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Preliminary Crystallographic Study of *Streptomyces coelicolor* Single-stranded DNA-binding Protein

Zoran Štefanić,[a] Dušica Vujaklija,[b] Luka Andrišić,[b] Goran Mikleušević,[a] Miha Andrejašič,[c] Dušan Turk,[c] and Marija Luić[a,*]

[a]*Department of Physical Chemistry, Ruđer Bošković Institute, P.O. Box 180, 10002 Zagreb, Croatia*

[b]*Department of Molecular Biology, Ruđer Bošković Institute, P.O. Box 180, 10002 Zagreb, Croatia*

[c]*Department of Biochemistry and Molecular Biology, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia*

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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 dm^3 of culture was obtained in one-step procedure applying Ni^{2+} 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 $I222$ or $I2_12_12_1$ 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.[1] Despite minimal sequence homology and highly diverse structures, different SSBs have similar roles in DNA metabolism.[2] 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.[3] Due to its importance in maintaining genomic integrity, SSB is one of the essential gene products required for life.[3] Depending on their oligomeric state, SSBs can be classified as monomeric, homo-dimeric, hetero-trimeric and homo-tetrameric.[4] 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.[5] 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.[6] 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.[7] It has been reported that the C-terminal tail of SSB mediates protein-protein interactions.[8] This part exhibits low sequence

* Author to whom correspondence should be addressed. (E-mail: marija.luic@irb.hr)

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 cm^3 of the Luria-Bertrani (LB) medium containing ampicillin (100 $\mu g/cm^3$). The overnight culture was used to inoculate 1 dm^3 of the LB medium supplemented with the antibiotic and the cells were grown as described. The isopropyl-β-D-thiogalactopyranoside (IPTG) inducer was added at a final concentration of 0.5 mmol/ dm^3 when the optical density of the cells reached 0.7 at OD_{600}. The cells were further incubated for 6 hours, harvested at 4000 g for 15 min and resuspended in 25 cm^3 of Lysis buffer (50 mmol/dm^3 TRIS-HCl, pH = 8.0; 300 mmol/dm^3 NaCl; 10 mmol/dm^3 imidazole). The suspension was supplemented with DNase (1 $\mu g/cm^3$), 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 cm^3 Ni^{2+}-nitrilotriacetic acid agarose column (Qiagen) equilibrated with the Lysis buffer. The column was washed with Wash buffer (50 mmol/dm^3 TRIS-HCl, pH = 8.0; 300 mmol/dm^3 NaCl; 20 mmol/dm^3 imidazole) and the bound protein was eluted with elution buffer (50 mmol/ dm^3 TRIS-HCl, pH = 8.0; 300 mmol/dm^3 NaCl; 300 mmol/dm^3 imidazole). Fractions with purified SSB protein were pooled; the protein buffer was exchanged on a PD10 column (Pharmacia) equilibrated with 50 mmol/dm^3 TRIS-HCl, pH = 7.0 and concentrated for microcrystallisation experiments.

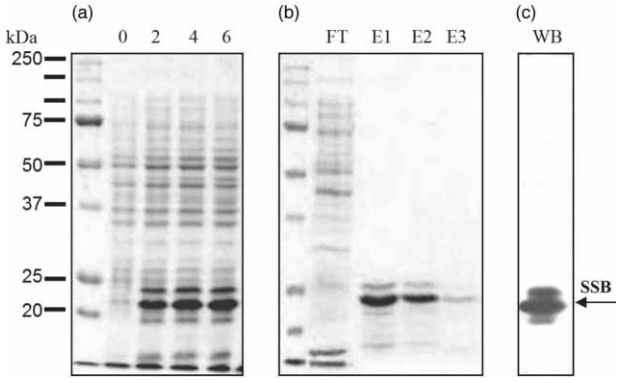

Figure 1. Polyacrylamide gel electrophoresis of: (a) cell free extracts collected at 0, 2, 4 and 6 hours after IPTG induction, and (b) protein fractions of the flow through (FT) and eluates. (c) Purified *S. coelicolor* SSB was analysed by Western blot.

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 mm^3 of protein solution (15.5 mg/cm^3 in 50 mmol/dm^3 TRIS-HCl, pH = 7.0) with 1 mm^3 of precipitant solution and equilibrated against 0.7 cm^3 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 *Mycobacterium* 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),

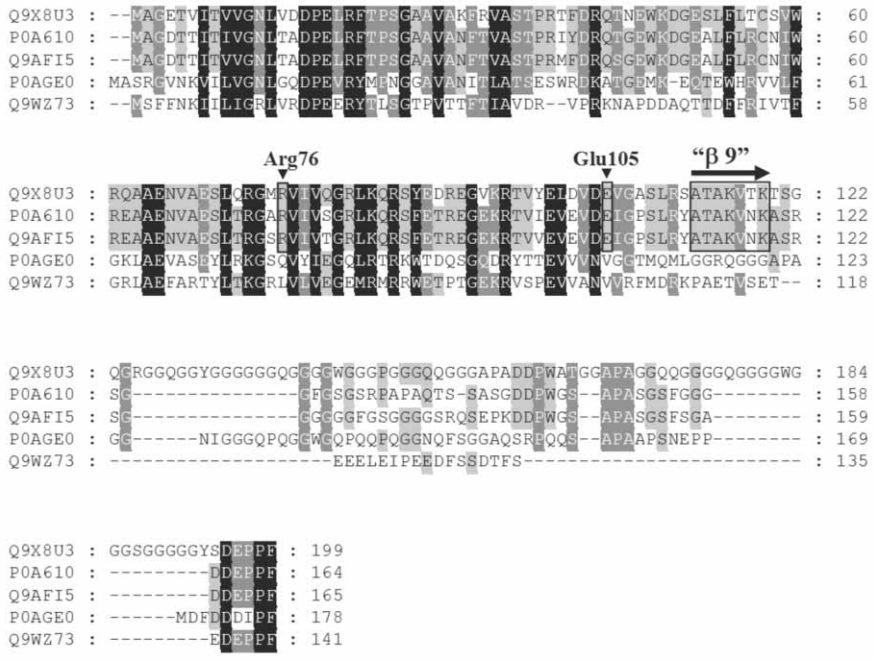

Figure 2. Multiple sequence alignment of SSBs in the following order: *S. coelicolor*, *M. tuberculosis* (PDB code 1UE1), *M. smegmatis* (PDB code 1X3E), *Escherichia coli* (PDB code 1QVC) and *Thermotoga maritima* (PDB code 1Z9F).

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.[8,10] SSBs of *Mycobacterium* [10] have a short sequence of highly conserved amino acid residues at the C-terminus of the OB-fold. Further, these residues compose an extra β-strand (strand 9), forming a hook-like structure in mycobacterial SSBs.[10] 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 dm^3 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/cm^3. 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/dm^3 HEPES-Na, pH = 7.5; 0.6 mol/dm^3 sodium dihydrogen phosphate; 0.6 mol/dm^3 potassium dihydrogen phosphate and 25 % (vol. ratio, ψ) anhydrous glycerol (condition 1) and of 0.085 mol/dm^3

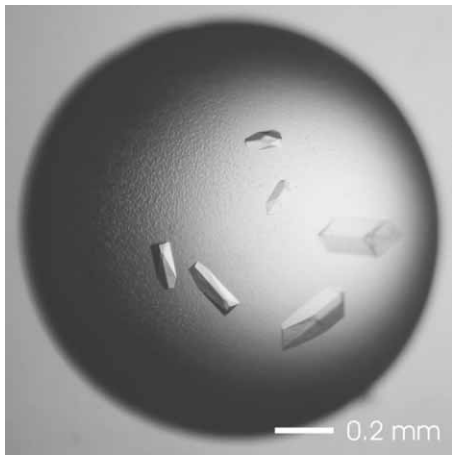

Figure 3. Morphology of the *S. coelicolor* SSB crystals.

HEPES-Na, pH = 7.5; 1.7 % (ψ) polyethylene glycol 400; 1.7 mol/dm^3 ammonium sulphate and 15 % (ψ) 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. coli*[15] 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/dm^3 TRIS-HCl, pH = 8.0; 2 mmol/dm^3 $CaCl_2$) 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/dm^3 trypsine 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 $\Delta\varphi = 1°$ oscillation frame. Dataset 3 was collected on a Siemens rotating anode generator, MAR300 image plate detector at Cu-Kα wavelength and $\Delta\varphi = 1°$ 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 $I222$ or $I2_12_12_1$ 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[17] coefficient ranges from 2.6–2.7 Å^3 Da^{-1}, which corresponds to a solvent content of 52–55 %. Data were processed using the HKL programme package[18] for dataset 1 and using the Mosflm[19] and Scala[20] programmes for datasets 2 and 3. Measured intensities were converted to structure-factor amplitudes using the Truncate[21] 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.

TABLE I. Crystallographic parameters and data-processing statistics for *S. coelicolor* SSB protein[(a)]

	Dataset 1	Dataset 2	Dataset 3
Crystallisation conditions	2	2	1
Crystal dimensions / mm	0.1 × 0.1 × 0.3	0.05 × 0.1 × 0.2	0.1 × 0.2 × 0.3
Resolution range / Å	50–3.5 (3.6–3.5)	50.44–3.8 (4.0–3.8)	40.5–3.7 (3.9–3–7)
Space group	$I222$ or $I2_12_12_1$	$I222$ or $I2_12_12_1$	$I222$ or $I2_12_12_1$
Unit cell / Å	a = 100.7	a = 100.8	a = 102.4
	b = 102.5	b = 102.1	b = 105.9
	c = 163.5	c = 164.2	c = 161.9
Total observations	37688 (5073)	30292 (4175)	62096 (8224)
Independent observations	10917 (1399)	7289 (1093)	9711 (1389)
Completeness / %	85.7 (78.2)	85.3 (88.5)	99.9 (100.0)
Multiplicity	4.0 (4.0)	4.2 (3.8)	6.4 (5.9)
Mean $I/\sigma(I)$	8.2 (3.4)	10.5 (4.4)	9.8 (4.0)
R_{merge}	0.14 (0.30)	0.12 (0.31)	0.23 (0.41)
Matthews coeff. / Å^3 Da^{-1}, M = 79.9 kDa	2.6 with 52.7 % solvent	2.6 with 52.8 % solvent	2.7 with 54.8 % solvent

[(a)] Values in parentheses refer to the highest resolution shell.

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SAŽETAK

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

Zoran Štefanić, Dušica Vujaklija, Luka Andrišić, Goran Mikleušević, Miha Andrejašič, Dušan Turk i Marija Luić

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 *ssb* iz bakterije *Streptomyces coelicolor* prekomjerno je eksprimiran u heterolognom domaćinu *E. coli* NM522. U samo jednom koraku, primjenom Ni^{2+} kelatne kromatografije, iz 1 dm^3 kulture dobiveno je 15 mg pročišćenog proteina. Ovaj protein pokazuje značajnu sličnost s proteinskim sekvencijama SSB proteina iz bakterija *Mycobacterum tuberculosi*s i *Mycobacterum smegmatis.* Očuvan aminokiselinski slijed koji kod mikobakterijskih SSB proteina čini dodatnu ß vrpcu, prisutan je i kod SSB proteina iz bakterije *S. coelicolor.* Cijeli SSB protein lako kristalizira u prostornoj grupi $I222$ ili $I2_12_12_1$ s dimenzijama jedinične ćelije a = 100,8, b = 102,1, c = 164,2 Å. Asimetrična jedinica najvjerojatnije sadrži četiri monomera, a sadržaj otapala je 52–55 %.