Identification and properties of the crenarchaeal single-stranded DNA binding protein from Sulfolobus solfataricus

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ABSTRACT

Single-stranded DNA binding proteins (SSBs) play central roles in cellular and viral processes involving the generation of single-stranded DNA. These include DNA replication, homologous recombination and DNA repair pathways. SSBs bind DNA using four ‘OB-fold’ (oligonucleotide/oligosaccharide binding fold) domains that can be organised in a variety of overall quaternary structures. Thus eubacterial SSBs are homotetrameric whilst the eucaryal RPA protein is a heterotrimer and euryarchaeal proteins vary significantly in their subunit compositions. We demonstrate that the crenarchaeal SSB protein is an abundant protein with a unique structural organisation, existing as a monomer in solution and multimerising on DNA binding. The protein binds single-stranded DNA distributively with a binding site size of ~5 nt per monomer. Sulfolobus SSB lacks the zinc finger motif found in the eucaryal and euryarchaeal proteins, possessing instead a flexible C-terminal tail, sensitive to trypsin digestion, that is not required for DNA binding. In comparison with Escherichia coli SSB, the tail may play a role in protein–protein interactions during DNA replication and repair.

INTRODUCTION

Single-stranded DNA binding proteins (SSB in eubacteria; RPA in eucarya and euryarchaea) are required by all cellular life forms, as well as many viruses, in order to bind, sequester and protect DNA that has had its natural double-stranded structure disrupted. Such disruptions occur during vital information processing pathways such as DNA replication, recombination and repair, as a result of DNA damage sustained from exposure to cellular or environmental factors, or simply due to DNA ‘breathing’ (1). Although there is little conservation at sequence level, the crystal structures of human RPA (2,3), Escherichia coli SSB and human mitochondrial SSB (4–6) indicate the presence of a core ‘OB-fold’ (oligonucleotide/oligosaccharide binding fold) (7). This domain comprises approximately 100 amino acids and is responsible for the major interactions with single-stranded DNA (ssDNA). The universality of OB-fold domains in SSB proteins suggests that an ancestral SSB existed in the Last Universal Cellular Ancestor (LUCA) (8) and therefore that all extant SSB and RPA proteins are derived from a common ancestral SSB. In the eubacteria, SSBs exist as homotetramers, whilst eucaryal RPA is a heterotrimer and euryarchaeal SSBs exist as monomers, heterodimers and heterotrimers (9).

Both E.coli SSB and the yeast and human RPA proteins have been characterised extensively, both biochemically and at a structural level. Comparisons of amino acid sequence and protein domain organisation suggest strongly that the eucaryal and euryarchaeal RPA proteins are more closely related to one another than either is to the eubacterial SSBs (9,10). By way of an illustration, the large subunits of both eucaryal and euryarchaeal RPA contain a putative zinc finger domain that is absent in the eubacterial and mitochondrial SSBs. This tallies with an extensive body of evidence that suggests that the archaea and eucarya share information processing pathways, including DNA replication and transcription, in common (reviewed in 11). Protein domains outwith the core OB-fold regions are thought to function as recognition surfaces for protein–protein interactions, for example RPA70 has an extensive N-terminal sequence that has also been postulated to interact with other repair proteins (Discussion).

We are investigating DNA recombination and repair pathways in the crenarchaeote Sulfolobus solfataricus. The crenarchaeal lineage is highly diverged from that of the euryarchaea, and has some distinct features, notably the lack of true histone proteins for double-stranded DNA (dsDNA) binding and compaction (12). To date, only one crenarchaeal genome sequence, that of Aeropyrum pernix, has been published (13), though several others are nearing completion. As there is no annotated open reading frame (ORF) encoding an SSB protein in the A.pernix genome, we undertook the identification of the Sulfolobus SSB using an assay for ssDNA binding activity. We report here the purification, identification and characterisation of Sulfolobus SSB, and its relationship with other members of this important class of proteins.

MATERIALS AND METHODS

Oligonucleotides

The following oligonucleotides were used for binding and cross-linking studies, and were radioactively 5',32P-labelled

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using polynucleotide kinase where appropriate: 18mer, 5'-CGT-GGATCCCATG GCC- 69mer, 5'-GGCGGAAAATGAG-AAATTCGACCTATCCTTGCGCAGCTCGAGAAGCTC-G GATCCCCATGGC; 69mer, 5′

Protein expression was induced by addition of 0.2 mM IPTG at 37°C for 30 min in a water bath, and denatured proteins were precipitated by centrifugation at 40 000 g at 4°C. The supernatant was analysed by SDS–PAGE, and shown to contain recombinant SSB, which migrated as a band of ~16 kDa as expected. The supernatant was diluted 4-fold with buffer A (50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM DTT) and applied to an SP-Septahose High Performance 26/10 column equilibrated with buffer A. A 500 ml linear gradient comprising 0–1000 mM NaCl was used to elute cationic proteins. Fractions corresponding to a distinct absorbance peak were analysed by SDS–PAGE, pooled, concentrated and loaded onto a 26/70 gel filtration column and developed with buffer A containing 300 mM NaCl. This step removes endogenous E.coli SSB, which is tetrameric in solution (Mr ~75 kDa). Active fractions were pooled and shown to contain essentially homogeneous SSB protein (Fig. 3). This protein was used for all subsequent analyses.

Digestion of recombinant SSB with proteolytic enzymes

Recombinant SSB was incubated in digest buffer (50 mM Tris–HCl pH 8.0, 2 mM CaCl₂) at 37°C for 1 h in the presence of a 1:250 (w/w) ratio of porcine trypsin (Promega), or a 1:250 (w/w) ratio of Glu-C (Promega). Following digestion, products were analysed by SDS–PAGE and MALDI-TOF. Trypsin was inhibited irreversibly by the addition of 1 mM AEBSF.

Antibody production and immunodetection

Purified recombinant SSB was used as an antigen to raise polyclonal antibodies in sheep (antibody production by Diagnostics Scotland, Law Hospital, Carluke, Scotland). The specificity of the resulting antibodies was tested by western- and dot-blot analyses, using an anti-goat HRP-conjugated secondary antibody and chemiluminescent detection with ECL-Plus reagents (Amersham Pharmacia). The immune serum at a dilution of 1:1000 allowed detection of 2 ng SSB in a dot-blot, and 10 ng SSB in a western blot. SSB concentrations in Sulfolobus cell extracts were estimated by western blotting, comparing the signal obtained from a set of known concentrations of the pure recombinant protein with that from a known volume of cell extract. Total protein in the extracts was estimated by Bradford analysis.

Agarose gel electrophoretic retardation analysis

A range of concentrations of purified SSB protein was incubated with a known concentration of single-stranded phage phiX174 DNA in binding buffer (20 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA and 0.1 mg/ml bovine serum albumin), in 10 µl total volume. After 15 min at 20°C, one-sixth vol loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 35% Ficoll type 400) was added, and samples were loaded onto 0.7% agarose gels and electrophoresed in 1× TBE buffer for 90 min. After electrophoresis, gels were stained in ethidium bromide and visualised under UV light.

Acrylamide gel electrophoretic retardation analysis

During protein purification, fractions eluting from chromatography columns were tested for the ability to retard a ssDNA oligonucleotide. An aliquot (1 µl) of each fraction was incubated with a known concentration of a radioactively 5'-32P-labelled oligonucleotide in binding buffer (20 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA and 0.1 mg/ml bovine serum albumin) in 10 µl total volume. After 15 min at 20°C, one-sixth vol loading
buffer was added, and samples were loaded onto 8% polyacrylamide gels (19:1 acrylamide to bisacrylamide) and electrophoresed in 1× TBE buffer. After electrophoresis, gels were dried and exposed to X-ray film for documentation or phosphor screens for quantitation as described previously (14).

**Fluorescence titrations**

The fluorimetric titration experiments were performed on a Perkin-Elmer LS50B Luminescence Spectrometer. The tryptophan emission spectra of *Sulfolobus* SSB were obtained by excitation at 290 nm (excitation and emission slit widths 5 nm) in reaction buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl) at 20°C. Fluorescence quenching at the emission peak of 345 nm was monitored whilst titrating ssDNA species into a fixed concentration of SSB protein. Relative fluorescence values were corrected for dilution effects and subtraction of fluorescence due to DNA components.

**Glutaraldehyde cross-linking**

Chemical cross-linking of SSB protein with glutaraldehyde was carried out according to the method of Jaenicke and Rudolph (15). SSB protein (50 µg) was incubated in the presence or absence of 1 µM 18mer oligonucleotide in a 50 µl final volume in X-link buffer (50 mM NaPO₄ pH 7.6, 50 mM NaCl) for 10 min at 20°C. After addition of 20 µl glutaraldehyde (25%, w/v) and incubation for 2 min at 20°C, the reaction was quenched by the addition of 25 µl NaBH₄ (2 M in 0.1 M NaOH, freshly prepared), and incubation at 20°C for a further 20 min. Protein was precipitated by the addition of 1.5 µl 10% sodium deoxycholate followed by 22.5 µl 78% (w/v) TCA and incubation on ice for 5 min, followed by centrifugation. The supernatant was discarded, and the pellet resuspended in 600 µl cold acetone, followed by a final centrifugation step (13,000 g at 4°C for 10 min). The pellet was drained and allowed to air dry. Cross-linked proteins were solubilised by the addition of 20 µl 1× SDS–PAGE sample loading buffer and heating to 80°C for 10 min, followed by SDS–PAGE.

**RESULTS**

**Purification, identification and cloning of the Sulfolobus SSB protein**

Using a single-stranded oligonucleotide probe in an EMSA, we detected a ssDNA binding activity in *S.solfataricus* protein extracts fractionated by anion exchange chromatography (Fig. 1). The protein responsible for this binding activity was purified through two further columns until it was essentially homogeneous as assessed by SDS–PAGE (Fig. 3). The purified protein was identified by MALDI-TOF mass spectroscopy of tryptic peptides, and interrogation of the public release of the *Sulfolobus* genome sequence. This resulted in the unambiguous identification of a single ORF (bac19_023) encoding a 16 184 Da polypeptide. The protein eluted from a calibrated gel filtration column with an estimated size of 20 ± 4 kDa (data not shown), consistent with a monomeric subunit composition in solution. Analysis of the primary structure revealed the presence of a putative OB-fold domain within the first 100 amino acids that is common to all known SSB proteins, together with an approximately 40 amino acid C-terminal extension. No N-terminal extension was apparent, and the sequence does not include the putative zinc finger found in RPA70 and several euryarchaeal RPA sequences (9). Polyclonal antibodies raised against the purified recombinant protein were used to estimate the *in vivo* concentration of SSB in *Sulfolobus* cells as ~2–5% of total soluble protein. This apparent abundance is consistent with the important role of SSB in many DNA transactions in cells, and with previous estimates of the abundance of human RPA (1,16). It may also reflect an important role for SSB proteins in ssDNA stabilisation in hyperthermophilic organisms.

The closest homolog to *Sulfolobus* SSB was found in an unannotated ORF (accession no. Q9YCD3) present in *A.pernix*, the only other crenarchaeote for which substantial sequence data is in the public domain. Neither *Sulfolobus* nor *Aeropyrum* contain any other ORFs that are recognisable as SSB domains. BLAST searches suggest that the OB-fold in the crenarchaeal SSBs is most similar to those in euryarchaeal SSBs, supporting the monophyly of the archaea. The next closest matches were to hypothetical ORFs in the genome sequences of the higher plant *Arabidopsis thaliana* and the metazoan *Caenorhabditis elegans* (Fig. 2A). In contrast, alignment of the eucaryal, euryarchaeal and newly discovered crenarchaeal SSB proteins with eubacterial sequences required the insertion of several gaps in the OB-fold domain.

Outwith the OB-fold, the crenarchaeal SSB sequences contain surprising similarities to the equivalent C-termini of the eubacterial SSBs. This includes the presence of a large number of glycine and proline residues that are expected to confer flexibility on the C-terminal ‘tail’ of the protein (shown in green in Fig. 2B), together with areas of positively and negatively charged residues (blue and red, respectively, in Fig. 2B).

The gene encoding the putative SSB was cloned from *S.solfataricus* P2 genomic DNA by PCR amplification, and cloned into a plasmid allowing heterologous expression in *E.coli*. The recombinant protein was purified by a heat denaturation and two column chromatography steps as...
described in Materials and Methods (Fig. 3). In order to test the predicted flexibility of the C-terminal tail of *Sulfolobus* SSB, the pure recombinant protein was subjected to partial digestion with a 250:1 ratio of SSB:porcine trypsin. SDS–PAGE and MALDI-TOF analysis of the digested protein showed a single major peak with a molecular mass of 12 933 Da. This allowed the unambiguous identification of the site of cleavage by trypsin as Arg-119, the first arginine present in the Gly/Pro-rich C-terminal region, suggesting that the C-terminal 30 amino acids of *Sulfolobus* SSB exist in a flexible conformation.

Digestion of intact recombinant SSB with the enzyme Glu-C, which cleaves C-terminal to glutamate residues, did not result in the formation of any further-truncated species, despite the presence of several potential target sites (data not shown), suggesting that the remainder of the protein has a defined folded structure.

DNA binding characteristics of native, recombinant and truncated SSBs

*Sulfolobus* SSB was identified on the basis of its ability to bind and retard a ssDNA oligonucleotide during acrylamide gel electrophoresis (Fig. 1). However, for the experimental determination of binding affinities and stoichiometries, the EMSA technique was not ideal for this protein, probably due to DNA–protein dissociation over the lifetime of the electrophoretic separation. Accordingly, we investigated alternative procedures for the characterisation of the interaction of SSB with nucleic acids. Using a circular ssDNA molecule, phage phiX174, composed of 5386 nt, we demonstrated DNA binding using an agarose gel electrophoretic assay (Fig. 4). As greater numbers of protein molecules bind to the ssDNA MALDI-TOF analysis of the digested protein showed a single major peak with a molecular mass of 12 933 Da. This allowed the unambiguous identification of the site of cleavage by trypsin as Arg-119, the first arginine present in the Gly/Pro-rich C-terminal region, suggesting that the C-terminal 30 amino acids of *Sulfolobus* SSB exist in a flexible conformation. Digestion of intact recombinant SSB with the enzyme Glu-C, which cleaves C-terminal to glutamate residues, did not result in the formation of any further-truncated species, despite the presence of several potential target sites (data not shown), suggesting that the remainder of the protein has a defined folded structure.

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circles, their mobility is gradually reduced in the gel, until an end-point is reached where all available ssDNA binding sites are saturated by protein. This end-point coincided with a dramatic reduction in fluorescence intensity after staining with ethidium bromide. We interpret this effect as being due to the occlusion of ethidium bromide from potential binding sites on the DNA when it is fully bound by the protein, suggesting that Sulfolobus SSB can intercalate side chains between DNA bases.

The agarose gel retardation assay was repeated for the purified native SSB protein, the recombinant SSB protein and the recombinant protein after truncation by trypsin. By calculating the molar ratios of DNA nucleotides:SSB molecules at the point of saturation, we estimated the binding site size of Sulfolobus SSB as 4–6 nt per SSB monomer for the native protein, ~8–12 nt per monomer for the recombinant protein and 7–10 nt per monomer for the truncated protein. Thus the truncated protein retains the ability to bind DNA, as seen for *E. coli* SSB (17,18). The slight differences in binding site size calculated for the native, recombinant and truncated species are more likely to be due to experimental error rather than having a functional significance, particularly in light of the fluorescence quenching data described below. This experiment also demonstrated that DNA binding is not highly co-operative, but has some distributive character, otherwise we would not expect to see a gradual reduction in the mobility of the bulk of the DNA, but rather an interconversion between non-retarded and fully-retarded species.

**DNA binding monitored by quenching of intrinsic tryptophan fluorescence**

*Sulfolobus* SSB has two tryptophan residues in the OB-fold domain, allowing analysis of ssDNA binding by tryptophan fluorescence quenching (Fig. 5). The protein displayed strong intrinsic fluorescence with a peak wavelength of 345 nm when excited at 290 nm. As ssDNA was titrated into the cuvette, the intrinsic fluorescence of the protein was progressively quenched, reaching an end-point at ~20–40% of the unquenched value. The binding isotherms suggest a dissociation constant in the low nanomolar concentration range. Regardless of the length of ssDNA strand added (18, 69 or 5386 nt), binding was saturated at a ratio of ~4–5 nt ssDNA per monomer of *Sulfolobus* SSB, or 20–25 nt per tetramer. This ratio was unaffected by the presence or absence of the C-terminal tail and is in broad agreement with the values estimated by agarose gel electrophoresis. In comparison, *Methanococccus jannaschii* RPA binds 20 nt ssDNA (10) and human RPA binds DNA non-cooperatively, with a binding site size of 30 nt per heterotrimer under most conditions (19). *Escherichia coli* SSB displays multiple binding modes, with 35 or 65 nt bound per tetramer (20–22).

**Glutaraldehyde cross-linking**

To address the question of the subunit composition of *Sulfolobus* SSB, we incubated the protein with glutaraldehyde in order to cross-link lysine residues in the presence and absence of an 18mer oligonucleotide. Conditions were chosen in order to maximise inter-subunit cross-links whilst minimising cross-links between non-associated protein subunits. Cross-linked species were precipitated and analysed by SDS–PAGE (Fig. 6). Whilst there was a suggestion of the presence of higher molecular species in the absence of DNA (lanes 3 and 4), addition of a roughly equimolar concentration of a short oligonucleotide resulted in a significant increase in the amount of cross-linked species, which may correspond to the presence of dimers and tetramers of SSB. No further-retarded complexes were observed. Lane 1, markers; lane 2, 5 µM recombinant SSB, no glutaraldehyde; lane 3, 5 µM SSB without DNA; lane 4, 1 µM SSB without DNA; lane 5, 5 µM SSB with DNA; lane 6, 1 µM SSB with DNA.

**DISCUSSION**

DNA is subjected to a continual assault by cellular and environmental factors, and must be repaired efficiently to preserve the genetic integrity of all DNA-based life forms.
Many of these stresses encountered by cells are likely to be highly exacerbated by adaptation to life in extreme conditions, and this has led to the evolution of highly efficient DNA repair pathways in extremophiles. For example, the extreme radiation-resistance of the bacterium *Deinococcus radiodurans* probably arose in response to the high levels of DNA damage suffered upon desiccation (23). Adaptation to growth at elevated temperatures presents a unique set of challenges, as the rates of chemical reactions resulting in DNA modification are correspondingly higher, and the structural integrity of all cellular components, including nucleic acids and proteins, must be preserved. Most of the hyperthermophilic organisms known at present are archaeal, and we still have only a very patchy knowledge of DNA repair pathways in these organisms. The tantalising suggestion that some archaeal DNA repair pathways may, like those of DNA replication and transcription, resemble their eucaryal equivalents deserves further study. Accordingly, we have undertaken the identification and characterisation of archaeal DNA repair proteins, using genomic information where possible, and biochemical alternatives when necessary. The latter approach has led to the identification of the first crenarchaeal ssDNA binding protein, reported here.

Analysis of the domain and subunit composition of *Sulfolobus* SSB has yielded some surprises. Whilst the *Sulfolobus* OB-fold domain responsible for DNA binding is clearly related most closely to other archaeal SSB domains, the C-terminal region of the protein has unexpected similarities to eubacterial SSBs. The role of the C-terminal tail of *E. coli* SSB, which consists of a flexible spacer region and a highly acidic C-terminal α-helix, has been investigated in some detail. Deletions in the C-terminal α-helix result in severe repair-deficient phenotypes that are not caused by alterations in DNA binding characteristics, suggesting the tail has a role in protein–protein interactions. *Escherichia coli* SSB interacts directly with the base excision repair protein exonuclease I via the C-terminal helix (24,25). It is tempting to speculate that the C-terminal tail of *Sulfolobus* SSB plays a similar role *in vivo*. If so, the clear differences apparent in the DNA binding domains of *Sulfolobus* and *E. coli* SSB suggest that the evolution of similar C-terminal domains is an example of convergent rather than divergent evolution. In the eucaryal and euryarchaeal RPA proteins, other domains probably fulfil similar roles, and it is notable that these proteins have N-terminal extensions and a zinc binding domain that are not present in the eubacterial and crenarchaeal proteins. The crenarchaeota possess some unique features in dsDNA as well as ssDNA binding proteins. They lack the histones found in most of the euryarchaeota, and instead rely on small non-histone dsDNA binding proteins such as Sso7 and Sso10 in *Sulfolobus*. The roles of all of these proteins in DNA stability, replication and repair in hyperthermophilic archaea are currently under scrutiny.

The DNA binding characteristics of *Sulfolobus* SSB appear similar to most other SSB proteins. The analyses described here have all been undertaken at 20°C, some 60°C below the optimal growth temperature of *Sulfolobus*. Our studies of other *Sulfolobus* proteins such as the Holliday junction resolving enzyme Hjc (26) and the dsDNA binding protein Sso10b (B.N.Wardleworth and M.F.White, unpublished data) suggest that DNA binding by thermostable proteins is not highly temperature dependent in general, whereas enzymatic activity clearly obeys the Arrhenius equation. Nevertheless, more detailed studies of the interaction of *Sulfolobus* SSB with ssDNA at more physiological temperatures may identify some significant effects. The binding site footprint of 5 nt ssDNA per monomer most likely reflects the amount of DNA accommodated by each OB-fold domain, which is known to be 4 nt for the co-crystal structure of RPA70 (i.e. 8 nt ssDNA are bound by the two OB-folds of RPA70) (2). However, *Sulfolobus* SSB appears unique amongst cellular SSB proteins in being monomeric in solution. Chemical cross-linking experiments suggest that the protein multimerises on DNA binding, and by analogy with other SSB proteins we would expect a tetramer to represent the functional subunit structure. Further analysis of the subunit composition of *Sulfolobus* SSB using analytical ultracentrifugation is currently underway.

The central role of SSB proteins in DNA replication and repair is emphasised by the large number of publications reporting functional interactions of RPA with other proteins. For example, RPA has been shown to interact with the nucleotide excision repair protein XPA (27–29), the Bloom’s syndrome helicase BLM (30), the strand exchange proteins Rad51 (31) and Rad52 (32–34) and P53 (35). The identification of the crenarchaeal ssDNA binding protein reported here brings us a step closer to the reconstitution of crenarchaeal DNA replication and repair pathways *in vitro*, and may aid the identification of further archaeal repair proteins that are not detectable solely on the basis of their protein sequence.

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