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Cloning, expression, purification, crystallization and preliminary X-ray characterization of the full-length single-stranded DNA-binding protein from the hyperthermophilic bacterium *Aquifex aeolicus*

Single-stranded DNA-binding (SSB) proteins stabilize single-stranded DNA, which is exposed by separation of the duplex during DNA replication, recombination and repair. The SSB protein from the hyperthermophile *Aquifex aeolicus* has been overexpressed in *Escherichia coli*, purified and characterized and crystals of the full-length protein (147 amino acids; $M_r$ 17 131.20) have been grown by vapour diffusion from ammonium sulfate pH 7.5 in both the absence and presence of ssDNA [dT(pT)$_n$]. All crystals diffract to around 2.9 Å resolution and those without bound DNA (native) belong to space group $P_2_1$, with two tetramers in the asymmetric unit and unit-cell parameters $a = 80.97$, $b = 73.40$, $c = 109.76$ Å, $\beta = 95.11$. Crystals containing DNA have unit-cell parameters $a = 108.65$, $b = 108.51$, $c = 113.24$ Å and could belong to three closely related space groups ($I\overline{2}2_1$, $I\overline{2}1_1\overline{2}1_2$ or $I_4$) with one tetramer in the asymmetric unit. Electro spray mass spectrometry of the crystals confirmed that the protein was intact. Molecular replacement with a truncated *E. coli* SSB structure has revealed the position of the molecules in the unit cell and refinement of both native and DNA-bound forms is under way.

1. Introduction

Single-stranded DNA-binding (SSB) proteins have been shown to play an essential role in many aspects of DNA metabolism (Chase & Williams, 1986). They preferentially bind and protect vulnerable single-stranded DNA (ssDNA), which is formed transiently during DNA replication, recombination and repair. SSB proteins are characterized by the presence of a conserved OB-fold motif (oligonucleotide/oligosaccharide/oligopeptide-binding fold), which is typically 100 amino acids in length (Murzin, 1993).

SSB proteins can be divided into two distinct groups based on their quaternary structure. Eukaryotic SSB proteins, known as replication protein A (RPA), are exemplified by human RPA, which has a heterotrimERIC structure comprising three subunits: RPA70, RPA32 and RPA14 (of molecular weights 70, 32 and 14 kDa, respectively; Wold, 1997). The RPA complex contains six OB folds, four of which bind DNA: three on RPA70 and one on RPA32 (Bastin-Shanower & Brill, 2001). An N-terminal domain on RPA70 has also been shown to be involved in protein–protein interactions (Jacobs et al., 1999). In contrast, bacterial SSB proteins form homotetramers, with each subunit containing one DNA-binding domain (Raghunathan et al., 1997). These DNA-binding domains are located at the N-termini of the individual SSB protein subunits and form the characteristic OB folds. While the N-terminus of each subunit binds ssDNA and contains the homotetramer interface, it is thought that the C-terminal domain is involved in interactions with other protein components of DNA metabolism. The C-terminal domain of bacterial SSB proteins exhibits low sequence homology across species, with the exception of the terminal six residues, which form a highly conserved negatively charged DDDIPF motif.

This motif is essential for the function of *Escherichia coli* SSB protein in vivo (Curth et al., 1996) and has been shown to interact directly with the 3’–5’ ssDNA-degrading exonuclease I (Genschel et al., 2000). The tails of both the *E. coli* and the *Sulfolobus solfataricus* SSB proteins are not involved in DNA binding, but are thought to play roles in mediating protein–protein interactions with other subunits within the DNA polymerase complex (Bruck et al., 2002). There is also evidence that a mutually exclusive interaction between the C-terminal domain of *E. coli* SSB protein, DNA polymerase and primase is utilized as a three-point switch to initiate the exchange of places of these two proteins on DNA (Yuzhakov et al., 1999). Furthermore, a recent report suggests that the interaction between the DNA polymerase and SSB from RB69 (a T4-like bacteriophage) results in an increase in the overall affinity of the SSB protein for ssDNA (Sun & Shamoo, 2003). Finally, Gulbis and coworkers have recently...
proposed a positively charged patch on the χ subunit of Pol III holoenzyme which may interact with the C-terminal acidic region of SSB (Gulbis et al., 2004).

The DNA-binding domains and OB folds from SSB proteins have been well studied and structural information is available from a variety of organisms spanning all three kingdoms of life (Bochkareva et al., 2001; Bochkarev et al., 1997, 1999; Webster et al., 1997; Raghanathan et al., 2000; Yang et al., 1997; Kerr et al., 2003). However, crystallization of a full-length bacterial protein has proved problematic and most studies have used proteolytic N-terminal fragments of SSB proteins; consequently, little is known about the structure of its C-terminal domain.

Fig. 1) from the hyperthermophilic bacterium A. aeolicus (SSB Aae) in both the free and the DNA-bound forms. In contrast to E. coli SSB, primary structure analysis of the SSB Aae reveals a polyglutamic acid region at its C-terminus and a EDEIPF motif (Fig. 1). We hope that the crystal structure of the A. aeolicus SSB protein will facilitate the study of the complex protein–protein interactions mediated through the C-terminus of bacterial SSB proteins and the data may also reveal the structural basis for the increased stability of this SSB at elevated temperatures. Further, the DNA-bound structure may reveal details of the (SSB)₆₅ binding mode.

2. Cloning, expression and purification

The ssb gene was identified from the complete A. aeolicus genome sequence (Deckert et al., 1998), amplified by polymerase chain reaction and the resulting 451 bp fragment was subsequently inserted into the pET-23a expression vector (Novagen) using NdeI/HindIII restriction sites. The fidelity of the construct, pET23a/ ssb, was verified by DNA sequencing before transformation of E. coli BL21(DE3)/pLysS (Novagen). Cells were grown in 2YT growth media supplemented with ampicillin (100 μg ml⁻¹) in shake flasks at 310 K and 250 rev min⁻¹ to OD₆₀₀ = 0.8 prior to induction with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After a further 4 h of growth, cells were collected by centrifugation and stored at 253 K.

The cell pellet was resuspended in 10 ml buffers (50 mM Tris–HCl pH 7.0) per liter of cell culture. The purified protein was a single high molecular weight species of 17310 ± 150 Da by ESI mass spectrometry, which is in agreement with the

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**SSB Aae**

1. **SSB Eco**

2. **SSB Sso**

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Figure 1: Sequence alignment of the SSB proteins from A. aeolicus (SSB Aae), E. coli (SSB Eco) and S. solfataricus (SSB Sso). The asterisk (*) under residue L112 of E. coli SSB (SSB Eco) denotes the limit of the resolution of the crystallographic dataset (SSBe, residues 1–135, represented by a $ symbol under W135) used to determine the structure of the native and DNA-bound SSB complex described by Raghunathan et al. (1997, 2000) (PDB codes 1kaw and 1eyg, respectively). The hash symbol (#) under residue R119 indicates the C-terminus of the trypsin fragment of S. solfataricus SSB (SSB Sso) crystallized by Kerr et al. (2003) (residues 1–119; PDB code 1o73). The addition sign (+) under N145 of SSB Eco denotes the limit of structure determination from the autolytic fragment crystallized by Matsumoto et al. (2000) (residues 1–145; PDB code 1qve). Notice the glutamate-rich C-terminus of SSB Aae in comparison to the glutamine-rich tail of SSB Eco.
3. Crystallization

Initial crystals were obtained using Molecular Dimensions Structure Screens 1 and 2 and the sitting-drop vapour-diffusion method at 290 K. The drop consisted of 5 μl protein solution (7 mg ml⁻¹ in buffer C) and 5 μl precipitant. Over two weeks, small crystals of native protein were observed under three different conditions, with the best quality obtained using 100 mM HEPES pH 7.5, 2% (v/v) PEG 400, 2.0 M (NH₄)₂SO₄ pH 7.5 as the precipitant. After refining the crystallization conditions, larger crystals were obtained after four weeks using the same precipitant at a pH of 7.0. Co-crystallization of the DNA-bound protein was achieved by mixing 7.5 mg ml⁻¹ protein in a 1:1 molar ratio (tetramer:ssDNA) with 69-mer dT(pT)₆₈ (MWG Biotech). The complex was incubated on ice for 60 min and centrifuged (10 min, 35 000g) prior to crystallizations being set up. Each crystallization drop comprised 1.5 μl protein in 50 mM Tris pH 7.0, 0.1 M NaCl and 1.5 μl precipitant. All were set up at 290 K. Crystals grew within one week; the best quality crystals were obtained using 100 mM HEPES pH 7.5, 2.3 M (NH₄)₂SO₄.

4. Data collection and processing

Crystals of native protein of approximate dimensions 0.1 x 0.2 x 0.2 mm were flash-cooled in a 20% glycerol well solution and X-ray data for the native SSB were collected at 100 K (Cryostream cooler; Oxford Cryosystems, Oxford, England) on a MAR Research 345 imaging plate mounted on an Enraf–Nonius FR591 rotating-anode generator, λ = 1.5418 Å, fitted with Osmonic mirrors and operating at 40 kV, 110 mA. Crystals of similar dimensions were obtained for the DNA-bound form and data were also collected at 100 K on station 14.2 (λ = 0.978 Å) at the SRS, CLRC Daresbury Laboratory. Analysis of the diffraction data for both crystals using MOSFLM/SCALA (Leslie, 1992; Collaborative Computational Project Number 4, 1994) produced the data shown in Table 1 and allowed the assignment of the native crystals to space group P₂₁. For the DNA-bound data, similar processing statistics were obtained with space groups P₂₁, P2₁2₁2₁ and P4₁.

For the native SSB data set, a model of the SSB from Escherichia coli (PDB code 1qv; Matsumoto et al., 2000) was used to search for an initial solution using MOLREP (Vagin & Teplyakov, 1997). The search molecule was trimmed of its flexible loops and amino-acid side chains to produce a tetrameric polyalanine structure. The top rotation-function solution produced a satisfactory translation-function solution that was then used to aid location of the second tetramer. No solution was obtained using the S. solfataricus structure as a search model. For the DNA-bound SSB, a multi-copy search with MOLREP using the partially refined SSB Aae dimer (R = 0.272, Rfree = 0.308) provided solutions, the best of which contained two dimers per asymmetric unit in each of the three space groups. 20 cycles of rigid-body refinement were followed by ten cycles of restrained refinement. The statistics for this process are also shown in Table 1. Refinement of both crystal forms is currently in progress while attempts are being made to improve the diffraction quality of the crystals.

Table 1

Data-collection and reduction statistics.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Native DNA-bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data collection</td>
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<tr>
<td>Oscillation range (°)</td>
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<tr>
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<td>P₂₁</td>
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<tr>
<td>Unicell parameters</td>
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</tr>
<tr>
<td>a (Å)</td>
<td>80.97</td>
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<tr>
<td>b (Å)</td>
<td>73.40</td>
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<tr>
<td>c (Å)</td>
<td>109.76</td>
</tr>
<tr>
<td>β (°)</td>
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</tr>
<tr>
<td>Tetramers per AU</td>
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</tr>
<tr>
<td>No. observations</td>
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</tr>
<tr>
<td>No. unique reflections</td>
<td>27790 (3680)</td>
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<tr>
<td>Completeness (%)</td>
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<tr>
<td>Rmerge †</td>
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<tr>
<td>MOLREP R factor</td>
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<td>MOLREP correlation coefficient</td>
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<tr>
<td>Restrained refinement Rmerge</td>
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</tr>
<tr>
<td>Restrained refinement Rfree</td>
<td>0.487</td>
</tr>
</tbody>
</table>

† For one SSB tetramer (Mᵣ = 68 400) and one DNA 69-mer (Mᵣ = 20 927). 
For one SSB tetramer (Mᵣ = 68 400) and one DNA 69-mer (Mᵣ = 20 927). 
⊥ Rmerge = ∑ᵢ ∑ᵣ |Iᵢ(h) − Iᵢ|/|Iᵢ|, where (Iᵢ(h)) is the mean intensity of the i symmetry-equivalent reflections

Figure 2

Analysis of the crystallized DNA-bound SSB protein by ESI–MS. The main figure shows the deconvoluted mass of 17 127.3 (obtained using Transform software; Micromass UK), consistent with the theoretical value of 17 131.2; right inset, ion envelope of crystallized SSB protein; left inset, SDS–PAGE analysis of crystallized SSB protein results in a single band running at an anomalous weight of ~23 kDa.
single band around 23 kDa in keeping with the observed anomalous mobility of the native SSB. Only one major species was observed by ESI–MS with a mass of 17 127.3 ± 2.7 (Fig. 2), in good agreement with the predicted weight. No LCMS data could be obtained from the dissolved native SSB crystal.

5. Discussion

It is clear from the sequence alignment of the SSB proteins that the A. aeolicus and the E. coli proteins are more closely related to each other than either is to S. solfataricus SSB (Fig. 1). This is borne out by the fact that a molecular-replacement solution using the E. coli structure was obtained relatively easily, whilst no satisfactory solution could be obtained with the S. solfataricus structure. The very high resolution of S. solfataricus reveals why this should be so in that the actual molecular structure is much more closely related to the eukaryotic SSB fold than that of E. coli SSB (Kerr et al., 2003). Consequently, despite a modest sequence identity, the structures are distinct.

For the DNA-bound crystals reported here, there is an ambiguity as regards the space group. Given an SSB tetramer in the asymmetric unit, using the monomer M, of 17 100 and that of the DNA as 20 927, the \( V_M \) can be calculated to be 2.43, 2.11, 1.86 or 1.51 \( \text{Å}^3 \text{Da}^{-1} \) for zero, 0.5, one or two bound DNA 69-mers per tetramer. The expected 1:1 complex requiring one DNA oligomer per asymmetric unit corresponds to a \( V_M \) of 1.86 \( \text{Å}^3 \text{Da}^{-1} \), which is within the range found by Matthews (1968), albeit quite close to the lower limit. The physiological tetramer as observed in the native structure sits on a crystallographic dyad in both \( I_4_1 \) and \( I_2_2_1_2_1 \), whereas the tetramer sits on a screw dyad axis in \( I_2_2_2 \). It is impossible for there to be exact twofold symmetry for the SSB tetramer with a single DNA oligomer bound, although a pseudo-twowofold arrangement is possible. Given the limited resolution of the present X-ray data and the current state of the refinement, such a situation cannot yet be ruled out. However, if the DNA oligomer is shared between two tetramers in some fashion, this could permit the DNA-bound tetramer to lie upon a crystallographic dyad, while maintaining four subunits and a single DNA molecule in the asymmetric unit (Ferrari et al., 1994). The initial electron-density maps in each of the three space groups all show extra electron density near regions of the protein expected to bind DNA (Raghunathan et al., 2000). Examination of the maps together with the statistics in Table 1 leads us to prefer \( I_2_2_2 \) as the space group, but we are continuing to refine all three possibilities. These refinements should clarify this uncertainty and also allow us to estimate the occupancy of the DNA.

In summary, our expression and purification strategy has produced full-length SSB from the hyperthermophile A. aeolicus with no autolysis observed by mass spectrometry and SDS–PAGE. The flexible C-terminal tail is present in the crystals reported here, unlike the truncated SSB used in both the E. coli and S. solfataricus structure determinations. Our initial refinement of the structures of both forms has allowed the clear assignment of the electron density to residues 1–38 and 41–108 and we are currently refining the models in an effort to distinguish the C-terminal residues.

Note added in proof: During the processing of this manuscript a report has been published describing the crystallization of full-length E. coli SSB (Savvides et al., 2004).

We wish to thank Professors Karl Stetter and Robert Huber (University of Regensburg) for the kind gift of A. aeolicus chromosomal DNA and Professor Jim Naismith and Dr Iain Kerr (University of St Andrews) for kindly providing the coordinates of the SSB fragment from S. solfataricus prior to publication. The Biotechnology and Biological Sciences Research Council UK and the University of Edinburgh supported this work (DJC, LAM).

References