco, Mosè Rossi, and Francesca M. Pisani‡

From the Istituto di Biochimica delle Proteine, Consiglio Nazionale delle Ricerche, Via P. Castellino, 111, 80131 Napoli, Italy

Cdc6 proteins play an essential role in the initiation of chromosomal DNA replication in Eukarya. Genes coding for putative homologs of Cdc6 have been identified in the genomic sequence of Archaea, but the properties of the corresponding proteins have been poorly investigated so far. Herein, we report the biochemical characterization of one of the three putative Cdc6-like factors from the hyperthermophilic crenarchaeon Sulfolobus solfataricus (SsoCdc6-1). SsoCdc6-1 was overproduced in Escherichia coli as a His-tagged protein and purified to homogeneity. Gel filtration and glycerol gradient ultracentrifugation experiments indicated that this protein behaves as a monomer in solution (molecular mass of about 45 kDa). We demonstrated that SsoCdc6-1 binds single- and double-stranded DNA molecules by electrophoretic mobility shift assays. SsoCdc6-1 undergoes autophosphorylation in vitro and possesses a weak ATPase activity, whereas the protein with a mutation in the Walker A motif (Lys-59 → Ala) is completely unable to hydrolyze ATP and does not autophosphorylate. We found that SsoCdc6-1 strongly inhibits the ATPase and DNA helicase activity of the S. solfataricus MCM protein. These findings provide the first in vitro biochemical evidence of a functional interaction between a MCM complex and a Cdc6 factor and have important implications for the understanding of the Cdc6 biological function.

The initiation of chromosomal DNA replication is of fundamental importance for the inheritance of genetic material and cell cycle regulation. In all organisms it requires the sequential assembly of macromolecular complexes at the replication origins. Genetic and biochemical studies highlight general properties of DNA replication initiation in a variety of model systems. In particular, bacterial and viral models greatly contribute to our understanding of the mechanistic details of this process (1). Several factors involved in the early steps of DNA replication are AAA⁺ proteins, a large superfamily of ATPases that are associated with various cellular activities (2). These factors contain a nucleotide-binding domain of the Rossmann fold family and can bind (and, in some instances, hydrolyze) ATP. Several AAA⁺ proteins involved in DNA replication are able to adopt two conformational states (ATP- or ADP-bound form) with different functions, and the switching between one conformation to the other is promoted by ATP hydrolysis (3, 4).

In Escherichia coli the first step of DNA replication is the binding of the initiator protein, DnaA, to the origin of chromosomal replication, oriC (5). DnaA is a member of the AAA⁺ family and utilizes ATP hydrolysis to promote DNA unwinding at the origin (6, 7). The melting activity of DnaA is tightly regulated by its nucleotide-bound state: DnaA-ATP is able to perform this function, whereas DnaA-ADP does not (6). After DnaA has bound oriC and unwound an A+T-rich region, the replicative helicase, DnaB, is loaded onto the ssDNA bubble in an ATP-dependent process that requires an additional AAA⁺ protein, DnaC (6, 8). This latter behaves as a monomer in solution and forms hexamers upon association with the hexameric DnaB (9–11).

Similarly, in the eukaryotic organisms AAA⁺ proteins play critical roles in DNA replication initiation (12). These include three (Orc1, Orc4, and Orc5) out of the six subunits of the origin recognition complex (13), the Cdc6 factor (Cdc18 in Schizosaccharomyces pombe (14, 15)), and the hetero-hexameric MCM complex (16–18). On the basis of genetic studies carried out mainly in Saccharomyces cerevisiae, it was postulated that at the onset of mitosis Cdc6 recruits and loads MCM onto DNA at the replication origins in an ATP-dependent process (19). Thus, Cdc6 might function as a DNA helicase-loader, likewise the E. coli DnaC factor. Nevertheless, the biochemical properties of the eukaryotic Cdc6 are largely unknown, and there is no direct biochemical evidence of its physical and/or functional interaction with the MCM DNA helicase.

The replication systems of Archaea are thought to function analogously to those of eukaryotes (20). The sequenced archaeal genomes contain ORFs coding for putative homologs of several eukaryotic replication proteins, including the initiation factors Cdc6 and MCM, but no homologs of the origin recognition complex subunits are evident. However, archaeal Cdc6 proteins share some sequence similarity with certain regions of the eukaryotic Orc1 subunit (21, 22). Furthermore, the crystallographic structure of the Cdc6 protein from the crenarchaeon Pyrobaculum aerophilum (PaeCdc6) revealed that it is composed of an N-terminal AAA⁺ nucleotide-binding module linked to a C-terminal winged-helix (WH) domain that is be-

* This work was supported by grants from the European Union (Contract QLK3-CT-2002-0207) (to F. M. P.), the Ministero Istruzione Università Ricerca/Consiglio Nazionale delle Ricerche (Biomolecole per la salute umana Legge 85/95 and Progetto Legge 449/97-DM 50/102/600), and the Agenzia Spaziale Italiana (Contract X/0B/2566/022) (to M. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 39-081-613-2292; Fax: 39-081-613-2248; E-mail: pisani@dafne.ibpe.na.cnr.it.

‡ The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; MCM, mini-chromosome maintenance; BSA, bovine serum albumin; TBE, Tris-Borate-EDTA; ORF, open reading frame; Pae, P. aerophilum; Aae, A. aeolicus; Sso, S. solfataricus; Mth, M. thermoautotrophicum; Est, A. acidocaldarius esterase.
lied to be responsible for DNA-binding activity (23). A similar modular organization was also observed in the three-dimen-
sional structure of the DnaA protein from the bacterium *Aquifex aeolicus* (AeneA470 (24)). Based on these structural similarities, it was hypothesized that the archaeal Cdc6 factors may have a dual function as the replication initiator (by spe-
cifically binding the chromosomal replication origin) and as the DNA helicase-loader (by recruiting the MCM complex at the origin (25)). However, despite this knowledge, the biochemical properties of the archaeal Cdc6 factors have been poorly investi-
gated so far. Therefore, we have undertaken the biochemical analysis of three putative Cdc6-like factors from the hyperther-
mophilic crenarchaeon *Sulfolobus solfataricus* (26). Herein we report the biochemical characterization of one of these proteins, named as SsoCdc6-1. The recombinant SsoCdc6-1 is a mon-
omer in solution, is able to autophosphorylate in vitro, has a weak ATPase activity, and binds either ssDNA or dsDNA. We found that SsoCdc6-1 strongly inhibits the ATPase and DNA helicase activity of the *S. solfataricus* MCM-like protein (SsoMCMC (27)).

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were of reagent grade. Restriction and modification enzymes were from New England Biolabs. Radioactive nucleotides were purchased from Amersham Biosciences. Oligonucleo-
tides were synthesized by Prologi (Paris, France). The homogeneous thermostable esterase from *Alcyococcus ocidivorans* (Est (28)) was a gift of Dr. G. Manco.

**Cloning of SsoCdc6-1**—The SsoCdc6-1 gene was amplified from a *S. solfataricus* genomic DNA by PCR using the High Fidelity PCR system (Roche Applied Science) with oligonucleotide Cdc6-1-for (5'-TTGGGAG-
ATTCATCAGTGTAGTAAATTTAACATAGGAC-3') as the 5' primer (the engineered EcoRI site is underlined) and oligonucleotide Cdc6-1-rev (5'-TTGGGAGTCAGTGTAGTAAATTTAACATAGGAC-3') as the 3' primer (the engineered XbaI site is underlined). The PCR product was cloned into EcoRI/XbaI-linearized *E. coli* H11032 Cdc6-1-for, Cdc6-1Xba-rev (5'-TATTTCTTCTATCTAGATATTCATGG-
0.2 mM. The bacterial culture was incubated at 37 °C for an additional 2 h. Then cells were harvested by centrifugation, and the pellet was thawed and resuspended in 1 liter of LB medium prepared that contained 200 fmol of 32P-labeled ssDNA or dsDNA in 20 mM Tris-HCl, pH 8.0, 2.5 mM 2-mercaptoethanol, and the indicated amounts of SsoCdc6-1 (or the thermostable esterase used in the control reactions). These samples were incubated at 60 °C for 30 min. The thermoprecipitated proteins were removed by centrifugation for 30 min at 30,000 rpm (Sorvall rotor 70.2 Ti) at 10 °C. The supernatant was passed through a 0.22-μm filter (Millipore) and loaded onto a nickel-nitrilotriacetic acid Superflow-agarose column (*Qiagen*) pre-
equilibrated with buffer A. After a washing step with buffer A, the loaded protein was eluted with 60 ml of an imidazole step gradient (50–500 mM) in buffer A. 1.5-ml fractions were collected and analyzed by SDS-PAGE to detect the SsoCdc6-1 polypeptide. Fractions containing the recombi-
nant protein were pooled and centrifuged for 10 min at 30,000 rpm (Sorvall rotor 70.2 Ti) to remove some precipitated material that ap-
peared shortly after elution from the column. The supernatant was dialyzed overnight against buffer A. The dialyzed solution was filtered and stored at ~80 °C. The final yield of the recombinant protein after this purification procedure was of about 20 mg. The KA mutant SsoCdc6-1 was purified using the above protocol.

**ATPase Assay**—The ATPase assay reactions contained 25 mM Tris-HCl, pH 8.0, 2.5 mM 2-mercaptoethanol, 50 mM sodium acetate, 5 mM MgCl2, and 100 μM [γ-32P]ATP (0.5–1 μCi). Incubations were performed for 90 min at 60 °C in a heated-top PCR cycler. A 1-μl aliquot of each mixture was spotted onto a polyethyleneimine-cellulose thin layer plate (Merck) and run in 0.5 M LiCl, 1 mM formic acid. The amount of [γ-32P]ATP hydrolyzed to [γ-32P]orthophosphate was quantified using a Phosphorimager (Amersham Biosciences). The rate of ATP hydrolysis was determined in the linear range of reaction time and protein con-
centration dependence. The specific activity of the recombinant ATPase was determined using blank reactions without enzyme and subtracted from the reaction rate values calculated as above.

In *Viro Autophosphorylation of SsoCdc6-1*—Samples of the purified wild type and KA mutant SsoCdc6-1 (1 μg in 100 μl) were applied to a 4.6-ml 15–50% glycerol gradient in 25 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 20 mM NaCl, 5 mM ATP. After centrifugation at 43,000 rpm for 16 h in a Beckman SW 55 Ti rotor at 10 °C, fractions (155 μl) were collected from the bottom of the tube. The proteins distribution was detected after SDS-10% PAGE and staining with Coomassie Brilliant Blue (R-250). A mixture of protein markers (tyroglobulin, 669 kDa; ferritin, 440 kDa; BSA, 69 kDa), and ribonuclease A (13.7 kDa) was loaded to the parallel gradient.

Quantitative Western Blot Analysis—S. *solfataricus* (strain P2) cells were grown aerobically at 80 °C, pH 3.5, in 100 ml of Brock’s basal salt medium supplemented with 2% glycerol (200 μg/ml ampicillin) and 20 μg/ml chloramphenicol. 50 μl of overnight culture (800,000 cells/ml) were present in a culture of *S. solfataricus* Cdc6 Biochemical Properties. The thermoprecipitated proteins were removed by centrifugation for an additional 2 h. Then cells were harvested by centrifugation, and the pellet was thawed and resuspended in 1 liter of LB medium prepared that contained 200 fmol of 32P-labeled ssDNA or dsDNA in 20 mM Tris-HCl, pH 8.0, 2.5 mM 2-mercaptoethanol, and the indicated amounts of SsoCdc6-1 (or the thermostable esterase used in the control reactions). These samples were incubated at 60 °C for 30 min and then subjected to electrophoresis in a 5% polyacrylamide/6 M urea gel (29-1) gel in 0.5 × TE buffer at a constant voltage of 100 V. The gel was then dried, and the radio-
active bands were detected using a Phosphorimager (Amersham Biosciences).

**ATPase Assay**—The standard ATPase assay reaction mixture (10 μl) contained 25 mM Tris-HCl, pH 8.0, 2.5 mM 2-mercaptoethanol, 50 mM sodium acetate, 5 mM MgCl2, and 100 μM [γ-32P]ATP (0.5–1 μCi). Incubations were performed for 90 min at 60 °C in a heated-top PCR cycler. A 1-μl aliquot of each mixture was spotted onto a polyethyleneimine-cellulose thin layer plate (Merck) and run in 0.5 M LiCl, 1 mM formic acid. The amount of [γ-32P]ATP hydrolyzed to [γ-32P]orthophosphate was quantified using a Phosphorimager (Amersham Biosciences). The rate of ATP hydrolysis was determined in the linear range of reaction time and protein con-
centration dependence. The specific activity of the recombinant ATPase was determined using blank reactions without enzyme and subtracted from the reaction rate values calculated as above.

In *Viro Autophosphorylation of SsoCdc6-1*—Samples of the purified wild type and KA mutant SsoCdc6-1 (1 μg in 100 μl) were applied to a 4.6-ml 15–50% glycerol gradient in 25 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 20 mM NaCl, 5 mM ATP. After centrifugation at 43,000 rpm for 16 h in a Beckman SW 55 Ti rotor at 10 °C, fractions (155 μl) were collected from the bottom of the tube. The proteins distribution was detected after SDS-10% PAGE and staining with Coomassie Brilliant Blue (R-250). A mixture of protein markers (tyroglobulin, 669 kDa; ferritin, 440 kDa; BSA, 69 kDa) was loaded to the parallel gradient.
at 70 °C in a reaction mixture (volume: 20 μl) containing 1.66 pmol of [γ-32P]ATP in 25 mM Tris-HCl, pH 8.0, 2.5 mM 2-mercaptoethanol, 50 mM sodium acetate, 5 mM MgCl2, in the absence or presence of 0.5 μg of M13 ssDNA as dsDNA (Amersham Biotech). The proteins were then separated on SDS-10% PAGE, and 32P-labeled bands were detected using a PhosphorImager.

**DNA Helicase Activity Assay**—A 85-mer oligonucleotide was used for the preparation of the DNA helicase substrate. This oligonucleotide (5′-TTGAAACCACCCCCCTTGTAAATACCTCTACTTGATCTCGCT-GCATGCTGAGTCTGAACTCCGGGAGTCGTTATTCCATCCCG-3′) was complementary to the M13mp18(−298) strand (Fig. 1, A) and was labeled with [γ-32P]ATP and T4 polynucleotide kinase and purified as previously described (27).

**Gel Filtration Analysis of SsoCdc6-1**—The analysis was performed using a Superose 6 column equilibrated with buffer B (see above). 0.5-ml fractions were collected, and the presence of each protein was examined by Western blot analysis of 20-μl aliquots of the indicated fractions, as previously described (27).

**RESULTS**

**Identification and Purification of SsoCdc6-1**—The analysis of the S. solfataricus genomic sequence revealed the presence of three ORFs coding for putative homologs of the eukaryotic Cdc6 proteins (26). They were named by us SsoCdc6-1 (ORF #SS00771), SsoCdc6-2 (ORF #SS02184), and SsoCdc6-3 (ORF #SS00257). In pairwise global sequence alignments the SsoCdc6 proteins were found to share about 35% similarity with the S. cerevisiae Cdc6 factor and to be similar to the eukaryotic Orc1 subunit showing about 20% similarity with the S. cerevisiae Orc1.2 The SsoCdc6-1 is encoded by the gene annotated as SSO0771 in the S. solfataricus genome web site (available at www-archbac.u-psud.fr/projects/sulfolobus). Black boxes indicate conserved sequence motifs. An asterisk indicates the position of the lysine residue 59 that was changed to alanine in the KA mutant SsoCdc6-1.

**DNA Helicase Activity Assay**—A 85-mer oligonucleotide was used for the preparation of the DNA helicase substrate. This oligonucleotide (5′-TTGAAACCACCCCCCTTGTAAATACCTCTACTTGATCTCGCT-GCATGCTGAGTCTGAACTCCGGGAGTCGTTATTCCATCCCG-3′) was complementary to the M13mp18(−298) strand (Fig. 1, A) and was labeled with [γ-32P]ATP and T4 polynucleotide kinase and purified as previously described (27).

**Gel Filtration Analysis of SsoCdc6-1**—The analysis was performed using a Superose 6 column equilibrated with buffer B (see above). 0.5-ml fractions were collected, and the presence of each protein was examined by Western blot analysis of 20-μl aliquots of the indicated fractions, as previously described (27).
chromatography, as described under “Experimental Procedures.” As shown in Fig. 4, SsoCdc6-1 was able to bind either ssDNA or dsDNA. The DNA-binding capability is a specific property of SsoCdc6-1, because we did not observe DNA band-shift activity by a thermostable nonspecific recombinant protein (the esterase 2 from A. acidocaldarius (28)) in the same experimental conditions (see Fig. 4C).

**ATPase Activity of SsoCdc6-1**—As shown in Fig. 1, the SsoCdc6-1 primary structure contains the Walker A and Walker B motifs that are typically found in proteins endowed with ATPase activity (2). Therefore, we tested the ATP hydrolysis catalyzed by the wild type and KA mutant SsoCdc6-1, in which the lysine residue 59 of the Walker A motif was replaced by alanine. The reaction mixtures were incubated at 60 °C and not at the optimal growth temperature for *S. solfataricus* (87 °C) to limit the thermally induced auto hydrolysis of ATP. Release of ([γ-32P]orthophosphate was measured by thin layer
Fig. 4. DNA-binding activity of SsoCdc6-1. The ability of SsoCdc6-1 to bind a $^{32}$P-labeled 51-mer synthetic oligonucleotide in single (A)- and double (B)-stranded form was analyzed by band-shift assays, as described under “Experimental Procedures.” In these experiments increasing amounts of SsoCdc6-1 were used (0, 100, 200, 300, 400, 500, 600, 700, 800, and 1000 ng of protein in the lanes 1-10 of each gel). C, control experiments were carried out with a thermostable esterase (Est, 33 kDa) as a nonspecific recombinant protein. In these assays increasing amounts of homogeneous esterase were added (0, 0.5, 1, 2, and 4 µg of protein in lanes 1-5 with ssDNA and from 5 to 10 with dsDNA). No appreciable DNA band-shift was observed in these control reactions. D, the shifted DNA is plotted versus the amount of protein used in the experiments shown in A and B. Detection and quantitation of the radioactive bands was carried out by phosphorimaging.

Fig. 5. ATPase activity of the wild type and the KA mutant SsoCdc6-1. ATPase activity assays were carried out with $\left[{\gamma}^{32}\right]$P-ATP at 60 °C for 30 min using increasing amounts of wild type and KA mutant SsoCdc6-1, as described under “Experimental Procedures.” The orthophosphate released during the hydrolysis reaction was plotted versus the amount of protein used. Data reported are mean values of at least three independent experiments.

Fig. 7, we found that the mutant protein had an effect similar to the one observed with the wild type protein. This latter finding clearly demonstrated that the reduction of the ATP hydrolysis in the above described mixtures was due to the inhibition of the SsoMCM activity, because the KA mutant SsoCdc6-1 is completely devoid of ATPase activity (see Fig. 5). This inhibitory effect was demonstrated to be a specific property of SsoCdc6-1, because we observed a lower reduction of the SsoMCM ATPase in assays carried out in the presence of a thermostable esterase (28) as a control recombinant protein (Fig. 7).

Effect of SsoCdc6-1 on the SsoMCM DNA Helicase Activity—Because the ATPase and DNA displacement activity of DNA
helicas are strictly correlated functions, we decided to analyze the effect of SsoCdc6-1 on the SsoMCM DNA-unwinding capability. The SsoMCM DNA helicase was tested with a strand-displacement assay performed at 70 °C for 30 min. The substrate utilized was prepared by annealing to M13mp18 ssDNA a 32P-labeled synthetic oligonucleotide of 85 (or 64) nucleotides, which gave rise to partial duplexes having a 30-nt (or 9-nt) 5′-tail. The SsoMCM helicase activity was assayed in the presence of increasing amounts of wild type SsoCdc6-1 (or KA mutant), as shown in Fig. 8. When SsoCdc6-1 (wild type or mutant) was added at a concentration of 5 pmol/μl, an almost total inhibition was observed on the unwinding of the DNA substrate with the 30-nt 5′-tail. Similar results were obtained when the substrate with the 9-nt 5′-tail was used. Inhibition of the SsoMCM DNA helicase activity was not observed when the strand displacement assays were performed in the presence of a thermostable esterase (28) as a control recombinant protein, indicating that the inhibition is a specific effect exerted by SsoCdc6-1 (Fig. 8).

Analysis of the Physical Interaction between SsoCdc6-1 and SsoMCM—The inhibition of the SsoMCM ATPase and DNA helicase activity by SsoCdc6-1 suggested that the two proteins could physically interact with one another. Gel filtration analyses of mixtures of the two proteins were carried out to test for protein-protein interaction. As shown in Fig. 9 (Gel filtration 1), SsoMCM eluted from the Superose 6 column forming a quite broad peak that extended from the elution volume corresponding to the hexameric form of the protein (molecular mass of about 470 kDa) to that of the monomeric species (molecular mass of about 77 kDa). When loaded alone onto the chromatographic column, SsoCdc6-1 formed a sharp protein peak (Gel filtration 3 in Fig. 9; see also Fig. 2) and was detected only in the fractions that corresponded to species of higher molecular mass (Gel filtration 2 in Fig. 9). Similar results were also obtained with the KA mutant SsoCdc6-1 instead of the wild type protein. These findings suggest that SsoCdc6–1 could physically interact with SsoMCM. However, this interaction appeared to be unstable, because in the above described gel filtration experiment the SsoCdc6-1 protein peak was not completely shifted toward the elution volume of species with a higher molecular mass.

The physical interaction between SsoCdc6-1 and SsoMCM was also investigated by glycerol gradient ultracentrifugation experiments, and a corresponding change was observed in the hydrodynamic properties of the SsoCdc6-1 protein. In addition, in vitro cross-linking experiments carried out with the bi-functional reagent dimethyl suberimidate in mixtures containing SsoMCM and SsoCdc6-1 at a molar ratio of 1:5 (MCM as a monomer/Cdc6-1 as a monomer) suggested that a direct physical interaction takes place between the two proteins.

DISCUSSION

In this report we describe the biochemical properties of a Cdc6 homolog from the thermoacidophilic crenarchaeon S. solfataricus (SsoCdc6-1). Analysis of the SsoCdc6-1 primary structure revealed that it contains all the sequence motifs typically found in the proteins of the AAA+ family, and in accordance with this similarity the homogeneous recombinant protein was...
the glutathione Walker A and B boxes, critical for this function (33). On the activity, although their amino acid sequence contains the arginine residues, likewise the SsoCdc6-1 undergoes phosphorylation on serine and threonine residues, whereas the MthCdc6 proteins were shown to be phosphorylated only on serine residues, we have recently found that the SsoCdc6-1 autophosphorylation in the presence of nucleic acid (32). In contrast, we observed only a slight reduction of the MthCdc6 factors is strongly inhibited by ssDNA and dsDNA molecules, as also reported for the PaeCdc6 (23), and ATP does not affect the protein oligomeric state. Because this deleted form lacks the C-terminal winged-helix (WH) domain, our results suggest that this domain could be responsible not only for the DNA-binding activity of SsoCdc6-1 but also for its ability to modulate the SsoMCM catalytic functions. It was reported that the ATPase and DNA helicase activities of E. coli DnaB are inhibited by DnaC when the two proteins are present at a molar ratio of 1:1 (11). However, unlike SsoCdc6-1, DnaC requires ATP to inhibit the DnaB helicase activity, because a Walker A mutant of DnaC, which is unable to bind ATP, does not exert any inhibitory effect. DnaC, which is a monomer in solution, was shown to associate with the hexameric DnaB to form a stable DnaB6-DnaC6 complex, and ATP is not required for this interaction (9–11). In contrast, we were unable to detect a stable association between the SsoCdc6-1 and SsoMCM proteins by gel filtration, glycerol gradient ultracentrifugation, and immunoprecipitation experiments. The SsoCdc6-1/SsoMCM molar ratio at which the DNA helicase is completely abolished is about 10-fold higher than that required by E. coli DnaC to completely inactivate the DnaB helicase (11). At the moment we do not have a clear explanation for this finding, and additional biochemical studies are needed to elucidate the molecular mechanism by which SsoCdc6-1 inhibits the SsoMCM catalytic functions. However, quantitative Western blot analyses carried out on the S. solfataricus cell extracts revealed that a large molar excess of the SsoCdc6-1 (about 2000–4000 molecules/cell) over the SsoMCM (about 300–600 molecules of hexamer/cell) is present also inside the cells.

Our gel filtration and glycerol gradient ultracentrifugation analyses indicated that the SsoCdc6-1 is a monomer in solution, as also reported for the PaeCdc6 (23), and ATP does not affect the protein oligomeric state. Because S. solfataricus is likely to possess two other Cdc6-like proteins, as indicated by the analysis of its genomic sequence (26), an intriguing possibility is that these proteins could form hetero-oligomers. To investigate this issue we have overexpressed the other S. solfataricus Cdc6-like factors as His-tagged proteins and purified them to homogeneity. Our preliminary data indicate that the SsoCdc6 factors have similar biochemical properties and that each of them is able to inhibit the SsoMCM enzymatic activities. In addition, gel filtration chromatography and immunoprecipitation experiments suggest that the SsoCdc6 proteins do not physically interact each other in vitro. It is presently not known whether the SsoCdc6 factors play distinct biological functions and whether they are differently expressed in the Sulfolobus cells. However, it was recently hypothesized that S. solfataricus could have three distinct chromosomal replication origins, which were mapped in proximity to each of the three Cdc6 genes, based on an in silico analysis of its genomic sequence carried out with a novel bioinformatic method (35). Therefore, it is likely that these proteins could perform a similar but independent function, each at a different replication origin.

Acknowledgments—The Istituto di Genetica e Biofisica, Consiglio Nazionale Ricerche/Tigem Sequencing core is acknowledged for the excellent assistance in DNA sequencing. The authors are grateful to Dr. Carlo A. Raia for helpful discussions.
REFERENCES

1. Kornberg, A., and Baker, T. A. (1992) *DNA Replication*, 2nd Ed., pp. 471–510, W. H. Freeman & Co., New York
2. Newald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999) *Genome Res.* 9, 27–43
3. Lee, D. G., and Bell, S. P. (2000) *Curr. Opin. Cell Biol.* 12, 280–285
4. Davey, M. J., Jeruzalmi, D., Kuriyan, J., and O'Donnell, M. (2002) *Nat. Rev. Mol. Cell. Biol.* 3, 1–10
5. Fuller, R. S., Funnell, B. E., and Kornberg, A. (1984) *Cell* 38, 889–900
6. Brach, D., and Kornberg, A. (1998) *Cell* 92, 743–755
7. Gille, H., and Messer, W. (1991) *EMBO J.* 10, 1579–1584
8. Carr, K. M., and Kaguni, J. M. (2002) *J. Biol. Chem.* 277, 39815–39822
9. Liang, C., Weinreich, M., and Stillman, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 441–446
10. Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A., and Nurse, P. (1993) *Cell* 74, 371–378
11. Labib, K., Tercero, J. A., and Diffley, J. F. (2000) *Science* 288, 1643–1647
12. Lee, J. K., and Hurwitz, J. (2000) *J. Biol. Chem.* 275, 18871–18878
13. Ishimi, Y. A. (1997) *J. Biol. Chem.* 272, 24508–24513
14. Weinreich, M., Liang, C., and Stillman, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 441–446
15. Zhang, R., and Zhang, C.-T. (2003) *Biochem. Biophys. Res. Commun.* 302, 728–734
16. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* 25, 3389–3402
17. Liu, J., Smith, C. L., DeRyckere, D., DeAngelis, K., Martin, G. S., and Berger, J. M. (2000) *Mol. Cell* 6, 637–648
18. Erzberger, J. P., Pirruccello, M. M., and Berger, J. M. (2002) *EMBO J.* 21, 4763–4773
19. Kelman, L. M., and Kelman, Z. (2003) *Mol. Microbiol.* 46, 605–615
20. She, Q., Singh, R. K., Confalonieri, F., Zivanovic, Y., Allard, G., Awaysz, M. J., Chan-Weber, C. C., Clausen, I. G., Curtis, B. A., De Mours, A., Erazo, G., Fletcher, C., Gordon, P. M., Heikamp-de Jong, L., Jeffreis, A. C., Kozera, C. J., Medina, N., Peng, X., Thi-Ngoc, H. P., Redder, P., Schenk, M. E., Theriault, C., Tolstrup, N., Charlebois, R. L., Doolittle, W. F., Duguet, M., Gaasterland, T., Garrett, R. A., Ragan, M. A., Sersen C. W., and Van der Oost, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 7835–7840
21. Carpentieri, F, De Felice, M., De Falco, M., Rossi, M., and Pisani, F. (2002) *J. Biol. Chem.* 277, 12118–12127
22. Liu, J., Smith, C. L., DeRyckere, D., DeAngelis, K., Martin, G. S., and Berger, J. M. (2000) *Mol. Cell* 6, 637–648
23. Erzberger, J. P., Pirruccello, M. M., and Berger, J. M. (2002) *EMBO J.* 21, 4763–4773
24. Kelman, L. M., and Kelman, Z. (2003) *Mol. Microbiol.* 46, 605–615
25. She, Q., Singh, R. K., Confalonieri, F., Zivanovic, Y., Allard, G., Awaysz, M. J., Chan-Weber, C. C., Clausen, I. G., Curtis, B. A., De Mours, A., Erazo, G., Fletcher, C., Gordon, P. M., Heikamp-de Jong, L., Jeffreis, A. C., Kozera, C. J., Medina, N., Peng, X., Thi-Ngoc, H. P., Redder, P., Schenk, M. E., Theriault, C., Tolstrup, N., Charlebois, R. L., Doolittle, W. F., Duguet, M., Gaasterland, T., Garrett, R. A., Ragan, M. A., Sersen C. W., and Van der Oost, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 7835–7840
26. Carpentieri, F, De Felice, M., De Falco, M., Rossi, M., and Pisani, F. (2002) *J. Biol. Chem.* 277, 12118–12127
27. Manco, G., Maudrich, L., and Rossi, M. (2001) *J. Biol. Chem.* 276, 37482–37490
28. Ho, S. N., Hunt, D. H., Horton, R. M., Pullen J. K., and Pease, L. R. (1989) *Gene (Amst.)* 77, 51–59
29. Brock, T. D., Brock, K. M., Belly, R. T., and Weiss, R. L. (1972) *Arch. Microbiol.* 84, 54–68
30. Feng, L., Wang, B., Driscoll, B., and Jong, A. (2000) *Mol. Biol. Cell* 11, 1673–1685
31. Gaborowski, B., and Kelman, Z. (2001) *J. Bacteriol.* 183, 5459–5464
32. Weinreich, M., Liang, C., and Stillman, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 441–446
33. Herbig, U., Marlar, C. A., and Fanning, E. (1999) *Mol. Biol. Cell* 10, 2631–2645
34. Zhang, R., and Zhang, C.-T. (2003) *Biochem. Biophys. Res. Commun.* 302, 728–734
35. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* 25, 3389–3402
Biochemical Characterization of a CDC6-like Protein from the Crenarchaeon Sulfolobus solfataricus
Mariarita De Felice, Luca Esposito, Biagio Pucci, Floriana Carpentieri, Mariarosaria De Falco, Mosè Rossi and Francesca M. Pisani

J. Biol. Chem. 2003, 278:46424-46431.
doi: 10.1074/jbc.M306075200 originally published online September 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306075200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 18 of which can be accessed free at http://www.jbc.org/content/278/47/46424.full.html#ref-list-1