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

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Cdc6 proteins play an essential role in the initiation of chromosomal DNA replication in Eukarya. Genes coding for putative homologs of Cdc6 have been also 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 from 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[1] 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-

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[1] 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.

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lieved to be responsible for DNA-binding activity (23). A similar modular organization was also observed in the three-dimensional structure of the DnaA protein from the bacterium *Aquifex aeolicus* (AaeDnaA (24)). Based on these structural similarities, it was hypothesized that the archaeal Cdc6 factors may have a dual function as the replication initiator (by specifically 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 investigated so far. Therefore, we have undertaken the biochemical analysis of three putative Cdc6-like factors from the hyperthermophilic crenarchaeon *Sulfolobus solfataricus* (26). Herein we report the biochemical characterization of one of these proteins, named by us SsoCdc6-1. The recombinant SsoCdc6-1 is a monomer 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 (SsoMCM (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. Oligonucleotides were synthesized by Proligo (Paris, France). The homogeneous thermostable esterase from *Alicyclobacillus acidocaldarius* (Est (28)) was a gift of Dr. G. Manco.

Cloning of SsoCdc6-1—The SsoCdc6-1 gene was amplified from *S. solfataricus* genomic DNA by PCR using the High Fidelity PCR system (Roche Applied Science) with oligonucleotide Cdc6-1-for (5′-TTGGGAATTCTCATCTGTATTGATAATTAAACATAAGGAC-3′) as the 5′ primer (the engineered EcoRI site is underlined) and oligonucleotide Cdc6-1-rev (5′-TTGGCTGCAGTTAAGCTCTCGATTTTAACCTCACCATTAT-3′) as the 3′ primer (the engineered PstI site is underlined). The PCR product was cloned into EcoRI/PstI-linearized *E. coli* expression vector pProEX-Hta (Invitrogen) and sequenced.

The SsoCdc6-1 gene was mutated at lysine 59 to alanine by PCR-based mutagenesis (29) using the following synthetic oligonucleotides: Cdc6-1-for, Cdc6-1Xba-rev (5′-TATTTCTTCTATCTAGATATTCATGGATCATTT-3′; the XbaI site is underlined), Cdc6-1KA-for (5′-GGTAGGACAGGTACTGGGGCAACAGCTACTGTTAGATTGTTTGGT-3′) and Cdc6-1KA-rev (5′-ACCAAACAATCTAACAGTAGCTGTTGCCCCAGTACCTGTCCTACC-3′). The final PCR product was subcloned back into the plasmid pProEX-Hta-SsoCdc6-1 at the unique EcoRI and XbaI sites. The presence of the desired mutation was checked by sequencing the amplified DNA fragment.

Expression and Purification of Recombinant Proteins—E. coli BL21-CodonPlus(DE3)-RIL cells (Novagen) transformed with the plasmid pProEX-Hta-SsoCdc6-1 were grown at 37 °C in 1 liter of LB medium containing 30 μg/ml chloramphenicol and 100 μg/ml ampicillin. When the culture reached an $A_{600\ nm}$ of 0.7 optical density, protein expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to 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 stored at −20 °C until use. The pellet was thawed and resuspended in 40 ml of buffer A (25 mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, 100 mM NaCl, 30% glycerol, 2 mM imidazole) supplemented with some protease inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 0.2 μg/ml benzamidine, 1 μg/ml aprotinin). Cells were broken by two consecutive passages through a French pressure cell apparatus (Aminco Co., Silver Spring, MD) at 1500 p.s.i. The resulting lysate was centrifuged for 30 min at 30,000 rpm (Sorvall rotor 50.2 Ti) at 10 °C. The supernatant was subjected to heat treatment at 70 °C for 5 min, then incubated in ice for 10 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 in buffer A. After a washing step with buffer A, the elution was carried out 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 recombinant protein were pooled and centrifuged for 10 min at 30,000 rpm (Sorvall rotor 70.2 Ti) to remove some precipitated material that appeared shortly after elution from the column. The supernatant was dialyzed overnight against buffer A. The dialyzed sample was aliquoted 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.

Gel Filtration Chromatography—Samples of the purified wild type and KA mutant SsoCdc6-1 (50 μg in 100 μl) were subjected to analytical gel filtration chromatography on a Superose 6 HR 10/30 fast protein liquid chromatography column (Amersham Biosciences) equilibrated with buffer B (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$). The chromatographic run was carried out at a flow rate of 0.3 ml/min at room temperature. The column was calibrated by running a set of gel filtration markers that included: tyroglobulin (669 kDa), ferritin (440 kDa), BSA (69 kDa), and ribonuclease A (13.7 kDa).

Glycerol Gradient Centrifugation—Samples of the purified wild type and KA mutant SsoCdc6-1 (35 μg in 100 μl) were applied to a 4.6-ml 15–30% glycerol gradient in 25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 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 (185 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 applied to a 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 (30) supplemented with glucose (2 g/liter). Growth was monitored spectrophotometrically at 600 nm, and, when the absorbance reached a value of O.7 OD, 10-ml aliquots of the culture were withdrawn and centrifuged. Each pellet was resuspended in 500 μl of SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 100 mM 2-mercaptoethanol, 0.6% SDS, 0.01% Blue Bromphenol). Aliquots of the extract were subjected to SDS-PAGE together with samples of the purified SsoCdc6-1. Then the gels were electroblotted onto polyvinylidene difluoride membranes, and Western blot analyses were carried out with rabbit polyclonal antisera raised against the SsoCdc6-1, as previously described (27). Antigen-antibody interactions were detected with horseradish peroxidase-conjugated secondary antibodies and the ECL^+ kit (Amersham Biosciences). Chemiluminescence was analyzed using a Chemi Doc 2000 System with the Quantity One software (Bio-Rad Laboratories). For calculating the number of SsoCdc6-1 molecules, it was assumed that 1.1×10^9 cells/ml were present in a culture of *Sulfolobus* when the absorbance was 600 nm at 1 optical density.

DNA Band-shift Assays—A 51-mer oligonucleotide (51nt-4U; 5′-CCCAGTCACGACGTTGTAAAACGACGGCCAGTGCGAGGCGCGCGAAGACCG-3′) was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, purified using Quantum Prep PCR Kleen Spin columns (Bio-Rad Laboratories), and used in ssDNA band-shift assays. To prepare dsDNA molecules a 51-mer oligonucleotide complementary to 51nt-4U (51nt-4Urev; 5′-CGGTCTTCGCGCGCCTCGCACTGGCCGTCGTTTTACAACGTCGTGACTGGG-3′) was mixed with equal molar amounts of 51nt-4U; the mixture was incubated for 5 min at 95 °C and then slowly cooled at room temperature. These molecules were labeled with [γ-^{32}P]ATP, purified as above described, and then used in dsDNA band-shift assays.

For the ssDNA and dsDNA band-shift assays, 10-μl mixtures were prepared that contained 200 fmol of ^{32}P-labeled ssDNA or dsDNA in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5 mM $MgCl_2$, 0.7 mM 2-mercaptoethanol, and the indicated amounts of SsoCdc6-1 (or the thermostable esterase used in the control reactions). These samples were incubated at room temperature for 10 min and then subjected to electrophoresis in a 5% polyacrylamide/bis (29:1) gel in 0.5× TBE buffer at a constant voltage of 100 V. The gel was then dried, and the radioactive 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 $MgCl_2$, and 100 μM [γ-^{32}P]ATP (0.5–1 μCi). Incubations were performed for 30 min at 60 °C in a heated-top PCR machine to prevent evaporation and stopped in ice. 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 M formic acid. The amounts of [γ-^{32}P]ATP hydrolyzed to [^{32}P]orthophosphate were quantitated using a PhosphorImager (Amersham Biosciences). The rate of ATP hydrolysis was determined in the linear range of reaction time and protein concentration dependence. The amount of spontaneously hydrolyzed ATP was determined using blank reactions without enzyme and subtracted from the reaction rate values calculated as above.

In Vitro Autophosphorylation of SsoCdc6-1—Samples of the purified wild type and KA mutant SsoCdc6-1 (1 μg) were incubated for 30 min

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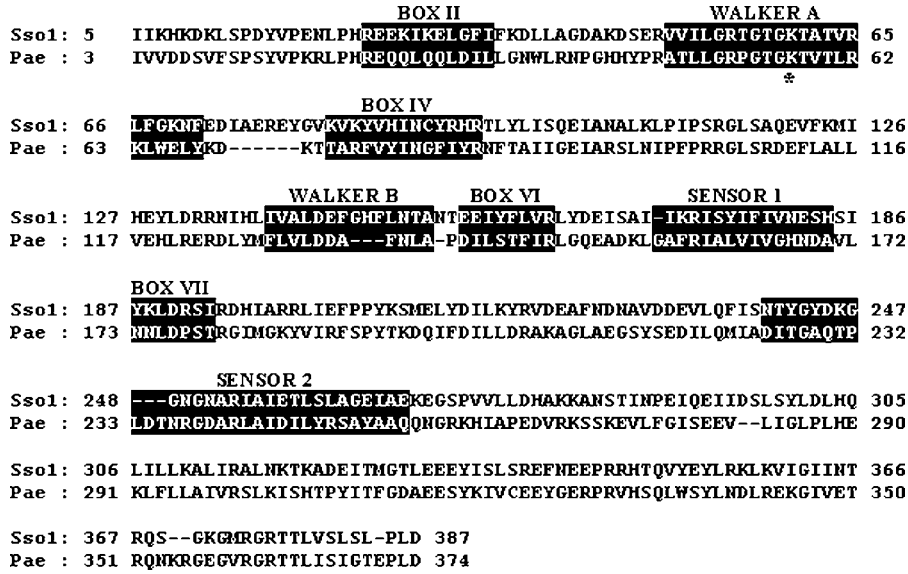

FIG. 1. **Pairwise sequence alignment of *S. solfataricus* Cdc6-1 (*Sso1*) and *P. aerophilum* Cdc6 (*Pae*).** The BlastP program (version 2.2.3 (36)) was used. The GenBank™ accession number of the Cdc6 protein from *P. aerophilum* is NP_558810. The SsoCdc6-1 is encoded by the gene annotated as SSO0771 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.

at 70 °C in a reaction mixture (volume: 20 μl) containing 1.66 pmol of [γ-^{32}P]ATP in 25 mM Tris-HCl, pH 8.0, 2.5 mM 2-mercaptoethanol, 50 mM sodium acetate, 5 mM $MgCl_2$, in the absence or presence of 0.5 μg of M13 ssDNA or dsDNA (Amersham Biotech). The proteins were then separated on SDS-10% PAGE, and ^{32}P-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′-TTGAACCACCCCCTTGTTAAATCACTTCTACTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCG) was complementary to the M13mp18(+) strand except for a 30-nt 5′-tail (the tail is underlined). The oligonucleotide was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and purified as previously described for the 51nt-4U oligonucleotide. To prepare partial duplexes, mixtures containing equal molar amounts of the labeled oligonucleotide and M13mp18(+) strand were incubated for 5 min at 95 °C and then slowly cooled at room temperature. Helicase assay reaction mixtures (20 μl) contained 50 fmol of ^{32}P-labeled substrate (about 1×10^3 cpm/fmol) in 25 mM Tris-HCl, pH 8.0, 2.5 mM 2-mercaptoethanol, 50 mM sodium acetate, 5 mM $MgCl_2$. The reactions were incubated for 30 min at 70 °C in a heated-top PCR machine to prevent evaporation and stopped by addition of 5 μl of 5× stop solution (0.5% SDS, 40 mM EDTA, 0.5 mg/ml proteinase K, 20% glycerol, 0.1% bromphenol blue), then run on a 8% polyacrylamide gel in TBE containing 0.1% SDS at a constant voltage of 150 V. After the electrophoresis the gel was soaked in 20% trichloroacetic acid and analyzed by means of a PhosphorImager. The reaction products were quantitated, and any free oligonucleotide in the absence of enzyme was subtracted.

Gel Filtration Analysis of SsoCdc6-1/SsoMCM Interaction—The physical interaction of SsoCdc6-1 with SsoMCM was analyzed by gel filtration chromatography. A mixture of SsoCdc6-1 (45 μg) and SsoMCM (90 μg) was gel-filtered on 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 #SSO0771), *SsoCdc6-2* (ORF #SSO2184), and *SsoCdc6-3* (ORF #SSO0257). 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] In Fig. 1 an alignment of the SsoCdc6-1 and the PaeCdc6 protein is reported. The motifs typically found in the AAA$^+$ proteins are present in the *Sulfolobus* Cdc6-1 sequence, including the Walker A and B and Sensor 1 and 2 boxes (2, 4). It should be noted that SsoCdc6-1 (and other archaeal Cdc6-like factors) lack the N-terminal extension of about 50 amino acid residues that is found in the eukaryotic counterparts. It was demonstrated that in the *S. cerevisiae* Cdc6 this portion of the polypeptide chain is responsible for binding to dsDNA (31).

SsoCdc6-1 was overproduced in *E. coli* as a soluble hexahistidine-tagged protein and was purified using a procedure that included a thermal treatment of the cell extracts and a chromatographic step on a Ni$^+$-chelate column, as described under "Experimental Procedures" (see Fig. 2*A*). N-terminal sequence analysis of the purified SsoCdc6-1 confirmed the identity of the recombinant polypeptide.[2]

To assess the oligomeric state of SsoCdc6-1, we carried out gel filtration experiments using three columns: Superose 6 (Fig. 2, *B* and *C*, see "Experimental Procedures"), Superdex 200 (Amersham Biosciences) and Protein Pak 300 SW Glass (Bio-Rad Laboratories).[2] A molecular mass of 45 kDa was calculated for the purified recombinant protein using all these different chromatographic supports. Samples of homogeneous SsoCdc6-1 were also subjected to glycerol gradient ultracentrifugation, as described under "Experimental Procedures," and the results obtained were consistent with the gel filtration data. Therefore, we concluded that SsoCdc6-1 is a monomer in solution. We also found that the oligomeric state of SsoCdc6-1 was not affected by addition of ATP (at 100 μM) in the buffered solutions used to prepare the glycerol gradients for the ultracentrifugation experiments.[2]

Level of Expression of SsoCdc6-1 in S. solfataricus Cells—The presence of multiple ORFs encoding putative homologs of the initiation factor Cdc6 in the *S. solfataricus* genome raised the question of whether all these genes are truly expressed and the corresponding proteins play a physiological role *in vivo*. Our Western blot experiments indicate that the SsoCdc6-1 protein is indeed present in the *Sulfolobus* cells (see Fig. 3). We estimated that about 2000–4000 molecules/cell of SsoCdc6-1 are present when the total cell extracts were prepared from *S. solfataricus* cultures taken at $A_{600\,nm} = 0.7$.

Binding of SsoCdc6-1 to ssDNA or dsDNA—To determine whether the purified SsoCdc6-1 could bind ssDNA and/or dsDNA, electrophoretic mobility shift assays were carried out using as the probe a γ-^{32}P-labeled 51-mer synthetic oligonucleotide in single- and double-stranded form, as described un-

[2] M. De Felice, M. Rossi, and F. M. Pisani, unpublished observations.

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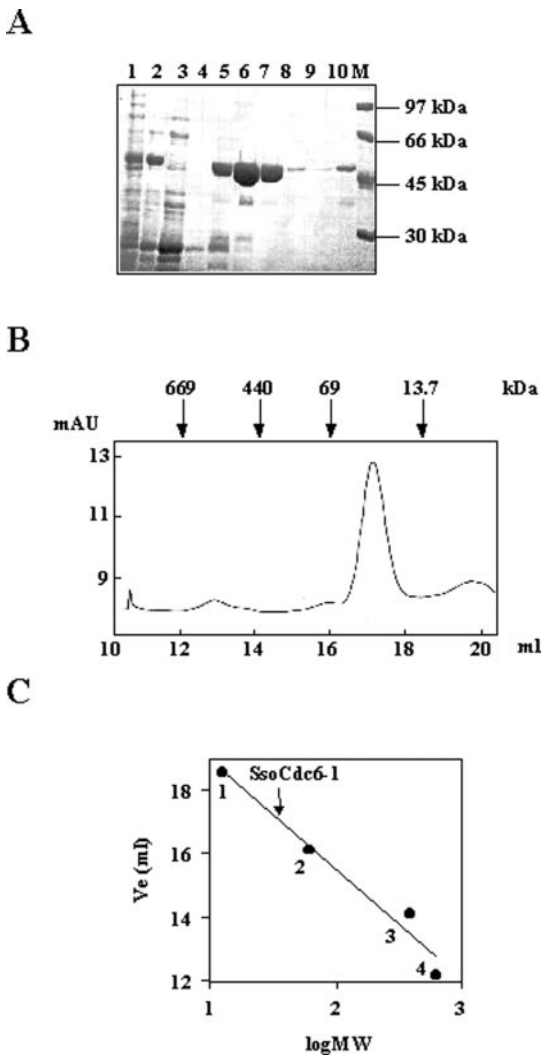

FIG. 2. **Purification and gel filtration analysis of SsoCdc6-1.** *A*, Coomassie Blue-stained SDS-polyacrylamide gel of the SsoCdc6-1 protein samples taken during the purification procedure: cell extract (*lane 1*), heat-treated cell extract (*lane 2*), Ni^{+}-chelate chromatography flowthrough (*lane 3*) and wash (*lane 4*) fractions, and fractions of the elution steps with 50, 100, 200, 300, 400, and 500 mM imidazole in buffer A (*lanes* from *5* to *10*, respectively). The native molecular mass of the SsoCdc6-1 was estimated by gel filtration on a Superose 6 column: *B*, elution profile of the purified protein from the chromatographic column (*arrows* indicate the position of some protein markers used to calibrate the column); *C*, calibration curve of the gel filtration column; the following standard proteins were used: ribonuclease A (*1*; Ve: 18.56 ml, 13.7 kDa), BSA (*2*; Ve: 16.11 ml, 69 kDa), ferritin (*3*; Ve: 14.11 ml, 440 kDa), tyroglobulin (*4*; Ve: 12.12 ml, 669 kDa).

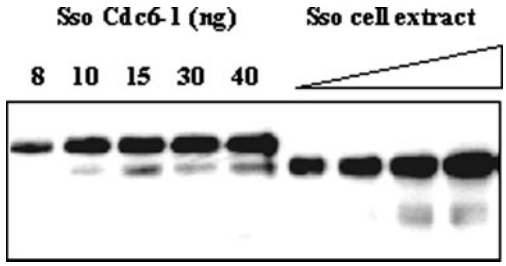

FIG. 3. **Detection of SsoCdc6-1 in *S. solfataricus* cells.** Quantitative Western blot showing detection of SsoCdc6-1 in total cell extracts, including standards with different amounts of the recombinant purified protein. The *S. solfataricus* cell extract, prepared as described under "Experimental Procedures," was applied at 2.5, 5, 8, and 10 μl per lane. The chemiluminescence values were expressed as the sum of the intensities of the pixels inside the volume boundary manually selected around each band per area of a single pixel, as described under "Experimental Procedures." The values obtained for the amounts of 8, 10, and 15 ng were used to construct a linear titration curve. The protein concentration values for the lanes loaded with the *Sulfolobus* total cell extract were extrapolated from this titration curve. It should be noticed that the slower migration of the recombinant SsoCdc6-1 is due to the presence of the tag added by the pProEX-Hta (Invitrogen) expression vector to the protein N-terminal end.

der "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. 4*C*).

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-^{32}P]orthophosphate was measured by thin layer chromatography, as described under "Experimental Procedures." As shown in Fig. 5, SsoCdc6-1 displays a low level of ATPase activity: am amount, 80 pmol of purified recombinant protein (in a 20-μl reaction volume), was able to hydrolyze about 160 pmol of ATP in 30 min at 60 °C. On the other hand, the KA mutant was found to be completely devoid of ATPase activity. Furthermore, no significant stimulation of the ATP hydrolysis catalyzed by SsoCdc6-1 was observed upon addition of various kinds of ssDNA or dsDNA molecules to the assay reaction mixtures.[2]

Autophosphorylation of SsoCdc6-1—It was reported that the two Cdc6 proteins from the euryarchaeon *Methanobacterium thermoautotrophicum* (Mth) are able to autophosphorylate *in vitro*. Because the MthCdc6 factors did not show any appreciable ATPase activity, this finding was taken as an indication that these proteins are able to bind ATP (32). In addition, the autophosphorylation reaction of the MthCdc6 proteins was shown to be completely inhibited in the presence of ssDNA or dsDNA. We found that even SsoCdc6-1 is able to autophosphorylate when incubated with [γ-^{32}P]ATP at 70 °C (Fig. 6). This ability requires an integral Walker A motif, because the KA mutant SsoCdc6-1 did not undergo autophosphorylation (see Fig. 6). However, in contrast with what was reported for the MthCdc6 factors (32), SsoCdc6-1 autophosphorylation is only slightly inhibited in the presence of ssDNA or dsDNA.

Effect of SsoCdc6-1 on the SsoMCM ATPase Activity—In the eukaryotic organisms the Cdc6 and MCM factors are both believed to play critical roles in DNA replication initiation (12). Cdc6 is thought to act as the loader of the MCM DNA helicase onto DNA at the replication origins (19, 21). In a previous report we showed that the SsoMCM protein is endowed with an efficient ATPase and a robust ATP-dependent DNA helicase activity *in vitro* (27). To test whether SsoCdc6-1 has any effect on the ATP hydrolysis reaction catalyzed by SsoMCM, the ATPase activity of a fixed amount of SsoMCM (1.5 pmol as an hexamer, Fig. 7) was assayed in the presence of increasing amounts of SsoCdc6-1. We observed that ATP hydrolysis in these mixtures was greatly reduced in comparison with the control reaction that did not contain the SsoCdc6-1 protein. The extent of this inhibition was proportional to the amount of SsoCdc6-1 added: a 34% residual ATPase activity was detected in the presence of SsoCdc6-1 at 5 pmol/μl. To better investigate this phenomenon we tested the influence of the KA mutant SsoCdc6-1 on the SsoMCM ATPase activity, and, as shown in

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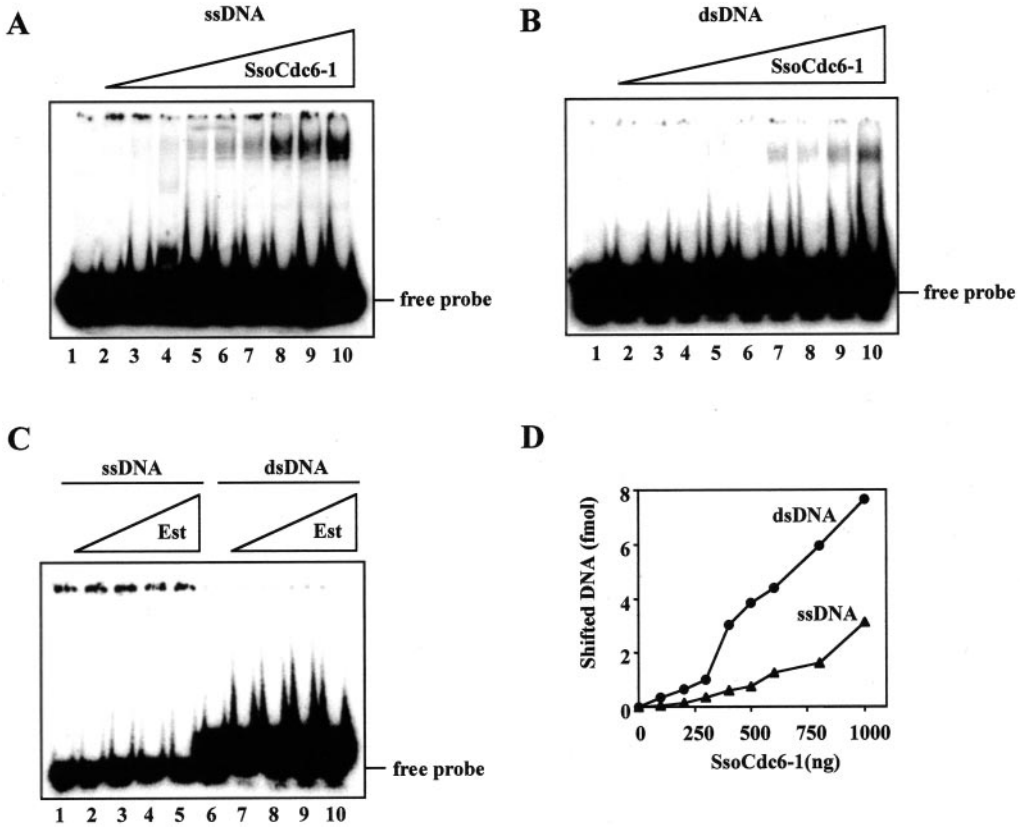

FIG. 4. **DNA-binding activity of SsoCdc6-1.** The ability of SsoCdc6-1 to bind a ^{32}P-labeled 51-mer synthetic oligonucleotide in single (*A*)- and double (*B*)-stranded form was analyzed by band-shift assays, as described under "Experimental Procedures." In these experiments increasing amounts of 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.

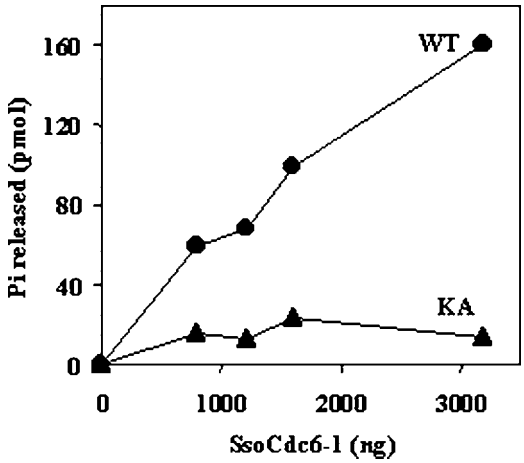

FIG. 5. **ATPase activity of the wild type and the KA mutant SsoCdc6-1.** ATPase activity assays were carried out with [γ-^{32}P]ATP at 60 °C for 30 min using increasing amounts of wild type and KA mutant SsoCdc6-1, as described under "Experimental Procedures." The orthophosphate released during the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

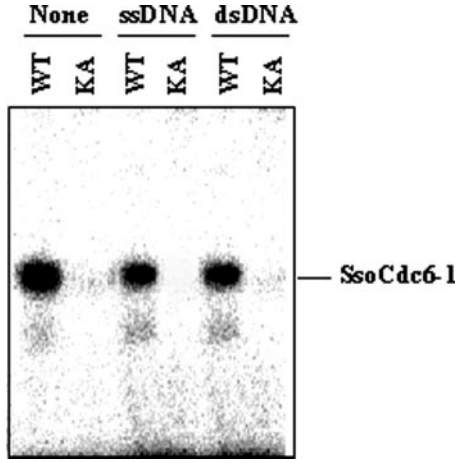

FIG. 6. **Autophosphorylation of the wild type and the KA mutant SsoCdc6-1.** Each protein (1 μg) was incubated at 70 °C for 30 min in a mixture (20 μl) containing [γ-^{32}P]ATP in the absence (*lanes* indicated with *None*) or in the presence of 0.5 μg of single- or double-stranded M13 DNA, as described under "Experimental Procedures." After incubation, the samples were subjected to SDS-10% polyacrylamide gel electrophoresis. The radioactive bands were visualized by phosphorimaging.

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

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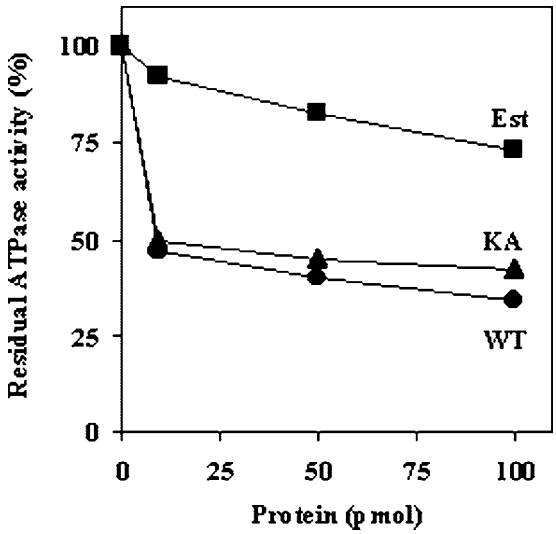

FIG. 7. **Effect of the wild type and the KA mutant SsoCdc6-1 on the SsoMCM ATPase activity.** The ATPase activity of SsoMCM (700 ng, 1.5 pmol of hexamer) was tested at 60 °C for 30 min in the presence of increasing amounts of the wild type and KA mutant SsoCdc6-1 or a thermostable esterase (*Est*, used as control recombinant protein; see "Experimental Procedures"). The ATPase activity of SsoMCM alone was taken as 100% (600 pmol of ATP are hydrolyzed by 1.5 pmol of hexamer in 30 min). The negligible contribution of the wild type SsoCdc6-1 to the total ATPase activity was subtracted. Data are mean values of at least three independent experiments.

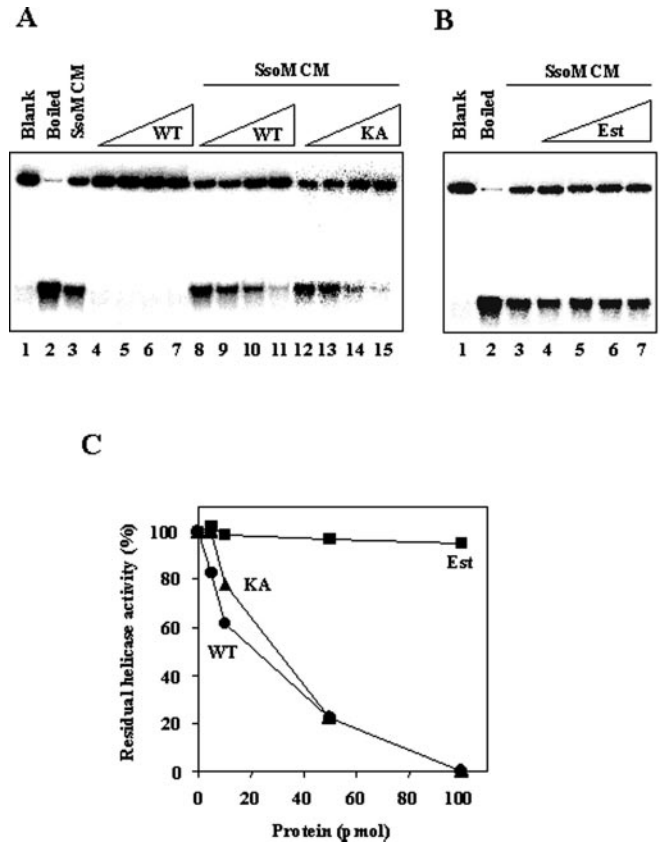

FIG. 8. **Effect of the wild type and the KA mutant SsoCdc6-1 on the SsoMCM DNA helicase activity.** *A*, the DNA-unwinding activity of SsoMCM (700 ng, 1.5 pmol of hexamer) was tested at 70 °C for 30 min in the presence of 5, 10, 50, and 100 pmol of the wild type (*lanes 8–11*) and the KA mutant (*lanes 12–15*) SsoCdc6-1. The DNA helicase activity of the wild type SsoCdc6-1 was tested as a control (*lanes 4–7*). *B*, same reactions as in *A* but in the presence of 0, 5, 10, 50, and 100 pmol of a thermostable esterase used as a control protein (*lanes 3–7*). *C*, the residual DNA helicase activity of SsoMCM was plotted *versus* the amount of the wild type and the KA mutant SsoCdc6-1, or the thermostable esterase, present in each enzymatic assay. The unwinding activity of SsoMCM alone was taken as 100%. Data shown are mean values of at least three independent determinations.

helicases 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 ^{32}P-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.[2] 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 one 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 the elution volume of a monomeric species (molecular mass of about 45 kDa). In contrast, a noticeable change in the elution profile of SsoCdc6-1 was observed when this protein was mixed with SsoMCM before loading onto the column: the protein peak appeared broadened, and SsoCdc6-1 was detected also 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.[2] 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.[2]

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

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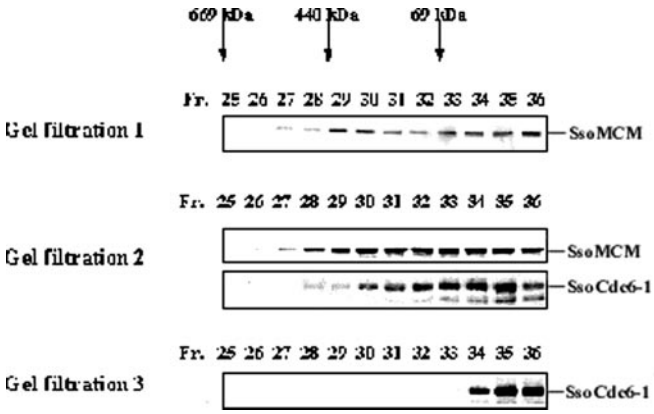

FIG. 9. **Gel filtration analysis of the SsoMCM-SsoCdc6-1 interaction.** Samples of SsoMCM (90 μg; *gel filtration 1*), SsoCdc6-1 (45 μg; *gel filtration 3*), and a mixture of SsoMCM and SsoCdc6-1 (90 and 45 μg, respectively; *gel filtration 2*) were analyzed by gel filtration on a Superose 6 column, as described under "Experimental Procedures." 0.5-ml fractions were collected and 20-μl aliquots of the indicated fractions were analyzed by Western blot using anti-SsoMCM or anti-SsoCdc6-1 antisera raised in rabbits. The positions of some protein markers used to calibrate the chromatographic column are indicated by *arrows* in the *upper part* of the figure.

found to be able to hydrolyze ATP. It has been long debated whether the eukaryotic Cdc6 factors possess the capability to hydrolyze ATP. In fact, biochemical studies indicated that *S. cerevisiae* Cdc6 and *S. pombe* Cdc18 are devoid of ATPase activity, although their amino acid sequence contains the Walker A and B boxes, critical for this function (33). On the other hand, the human Cdc6 protein produced as a fusion with the glutathione *S*-transferase in baculovirus-infected cells was reported to bind and hydrolyze ATP (34).

We found that SsoCdc6-1 undergoes autophosphorylation and after incubation at high temperature (70 °C) in the presence of [γ-^{32}P]ATP, its polypeptide chain was shown to be radiolabeled. On the other hand, a Walker A mutant SsoCdc6-1 does not undergo autophosphorylation and is completely devoid of ATPase activity, being unable to bind ATP. It was reported that the *in vitro* autophosphorylation of the PaeCdc6 and the MthCdc6 factors is strongly inhibited by ssDNA and dsDNA (32). In contrast, we observed only a slight reduction of the SsoCdc6-1 autophosphorylation in the presence of nucleic acid molecules, as also reported for the *S. pombe* Cdc18 (32). Furthermore, whereas the MthCdc6 proteins were shown to be phosphorylated only on serine residues, we have recently found that SsoCdc6-1 undergoes phosphorylation on serine and threonine residues,[2] likewise the *S. pombe* Cdc18 (32).

SsoCdc6-1 is able to bind ssDNA, and, although less efficiently, dsDNA and this activity are not affected by ATP. Similarly, the MthCd6 factors are likely to bind either ssDNA or dsDNA, because their autophosphorylation is modulated by these nucleic acid molecules, as previously discussed. In contrast, it was reported that the *S. cerevisiae* Cdc6 is able to bind only dsDNA not ssDNA, and the first 50 amino acid residues of its polypeptide chain were found to be responsible for this activity (31). It is interesting to observe that the *E. coli* DnaC protein is able to bind ssDNA, and this ability is greatly enhanced in the presence of ATP (11). These data suggest that the DNA-binding properties of the archaeal Cdc6-like factors might be substantially different from those of the eukaryotic and bacterial counterparts.

One important finding of our analysis is the noticeable inhibitory effect exerted by SsoCdc6-1 on the ATPase and DNA helicase activity of the *S. solfataricus* MCM protein. Whereas the SsoMCM ATPase activity is not completely inhibited in the presence of SsoCdc6-1, the DNA unwinding activity is completely abolished when the molar ratio Cdc6 (monomer)/MCM (monomer) is of about 10:1. A similar effect was also observed in the presence of the KA mutant SsoCdc6-1, indicating that ATP binding by Cdc6 is not needed for this inhibition. Furthermore, we have recently produced a truncated form of SsoCdc6-1 that lacks 103 residues at the C terminus and found that it is able to bind ATP (it undergoes autophosphorylation) but does not retain the DNA-binding capability and does not inhibit the SsoMCM enzymatic activities.[2] 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 $DnaB_6$·$DnaC_6$ 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.[2] 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)[2] 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*.[2] 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.

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

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