CHARACTERISATION OF THREE SINGLE STRANDED DNA BINDING (SSB)-LIKE PROTEINS FROM THE NITROGEN-FIXING CYANOBACTERIUM ANABAENA PCC7120

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Abstract

*Anabaena sp.* strain PCC 7120 is a filamentous, nitrogen-fixing radioresistant cyanobacterium with a robust DNA repair system. Of the several DNA repair proteins involved in maintaining the integrity of genome, single stranded DNA binding (SSB) protein plays a central role. Unlike most other bacteria, *Anabaena* 7120, has three genes possibly coding for SSB-like protein, namely *alr0088* and *alr7579* (both annotated as *ssb*) and *all4779* (annotated as a hypothetical protein), and designated as *ssb1*, *ssb2* and *ssb3* respectively. While the 13 kDa *ssb1* and 14 kDa *ssb2* proteins have only the N-terminal oligonucleotide binding (OB)-fold region corresponding to bacterial SSBs, *all4779* possesses all three regions, namely N-terminal OB-fold, C-terminal acidic tail separated by a Proline/Glycine-rich linker, typical of bacterial SSBs. In fact the *ssb1* and *ssb2* proteins are the smallest reported SSB-like proteins among prokaryotes and eukaryotes. Owing to the OB-fold, all three *Anabaena* SSB proteins bind single-stranded DNA (ssDNA), but differ in their binding affinity and length of ssDNA bound. Expression of all three SSB proteins in *Anabaena* indicates that these are functional genes and possibly have distinct physiological roles.

Introduction

Single stranded DNA (ssDNA) binding proteins (SSBs) are essential for all DNA metabolic processes, such as DNA replication, recombination as well as repair for genome maintenance (Shereda et al, 2008), all of which require presence of ssDNA. This is achieved by the non-specific binding of SSB protein to ssDNA, thereby preventing them from forming secondary structures. These are ubiquitous proteins, present across all organisms from viruses to bacteria to humans, show significant degree of homology in the functional domains and are essential for cell survival. A typical bacterial SSB protein has three domains, the N-terminal oligonucleotide-binding (OB)-fold for interacting with ssDNA, a C-terminal acidic tail to interact with other proteins, and a proline/glycine (P/G)-rich linker separating the positively charged N-terminus from the negatively charged C-terminus (Lohman and Ferrari 1984). The P/G-rich region results in kinks in the ordered structure of protein allowing better spatial separation of oppositely charged ends (Belts and Russell, 2003). Though SSB protein is functionally, biochemically and structurally well studied in other organisms including the radioresistant *Deinococcus* (Eggington et al 2004; Basu and Apte 2012), very little is known about them in cyanobacteria. The photosynthetic nitrogen-fixing *Anabaena* 7120, exhibits high tolerance towards radiation stresses (LD$_{50}$ = 6 kGy) (Singh et al, 2011). This offers an advantage over *Deinococcus*, since *Anabaena* can survive with minimal nutritional assistance, requiring mainly sunlight and air. Thus, as a first step in this direction the three SSB proteins of *Anabaena*, central to all DNA metabolism, were characterised. The presence of multiple SSB-like proteins, which is rare in bacteria, raises several questions, such as whether they are real or pseudo genes, whether they are expressed *in vivo* or not,
differences in their biochemical activities and role of each of these proteins in *Anabaena*.

**Results and Discussion**

Schematic representation of the domains of the three SSB proteins of *Anabaena* 7120 indicated termination of the protein sequence immediately after the N-terminal OB-fold in SSB1 and SSB2 (Fig. 1). The SSB3 protein, on the other hand, exhibited all three domains and was as large as *E. coli* SSB (Fig. 1). Thus, SSB1 and SSB2 are the smallest reported SSB proteins till date. The smallest prokaryotic SSB reported earlier was 140 amino acid long SSB from *Thermatoga* sp. (Olszewski et al, 2010) and the 130 amino acid long human mitochondrial SSB (Curth et al, 1994). The amino acid sequence of the three *Anabaena* SSB proteins bear low homology and thus could not have arisen by gene duplication.

**Single Stranded DNA Binding Protein**

<table>
<thead>
<tr>
<th>Domain</th>
<th>Starting Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>OB-fold</td>
<td>1</td>
</tr>
<tr>
<td>spacer region</td>
<td>100</td>
</tr>
<tr>
<td>acidic tail</td>
<td>185</td>
</tr>
<tr>
<td>ssDNA binding</td>
<td>37</td>
</tr>
<tr>
<td>P/G-rich flexibility</td>
<td>119</td>
</tr>
<tr>
<td>interaction with protein</td>
<td>127</td>
</tr>
<tr>
<td>E. coli SSB (178 aa)</td>
<td></td>
</tr>
<tr>
<td>SSB1 (119 aa)</td>
<td></td>
</tr>
<tr>
<td>SSB2 (127 aa)</td>
<td></td>
</tr>
<tr>
<td>SSB3 (182 aa)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1: Domain organization of *Anabaena* and *E. coli* SSB proteins. The Blue box indicates the OB-fold, the yellow box indicates the P/G-rich region and the magenta box indicates the acidic tail. The region in SSB1 and SSB2 beyond the OB-fold is indicated as an orange box.

Presence of OB-fold is indicative of the ability to bind to ssDNA. To validate this bioinformatically obtained information, the three *Anabaena* SSB proteins were characterised *in vitro* for their ability to bind ssDNA. To obtain pure proteins for biochemical analysis, the respective genes were obtained from the genomic DNA of *Anabaena* 7120 by Polymerase Chain Reaction-based amplification using gene specific primers. Design of such DNA primers was facilitated by the availability of the entire genome sequence of *Anabaena* 7120 (http://genome.microbedb.or.jp/cyanobase). DNA sequencing of these fragments confirmed amplification of the desired genes and the DNA sequences were submitted to GENBANK (Accession Nos. GU225949, GU225950 and GU225951). These DNA fragments were cloned in an expression vector, pET16b, which provides a His\(_6\)-tag to the N-terminal of the overexpressed protein. This assists in affinity based purification of proteins. Overexpression of the protein coded by the cloned gene was achieved in *E. coli* using Isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer. The overexpressed protein was purified from the recombinant *E. coli* cell lysate by Ni-NTA based affinity chromatography (Fig. 2A), and the purified protein was used to generate polyclonal antibodies. Analysis of *Anabaena* cell free extracts with these antibodies indicated that all three SSB proteins expressed in *Anabaena*, and thus are all functional genes.

![Purification and Glutaraldehyde cross-linking of Anabaena SSB proteins. (A) The three SSB proteins of Anabaena were purified by Ni-NTA chromatography. (B) Native SSB proteins were cross-linked with glutaraldehyde. The proteins were electrophoretically separated on SDS-polyacrylamide gel and stained with Coomassie brilliant blue G-250. Molecular mass of different molecular form is indicated.](http://)
Binding of SSB to ssDNA requires the availability of at least two OB-folds, while the wrapping around of the ssDNA by SSB requires simultaneous availability of four OB-folds. This is aided by the tetramerisation of bacterial SSB proteins (Lohman and Ferrari, 1984), or dimerisation of Deinococcal SSB protein, which has two OB-folds per monomer (Eggington et al, 2004). HPLC based size determination of the Anabaena SSB proteins in their native state indicated formation of a dimer of 26 and 27 kDa for SSB1 and SSB2 respectively, while the SSB3 was found to exist as a monomer of about 20 kDa. Further confirmation of the oligomeric status of Anabaena SSB proteins was achieved by allowing cross-linking of the individual purified Anabaena SSB proteins using glutaraldehyde followed by analysis by SDS-polyacrylamide gel electrophoresis. The existence of the dimeric forms in addition to the monomeric forms of SSB1 and SSB2 was further confirmed, while a dimeric form of about 41 kDa was also detected for SSB3 (Fig. 2B). Thus, Anabaena SSB proteins exhibit an unique feature in existing as a dimer.

The ability of the dimeric SSB proteins of Anabaena to bind ssDNA was evaluated by (i) Electrophoretic Mobility Shift Assay (EMSA) and (ii) Fluorescence Quenching techniques using ssDNA as substrate. In EMSA, binding of the SSB protein with ssDNA (digoxigenin-labeled 27 base long ssDNA) would result in retardation in the mobility of the ssDNA compared to the free DNA. Decreased mobility of the 27-mer ssDNA was observed in the presence of SSB1 and SSB3, but not SSB2 even at high concentrations of the proteins (Fig. 3). This indicated that the OB-fold of SSB1 and SSB3 was active, but does not discount that of SSB2, since it may require different binding conditions.

To assess this further, a quantitative estimation of binding affinity was carried out using fluorescence quenching technique. The presence of aromatic amino acids, such as tyrosine, tryptophan and phenylalanine render a characteristic fluorescence pattern for each protein, when excited at a particular wavelength based on the spatial arrangement of these amino acids within the protein. And when the protein interacts with DNA, the changes in conformation are reflected by decrease in the intrinsic protein fluorescence, which is measured as fluorescence quenching. In this experiment, two kinds of substrate were used, poly(dT) or 7 kb long M13 ssDNA, as shown in Fig. 4.

The ability of the Anabaena SSB proteins to bind ssDNA was quantitatively analysed by determining the binding affinity using fluorescence quenching technique. Small oligonucleotides (30-100 bases) and the 7 kDa M13 ssDNA were used as substrate. Quenching of fluorescence of SSB1 and SSB3 was achieved with short oligonucleotides (polydT) (Fig. 4A) as well as long M13 ssDNA (Fig. 4B), while that of SSB2 could be achieved with only M13 ssDNA (Fig. 4B).
The fluorescence quenching experiments showed that the SSB3 protein has maximum affinity to ssDNA. Due to the presence of the C-terminal acidic tail, which would allow it to interact with other DNA binding proteins, it may be the true or real functional SSB protein of *Anabaena*, instead of the other two annotated but truncated SSB proteins. Of the two naturally C-terminal truncated *Anabaena* SSB proteins, SSB1 binds short ssDNA preferably, while SSB2 prefers long ssDNA. This indicates different physiological roles for all three proteins in *Anabaena*. Instead of a single SSB protein catering to the various needs of SSB for replication, repair and recombination, the ancient organism *Anabaena*, synthesises three different proteins, possibly for each of these distinct roles. The *in vivo* functional roles of the three SSB proteins is currently under investigation.

Fig. 4: Measurement of change in fluorescence of SSB proteins in the presence of ssDNA. Fluorescence of SSB proteins was measured in the presence of increasing concentration of (A) poly (dT) or (B) 7 kb M13 ssDNA.

References