A distinctive single-stranded DNA-binding protein from the Archaeon *Sulfolobus solfataricus*

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Summary

Single-stranded DNA binding proteins (SSBs) have been identified in all three domains of life. Here, we report the identification of a novel crenarchaeal SSB protein that is distinctly different from its euryarchaeal counterparts. Rather than comprising four DNA-binding domains and a zinc-finger motif within a single polypeptide of 645 amino acids, as for *Methanococcus jannaschii*, the *Sulfolobus solfataricus* SSB protein (SsoSSB) has a single DNA-binding domain in a polypeptide of just 148 amino acids with a eubacterial-like acidic C-terminus. SsoSSB protein was purified to homogeneity and found to form tetramers in solution, suggesting a quaternary structure analogous to that of *E. coli* SSB protein, despite possessing DNA-binding domains more similar to those of eukaryotic Replication Protein A (RPA). We demonstrate distributive binding of SsoSSB to ssDNA at high temperature with an apparent site size of approximately five nucleotides (nt) per monomer. Additionally, the protein is functional both *in vitro* and *in vivo*, stimulating RecA protein-mediated DNA strand-exchange and rescuing the *ssb-1* lethal mutation of *E. coli* respectively. We discuss possible evolutionary relationships amongst the various members of the SSB/RPA family.

Introduction

Archaea are a separate group of organisms distinguished from the eubacteria through 16S rDNA sequence analysis. These prokaryotes are further subdivided into three diverse groups named the crenarchaeota, the euryarchaeota and the korarchaeota (Woese *et al.*, 1990; Woese and Fox, 1977; Barns *et al.*, 1996). Only members of the crenarchaeal and euryarchaeal groups, however, have been cultivated. Genomic studies suggest a significant evolutionary division between metabolic and informational processes in archaea. Whereas most intermediary metabolic processes strongly resemble those observed in eubacteria, genomic informational processes are generally thought to be more closely related to those found in eukaryotes (reviewed in Doolittle and Logsdon, 1998). Archaea utilize eukaryotic B-type DNA polymerases for replication, and their ribosomal proteins, as well as translation initiation factors, are remarkably eukaryotic. The recent identification of archaeal snoRNA genes reveals an unexpected eukaryotic connection (Omer *et al.*, 2000). Transcription also involves eukaryotic protein homologues, but the discovery of multiple TBP and TFB proteins in halophiles hints at a unique archaeal transcription mechanism (Baliga *et al.*, 2000). Examination of archaeal recombination proteins suggests a definite similarity with eukaryotes. The archaeal DNA strand exchange protein, RadA, is more similar to its eukaryotic counterpart, Rad51 protein, than to the eubacterial RecA protein, both at the amino acid level (Sandler *et al.*, 1999) and at the biochemical level (Seitz *et al.*, 1998). The associated RadB protein is proposed to serve as a simpler archaeal version of eukaryotic Rad55/57 protein (Rashid *et al.*, 1996; DiRuggiero *et al.*, 1999; Komori *et al.*, 2000), and archaeal Holliday junction resolvase protein characterization suggests they may also be eukaryotic in nature (Komori *et al.*, 1999; Kvaratskhelia and White, 2000).

Single-stranded DNA (ssDNA) binding proteins (SSBs) are essential in most intracellular interactions that involve DNA, including replication, repair and recombination (Kowalczykowski *et al.*, 1994; Lohman and Ferrari, 1994). Homologues of this class of proteins were identified in all three domains of life, as well as in viral genomes (Kowalczykowski *et al.*, 1981; Lohman and Ferrari, 1994; Wold, 1997; Chedin *et al.*, 1998; Kelly *et al.*, 1998; Iftode *et al.*, 1999). Despite the lack of strong homology at the amino acid level, preservation of both structural and domain organization suggests that SSBs are derived from a common evolutionary ancestor (Pfuetzner *et al.*, 1997; Chedin *et al.*, 1998; Raghunathan *et al.*, 2000). While functionally equivalent, eubacterial SSB and the eukaryotic version, RPA, have distinctly different quaternary structures. In eubacteria, SSB is encoded by a single

gene and the active form of the protein is a homotetramer in which each monomer provides one ssDNA-binding domain (Sancar *et al.*, 1981; Lohman and Ferrari, 1994). In eukaryotes, the RPA complex from both humans and yeast is composed of three distinct subunits that together provide a total of four ssDNA-binding domains (Brill and Bastin-Shanower, 1998). Recent descriptions of archaeal SSB homologues from the euryarchaeal branch of the archaeal domain demonstrate their amino acid sequences are more similar to eukaryotic RPA than to eubacterial SSB (Chedin *et al.*, 1998; Kelly *et al.*, 1998). However, these archaeal proteins maintain multiple ssDNA-binding domains within one or just a pair of polypeptides and, therefore, are expected to function as monomers or heterodimers rather than as a homotetramer (as does *E. coli* SSB). It was proposed that these archaeal RPA homologues are evolutionarily related to eukaryotic RPA through gene duplication and recombination events (Chedin *et al.*, 1998).

Genome sequencing of several archaeons simplified molecular analysis in these organisms. Whereas a number of euryarchaeal genome sequences have been determined, to date, the only publicly available crenarchaeal genome sequences are that of *Aeropyrum pernix* (Kawarabayasi *et al.*, 1999) and *Sulfolobus solfataricus* (She *et al.*, 2001). Here we describe the identification of a novel SSB with distinct genetic organization and sequence from *S. solfataricus* as well as a closely related open reading frame from *A. pernix*. To demonstrate that the proteins encoded by the identified open reading frames represent true SSB homologues, we heterologously expressed and purified *S. solfataricus* SSB protein, and we investigated its structure and ssDNA-binding activity, its ability to complement a eubacterial SSB mutation *in vivo*, and its effect on DNA strand exchange.

Results

Crenarchaeal homologues of ssDNA binding proteins

Previous searches of genome sequences identified open reading frames (ORFs) with homology to human RPA70 from the euryarchaeons *Methanococcus jannachii*, *Methanobacterium thermoautrophicum* and *Archaeoglobus fulgidus* (Chedin *et al.*, 1998; Kelly *et al.*, 1998). Each of these sequences consists of multiple ssDNA-binding domains contained in one or a pair of ORFs, which share a significant degree of sequence homology among themselves. In one case, the protein encoded by this homologous sequence was shown to be an RPA homologue, both structurally and functionally (Kelly *et al.*, 1998). However, there has been no corresponding homologue of RPA identified in members of the other branch of the archaeal domain, the crenarchaea. The availability of sequence information from both *Aeropyrum pernix* (Kawarabayasi *et al.* (1999) and *Sulfolobus solfataricus* (She *et al.*, 2001) permitted a search for ssDNA-binding protein sequences in the crenarchaea.

A survey of genome sequences from *A. pernix* and *S. solfataricus* with the program BLAST (Altschul *et al.*, 1990) revealed a single small ORF in each genome with sequence similarity to MJ1159, the RPA homologue from *M. jannaschii*. Both ORFs code for proteins that are strikingly similar to the first ssDNA-binding domain of the *M. jannaschii* RPA (Fig. 1A), but are much shorter than the *M. jannaschii* protein. The *S. solfataricus* sequence (SSO2364) is 477 basepairs in length and encodes
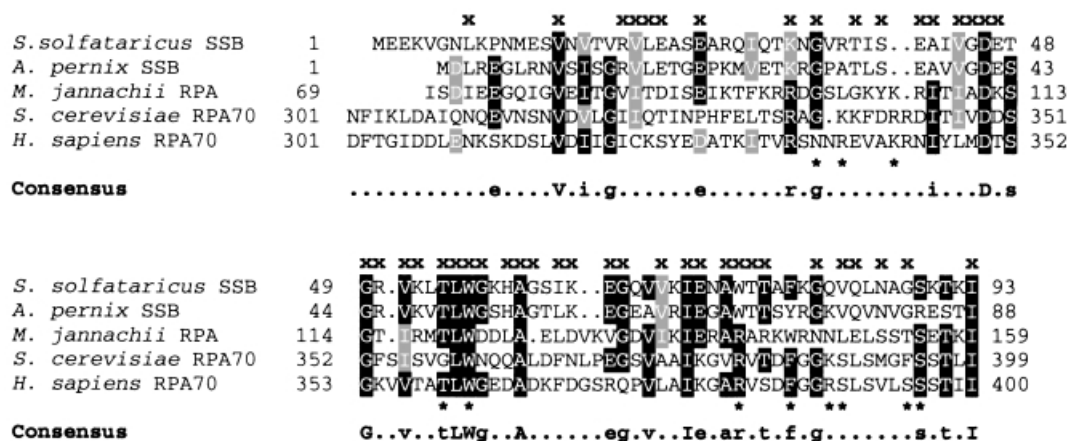

Fig. 1. Alignment of ssDNA-binding domain protein sequences. Crenarchaeal sequences are aligned with the first ssDNA-binding domains of *M. jannachii* RPA, *S. cerevisiae* RPA70 and *H. sapiens* RPA70. Identical residues are shaded black and conserved residues are shaded grey. The asterisk symbol (*) indicates residues identified in the *H. sapiens* RPA70 that interact with DNA (Bochkarev *et al.*, 1997), whereas an 'x' indicates residues that are identical between the two crenarchaeal sequences. The consensus is shown beneath the alignment. Sequence accession nos. are as follows: *S. solfataricus* SSB, SSO2364; *A. pernix* SSB, gi5105001; *M. jannachii* RPA, MJ1159; *S. cerevisiae* RPA70, gi6319321; *H. sapiens* RPA70, gi1350579.

a protein of 148 amino acids whereas the *A. pernix* sequence (gi5105001) is 429 basepairs in length and encodes a protein of 143 amino acids; in contrast, the *M. jannaschii* protein is 645 amino acids in length. The *S. solfataricus* protein has a predicted pI of 9.0 and the *A. pernix* protein also has a predicted pI of 9.0. For comparison, the pIs of the T4 phage SSB homologue gp32, *E. coli* SSB and *M. jannaschii* RPA are 4.8, 5.4 and 4.7 respectively. The *S. solfataricus* protein has 52% similarity and 26% identity with MJ1159 from amino acids 68–170 and the *A. pernix* protein has 52% similarity and 25% identity with MJ1159 from amino acids 70–173. The two residues conserved in all archaeal sequences correspond to amino acids involved in DNA binding in human RPA70 protein domain B (Thr 359 and Trp 361 in the hsRPA70 sequence) (Bochkarev *et al.*, 1997) (Fig. 1A). Additionally, the *S. solfataricus* sequence shares two other amino acids with human RPA70 that are implicated in DNA binding, Phe 386 and Ser 396 (Bochkarev *et al.*, 1997).

The two crenarchaeal protein sequences show a remarkable level of homology between each other, exhibiting 69% similarity and 47% identity (Fig. 1). Both of the newly identified ORFs are significantly shorter than their euryarchaeal and eukaryotic counterparts, each comprising only a single ssDNA-binding region as opposed to closely related tandem repeats (four, in the case of the *M. jannaschii* protein) of multiple binding regions in a larger polypeptide. Examination of nearby regions in each genome revealed no further sequences that might encode for portions of a ssDNA-binding protein, suggesting either that *S. solfataricus* SSO2364 and *A. pernix* gi5105001 are the only genes responsible for producing SSB for these crenarchaeons or that there were other genes elsewhere in the genome that encoded the remainder of the subunits for an RPA-like protein. No other sequence with significant similarity to MJ1159 is apparent elsewhere in the complete genomes of either *A. pernix* or *S. solfataricus*, suggesting that these proteins may serve as the sole SSBs in these organisms.

The crenarchaeal proteins structurally resemble E. coli *SSB protein*

In contrast to other archaeal SSB homologues that resemble eukaryotic RPAs, it appears that the overall architecture of the crenarchaeal versions is distinctly different. Euryarchaeal RPA homologues are comprised of multiple DNA-binding domains, within one or a pair of proteins, and presumably function in monomeric or heterodimeric forms respectively (Fig. 2). The ORFs from both *A. pernix* and *S. solfataricus*, however, are distinguished by a single core ssDNA-binding domain of about 100 amino acids in length, identical to that observed in eubacterial ssDNA-binding proteins. This suggests that the quaternary structure of the crenarchaeal SSB protein might be analogous to the eubacterial version, and could
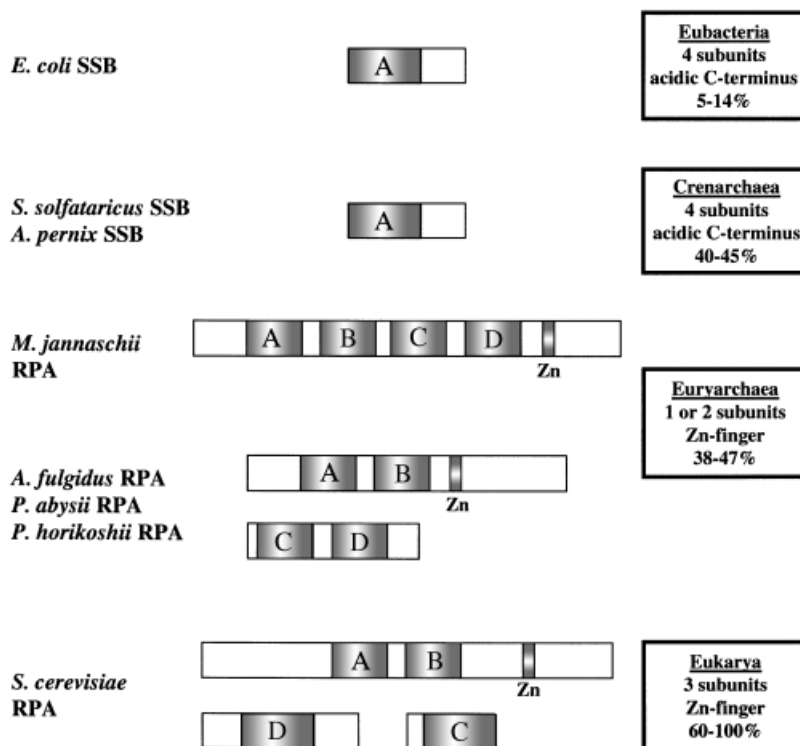

Fig. 2. Schematic representation of the domainal architecture of ssDNA-binding proteins in all three domains of life. Homologous DNA-binding domains are represented by shaded boxes and the location of the zinc-finger motif is indicated. A summary of the attributes of each type of ssDNA-binding protein is represented in the boxes. A range of percentage similarities for proteins from each domain to the first single-stranded DNA-binding domain of *S. cerevisiae* RPA70 was determined using the BestFit program and is indicated.

be tetrameric. Additionally, the crenarchaeal proteins lack the C-terminal zinc finger of the euryarchaeal RPAs and, instead, contain a number of acidic residues in this region. This is analogous to the C-terminal structure of *E. coli* SSB, which is required for protein–protein interactions (Williams *et al.*, 1983; Chase *et al.*, 1985; Kelman *et al.*, 1998). Also absent in the crenarchaeal sequences is an approximately 100-residue N-terminal region found in euryarchaeal RPAs that is believed to be involved in protein binding (Wold, 1997).

The SSB protein homologue of phage T4, gp32, has little sequence homology to bacterial SSBs but displays both structural similarity with these proteins and an acidic C-terminus (Shamoo *et al.*, 1995). Much like gp32, however, the crenarchaeal SSBs share minimal sequence homology with *E. coli* SSB. BestFit alignment of the crenarchaeal sequences with *E. coli* SSB shows 35% similarity and 31% identity over only 48 amino acids for SsoSSB, and 43% similarity and 38% identity over only 60 amino acids for ApeSSB.

Expression and purification of the SSB homologue from S. solfataricus

To determine if SSO2364 from *S. solfataricus* did indeed encode a functional SSB protein homologue, we purified and characterized the candidate protein. The SSO2364 ORF was cloned into the pET21a bacterial expression vector and subsequently heterologously expressed in *E. coli.* The bacterial strain carried a plasmid encoding three rare tRNA genes in an effort to reduce translational pausing, as the predicted protein sequence contains 11 codons that are rare in *E. coli*, four of which are present in the final 50 bases of the ORF. The expressed protein is soluble and was purified to near homogeneity by first heat-treating the cell sonicate at 80°C to denature all mesophilic proteins. This step was followed by affinity chromatography on ssDNA-cellulose, and by anion exchange chromatography on Resource Q. The recombinant protein eluted from ssDNA-cellulose at 0.75 M NaCl, which is identical to the salt concentration necessary for elution of *E. coli* SSB (Lohman *et al.*, 1986). In contrast, elution of either yeast or *M. jannachii* RPA from the same matrix requires 1.5 M NaCl with the addition of 40% ethylene glycol, 1.3 M potassium thiocyanate or 1.5 M sodium thiocyanate (Heyer *et al.*, 1990;Henricksen and Wold, 1994; Kelly *et al.*, 1998), suggesting that the *S. solfataricus* SSB (SsoSSB) protein is chemically more similar to the bacterial protein than it is to RPA. The SsoSSB protein eluted at approximately 60 mM NaCl from Resource Q and pooled fractions yielded essentially pure protein (greater than 98% purity based on Coomassie brilliant blue-stained gels). Examination of the purified protein by SDS/PAGE revealed a single band with a
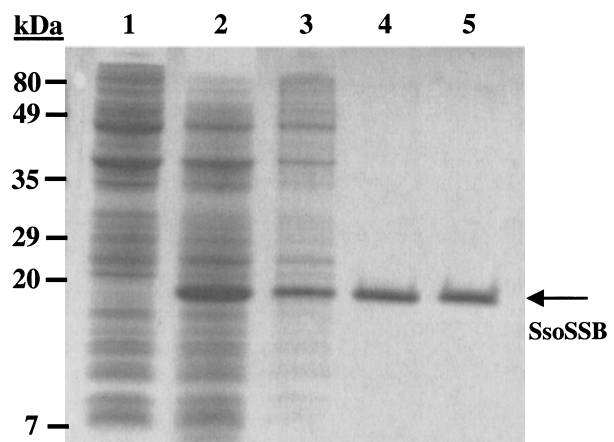

Fig. 3. SDS–PAGE of the purified SsoSSB protein. Samples were subjected to SDS–PAGE and gels were stained with Coomassie brilliant blue R250. The samples loaded were: uninduced crude cell sonicate (lane 1, 100 μg protein); induced crude cell sonicate (lane 2, 100 μg protein); heat-treated clarified sonicate (lane 3, 80 μg protein); pooled ssDNA-cellulose fractions (lane 4, 15 μg protein); and concentrated Resource Q fractions (lane 5, 15 μg protein). The arrow indicates the position of the SsoSSB protein.

molecular weight of approximately 16 kDa, in close agreement with the predicted molecular weight of 16 138 Da as determined from the amino acid sequence (Fig. 3).

SsoSSB protein forms tetramers in solution

As *E. coli* SSB protein assembles into a tetramer, it was consequently of interest to determine if the purified *S. solfataricus* protein would multimerize in solution. Analytical gel filtration was used to determine the native form of the recombinant protein. Examination of the SsoSSB protein in solution revealed that it was present in three distinct species: monomeric, dimeric and tetrameric forms, at 18 kDa, 36 kDa and 62 kDa respectively (Fig. 4A). As demonstrated by a representative elution profile (Fig. 4B), the composition of the purified material was primarily tetrameric, whereas somewhat less dimeric protein was present. A significantly smaller quantity of the monomeric form of SsoSSB protein was observed. This result indicates that the SsoSSB protein can indeed form tetramers in solution. As might have been expected, the *M. jannachii* RPA protein does not multimerize in solution (Kelly *et al.*, 1998; unpublished observation).

SsoSSB protein binds ssDNA

To determine the occluded binding site size of the protein and to evaluate the activity of the SsoSSB protein, we performed acrylamide gel mobility-shift assays with a radioactively labelled single-stranded oligonucleotide that was 63 nucleotides in length (Fig. 5). When a fixed quantity of radiolabelled oligonucleotide was incubated with
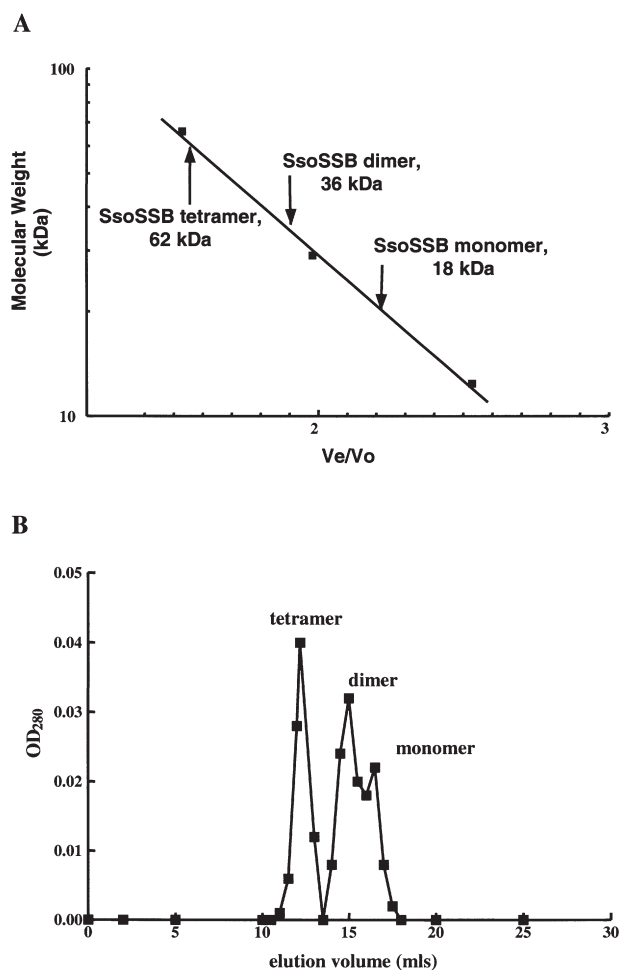
Fig. 4. Gel filtration chromatography of SsoSSB protein.
A. Elution of purified SsoSSB protein relative to molecular weight standards: BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa), which are represented by closed squares (■). Ve/Vo, elution volume divided by void volume.
B. A representative elution profile for purified SsoSSB protein. Closed squares (■) represent optical density at 280 nm (OD_{280}) for the elution volume indicated.

increasing quantities of protein, a band of reduced mobility was observed. The apparent mobility further decreased upon addition of more protein, finally achieving a constant mobility after the addition of 2 μM protein. The absence of discrete species implied rapid equilibration between bound and free forms of the complexes and, consistent with this possibility, more discrete species were visualized when electrophoresis was performed more rapidly (at high voltage; data not shown). The addition of linear pUC19 DNA to a 100-fold molar (nucleotide) did not alter the mobility-shift pattern, demonstrating that the SsoSSB has an, at least, 100-fold greater affinity for ssDNA than for dsDNA (data not shown). Saturation was achieved at a ratio of approximately one SSB protein molecule to five nucleotides. The *M. jannaschii* RPA protein has a site size

of 15–20 nucleotides (Kelly *et al.*, 1998) whereas the site size of *E. coli* SSB protein, depending on solution conditions, varies from 8–16 nucleotides per monomer. The apparent site size of SsoSSB protein is similar to the seven-nucleotide site size observed for phage T4 SSB protein, gp32. Binding of SsoSSB protein to ssDNA does not appear to be a co-operative process under these conditions, as intermediately shifted species are evident instead of the approximately two-state transitions that typify co-operative binding. Rather, a steady reduction in the mobility of protein-DNA complexes occurs as protein concentration is increased, suggesting that binding of SsoSSB protein to ssDNA is distributive in nature. The observed band retardation pattern is probably the result of a combination of this non-co-operative binding and rapid equilibration of protein–DNA complexes during electrophoresis.

SsoSSB protein is a functional SSB homologue

In *E. coli*, SSB is an essential protein. A number of mutations were used to elucidate the function of *E. coli* SSB protein; one mutation is the temperature-sensitive mutation called *ssb-1*. The *ssb-1* mutation is an alteration of amino acid 55 from a histidine to a tyrosine, which is believed to destabilize the tetrameric SSB protein complex upon shifting temperature from 30°C to the non-permissive temperature of 43°C, resulting in a lethal phenotype (Chase *et al.*, 1983). Overexpression of wild-type *E. coli* SSB protein encoded on a plasmid can overcome the lethality of the mutation (Chase *et al.*, 1983) as can the SSB protein of bacteriophage P1 (Lehnherr *et al.*, 1999). To test the *in vivo* functionality of SsoSSB, the protein was overexpressed in the *E. coli* strain KLC789 that carries the *ssb-1* allele. The *SsoSSB* plasmid was transformed into KLC789 along with a plasmid carrying an arabinose-inducible T7 polymerase gene. Cells were grown in the presence of arabinose for 16 h at the
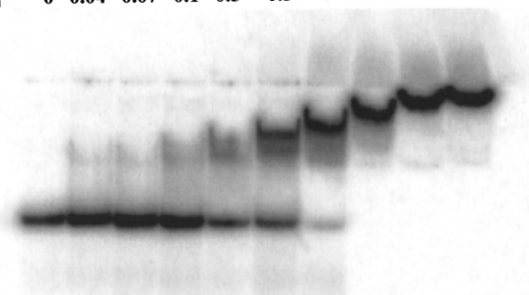

Fig. 5. Gel mobility-shift analysis of SsoSSB protein binding to ssDNA. Increasing concentrations of protein (0.04 to 20 μM) were added to a constant concentration of 63-mer oligonucleotide (10 μM nucleotides).

permissive temperature to allow overexpression of the SsoSSB protein, whereas control cultures were cultivated in the presence of glucose. Cells were then shifted to the non-permissive temperature and monitored for further growth (Fig. 6A). The presence of the ORF encoding SsoSSB protein in pET21a permitted continued growth of the *ssb-1* mutant strain whereas the pET21a vector alone was unable to rescue the lethal phenotype. Rescued growth required the presence of arabinose, as control experiments with glucose showed no increase in optical

A

B

Fig. 6. Overexpression of SsoSSB protein rescues the lethal phenotype of an *E. coli ssb-1* mutation. KLC789 cells containing the pTara arabinose-inducible T7 expression plasmid, and either pET21a (circles) or the *SsoSSB* expression vector (squares) were grown at 30°C in either arabinose (closed symbols) or glucose (open symbols). Cultures were shifted to 43°C at the point indicated by the arrow.
A. Optical densities were monitored spectrophotometrically at 600 nm.
B. Colony forming units (cfu) were determined by plating in triplicate and the points shown are averages of the replicates.

density that would be consistent with continued growth. To verify that the cells containing the *SsoSSB* plasmid were indeed still growing, viable counts for each timepoint were determined by plating (Fig. 6B). Following the temperature shift, the control cultures continued to grow for a brief period and then dramatically lost viability. In contrast, the cells containing the *SsoSSB* plasmid displayed a continuous increase in viable counts. These results indicate that SsoSSB protein is capable of replacing *E. coli* SSB protein *in vivo* and that it functions at 43°C.
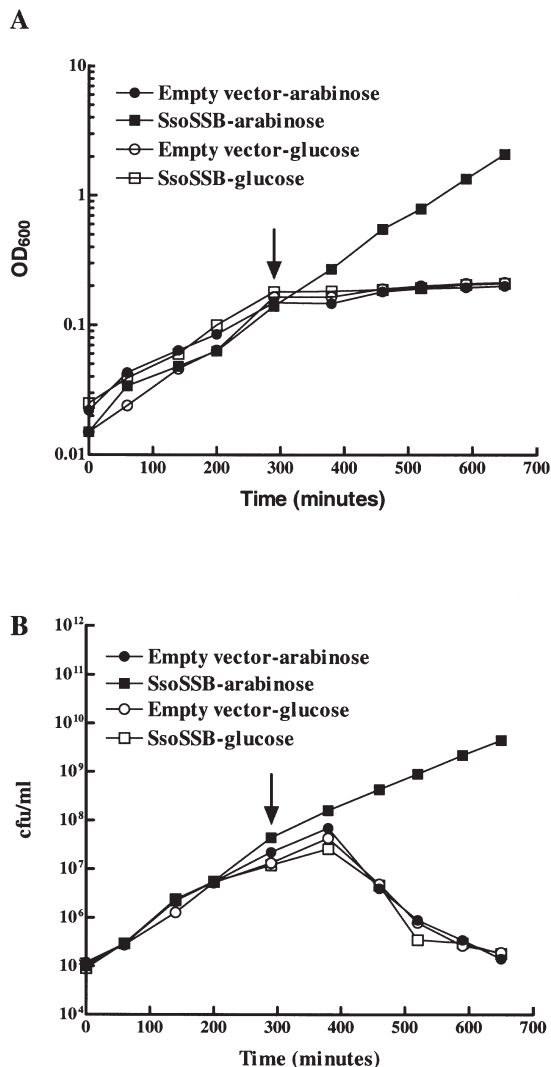
SsoSSB protein stimulated DNA strand exchange by E. coli *RecA protein*

It was shown previously that heterologously expressed SSB proteins can stimulate DNA strand exchange mediated by *E. coli* RecA protein (Egner *et al.*, 1987). To demonstrate the functionality of the SsoSSB protein in at least one nucleic acid metabolic function *in vitro*, DNA strand exchange reactions were performed (Fig. 7). Purified SsoSSB protein was capable of substituting for *E. coli* SSB protein in DNA strand exchange reactions mediated by *E. coli* RecA protein at 37°C using homologous φX174 ss- and dsDNA. Clear enhancement of DNA strand exchange, as determined by production of nicked circular product, was observed with SsoSSB protein (Fig. 7, lane 3). Interestingly, the *M. jannaschii* RPA protein can also promote RecA-mediated DNA-strand exchange (E.M. Seitz, C.A. Haseltine and S.C. Kowalczykowski, unpublished observations). The reduced amount of nicked circular product in reactions containing SsoSSB protein relative to levels with *E. coli* SSB protein may be the result of performing the experiment at temperatures at which SsoSSB protein is not as effective. The optimal temperature for the SsoSSB protein is expected to be near the growth temperature of *S. solfataricus*, which is 80°C. As yet, no enhancement of strand exchange activity through addition of SsoSSB protein to *S. solfataricus* RadA protein-containing reactions at high temperature has been observed (unpublished observations), though this may be a consequence of inappropriate assay conditions or may signify a postsynaptic role for SsoSSB protein at physiological temperatures.

Discussion

Divergence between the archaeal and eukaryotic lineages is more recent than the divergence of the bacterial and eukaryotic/archaeal groups (Olsen and Woese, 1997). Accordingly, a number of features shared by eukaryotes and archaea, but not bacteria, including replication and transcription proteins were probably obtained after evolutionary divergence of these three groups. In
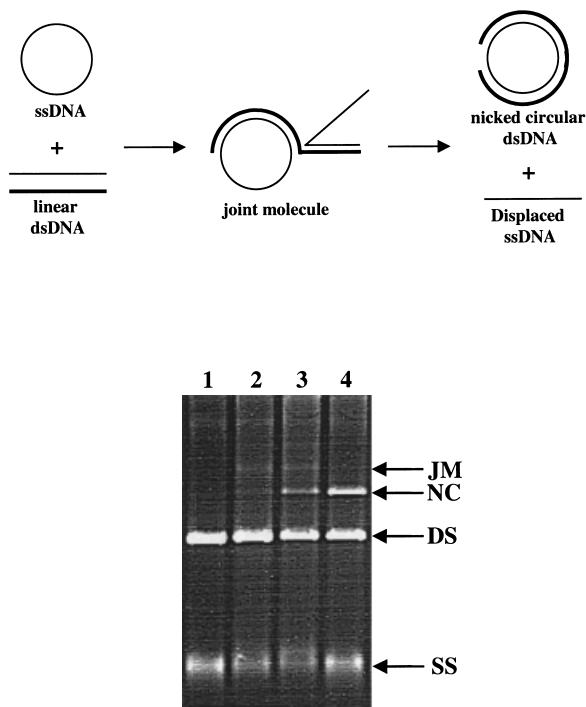
Fig. 7. SsoSSB protein stimulates DNA strand exchange by *E. coli* RecA protein. Lane 1, no protein; lane 2, RecA protein; lane 3, RecA protein and SsoSSB protein; lane 4, RecA protein and *E. coli* SSB protein. The abbreviations are: JM, joint molecules; NC, nicked circular dsDNA; DS, dsDNA; and SS, ssDNA. A schematic representation of the formation of nicked circular dsDNA and joint molecules from starting substrates is shown above the gel.

contrast, features found in archaea and bacteria, but not in eukaryotes, including morphological attributes (a lack of organelles and nucleus), coupling of transcription and translation, conjugative mechanisms and a single circular genome may be reminiscent of a more ancient, shared ancestor.

However, despite the logic of this viewpoint, the evolutionary behaviour of the archaea is not so simplistic. Our results indicate that, in contrast to the canonical expectation that all archaeal ssDNA binding proteins would be RPA-like, the crenarchaeal SSBs share sequence homology with eukaryal RPAs but structural homology with bacterial SSBs. Both *E. coli* SSB and SsoSSB proteins elute from ssDNA-cellulose at the same salt concentration, and both form tetramers in solution. The apparent binding site size is approximately five nucleotides, which is consistent with the site size observed for T4 g32 protein and the low site size binding mode of *E. coli* SSB protein, but is one quarter that of *M. jannachii* RPA protein, which has four DNA-binding domains. Another feature that makes the crenarchaeal SSB protein more eubacterial is that SsoSSB protein and ApeSSB protein are missing the zinc finger motif near the C-terminus, which is present in both euryarchaeal and eukaryal RPAs, suggesting that

this motif was acquired after the separation of the two archaeal branches. Furthermore, the acidic residues in the SsoSSB protein C-terminal region are similar to those found in bacterial proteins and may have been maintained from the last shared bacterial and archaeal ancestor. SsoSSB protein resembles *E. coli* SSB chemically in that they both elute from ssDNA-cellulose at 0.75 M NaCl whereas the euryarchaeal and eukaryal RPAs require higher salt concentrations and the addition of ethylene glycol, potassium thiocyanate or sodium thiocyanate for elution from this matrix. Finally, SsoSSB protein can functionally substitute for *E. coli* SSB protein both *in vivo* and *in vitro*. *In vivo*, overexpression of the SsoSSB protein can overcome the lethal *ssb-1* temperature-sensitive mutation in *E. coli*; *in vitro*, the SsoSSB protein can replace the *E. coli* SSB protein in DNA strand exchange reactions mediated by RecA protein.

Concurrent with our efforts, the same crenarchaeal protein was identified by another laboratory using biochemical criteria (Wadsworth and White, 2001). The DNA-binding characteristics of SsoSSB protein were found to be similar to those we describe here. No apparent multimerization of the protein was demonstrated, in contrast to our observation. In our experience, however, heating of the SsoSSB protein was necessary for formation of the tetrameric structure as observed by gel filtration and for activity in DNA strand-exchange. The absence of this preheating step by Wadsworth and White could account for this seemingly minor discrepancy.

Overall, the archaeal ssDNA binding proteins show more sequence similarity to eukaryotic RPA proteins than to *E. coli* SSB protein. However, the common sequences and DNA-binding domain utilization hint at a conserved evolutionary relationship between RPA and SSB (Fig. 2). Strong homology among the archaeal proteins suggests a common evolutionary origin, and the crenarchaeal SSB proteins may represent a link to ancient ssDNA-binding proteins. The crenarchaeal SSB protein, being one of the simplest in structure, may represent the earliest form of single-stranded DNA-binding proteins involved in recombination, and it may serve as a model for understanding structure, function and evolution of the conserved core ssDNA-binding domain.

Crenarchaea and euryarchaea are distinguished from each other not only by single-stranded DNA-binding proteins as described here, but also by double-stranded DNA-binding proteins. Whereas eukaryotic-like histone proteins have been identified and extensively studied in members of the euryarchaea (Sandman and Reeve, 2000), no such homologues are apparent in members of the crenarchaea. Instead, crenarchaea maintain sequences that code for small, basic dsDNA-binding proteins (Agback *et al.*, 1998; Faguy and Doolittle, 1999). These proteins are proposed to be similar to histone-like

proteins in eubacteria, especially Sso7d, which was shown to be analogous to the dsDNA-binding protein HU (Lopez-Garcia *et al.*, 1998). It appears that the crenarchaea have maintained both single-stranded and double-stranded DNA-binding proteins that are more similar to those found in bacteria than they are to eukaryotic homologues. Crenarchaeal dsDNA-binding proteins and SSB may have evolved separately from mechanisms employed by eukaryotes or, alternatively, crenarchaea diverged earlier, before the co-evolution of eukaryotic-like histones and RPA. The use of a eubacterial-like SSB may necessitate a eubacterial DNA-binding protein for the compaction of DNA. The discovery of an SSB homologue in the crenarchaea that is significantly more similar to eubacterial SSB, both structurally and biochemically, than it is to either euryarchaeal or eukaryotic RPA further establishes the evolutionary distance between the two archaeal phyla.

Experimental procedures

Alignment of protein sequences

The *Sulfolobus solfataricus ssb* sequence (SSO2364) encoding the SSB protein (SsoSSB) was identified using BLASTP at the *S. solfataricus* genome website: http://www-archbac. upsud.fr/projects/sulfolobus. The *Aeropyrum pernix ssb* sequence (gi5105001) encoding the SSB protein (ApeSSB) was identified using BLAST at the PEDANT website: http://pedant.gsf.de/. Both ORFs were recognized through their homology to MJ1159, encoding the *Methanococcus jannaschii* RPA protein (Chedin *et al.*, 1998). Subsequent alignments were performed using the ALIGN program at http://www.toulouse.inra.fr/multalin.html and additional features were highlighted by manual adjustment. BestFit comparisons were performed using the Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, Wisconsin and were between *S. cerevisiae* RPA70 single-stranded DNA-binding region 1 (gi6319321, amino acids 301–399) and the following single-stranded DNA-binding protein sequences; *Archaeoglobus fulgidus* (gi11497994), *A. pernix* (gi5105001), *Bacillus subtilis* (gi2127217), *Escherichia coli* (gi134913), *Homo sapiens* (gi1350579), *M. jannaschii* (MJ1159), *Methanobacterium thermoautotrophicum* (gi2622495), *Pyrococcus abyssii* (gi5457718), *Pyrococcus horikoshii* (gi3258332), *S. solfataricus* (SSO2364), as well as between *E. coli* SSB protein (gi134913) and *S. solfataricus* (SSO2364). The *A. fulgidus* sequence used was one of two identified as homologous to MJ1159 and is the most homologous to the N-terminus of the *M. jannaschii* protein (Chedin *et al.*, 1998). The *M. thermoautotrophicum* sequence was adjusted to account for the frameshift identified by Chedin and colleagues (Chedin *et al.*, 1998).

Strains and cultivation

Sulfolobus solfataricus strain P2 (DSM 1616 (Zillig *et al.*, 1980)) was the generous gift of Dennis Grogan (University of Cincinnati) and was grown at 80°C as described (Rolfsmeier *et al.*, 1998) at a pH of 3.0 in screw-cap flasks as described

(Rolfsmeier and Blum, 1995). Basal salts medium was Allen's medium (Allen, 1959), as modified by Brock (Brock *et al.*, 1972), and was supplemented with tryptone to a final concentration of 0.2% (w/v). Growth was monitored spectrophotometrically at a wavelength of 540 nm. *Escherichia coli* strains were DH5α (φ80 d*lacZ* ΔM15, *endA*1, *recA*1, *hsdR*17 (*r*$_k^-$,*m*$_k^+$)*, supE44, thi-1, gyrA96, relA1,* Δ(*lacZYA-argF)U169*); BL21(DE3) *(ompT [lon] hsdS$_B$* (r$_B^-$m$_B^-$; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene); and KLC789 (F⁻, *metA7, rha8, thyA36, amp50, deoC2, ssb-1*) (Chase *et al.*, 1983) from laboratory collections or BL21(DE3) CodonPlus ultracompetent cells from Stratagene. *Escherichia coli* was propagated in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) at 30°C in Ehrlenmeyer flasks shaken at 250 r.p.m.

PCR amplification and cloning of the SsoSSB gene

Genomic DNA was prepared from *S. solfataricus* cells as described previously (Rolfsmeier *et al.*, 1998). Polymerase chain reaction (PCR) was performed using 10 mM potassium chloride, 10 mM ammonium sulphate, 2 mM magnesium chloride, 20 mM Tris-Cl (pH 8.75), 0.1% Triton X-100, 100 μM dNTPs, 100 pmol primers, 2 ng template DNA and 2.5 U of recombinant *Pfu* DNA polymerase (Stratagene). The primers for amplification of *SsoSSB* were: 5′-CGGGATCCCCTTTCA TTAACACATAGATTTATAAATGG-3′ (SSB-F) and 5′-CGGGA TCCGGAGCAAGCTCGTATACTTTGTCTCTAGCC-3′ (SSB-R). All primer sequences were chosen based on sequence information presented at the *S. solfataricus* genome website: http://www-archbac.u-psud.fr/projects/sulfolobus. PCR was performed using a 55°C annealing temperature and the resulting PCR products were digested with *Bam*HI and ligated into the *Bam*HI site of pUC19. Ligated molecules were transformed into DH5α as described previously (Rolfsmeier *et al.*, 1998). Plasmids from transformants were isolated using the Qiagen midiprep system and DNA sequences were determined using BigDye dRhodamine Terminator chemistry (Perkin-Elmer) at the Division of Biological Sciences Automated DNA sequencing facility at UC Davis.

Overexpression of SsoSSB protein

Sequence information obtained from the pUC19 clones was used to design a forward PCR primer with an *Nde*I site at the starting ATG codon for *SsoSSB*. The gene sequence was re-amplified from the cloned template using the new forward primer (5′-GTGAGTCGAGTCATATGGAAG-3′) and the original reverse primer. The resulting product was digested using *Nde*I and *Bam*HI before ligation into pET21a (Novagen), which had been digested with the same enzymes to place the gene under the control of the T7 promoter. Ligation products were transformed into the CodonPlus strain (Stratagene) and transformants were cultivated at 30°C in LB containing 100 μg ml⁻¹ of ampicillin until mid-log phase.

Purification of SsoSSB protein

BL21(DE3) CodonPlus cells (Stratagene) harbouring the pET21a *SsoSSB* expression construct were grown at 30°C

in a 500 ml volume to an OD$_{600}$ of 1.0. IPTG was added to a final concentration of 1 mM and expression was allowed to continue for 2 h. Cells were harvested by centrifugation and stored at –20°C until processing. The frozen cell pellet was resuspended in 4 ml of 10 mM Tris-Cl (pH 7.5), 1 mM EDTA (TE) with 50 mM NaCl and sonicated to disrupt the cells. The sonicate was heat treated at 80°C for 1 h, and insoluble material was removed by centrifugation. Clarified sonicate was applied to a ssDNA cellulose column equilibrated in 30 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM DTT and 10% glycerol; the column was washed with the same buffer containing 0.5 M NaCl and 0.75 M NaCl at room temperature. Fractions eluting at 0.75 M NaCl were pooled and dialysed into buffer containing 20 mM Tris-Cl (pH 7.5), 1 mM DTT, 1 mM EDTA and 10% glycerol. This material was then applied to a Resource Q column (Pharmacia) equilibrated with the same buffer at room temperature. Protein was eluted using a gradient of 50 mM NaCl to 1 M NaCl in the same buffer; the SsoSSB protein eluted from the column at approximately 60 mM NaCl. The protein was pooled and concentrated using dry polyethylene glycol and then dialysed against 25 mM Tris-HCl (pH 7.5), 20 mM NaCl, 1 mM EDTA, 1 mM DTT and 10% spectral grade glycerol, and stored at 4°C. Protein concentrations were obtained by spectrophotometric absorbance at a wavelength of 280 nm, using an extinction coefficient of 12 660 M^{-1} cm^{-1} as determined with the ProtParam tool at the ExPASy website (http://expasy.cbr.nrc.ca/tools/protparam.html).

Gel filtration of SsoSSB protein

Fast protein liquid chromatography (FPLC) was performed at 4°C using a Superose 12 column (Pharmacia) and 25 mM Tris-HCl pH 7.5, 1 mM DTT, 100 mM NaCl and 1 mM EDTA as the running buffer. Molecular size standards were BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa) and were prepared in running buffer. A total of 10 µg of SsoSSB protein was loaded on the column in a volume of 100 µl. Elution profiles were determined by monitoring OD$_{280}$ readings and a standard curve was prepared by plotting elution volume (Ve)/void volume(Vo) against the molecular mass of the size standards. The value of Vo was determined by elution of Dextran blue from the Superose 12 column.

Gel mobility-shift analysis

The 63-mer oligonucleotide 5′-ACAGCACCAATG AAATCTA TTAAGCTCCTCATCGTCCGCAAAAATATCGTCACCTCAA AAGGA-3′ was end-labelled with ^{32}P using T4 polynucleotide kinase (NEB). SsoSSB protein was incubated at the indicated concentrations with 10 µM (nucleotides) of the ^{32}P-labelled oligonucleotide for 30 min at 75°C in buffer containing 30 mM Tris-OAc (pH 7.5), 10 mM MgOAc$_2$, 5 mM NaCl, 0.1 mM DTT and 50 µg ml^{-1} of BSA. Increasing concentrations of linearized pUC19 were used as the dsDNA competitor as indicated in the text. Loading dye was then added, and the samples were applied to a vertical 10% acrylamide gel prepared with 1× TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA).

In vivo *complementation*

The *SsoSSB* expression vector or pET21a (empty vector) was transformed into *E. coli* strain KLC789 (Chase *et al.*, 1983) containing pTara, a T7 polymerase expression vector that is inducible by arabinose addition. The pTara plasmid was the generous gift of Kathleen Mathews (Rice University) (Wycuff and Matthews, 2000). Transformants were propagated in LB medium lacking yeast extract with 0.2% (w/v) arabinose, 100 µg ml^{-1} of ampicillin and 30 µg ml^{-1} of chloramphenicol for 16 h at 30°C to allow phenotypic over-expression of SsoSSB protein. Control cultures were propagated identically, except 0.2% (w/v) glucose was substituted for arabinose. Cells were subcultured into fresh medium without chloramphenicol and grown at 30°C until they were shifted to the non-permissive temperature of 43°C. Optical densities were monitored spectrophotometrically at a wavelength of 600 nm. Colony forming units (cfu) per millilitre were determined by plating serial dilutions of each time-point in triplicate on LB medium. Plates were incubated overnight at 30°C before scoring for viable counts.

DNA strand exchange reactions

Escherichia coli RecA protein (11 µM) was incubated with φX174 ssDNA (New England Biolabs) at a concentration of 33 µM (nucleotides) in 30 mM Tris-OAc (pH 7.5), 10 mM DTT, 20 mM MgOAc, 2.5 mM ATP, and 5 µg ml^{-1} of BSA at 37°C for 10 min. After the addition of either 2.2 µM SsoSSB protein or *E. coli* SSB protein, reaction mixtures were incubated at 37°C for another 5 min before the introduction of *Pst*I-linearized φX174 dsDNA (New England Biolabs) at a concentration of 33 µM (nucleotides). The reaction mixtures were then incubated at 37°C for 90 min and were stopped by the addition of SDS to a final concentration of 0.6% and proteinase K to a final concentration of 1 µg ml^{-1}. Deproteinization of the reaction mixtures was carried out at 37°C for 10 min.

Gel electrophoresis

Agarose gels for evaluation of cloning procedures were prepared at 0.8% and run at approximately 150 V in TBE buffer before staining with ethidium bromide. Tricine SDS/PAGE, used to monitor protein purification, was prepared with a 4% stacking gel and a 20% separating gel as described (Price and Shand, 2000), and run at 100 V. Acrylamide gels for gel mobility-shift analysis were prepared at 10% in TBE buffer and run at 100 V for 3 h at 65°C before exposure to phosphorimaging screens and analysis with a Storm 840 PhosphorImager (Molecular Dynamics). Agarose gels for evaluation of DNA strand exchange products were prepared at 1% and run at approximately 30 V in TAE buffer (0.04 M Tris-OAc, 0.002 M EDTA) for 15 h before staining with ethidium bromide.

Acknowledgements

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