PRELIMINARY CRYSTALLOGRAPHIC STUDY OF *STREPTOMYCES COELICOLOR* SINGLE-STRANDED DNA-BINDING PROTEIN

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Single-stranded DNA-binding proteins (SSBs) play a crucial role in DNA processing such as replication, repair and recombination in all organisms, from bacteria to human. *Streptomyces coelicolor* ssb gene was overexpressed in a heterologous host, *Escherichia coli* NM522. 15 mg of purified protein from 1 dm³ of culture was obtained in one-step procedure applying Ni²⁺ chelating chromatography. Among bacterial SSBs with the solved crystal structure, the *S. coelicolor* SSB displayed significant sequence similarity with those from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*, slow growing bacteria with a high GC content. Moreover, conserved amino acid region that forms additional ß strand in mycobacterial SSBs was also found in *S. coelicolor* SSB. The full-length protein readily crystallises in space group *I*222 or *I*212121 with unit-cell parameters *a* = 100.8, *b* = 102.1, *c* = 164.2 Å. The asymmetric unit is expected to contain four monomers with solvent content of 52–55 %.

Keywords: single-stranded DNA-binding protein, SSB purification, *Streptomyces coelicolor*, crystallisation

INTRODUCTION

The SSB protein family contains more than 400 sequence homologues, mainly of bacterial, eukaryotic and viral origin.ι Despite minimal sequence homology and highly diverse structures, different SSBs have similar roles in DNA metabolism.ι SSB proteins protect the transiently formed single-stranded DNA (ssDNA), generated during DNA metabolism, from chemical and nuclease attacks and from forming aberrant secondary structures.ι Due to its importance in maintaining genomic integrity, SSB is one of the essential gene products required for life.ι Depending on their oligomeric state, SSBs can be classified as monomeric, homo-dimeric, hetero-trimeric and homo-tetrameric.ι The bacterial and mitochondrial SSBs are predominantly homo-tetrameric proteins. The only exceptions are bacteria of the *Thermus* and *Deinococcus* genera whose SSBs are homo-dimers, but each monomer encodes two OB folds.ι OB fold (oligonucleotide/oligosaccharide/oligopeptide binding fold) of SSB proteins is characterised by the presence of five-stranded ß-sheet coiled to form a closed ß-barrel capped by an α-helix.ι This fold typically comprises 100 amino acids located at the N terminus of SSB. Thus far, the only known SSB structure missing an OB-fold is adenoviral SSB.ι It has been reported that the C-terminal tail of SSB mediates protein-protein interactions.ι This part exhibits low sequence

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homology across species, with the exception of the highly conserved negatively charged motif at the C-terminus.9

Recently solved crystal structures of *Mycobacterium tuberculosis*10 and *Mycobacterium smegmatis*8 SSBs showed unexpected variations in quaternary structures of homo-tetrameric SSBs despite similarity in their tertiary structures.

Here, we report overexpression, single-step protein purification, crystallisation and preliminary X-ray diffraction analysis of the *Streptomyces coelicolor* single-stranded DNA-binding protein. Similarly to *Mycobacterium*, *Streptomyces* belong to Gram-positive bacteria with a high GC content. However, this genus exhibits a substantial difference in the life cycle growing much faster than *Mycobacterium*.11

To our knowledge, this is the first crystallographic study of SSB isolated from *Streptomyces*, bacteria that exhibit life-cycle complexity and have the ability to produce approximately 70 % of all known antibiotics, including many of great importance to medicine, pharmacology and biotechnology.

**EXPERIMENTAL SECTION**

**Protein Synthesis and Purification**

*Streptomyces coelicolor* SSB-encoding gene was PCR-amplified from the respective genomic DNA and the PCR product was inserted into vector pQE-30 (Qiagen), which introduced six adjacent histidine residues to the N-terminus of the recombinant protein, as described previously.12 For overexpression of the *S. coelicolor* *ssb* gene, this construct was introduced in *E. coli* NM522. The *E. coli* cells were grown overnight at 310 K in 10 cm³ of the *Luria-Bertani* (LB) medium containing ampicillin (100 µg/cm³). The *E. coli* was introduced in *S. coelicolor* ssb overexpression.

The isopropyl-β-D-thiogalactopyranoside (IPTG) inducer was added at a final concentration of 0.5 mmol/dm³ when the optical density of the cells reached 0.7 at OD₆₀₀. The cells were further incubated for 6 hours, harvested at 4000 g for 15 min and resuspended in 25 cm³ of Lysis buffer (50 mmol/dm³ TRIS-HCl, pH = 8.0; 300 mmol/dm³ NaCl, 10 mmol/dm³ imidazole). The suspension was supplemented with DNease (1 µg/cm³), cells were disrupted by sonication (6 x 30 s) and the suspension was centrifuged at 10000 g for 30 min to remove cell debris. The supernatant was loaded on a 2 cm³ Ni²⁺-nitrilotriacetic acid agarose column (Qiagen) equilibrated with the Lysis buffer. The column was washed with Wash buffer (50 mmol/dm³ TRIS-HCl, pH = 8.0; 300 mmol/dm³ NaCl; 20 mmol/dm³ imidazole) and the bound protein was eluted with elution buffer (50 mmol/dm³ TRIS-HCl, pH = 8.0; 300 mmol/dm³ NaCl; 300 mmol/dm³ imidazole). Fractions with purified SSB protein were pooled; the protein buffer was exchanged on a PD10 column (Pharmacia) equilibrated with 50 mmol/dm³ TRIS-HCl, pH = 7.0 and concentrated for microcrystallisation experiments.

Protein fractions collected during the purification procedure were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blotting as described.13

**Crystallisation**

Crystallisation experiments were performed at a constant temperature of 291 K by the hanging-drop vapour-diffusion method using VDX plates and siliconised glass cover slips. Crystal Screen Cryo (Hampton Research) was utilised to screen the initial conditions. The droplet was prepared by mixing 1 mm³ of protein solution (15.5 mg/cm³ in 50 mmol/dm³ TRIS-HCl, pH = 7.0) with 1 mm³ of precipitant solution and equilibrated against 0.7 cm³ reservoir solution.

**RESULTS AND DISCUSSION**

**Sequence Analysis**

The SSB protein from *Streptomyces coelicolor* consists of 199 amino acid residues with a molecular mass of 19.9 kDa.12 Sequence alignment of the *S. coelicolor* SSB with the proteins that have solved crystal structures and the final presentation were obtained using CLUSTALX 1.81.14 The result is depicted in Figure 2. Evolutionary related bacteria, *M. tuberculosis* and *M. smegmatis* (PDB codes 1UE1 and 1X3E, respectively), and distant, *E. coli* (PDB code 1QVC and *Thermotoga maritima* (PDB code 1Z9F), were selected. SSBs from *S. coelicolor* and *Myxococcus* share significant sequence identity: approximately 65 % over the entire sequence and 75 % of the 120 residues overlap at the N-terminal DNA binding domain. It is known that C-terminus of bacterial SSBs is often composed of many glycines and prolines and is therefore very flexible. Interestingly, *S. coelicolor* SSB has 50 % glycines in its C-terminus (residues 110–199),
approximately two times more compared to SSBs from the related bacteria *M. tuberculosis* and unrelated *E. coli*.

The majority of bacterial SSBs exist as homo-tetramers and we assume that *S. coelicolor* SSB forms the same oligomeric structure. However, it has been reported that the quaternary structure of Gram-negative *E. coli* and *T. maritima* is an approximate spheroid, while that of the Gram-positive *Mycobacterium* is an ellipsoid. SSBs of *Mycobacterium* have a short sequence of highly conserved amino acid residues at the C-terminus of the OB-fold. Further, these residues compose an extra \( \beta \)-strand (strand 9), forming a hook-like structure in mycobacterial SSBs. Consequently, this causes the previously mentioned unique quaternary structure. The motif that forms the hook-like structure in mycobacterial SSBs has been also found in the *S. coelicolor* SSB sequence (Figure 2), suggesting a similar quaternary structure. Moreover, arginins at positions 76 and glutamic acids at positions 105, conserved in *M. tuberculosis* and *M. smegmatis* SSBs, are also present in *S. coelicolor* (Figure 2). These polar amino acids form inter-subunit salt-bridges and add stability to the SSB dimer. All this indicates conservation of the SSB quaternary structure among Gram-positive bacteria with a high GC content, like *Mycobacterium* and *Streptomyces*.

**Protein Synthesis and Purification**

Addition of the His residues to the N-terminus of the *S. coelicolor* SSB facilitated protein purification by an affinity Ni-resin column, as described in the Experimental section. After induction with the IPTG, the *E. coli* was grown for an additional 6 hours. During that time, the cell lysates were collected and the accumulation of the heterologous protein was monitored in soluble *E. coli* cell extracts by SDS-PAGE, as shown in Figure 1a. Protein fractions obtained during the purification procedure are presented in Figure 1b. Purified histidine-tagged SSB protein was recognised by rabbit-serum with the polyclonal antibodies raised against *S. coelicolor* SSB; the result of the Western blot is presented in Figure 1c. We obtained about 15 mg of purified SSB protein from 1 dm³ of cell culture in a single step of immunoaffinity chromatography and this facilitated microcrystallisation experiments. Proteolytic modification of the SSB protein was not observed upon storage at 253 K, as reported for SSBs from *M. tuberculosis* or *E. coli*. Successful purification implied that the spaced His-tag was not buried in the interior of the protein, thus enabling purification by affinity chromatography. The N-termini of *S. coelicolor* SSB tetramer most likely occupy similar positions as those in the solved crystal structures of single OB-fold bacterial SSBs.

**Crystallisation**

Purified protein was concentrated to 15.5 mg/cm³. His tag at the N-terminus of SSB was not removed prior to crystallisation. Prismatic crystals (Figure 3) appeared after several days in two drops: with reservoir solution composed of 0.075 mol/dm³ HEPES-Na, pH = 7.5; 0.6 mol/dm³ sodium dihydrogen phosphate; 0.6 mol/dm³ potassium dihydrogen phosphate and 25 % (vol. ratio, \( \psi \)) anhydrous glycerol (condition 1) and of 0.085 mol/dm³...
HEPES-Na, pH = 7.5; 1.7 % (v) polyethylene glycol 400; 1.7 mol/dm³ ammonium sulphate and 15 % (v) glycerol anhydrous (condition 2), respectively. Unlike with Mycobacterium smegmatis and M. tuberculosis SSBs, no crystals were obtained in the presence of cadmium or zinc sulphate.

Full-length protein crystallised readily, but all tested crystals diffracted poorly. Crystallisation studies with different SSB proteins, e.g., from E. coli15 and Sulfolobus solfataricus,16 reported that limited degradation of SSB by trypsin or chymotrypsin yields stable tetramers lacking the flexible C-terminus. In order to get better diffracting crystals, partial trypsin digestion was performed. Purified SSB was incubated in digest buffer (50 mmol/dm³ TRIS-HCl, pH = 8.0; 2 mmol/dm³ CaCl₂) at 310 K for 15 minutes in the presence of a bovine trypsine (1:250 mass ratio). Trypsine was inhibited irreversibly by addition of 1 mmol/dm³ trypsin inhibitor from soya. No crystals were obtained by applying the same crystallisation conditions as those for full-length protein.

**Preliminary X-ray Studies**

Three datasets were collected on different crystals of full-length protein. Datasets 1 and 2 were collected on a Rigaku RU-H2R rotating anode source with a MAR345 image plate detector and Xenocs mirror optics at Cu-Kα wavelength and Δφ = 1° oscillation frame. Dataset 3 was collected on a Siemens rotating anode generator, MAR300 image plate detector at Cu-Kα wavelength and Δφ = 1° per image. Data collection parameters and data processing statistics are summarised in Table I. All data were collected at 100 K. All crystals belong to orthorhombic space group $I_222$ or $I_212_12_1$ with similar unit cell parameters. Datasets 1 and 2 on crystals from crystallisation conditions 2 showed very high mosaicity (more than 1.5°), which resulted in a high proportion of reflections being rejected during integration and in low completeness. Although dataset 3 (conditions 1) is of somewhat lower maximal resolution, its overall completeness and multiplicity are much better. The Matthews coefficient ranges from 2.6–2.7 Å³ Da⁻¹, which corresponds to a solvent content of 52–55 %. Data were processed using the HKL programme package18 for dataset 1 and using the Mosflm19 and Scala20 programmes for datasets 2 and 3. Measured intensities were converted to structure-factor amplitudes using the Truncate21 programme incorporated in the CCP4 suite.22 Determination of the crystal structure by the molecular replacement method using M. tuberculosis and M. smegmatis as search models is under way.

![Figure 3. Morphology of the S. coelicolor SSB crystals.](image)

**Table I. Crystallographic parameters and data-processing statistics for S. coelicolor SSB protein[^a]**

<table>
<thead>
<tr>
<th></th>
<th>Dataset 1</th>
<th>Dataset 2</th>
<th>Dataset 3</th>
</tr>
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<tr>
<td>Crystallisation conditions</td>
<td>2</td>
<td>2</td>
<td>1</td>
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<tr>
<td>Crystal dimensions / mm</td>
<td>0.1 × 0.1 × 0.3</td>
<td>0.05 × 0.1 × 0.2</td>
<td>0.1 × 0.2 × 0.3</td>
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<tr>
<td>Resolution range / Å</td>
<td>50–3.5 (3.6–3.5)</td>
<td>50.44–3.8 (4.0–3.8)</td>
<td>40.5–3.7 (3.9–3–7)</td>
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<tr>
<td>Space group</td>
<td>$I2_22$ or $I2_12_12_1$</td>
<td>$I2_22$ or $I2_12_12_1$</td>
<td>$I2_22$ or $I2_12_12_1$</td>
</tr>
<tr>
<td>Unit cell / Å</td>
<td>$a = 100.7$</td>
<td>$a = 100.8$</td>
<td>$a = 102.4$</td>
</tr>
<tr>
<td></td>
<td>$b = 102.5$</td>
<td>$b = 102.1$</td>
<td>$b = 105.9$</td>
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<tr>
<td></td>
<td>$c = 163.5$</td>
<td>$c = 164.2$</td>
<td>$c = 161.9$</td>
</tr>
<tr>
<td>Total observations</td>
<td>37688 (5073)</td>
<td>30292 (4175)</td>
<td>62096 (8224)</td>
</tr>
<tr>
<td>Independent observations</td>
<td>10917 (1399)</td>
<td>7289 (1093)</td>
<td>9711 (1389)</td>
</tr>
<tr>
<td>Completeness / %</td>
<td>85.7 (78.2)</td>
<td>85.3 (88.5)</td>
<td>99.9 (100.0)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>4.0 (4.0)</td>
<td>4.2 (3.8)</td>
<td>6.4 (5.9)</td>
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<tr>
<td>Mean $I/\sigma(I)$</td>
<td>8.2 (3.4)</td>
<td>10.5 (4.4)</td>
<td>9.8 (4.0)</td>
</tr>
<tr>
<td>$R_{merge}$</td>
<td>0.14 (0.30)</td>
<td>0.12 (0.31)</td>
<td>0.23 (0.41)</td>
</tr>
<tr>
<td>Matthews coeff. / Å³ Da⁻¹, $M = 79.9$ kDa</td>
<td>2.6 with 52.7 % solvent</td>
<td>2.6 with 52.8 % solvent</td>
<td>2.7 with 54.8 % solvent</td>
</tr>
</tbody>
</table>

[^a]: Values in parentheses refer to the highest resolution shell.

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REFERENCES


SAŽETAK

Preliminarno kristalografsko proučavanje proteina koji vežu jednolančanu DNA iz bakterije Streptomyces coelicolor

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Proteini koji vežu jednolančanu DNA (SSB proteini, iz engl. single-stranded DNA-binding proteins) imaju bitnu ulogu u metabolizmu DNA, poput replikacije, rekombinacije i popravka, i to u svim organizmima, od bakterije do čovjeka. Gen sbb iz bakterije Streptomyces coelicolor prekomjerno je eksprimiran u heterolognom domaćinu E. coli NM522. U samo jednom koraku, primjenom Ni²⁺ kaltele kromatografije, iz 1 dm³ kulture dobiveno je 15 mg pročišćenog proteina. Ovaj protein pokazuje značajnu sličnost s proteinima sekvencijama SSB proteina iz bakterija Mycobacterium tuberculosis i Mycobacterium smegmatis. Očuvan aminokiselinski slijed koji kod mikobakterijskih SSB proteina čini dodatnu β vrpcu, prisutan je i kod SSB proteina iz bakterije S. coelicolor. Cijeli SSB protein lako kristalizira u prostornoj grupi P22 ili P2_12,1_2 s dimenzijama jedinične češlije a = 100,8, b = 102,1, c = 164,2 Å. Asimetrična jedinica najvjerojatnije sadrži četiri monomera, a sadržaj otapala je 52–55 %.