A novel DNA helicase with strand-annealing activity from the crenarchaeon *Sulfolobus solfataricus*

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To protect their genetic material cells adopt different mechanisms linked to DNA replication, recombination and repair. Several proteins function at the interface of these DNA transactions. In the present study, we report on the identification of a novel archaeal DNA helicase. BlastP searches of the *Sulfolobus solfataricus* genome database allowed us to identify an open reading frame (SSO0112, 875 amino acid residues) having sequence similarity with the human RecQ5 β . The corresponding protein, termed Hel112 by us, was produced in *Escherichia coli* in soluble form, purified to homogeneity and characterized. Gel-filtration chromatography and glycerol-gradient sedimentation analyses revealed that Hel112 forms monomers and dimers in solution. Biochemical characterization of the two oligomeric species

revealed that only the monomeric form has an ATP-dependent 3'–5' DNA-helicase activity, whereas, unexpectedly, both the monomeric and dimeric forms possess DNA strand-annealing capability. The Hel112 monomeric form is able to unwind forked and 3'-tailed DNA structures with high efficiency, whereas it is almost inactive on blunt-ended duplexes and bubble-containing molecules. This analysis reveals that *S. solfataricus* Hel112 shares some enzymatic features with the RecQ-like DNA helicases and suggests potential cellular functions of this protein.

Key words: Archaea, DNA helicase, DNA-pairing activity, DNA recombination, genome stability, *Sulfolobus solfataricus*.

INTRODUCTION

The ability to faithfully duplicate genetic information is essential to all cells. To protect their genetic material cells adopt different mechanisms linked to DNA replication, recombination and repair. This is the reason why many human disorders are caused by defects in proteins involved in these processes. The importance of genome stability is evidenced by the difficulties that cells face when these mechanisms break down. There is a symbiotic relationship between DNA replication and recombination: the replication forks stalling due to damage on the genome is rescued through recombination events that ensure completion of the DNA replication process. Many aspects of DNA metabolic pathways, including both DNA replication and recombination, often involve steps that require unwinding of double-stranded DNA by the action of specific DNA helicases to form transient single-stranded intermediates. The DNA helicases possess a distinct polarity, either 3'–5' or 5'–3' with respect to the strand on which the protein is presumed to translocate, deriving the energy from nucleotide triphosphate hydrolysis [1]. Particular attention has been given to the DNA helicases of the RecQ superfamily [2]. They are named after the 3'–5' DNA helicase RecQ of *Escherichia coli* and are evolutionarily conserved from bacteria to humans [3]. RecQ-like DNA helicases share a centrally located helicase domain of about 400 amino acids that includes seven signature motifs characteristic of a wide variety of nucleic acid helicases. Included among these conserved sequences are the Walker A and B motifs, which are required for binding and hydrolysis of ATP. Outside of the helicase domain there is much less sequence similarity among the RecQ superfamily members. Nevertheless, there are two additional identifiable domains that are found in some, but not all, RecQ-like proteins.

A conserved region of about 80 amino acid residues, apparently unique to the RecQ superfamily, is located C-terminally to the helicase domain and is termed RQC (RecQ conserved; [4]). This domain has been suggested to be involved in protein interaction [5] and DNA binding [6,7]. X-ray crystallographic analysis of *E. coli* RecQ protein has shown that the RQC region consists of an α -helical Zn²⁺-binding module followed by a WH (winged-helix domain; [8]). This latter domain acts as a double-stranded DNA-binding motif in several proteins including the catabolite gene activator protein [9]. A second sequence feature found in certain RecQ-like DNA helicases is the HRDC (helicase and RNase D conserved) motif that is located C-terminally to the RQC domain. The HRDC motif is poorly conserved at the primary sequence level but has a well-defined structural fold comprising five α -helices and a number of basic residues that constitute a putative DNA-binding surface [10]. Whereas unicellular organisms express a single RecQ-like protein, more complex organisms express two or more. There are five human RecQ DNA helicases: (i) WRN (Werner syndrome helicase), defective in Werner syndrome; (ii) BLM (Bloom syndrome helicase), defective in Bloom syndrome; (iii) RTS (Rothmund–Thomson helicase), defective in Rothmund–Thomson syndrome; (iv) RecQ1; and (v) RecQ5 [11]. Only the biochemical properties of BLM [12], WRN [13], RecQ1 [14], a *Drosophila* variant of RecQ5 [15], *Saccharomyces cerevisiae* Sgs1 [16] and *E. coli* RecQ [17] have been carefully characterized. They all unwind DNA with a 3'–5' polarity, but their substrate specificity is not identical. Several mechanisms have been proposed by which RecQ-like DNA helicases can help to prevent the breakdown of replication. These enzymes are thought to promote resolution of D-loops and double Holliday junctions which originate when replication forks collapse due to the presence of a block in the

Abbreviations used: BLM, Bloom syndrome helicase; HRDC, helicase and RNase D conserved; MCM, mini-chromosome maintenance; ORF, open reading frame; RQC, RecQ conserved; TBE, Tris/borate/EDTA buffer; WRN, Werner syndrome helicase.

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lagging strand [18]. In addition, RecQ-like DNA helicases have been implicated in reverse-branch migration resolving the four-way junctions (or 'chicken-foot' structures) that originate when a DNA lesion in the leading strand produces regression of the replication fork [19]. Unwinding of these secondary structures results in the leading strand bypassing the damage and avoids their processing by the action of Holliday junction resolvases and subsequent homologous recombination. On the other hand, BLM has recently been proposed to promote the formation of the 'chicken-foot' structures *in vivo* on the basis of its ability to catalyse the regression of a model replication fork *in vitro* in an ATP-dependent manner [20].

Archaea, the third domain of life, share many similarities with Eukarya in their genetic information processing pathways even if they have a bacterial-like cellular structure [21]. Therefore they represent an attractive model system to understand the more complex mechanisms of the eukaryotic cell. Whereas the DNA replication machinery of both Archaea and Eukarya has been extensively investigated, still little is known about the biological function of the various DNA helicases that are involved in repair or restart at damaged replication forks and no clear RecQ homologue has been identified so far in Archaea.

In the present study we report the identification and biochemical characterization of a novel archaeal DNA helicase. This protein, referred to as Hel112, has been identified as a putative homologue of human RecQ5 β by sequence similarity searches of the *Sulfolobus solfataricus* genome database. Recombinant Hel112 shows two predominant stable oligomeric states: monomer and dimer. Only the monomeric form of Hel112 has a 3'–5' DNA-helicase activity, preferring a forked DNA as the substrate. Both the monomer and the dimer possess strand-annealing activity. The biochemical properties of purified Hel112 are described and discussed.

EXPERIMENTAL

Materials

All chemicals were of reagent grade. Restriction and modification enzymes were from New England Biolabs. Radioactive nucleotides were purchased from GE Healthcare Life Sciences. Oligonucleotides were synthesized by Proligo.

Production of Hel112 in *E. coli*

The Hel112 gene was amplified from *S. solfataricus* P2 genomic DNA by PCR using the Vent polymerase (New England Biolabs) with the oligonucleotide Hel112 NheI (5'-GGTTGCT-AGCTTGAGCAATACTTACTTCTACTCTGATGAAG-3') as the 5' primer (the engineered NheI site is underlined) and the Hel112 initiation codon is shown in bold) and the oligonucleotide Hel112 BamHI (5'-GGTTGGATCCTTAAACGGAAGTA-TACTCGAGCTCGATATTC-3') as the 3' primer (the engineered BamHI site is underlined). The PCR product was cloned into NheI/BamHI-linearized *E. coli* expression vector pET28a (Novagen) and sequenced. *E. coli* Rosetta cells (Novagen) transformed with the plasmid pET28a-Hel112 were grown at 37°C in 5 litres of LB (Luria–Bertani) medium containing 30 μ g/ml chloramphenicol and 100 μ g/ml kanamycin. When the culture reached an A_{600} of 0.8, protein expression was induced by adding isopropyl β -D-thiogalactoside at a concentration of 0.2 mM. The bacterial culture was incubated at 37°C for an additional 2 h. Cells were then harvested by centrifugation (10 800 g for 10 min at 4°C) and the pellet (10 g) was stored at –20°C until use. The cell pellet was thawed and suspended in

50 ml of buffer A [25 mM Tris/HCl (pH 8.0), 2.5 mM MgCl₂, 500 mM NaCl and 15 % glycerol] supplemented with a protease inhibitor cocktail (Sigma). Cells were broken by two consecutive passages through a French pressure cell apparatus (Aminco Co) at 1500 p.s.i. The resulting cell extract was treated with DNase I (at 0.25 mg/ml) and RNase A (at 0.1 mg/ml) for 30 min on ice with shaking. Protamine sulfate (at 2 mg/ml) was then added and incubation was continued for an additional 30 min on ice with shaking. The sample was centrifuged for 30 min at 30 000 rev./min (Beckman rotor 70.0 Ti) at 10°C. The supernatant was subjected to heat-treatment at 80°C for 20 min, then kept on ice for 10 min. The thermo-precipitated proteins were removed by centrifugation for 30 min at 30 000 rev./min (Beckman rotor 70.0 Ti) at 10°C. The supernatant was passed through a 0.22 μ m filter (Millipore), mixed with Ni²⁺-nitrilotriacetic acid Superflow-agarose resin (Qiagen) pre-equilibrated in buffer A and incubated for 1 h on ice with gentle shaking. The resin was washed with buffer A and elution was carried out with 100 ml of an imidazole gradient (25–500 mM) in buffer A. Fractions of 4 ml were collected and analysed by SDS/PAGE to detect the Hel112 polypeptide. Fractions containing the recombinant protein were pooled and concentrated up to 6 mg/ml using an Amicon system. The protein sample was dialysed overnight against buffer A containing 100 mM NaCl. The sample was loaded on to an heparin–Sephacrose column (8 ml column volume) at 0.5 ml/min using an AKTA system (GE Healthcare Life Sciences). The column was washed using the same buffer and bound protein was eluted using a linear gradient of NaCl in buffer (from 0.1 to 1 M NaCl). Fractions containing the recombinant Hel112 were pooled and concentrated. The sample was then loaded on to a Superdex 200 16/60 column (GE Healthcare Life Sciences) equilibrated in buffer A containing 200 mM NaCl. The column was developed at a flow rate of 0.3 ml/min. Fractions of 1 ml were collected. A 20 μ l sample of each fraction was analysed by SDS/PAGE. Fractions containing the Hel112 protein were stored at 4°C for subsequent biochemical analyses. The column was calibrated by running a set of gel-filtration markers that included tyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa) and BSA (69 kDa).

Glycerol-gradient sedimentation

Glycerol-gradient ultracentrifugation was performed by applying the protein sample (60 μ g of fraction #74 and 30 μ g of fraction #65 in 150 μ l) to a 8 ml preformed 20–40 % glycerol gradient in buffer containing 25 mM Tris/HCl (pH 8.0), 2.5 mM MgCl₂ and 200 mM NaCl. A parallel gradient was used to fractionate a mixture of standard proteins which included catalase (232 kDa, 11.3 S), aldolase (158 kDa, 7.3 S), BSA (67 kDa, 4.6 S) and ovalbumin (43 kDa, 3.5 S). After centrifugation at 38 000 rev./min for 18 h in a Beckman SW41 rotor at 10°C, 200 ml fractions were collected from the bottom of the tube and analysed by SDS/PAGE followed by Coomassie Blue staining of the gel.

DNA substrates

The sequence of the synthetic oligonucleotides used to prepare the DNA substrates is reported in Table 1.

Oligonucleotides were labelled using T4 polynucleotide kinase and [γ -³²P]ATP. After the labelling reaction oligonucleotides were purified using a Micro Bio-Spin P-30 Tris chromatography column (Bio-Rad), according to the manufacturer's protocol. To prepare duplexes of DNA molecules, mixtures were incubated for 5 min at 95°C which contained the labelled and the complementary unlabelled oligonucleotides at a 1:2 molar ratio. The samples were then slowly cooled at room temperature (25°C).

Table 1 Synthetic oligonucleotides used to prepare the DNA substrates used in the present study

dsDNA, double-stranded DNA; TSJ, three-strand junction.

Name used	Oligonucleotide sequence
dsDNA	Strand 1: 5'-GCTCGGTACCGGGGATCCTCTAGA-3' Strand 2: 5'-TCTAGAGGATCCCCGGGTACCGAGC-3'
3'-mis	Strand 1: 5'-CTTGCATGCCTGCAGTCTGACTCTAGAGGATCCCCGGGTACCGAGC-3' Strand 2: 5'-TCCTCTAGAGTCGACCTGCAGGCATGCAAG-3'
5'-mis	Strand 1: as strand 1 of 3'-mis Strand 2: 5'-TCACACAGGAACAGCTATGACCATGATTA-3'
Bubble	Strand 1: 5'-TCTACCTGGACGACCGGGTTTTTTTTTTTTTTTTTTTTTTGGGCCAGCAGGTCCATCA-3' Strand 2: 5'-TGATGGACCTGCTGGCCCTTTTTTTTTTTTTTTTTTTTCCCGGTCGTCAGGTAGA-3'
Fork	Strand 1: 5'-GCTCGGTACCGGGGATCCTCTAGATTTTTTTTTTTTTTTTTTT-3' Strand 2: 5'-TTTTTTTTTTTTTTTTTTTTTTCTAGAGGATCCCCGGGTACCGAGC-3'
TSJ	C80: 5'-GCTGATCAACCTACATGTGTAGGTAACCCCTAACCCCTAAGGACAACCCCTAGTGAAGCTTGTAACCCCTAGGAGCT-3' G80F: 5'-CTTCACAGTCAGAGTCACAGTCTGCCGGGTGTCCTTAGGGTAGGGTTAGGGTTACCTACACATGATAGGGTTGATCAGC-3' G80B: 5'-AGCTCTAGGGTTACAAGCTTCACTAGGGTTGTCCAGTCACAGTCAGAGTCACAGTCTACACATGATAGGGTTGATCAGC-3'

DNA band-shift assays

For each substrate, 10 μ l mixtures were prepared which contained 200 fmol of [32 P]-labelled DNA 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 Hel112. Following incubation for 10–15 min at room temperature, complexes were separated by electrophoresis through 5% polyacrylamide/bis gels (37.5:1) in 0.5 $\times$ TBE [Tris/borate/EDTA buffer (100 mM Tris/HCl, pH 8.3, 2 mM EDTA and 85 mM boric acid)]. Gels were dried down and analysed using a Storm PhosphorImager (Molecular Dynamics; Image Quant software).

DNA-helicase assay

Reaction mixtures (10 μ l) contained 50 fmol of [32 P]-labelled substrate (about 1×10^3 cpm/fmol) and the indicated amounts of Hel112 (gel-filtration peak fractions corresponding to the monomeric or the dimeric form of the protein) in helicase assay buffer [25 mM Tris/HCl (pH 8.0), 2.5 mM 2-mercaptoethanol, 50 mM sodium acetate and 5 mM $MgCl_2$] containing 5 mM ATP. Samples were incubated for 30 min at 60°C in a heated-top PCR machine to prevent evaporation and reactions were stopped by addition of 2.5 μ l of 5 $\times$ stop solution (0.5% SDS, 40 mM EDTA, 0.5 mg/ml proteinase K, 20% glycerol and 0.1% bromophenol blue). Reaction products were separated on a 8% polyacrylamide gel in TBE containing 0.1% SDS at a constant voltage of 150 V. After electrophoresis the gel was soaked in 20% trichloroacetic acid and analysed by a phosphorimager. Reaction products were quantified and any free oligonucleotide in the absence of enzyme was subtracted.

Strand-annealing assay

Reaction mixtures (10 μ l) contained partially or fully complementary synthetic oligonucleotides (each at a concentration of 2.5 nM), one of which was labelled at the 5'-end with T4 polynucleotide kinase and [γ - 32 P]ATP, and the indicated amounts of Hel112 in helicase assay buffer. Reaction mixtures were assembled on ice. The protein was then added to each mixture and the samples were incubated for the indicated times at 75°C in a heated-top PCR machine to prevent evaporation and reactions were stopped by addition of 2.5 μ l of 5 $\times$ stop solution. Samples were subjected to gel electrophoresis as described for the DNA-helicase assay. Reaction products were quantified and

any annealed oligonucleotide in the absence of enzyme was subtracted.

For the three-strand junction, samples (10 μ l) contained a mixture of oligonucleotides (2.5 nM C80, 5 nM G80F and 5 nM G80B; the C80 was labelled at the 5'-end with T4 polynucleotide kinase and [γ - 32 P]ATP), increasing amounts of Hel112 gel-filtration peak fraction corresponding to the monomer (5, 15, 30, 50, 100 and 200 ng) in helicase buffer. Samples were incubated for 10 min at 60°C. ATP was then added at 5 mM into each sample, incubation was continued for an additional 15 min at 60°C and stopped with 2.5 μ l of 5 $\times$ stop solution. Samples were subjected to gel electrophoresis as described for the DNA-helicase assay.

ATPase assay

The ATPase reaction mixture (10 μ l) contained 100 μ M [γ - 32 P]ATP (0.5–1 μ Ci) and the indicated amounts of Hel112 (gel-filtration peak fractions corresponding to the monomeric or dimeric form of the protein). Incubation was performed for 30 min at 60°C in a heated-top PCR machine to prevent evaporation and stopped in ice. A 1 μ l sample of each mixture was spotted on to a polyethyleneimine-cellulose thin layer plate (Merck) and run in 0.5 M LiCl and 1 M formic acid. The [γ - 32 P]ATP hydrolysed to [32 P]orthophosphate was quantified using a phosphorimager. The rate of ATP hydrolysis was determined in the linear range of reaction time and protein concentration dependence. The amount of spontaneously hydrolysed ATP was determined using blank reactions without enzyme and was subtracted from the reaction rate values calculated as above.

RESULTS

Identification of a novel *S. solfataricus* DNA helicase

The aim of the present study was to identify a DNA helicase belonging to the RecQ superfamily in the hyperthermophilic archaeon *S. solfataricus* and investigate its biochemical properties. Since the archaeal molecular machineries responsible for DNA replication/recombination/repair are evolutionarily related to the eukaryal counterparts [21], we carried out BlastP [22] searches of the *S. solfataricus* genome database using each of the five human RecQ-like DNA helicases as the query sequence in order to identify putative RecQ homologues in this archaeal species. Only when human RecQ5 β was used as the query sequence in these searches, ORFs (open reading frames) putatively coding for DNA helicases (SSO0112, 0017, 0965, and

Figure 1 Oligomeric state of Hel112

(A) The Hel112 pool from the heparin–Sepharose chromatography was run on a Superdex 200 HR 16/60 gel-filtration column, as described in the Experimental section. A 20 μ l sample of each 1 ml fraction was loaded on to a SDS polyacrylamide gel which was stained with Coomassie Blue. Arrows indicate the position of two protein markers used to calibrate the column [BSA and aldolase (Ald)]. (B) Samples of the gel-filtration fractions #65 and #74 were fractionated on a glycerol gradient as described in the Experimental section. Aliquots of each fraction were analysed by SDS/PAGE followed by Coomassie Blue staining. Arrows indicate the position of protein markers fractionated on a parallel gradient. (C) A plot of the S values versus the position in the gradient for each marker protein. S values for the protein in the peak fraction #65 and #74 were estimated to be 8.5 and 5.7 respectively.

0394) were identified. Among these sequences, SSO0112 (875 amino acid residues; referred to as Hel112) gave the highest similarity score with human RecQ5 β . The most conserved region between the two proteins was the N-terminal portion which contains the sequence similarity motifs typically found in the Superfamily 2 DNA helicases (sequence similarity 41 %; [23]). Unlike other RecQ proteins, Hel112 does not seem to contain the RQC and the HRDC sequence motifs that are located C-terminally to the conserved DNA helicase domain.

Hel112 was over-produced in *E. coli* as a protein containing an His₆ tag at the N-terminus using the vector pET28a. The protein was found to be expressed at a high level in a soluble form and was purified using a procedure that included a thermal treatment of the cell extracts, chromatographic steps on a Ni²⁺-chelate resin, Heparin Sepharose, and gel-filtration on a Superdex 200 column, as described in the Experimental section. The fractions of the gel-filtration chromatography, carried out in a buffer containing 200 mM NaCl, were analysed by SDS/PAGE (Figure 1A). This analysis revealed that Hel112 possessed two different oligomeric states with an apparent molecular mass of about 65 and 140 kDa. Since the Hel112 theoretical molecular mass is calculated to be 100 kDa, we predicted that the two oligomeric states corresponded to the monomeric and dimeric form of the protein in solution. In order to determine whether the two forms of Hel112 were stable, samples of the peak fractions (fraction #65 corresponding to the dimer and fraction #74 corresponding to the monomer) were subjected to analytical gel-filtration chromatography on a Superdex 200 column (V. Aria, M. De Felice and F. M. Pisani, unpublished work). They both eluted as single peaks corresponding to molecular globular

species of 65 and 140 kDa suggesting that both the monomeric or the dimeric form were stable in solution. When the gel-filtration analyses were carried out in different salt conditions (0.5 M and 1 M NaCl), the stability of the dimeric form was affected and it was converted into the monomeric form (results not shown). In addition, a sample of purified dimer was subjected to a prolonged incubation with 2-mercaptoethanol (at 5 mM) and then analysed by gel-filtration. Treatment with this sulfhydryl-reducing agent did not affect the oligomeric state of Hel112 revealing that disulfide bonds were not required for the formation of the protein dimeric assembly.

In order to analyse the biochemical properties of Hel112, DNA-unwinding assays were carried out using different kinds of DNA duplex molecules. The activity assays were performed at 60 °C (and not at 75 °C) to reduce the spontaneous melting of the probe. We first analysed the pool recovered from the Ni²⁺-chelate column but no DNA-helicase activity was detected even using 1.5 μ g of the protein sample on different substrates (results not shown). We next decided to test the DNA-unwinding activity of the peak fractions eluted from the gel-filtration column using two partially complementary oligonucleotides forming forked DNA molecules as the substrates (see Table 1). As shown in Figure 2(A), an evident displacement of the probe was observed when Hel112 in the monomeric state (gel-filtration fraction #74, see Figure 1) was assayed. In contrast, the dimeric species (corresponding to the gel-filtration fraction #65, see Figure 1) did not display the ability to melt the fork DNA molecules (Figures 2B and 2C). Surprisingly, fraction #65 seemed to catalyse annealing instead of unwinding of DNA molecules (Figures 2B and 2C). Furthermore, DNA-pairing activity was detected when

Figure 2 Catalytic activities of Hel112 dimeric and monomeric forms

Enzymatic assays were carried out using the Hel112 dimeric and monomeric forms. Increasing amounts of each protein (1.4, 7, 14, 70, 140 and 280 ng) were used either in DNA-helicase (**A** and **B**) or in DNA-pairing (**D** and **E**) assays. The labelled single-stranded DNA was quantified using a phosphorimager system and plotted versus the amount of protein used. The labelled single-stranded DNA present in the blank reaction was considered as 0 or 100% in the plots shown in (**C**) and (**F**) respectively. Plots obtained for the DNA-helicase (**C**) and DNA-pairing (**F**) assays are shown. ♦, monomeric Hel112; ●, dimeric Hel112. The enzymatic assays were performed in at least triplicate and the results were averaged. Values are means $\pm$ S.E.M. ssDNA, single-stranded DNA.

fraction #74 was assayed. To better investigate this issue, strand-annealing assays were carried out on both of the gel-filtration peak fractions using the fork DNA as the substrate. To avoid melting of double-stranded DNA due to the DNA-unwinding activity of the protein, ATP was not present in the reaction mixtures. This analysis revealed that Hel112 in either monomeric or dimeric form possesses DNA-pairing activity (see Figures 2D–2F).

To perform a more accurate analysis of the Hel112 oligomeric state, glycerol gradient sedimentation analyses were carried out. Samples of the gel-filtration peak fractions were subjected to ultracentrifugation through a preformed glycerol gradient. As shown in Figure 1(B), the protein of the peak fraction #74 behaved as a monomer (with an expected molecular mass of 100 kDa) and the protein of the peak fraction #65 was detected in a position of the gradient (between the catalase and aldolase protein markers) compatible with a dimeric structure.

The monomeric form of Hel112 has ATP-dependent 3'–5' DNA-helicase activity

The gel-filtration peak fraction corresponding to the Hel112 monomeric form (fraction #74 in Figure 1) was used to more deeply characterize the DNA-helicase activity. To determine the directionality of Hel112 helicase, DNA-unwinding assays were

carried out using a substrate with a single-stranded region at either the 3'- or 5'-end (termed 3'-mis and 5'-mis respectively). As shown in Figure 3(A), Hel112 was able to utilize only the substrate bearing a 3'-tail suggesting that the protein is able to translocate on to the DNA in the 3'–5' direction. In addition, in the absence of ATP Hel112 was found to be devoid of any DNA-unwinding capability indicating that the 3'–5 DNA-helicase activity is ATP-dependent (Figure 3B).

To analyse the substrate preference of Hel112, various kinds of DNA substrates were used in the unwinding assays, such as fork, bubble and blunt-ended molecules. As shown in Figure 4(A), fork and 3'-tail DNA are the Hel112 favourite substrates, whereas no activity was detected on bubble or blunt-ended DNA.

Both monomeric and dimeric forms of Hel112 possess ATPase activity

The ATPase activity of the two Hel112 oligomeric forms was analysed, as shown in Figure 5. The amount of [32 P] orthophosphate released was determined by means of a phosphorimager system and plotted versus the amounts of Hel112 used as either a monomer or a dimer (Figure 5B). As indicated by the plots, the two oligomeric forms of Hel112 had a comparable level of ATPase activity. The ATP-hydrolysis function was not stimulated

Figure 3 DNA-helicase directionality and ATP-hydrolysis dependency

(A) Increasing amounts of monomeric Hel112 (100, 200, 400 and 800 ng) were used in DNA-unwinding assays with 3'-mis or 5'-mis DNA molecules as the substrates. (B) To check the DNA-helicase dependency on ATP-hydrolysis, increasing amounts of monomeric Hel112 (100, 200, 400 and 800 ng) were assayed in the presence or absence of ATP on the same 3'-mis DNA substrate. The ● on the oligonucleotides represents the radiolabelled phosphate group at the 5'-end.

in the presence of either single- or double-stranded DNA (results not shown).

Hel112 has strand-annealing activity

The Hel112 strand-pairing activity was tested on different DNA substrates. An initial kinetic analysis was carried out using partially complementary oligonucleotides forming the bubble DNA and a fixed amount of monomeric Hel112 (100 ng). As reported in Figures 6(A) and 6(B), by 2 min Hel112 promoted annealing of about 50% of the single-stranded molecules. On the other hand, in the absence of protein, spontaneous melting of the paired oligonucleotides was observed, since samples were incubated at 75°C due to the thermophilic nature of Hel112. To verify that the observed strand pairing was truly due to a specific

Figure 5 ATPase activity of Hel112

ATPase assays were performed as described in the Experimental section. (A) Increasing amounts (5, 10, 25, 50, 100 and 250 ng) of Hel112 monomer (▲) or dimer (■) were used. The orthophosphate released during the ATP hydrolysis was plotted versus the amount of protein used. The enzymatic assays were performed at least in triplicate and the results were averaged. Values are means $\pm$ S.E.M.

catalytic function of Hel112, we carried out parallel control assays using *S. solfataricus* MCM (mini-chromosome maintenance) DNA helicase, produced and characterized previously in our laboratory [24]. In the control reactions performed with this hexameric DNA helicase no difference was observed with the sample not containing protein (results not shown). In addition, DNA pairing assays were performed with increasing amounts of either monomeric or dimeric Hel112 at 75°C for 2 min. Plots of annealed probe versus the amount of protein used are shown in Figures 6(D) and 6(E). It can be noticed that the strand-annealing activity of the Hel112 monomeric and dimeric forms are comparable on all the substrates used in these enzymatic assays

Figure 4 DNA-helicase activity of Hel112 on various DNA substrates

(A) Increasing amounts of Hel112 in the monomeric form (25, 50, 100, 200, 400 and 800 ng) were used to analyse the DNA-unwinding activity on various DNA molecules, as described in the Experimental section. (B) The displaced oligonucleotide was quantified by a phosphorimager system and plotted against the amount of protein used. ■, 3'-mis; ◆, fork; ●, bubble; ▲, double-stranded DNA. The enzymatic assays were performed at least in triplicate and the results were averaged. Values are means $\pm$ S.E.M.

Figure 6 DNA-annealing activity of Hel112

(A) Annealing of partially complementary oligonucleotides to form bubble DNA molecules was carried out in reaction mixtures containing (or not) monomeric Hel112 (100 ng). (B) The percentage of annealed probe was plotted versus the reaction time in the absence (●) or presence of monomeric Hel 112 (■). The annealed probe present in the blank reaction was considered as 0%. The enzymatic assays were performed at least in triplicate and the results were averaged. Values are means $\pm$ S.E.M. (C) Different DNA molecules were used to analyse the substrate specificity of monomeric or dimeric Hel112 in the annealing reaction. (D and E) The annealed probe was plotted versus the amount (0, 10, 25, 50 and 100 ng) of Hel112 in the monomeric (D) or dimeric (E) forms. The annealed probe present in the blank reaction was considered as 0%. ●, double-stranded DNA; ■, fork; ▲, 3'-mis; ◆, bubble DNA. The enzymatic assays were performed at least in triplicate and the results were averaged. Values are means $\pm$ S.E.M. (F) Increasing amounts of monomeric Hel112 (5, 10, 30, 50, 100 and 200 ng) were tested in strand-exchange assays as described in the text. TSJ, three-strand junction.

(Figure 6C). We then decided to determine whether Hel112 was able to combine DNA pairing and unwinding to catalyse strand-exchange reactions, as previously reported for RecQ-like DNA helicases [25]. To this end, we utilized a fork substrate containing a 54 base-pair duplex region and 26-nt single-stranded 5'- and 3'-tails, which was made from a 5'-labelled 80-mer oligonucleotide (termed C80) annealed to an unlabelled partially complementary 80-nt oligomer (termed G80F). Assays were performed with the Hel112 monomeric form using this fork substrate in combination with a third unlabelled 80-nt oligonucleotide (termed G80B). The G80B oligonucleotide is complementary to both ends of C80, apart from a central non-complementary stretch (21-nt), and its 5'-end can pair with the 3'-tail of C80 to form a three-strand junction structure, as previously described [25]. Displacement of the G80F

oligonucleotide from the three-strand junction structure together with annealing of the G80B 3'-end to the C80 5'-end produced a blunt-ended duplex with a central 21-nt bubble. In these assays, equimolar amounts of the fork substrate described above and the partially complementary G80B oligonucleotide were incubated with increasing amounts Hel112 monomer (5–200 ng) for 10 min at 60 °C in the absence of ATP. ATP was then added to the reaction mixtures and the samples were incubated for an additional 15 min. As shown in Figure 6(F) a slower migrating product was detected corresponding to a three-strand junction structure. On the other hand, the formation of the bubble-containing duplex was not observed as the result of enzyme-catalysed strand-exchange, even when the G80B was present in molar excess with respect to the forked DNA substrate and the reaction mixtures were incubated up

Figure 7 DNA-binding activity of Hel112

The ability of Hel112 to bind different ^{32}P -labelled synthetic oligonucleotides was analysed by gel-shift assays as described in the Experimental section. (A) Increasing amounts of Hel112 (50, 100, 200 and 400 ng) were used in the presence of the indicated DNA molecules. For each DNA ligand used a plot is reported of the shifted DNA versus the amount of monomeric (B) or dimeric (C) protein used. $\square$, bubble; $\blacksquare$, fork; $\blacktriangle$, double-stranded DNA; $\triangle$, single-stranded DNA; $\bullet$, 3'-mis. The enzymatic assays were performed at least in triplicate and the results were averaged. Values are means $\pm$ S.E.M.

to 30 min after the addition of ATP (results not shown). Therefore Hel112 can promote strand-annealing on DNA substrates with a more complex structure, but it seems unable to catalyse strand-exchange reactions, as previously reported for RecQ-like DNA helicases [25].

Hel112 monomer and dimer show different DNA-binding affinities

To further biochemically characterize Hel112, gel mobility-shift assays were performed using either the monomeric or the dimeric form of the protein and various kinds of DNA ligands. As shown in Figure 7, Fork and 3'-mis DNA were bound by Hel112 in either a monomeric or dimeric state with higher affinity. On the other hand, a clear difference in the DNA-binding capability was observed between the two forms of the protein on both single- and double-stranded DNA: the monomer bound these molecules with a very low affinity, whereas the DNA-binding capability of the Hel112 dimeric form was negligible on both these ligands.

Hel112 in the monomeric state was able to bind single-stranded DNA with an affinity lower than the one observed for the fork and 3'-mis DNA. In contrast, Hel112 in the dimeric form bound single-stranded DNA with very low affinity (only about 10% of the input DNA was shifted using 200 ng of protein; Figures 7B and 7C). The difference in the DNA-binding capability between

the two forms of the protein was more evident on double-stranded DNA: the monomer was able to bind these DNA molecules with low affinity (only 40% of the total DNA was shifted by 400 ng of protein), whereas the dimer did not display any DNA-binding capability on this ligand.

DISCUSSION

The Hel112 protein described in the present study has been identified by a sequence similarity search of the *S. solfataricus* genome database using human RecQ5 β as the query sequence. Our biochemical analysis revealed that Hel112 possesses catalytic properties shared by RecQ superfamily members. First, Hel112 can exist in two stable oligomeric states (monomer and dimer), but only the monomeric form displays a DNA-unwinding activity which operates in the 3'-5' direction. Similarly, RecQ proteins are monomeric, although *E. coli* RecQ, human BLM and WRN have also been reported to form oligomeric assemblies in certain experimental conditions [17,26,27]. Hel112 is able to unwind forked and 3'-tailed DNA structures with high efficiency, whereas it is almost inactive on blunt-ended duplexes and bubble-containing molecules. These properties are shared by human BLM and WRN and *Drosophila* and human RecQ5 β . In contrast, *E. coli* RecQ is able to unwind blunt-ended DNA as well as

bubble-containing structures [3]. Furthermore, *S. solfataricus* Hel112 is endowed with an efficient strand-annealing activity that promotes the formation of various duplex DNA molecules starting from fully or partially complementary synthetic oligonucleotides. This peculiar feature has been previously described for RecQ-like proteins of metazoans, including WRN, BLM, RecQ1 and RecQ5 β [28–30].

Nonetheless, Hel112 lacks both the QRC and HRDC sequence motifs that are typically found in RecQ superfamily members [3]. The sequence similarity between human RecQ5 β is limited to the N-terminal half of the polypeptide chain which contains the sequence similarity boxes of the Superfamily 2 DNA helicases [23]. With all that considered, Hel112 can not be strictly classified within the RecQ superfamily, although it displays biochemical features and catalytic properties that are a hallmark of the RecQ-like DNA helicases. Despite the proposed critical cellular role and their ubiquitous distribution among Bacteria and Eukarya, clear RecQ homologues have not yet been identified in Archaea. Nevertheless, proteins responsible for Holliday junction migration were characterized from *Pyrococcus furiosus* (Hjm; [31]) and *Methanobacterium thermoautotrophicus* (ORF Mth810; [32]). Both of these enzymes belong to the Mus308 family of DNA helicases/translocases that support genome stability in metazoans, but are absent in bacteria and yeasts. This group includes human and *Drosophila* Hel308, human Pol θ and *Drosophila* Mus308. The latter two contain a C-terminal polymerase module in addition to the N-terminal helicase domain. Hjm and Mth810 proteins, lacking the polymerase module, belong to the Hel308 subset. Similar sequences have been found in various sequenced archaeal genomes, including *S. solfataricus* (ORF #SSO2462) and *S. tokodaii* (ORF #STO0590). However, all of these DNA helicases do not belong to the RecQ superfamily.

Interestingly, Hel112 homologues are present in all the other archaeal species whose genome has been completely sequenced, suggesting a critical function for these proteins in DNA transactions of both crenarchaeal and euryarchaeal species. A deeper analysis of the physical and/or functional interaction of Hel112 with other DNA replication/repair factors will help to unravel the biological role of this novel DNA helicase with strand-pairing activity.

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