Tuberculosis 108 (2018) 16–25

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: www.elsevier.com/locate/tube

Molecular Aspects

Distinct properties of a hypoxia specific paralog of single stranded DNA binding (SSB) protein in mycobacteria

Amandeep Singh[a], M. Vijayan[a,**], Umesh Varshney[b,c,*]

[a] Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India
[b] Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India
[c] Jawaharlal Nehru Centre for Advamced Scientific Research, Jakkur, Bangalore 560064, India

ARTICLE INFO

Keywords:
DNA repair
Recombination repair
Hypoxia
RecA
SSBb
Stress response

ABSTRACT

In addition to the canonical Single Stranded DNA Binding (SSBa) protein, many bacterial species, including mycobacteria, have a paralogous SSBb. The SSBb proteins have not been well characterized. While in *B. subtilis*, SSBb has been shown to be involved in genetic recombination; in *S. coelicolor* it mediates chromosomal segregation during sporulation. Sequence analysis of SSBs from mycobacterial species suggests low conservation of SSBb proteins, as compared to the conservation of SSBa proteins. Like most bacterial SSB proteins, *M. smegmatis* SSBb (*Ms*SSBb) forms a stable tetramer. However, solution studies indicate that *Ms*SSBb is less stable to thermal and chemical denaturation than *Ms*SSBa. Also, in contrast to the 5–20 fold differences in DNA binding affinity between paralogous SSBs in other organisms, *Ms*SSBb is only about two-fold poorer in its DNA binding affinity than *Ms*SSBa. The expression levels of *ssbB* gene increased during UV and hypoxic stresses, while the levels of *ssbA* expression declined. A direct physical interaction of *Ms*SSBb and RecA, mediated by the C-terminal tail of *Ms*SSBb, was also established. The results obtained in this study indicate a role of *Ms*SSBb in recombination repair during stress.

1. Introduction

Mycobacterium tuberculosis is known to modulate its gene expression to adapt to the stress conditions encountered in the host cells and it may remain dormant in a non-replicating persistent (NRP) state, for decades in the host [1]. *M. tuberculosis* NRP is characterized by slow metabolism upon encountering hypoxia, starvation or acidic pH in the host cells [2–4]. DNA damages such as oxidation of nucleotides and DNA breaks, by host generated reactive oxygen species and reactive nitrogen intermediates threaten the genomic integrity during this dormant phase, necessitating a robust DNA repair in the early stages of exit from dormancy [5,6]. Recombination repair forms a major part of this repair system. Upon DNA break, RecA binds to the single stranded DNA (ssDNA) and stimulates autocatalytic cleavage of LexA, which regulates genes for damage repair and tolerance [7,8].

Single Stranded DNA Binding (SSB) proteins are highly conserved proteins implicated in protection of ssDNA from nucleases, and prevent formation of secondary structures during major DNA transactions [9,10]. During DNA break repair, SSBs play a crucial role in nucleoprotein filament formation and RecA loading on DNA [11–13]. Most bacterial SSBs are homo-tetramers, where tetramerization is mediated by the conserved N-terminal oligo-nucleotide binding (OB) domain followed by a less conserved, and highly disordered C-terminal domain or tail. The C-terminal tail of SSBs is known to interact with many other proteins involved in DNA repair, replication and recombination [14–18]. It has also been reported to have a role in modulating the DNA binding affinity [19,20].

Apart from the well-studied SSB (SSBa), several naturally competent bacteria possess a second SSB paralog (referred to as SSBb). SSB homologs are often encoded by nearly all conjugative plasmids found in bacteria. While SSBa (homologous to *E. coli* SSB) is attributed to perform all the canonical functions, the biological functions of the paralogous SSBb proteins are still ambiguous. In *Bacillus subtilis*, SSBb (*Bs*SSBb) shares the load of genetic recombination with SSBa and aids in natural-competence associated recombination [21]. In *Streptococcus pneumoniae*, SSBb (*Sp*SSBb) protects the naturally internalized ssDNA to improve the likelihood of crossover events [22]. Interestingly, deletion of *ssbB* gene in *Streptomyces coelicolor* leads to irregular chromosomal segregation during sporulation [23]. The members of genus mycobacteria, except for *M. leprae*, are also found to possess *ssbB* genes [24].

The interaction of DNA with the OB domain has been studied in detail in *E. coli* and is known to be sequence independent. However,

* Corresponding author. Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, Karnataka, India.
** Corresponding author.
E-mail address: varshney@iisc.ac.in (U. Varshney).

http://dx.doi.org/10.1016/j.tube.2017.10.002
Received 12 July 2017; Received in revised form 18 September 2017; Accepted 1 October 2017

depending on the concentration of the protein and ions in the solution, SSBs interact with DNA in either SSB_{35} (in which 35 nucleotides wrap around two subunits of the tetramer), or $SSB_{56/65}$ (in which 56/65 nucleotides wrap around all four subunits of the tetramer) modes [25,26]. The DNA binding affinities of the paralogous SSBs were found to be different. The *Sp*SSBb and *Sc*SSBb proteins were reported to have higher DNA binding affinity compared to their SSBa counterparts; although in the case of *B. subtilis* proteins the converse was observed [21,22].

On account of slow growth of *M. tuberculosis* and requirement of contained laboratory conditions, *M. smegmatis*, a fast growing mycobacterium, has often been used as a surrogate to study fundamental molecular processes in mycobacteria [27]. Sequence analysis of *M. tuberculosis* and *M. smegmatis* genomes suggests that the latter retains most of the genes involved in adaptation to hypoxia [28,29]. SSBa has been characterized from multiple mycobacterial species [30–33]. The three-dimensional structure of *M. smegmatis* SSBb (*Ms*SSBb), solved recently in our lab, revealed a classical homo-tetrameric structure [34]. The quaternary structure of *Ms*SSBb is highly similar to that observed for mycobacterial SSBa and *Sc*SSBa structures, retaining the 'clamp' like inter-subunit strand exchange [35,36]. To address the question of why there are two SSBs in mycobacteria, we have now studied DNA binding properties of *Ms*SSBb. We also performed the same experiments on *Ms*SSBa, for comparison of properties. Transcriptional regulation of the two *ssb* genes was tested and a possible role of SSBb in stress response has been established.

2. Materials and methods

2.1. Sequence analysis and DNA

The sequences of SSB proteins were obtained from Uniprot [37]. Multiple sequence alignment (MSA) of proteins was generated using three combined iterations, while keeping others settings as default, in Clustal Omega [38]. ESPript was used to generate the MSA [39]. M13 phage ssDNA was obtained from New England Biolabs (NEB). DNA oligomers, including 6FAM-tagged (fluorescein) were obtained from Sigma-Aldrich.

2.2. Cloning of SSBb open reading frames

Cloning of the gene encoding *Ms*SSBb (MSMEG_4701) has been described previously [34]. The gene encoding *Ms*SSBa (MSMEG_6896) was also cloned using a similar strategy. The details of the primers used are provided in Table S1. SSBbΔ7 and SSBbΔ27 constructs, with 7 and 27 residue truncations respectively, from the C-terminal tail of *Ms*SSBb were also made in pET14b vector. *Ms*RecA (MSMEG_2723) was cloned in pET His6 Sumo TEV LIC cloning vector (1S) (Addgene) using manufacturer's protocol. Cloning was confirmed by primer-based sequencing (Xcleris genomics).

2.3. Protein expression and purification

The expression constructs were transformed into *E. coli* BL21 (DE3) (Novagen Inc.). All SSBs, including the shorter constructs were purified as described previously [34]. For purification of RecA, cultures in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml) were grown for 8 h after induction, at 0.5 OD_{600}, with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 °C. The cells were harvested by centrifugation at 6000*g* for 15 min, re-suspended in buffer A (30 mM Tris-acetate, pH 7.5, 300 mM NaCl, 10 mM imidazole and 10% (v/v) glycerol) and lysed by sonication. The lysate was then centrifuged at 15,000 g for 60 min. The supernatant was loaded onto Ni-NTA column (GE Healthcare) equilibrated with buffer A, washed with the same buffer supplemented with 20 mM imidazole and eluted with a linear gradient of imidazole (30 mM–500 mM). The protein was dialyzed against 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.5 mM Na_2EDTA and 10% (v/v) glycerol for 12 h. The His-SUMO tag was cleaved using TEV protease (Sigma-Aldrich) for 6 h at 25 °C, in the same buffer. The protein was further purified by passing through Ni-NTA beads followed by size-exclusion chromatography using High load 16/600 Superdex 200 column (GE Healthcare) in buffer B (30 mM Tris-HCl, pH 7.5, 250 mM NaCl and 10% (v/v) glycerol). The purity was established by SDS-PAGE [Fig. S1]. *M. smegmatis* Ung (Uracil DNA glycosylase Family I) was purified as described previously [40].

2.4. Electrophoretic mobility shift assay (EMSA)

Standard reaction mixtures containing 200 ng M13 phage DNA or 2 μM fluorescein tagged DNA were incubated with increasing concentrations of SSBs (*Ms*SSBa or *Ms*SSBb) at 4 °C for 30 min. Different reaction buffers used are specified in the figure legends. Reaction was mixed with 4 μl loading dye [containing 0.12% (w/v) each of bromophenol blue and xylene cyanol FF in 20% glycerol], and resolved on 1% agarose gel, containing ethidium bromide, or 4–8% native PAGE in 1X TBE buffer. Agarose gels were visualized under UV light. The PAGE gels were visualized using fluorescein filter in the BIORAD gel doc system.

Interaction with Ung: Standard reaction mixtures (20 μl) containing 200 ng M13 ssDNA in 20 mM Tris-HCl, pH 8.0 and 50 mM NaCl were incubated with 500 nM *Ms*SSBa or *Ms*SSBb and increasing concentrations (500 nM–4 μM) of *Ms*Ung at 4 °C for 30 min. The reaction mixtures were mixed with 4 μl of loading dye and individual samples were loaded and resolved on a 0.8% agarose gel, containing ethidium bromide, in 1X TBE buffer.

2.5. Fluorescence measurements

Fluorescence measurements were made using a FP-6300 spectrometer from JASCO analytical instruments. A 10 μM solution of *Ms*SSBa or *Ms*SSBb was titrated against increasing concentrations of 35-mer DNA (poly-dT) and 76-mer DNA (poly-dT) and the quenching of fluorescence was recorded. Titrations were carried out in different buffers, as specified in figure legend and Results section. An excitation wavelength of 295 nm was used and emission spectra at 343 nm were recorded. After each titration, the solution was allowed to equilibrate for 1 min before the fluorescence was measured. Experiments were setup in duplicates and an average of 3 readings for each data point were used to plot the graph.

2.6. Thermal melt assay

Protein unfolding as a function of temperature was monitored using fluorophore SYPRO Orange (Sigma, S5692) in iQ5, BioRad iCycler Multicolor Real-Time PCR detection system. Increase in fluorescence of dye upon binding to the hydrophobic regions, upon thermal denaturation, was measured. Protein samples (10 μM) were mixed with buffer (20 mM Tris-HCl, pH 7.5 and 200 mM NaCl) and 1x SYPRO orange dye. The readings were taken in 96-well PCR microplates (BioRad) in the RT-PCR device. Samples were heated at 0.5 °C per min, ranging between 10 °C and 95 °C and the fluorescence intensity was measured using Cy5 filter with red-orange color intensity detection.

2.7. Chemical melt assay

A decrease in intrinsic tryptophan fluorescence was recorded as a function of chemical denaturation by guanidine hydrochloride (GndCl). Protein samples (10 μM) were mixed with increasing concentrations of GndCl (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 4.5, 5.0, 5.5 and 6 M) in 10 mM Tris-HCl, pH 7.5 and incubated for 30 min at 37 °C.