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*Review*

# Single-stranded DNA-binding proteins (SSBs) — sources and applications in molecular biology*

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**Single-stranded DNA-binding proteins (SSBs) play essential roles in DNA replication, recombination, and repair in bacteria, archaea and eukarya. The SSBs share a common core ssDNA-binding domain with a conserved OB (oligonucleotide/oligosaccharide binding) fold. This ssDNA-binding domain was presumably present in the common ancestor to all three major branches of life. In recent years, there has been an increasing interest in SSBs because they are useful for molecular biology methods and for analytical purposes. In this review, we concentrate on recent advances in the discovery of new sources of SSBs as well as certain aspects of their applications in analytical sciences.**

**Keywords:** SSB, DNA replication, recombination, PCR, *Escherichia coli*, *Thermus aquaticus*, *Thermus thermophilus*

Single-stranded DNA-binding proteins (SSBs) are indispensable elements in cells of all living organisms. Most SSBs bind non-specifically to single-stranded DNA (ssDNA), conferring a regular structure upon it, which is recognised and exploited by a variety of enzymes involved in the essential biological processes. SSBs are usually present in stoichiometric quantities with respect to the ssDNA substrate, and protect the transiently formed ssDNA against nuclease attack, also preventing the formation of secondary structures (Perales *et al*., 2003). In such a manner, SSB-binding proteins participate in all processes involving ssDNA such as replication, repair and recombination (Lohman & Overman, 1985; Greipel *et al*., 1989; Meyer & Laine, 1990; Moore *et al*., 1991; Alani *et al*., 1992).

## APPLICATIONS OF SINGLE-STRANDED DNA-BINDING PROTEINS

In recent years, there has been an increasing interest in SSBs because they find numerous applications in diverse molecular biology and analytical methods (Table 1). There are many natural and recombinant sources of SSBs. As a consequence of the increasing importance of such proteins, heterologous expression systems may be useful in providing large amounts of pure SSBs (Williams *et al*., 1983; Lohman *et al*., 1986; Dąbrowski *et al*., 1999; 2002b). Production from recombinant expression systems has several distinct advantages over production from natural sources. It is rapid and yields are much higher than using cultures of the original organism.

There are an increasing number of studies which report the usefulness of SSBs for the polymerase chain reaction (PCR). A number of modifications to the basic PCR format have been developed in an attempt to increase amplification efficiency and specificity. It was shown that the use of a native ssDNA-binding protein, gene 32 protein from bacteriophage T4 or SSB from *Escherichia coli*, increases amplification efficiency with a number of diverse templates (Rapley, 1994; Dąbrowski & Kur, 1999).

The SSB-encoding genes from *Thermus aquaticus* and *T. thermophilus* have been cloned and *Tth*SSB and *Taq*SSB proteins overexpressed in *E. coli* were easily prepared in milligram quantities and characterized (Dąbrowski *et al*., 2002a; 2002b). Based on their characteristics, the presence of SSBs during DNA replication is likely to improve the efficiency of PCR. SSBs of thermophilic origin would be ideal

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**Abbreviations:** CE, capillary electrophoresis; OB., oligonucleotide/oligosaccharide binding; PCR, polymerase chain reaction; RPA, replication protein A; SSB, single-stranded DNA-binding protein; ssDNA, single-stranded DNA.

candidates for such an application due to their high thermostability. *Thermus* SBBs increase amplification efficiency with a number of diverse templates (Dąbrowski *et al*., 2002a; 2002b; Perales *et al*., 2003).

Recently, the application of an SSB-like protein from *T. aquaticus* (*Taq*SSB) in multiplex PCR identification of human Y-STR markers was demonstrated (Olszewski *et al*., 2005). The use of thermostable *Taq*SSB prevents or reduces primer dimer formation, one of the problems known to cause inhibition of primer hybridization to the template and reduction of the number of primer molecules available for annealing.

The use of SSB proteins may prove to be generally applicable in improving PCR efficiency. It was also demonstrated that thermostable SSBs stimulate the mean rate of DNA synthesis by both bacterial and archaeal DNA polymerases, and the fidelity of the proof-reading-free DNA polymerase from *T. thermophilus* (Perales *et al*., 2003). The thermostable *Tth*SSB also interacts efficiently with RNA, allowing a dramatic increase in the size of the cDNA synthesised by the reverse transcriptase activity of *T. thermophilus* DNA polymerase (Perales *et al*., 2003). Inactivation assays demonstrated that *Tth*SSB can withstand short heating periods at 94°C, suggesting that it could be used to stimulate the activity of thermostable DNA polymerases at high temperatures. In fact, the presence of *Tth*SSB shortened the elongation time required to synthesise a specific DNA fragment by *Tth* DNA polymerase (Perales *et al*., 2003).

A large number of proteins involved in DNA replication, DNA damage control, DNA repair, and gene expression are capable of binding DNA and RNA with different affinities and sequence specificities. This ability of the DNA- and RNA-binding proteins has a yet-to-be realized potential in analytical sciences. It is suggested that they can be used as highly efficient and versatile tools in analyses of DNA, RNA, and proteins. It has been demonstrated that SSBs can facilitate quantitative analyses of DNA, RNA, and proteins in gel-free capillary electrophoresis (CE) (Drabovich & Krylov, 2004). The application of SSB-mediated gel-free CE for analyses of PCR products has also been reported (Drabovich & Krylov, 2004). The unique ability of SSB to bind ssDNA but not double-stranded DNA (dsDNA) allows efficient separation of three types of DNA molecules in the PCR reaction mixture: primers, products (amplified templates), and by-products, which originate from non-specific DNA hybridization. The ability of the method to distinguish between products and by-products makes it an indispensable tool in preparative PCR (e.g., in the development of nucleotide aptamers).

The affinity of SSB towards ssDNA has been successfully utilized for the detection of hybridization on gold surface with surface plasmon resonance imaging by Brockman *et al*. (1999). *In situ* surface plasmon resonance difference images clearly showed that significant binding of the protein occurred at the array locations with covalently bound single-stranded oligonucleotides, whereas very little binding occurred at the array locations which contained double-stranded hybrids. A novel hybridization detection protocol, which combines the advantages of the recognition ability of a protein and the electrochemical activity of metal nanoparticles, was also described (Kerman *et al*., 2004). SSB-coated Au nanoparticles accumulate on the probe-modified

**Table 1. Use of SSBs in molecular biology methods**

| SSB protein | Applications | Function | References |
|---|---|---|---|
| *Eco*SSB | PCR | Increased amplification efficiency | Rapley, 1994<br>Dąbrowski & Kur, 1999 |
| | Analyses of DNA, RNA and proteins in gel-free capillary electrophoresis | Quantitative analyses separation of DNA molecules in PCR reaction mixture | Drabovich & Krylov, 2004 |
| | Hybridization | Detection of hybridization products | Brockman *et al*., 1999<br>Kerman *et al*., 2004 |
| gp32 of T4 bacteriophage | PCR | Increased amplification efficiency | Rapley, 1994 |
| *Taq*SSB | PCR | Increased amplification efficiency | Dąbrowski *et al*., 2002b<br>Perales *et al*., 2003 |
| | Multiplex PCR | Prevention of primer dimer formation | Olszewski *et al*., 2005 |
| *Tth*SSB | PCR | Increased amplification efficiency stimulation of fidelity and the mean rate of DNA synthesis | Dąbrowski *et al*., 2002b<br>Perales *et al*., 2003 |
| | RT-PCR | Increased size of cDNA | Perales *et al*., 2003 |

electrode, then give rise to an amplified Au oxidation signal. This high signal of Au nanoparticles significantly lowered the detection limit of the target DNA. Thus, it can be concluded that custom fabrication of an electrochemical DNA chip based on SSB interactions, and also the design of new synthetic DNA-binding proteins for hybridization detection are promising tasks for future point-of-care tests.

We predict that many single-stranded DNA-binding proteins will find applications in analytical sciences.

## SOURCES OF SINGLE-STRANDED DNA-BINDING PROTEINS

SSB proteins, which are present in all three branches of organisms and in viruses, share sequences as well as biochemical and structural characteristics. In their soluble form, SSBs are found in different oligomeric states. They are found in different organisms as homodimers (SSBs from bacteriophages, *T. thermophilus*, *T. aquaticus* and *Deinococcus radiodurans*), heterotrimers (euryarchaeal and eukaryotic SSBs, alias RPAs) and homotetramers (mitochondrial, crenarchaeal and most prokaryotic SSBs) (Williams & Koningsberg, 1978; Shamoo *et al.*, 1995; Stassen *et al*., 1995; Webster *et al*., 1997; Wadsworth & White, 2001; Dąbrowski *et al*., 2002a; Eggington *et al.*, 2004). Although the sequences of SSB family members are highly variable, two common functional themes have emerged that link this class of proteins across evolution. The first is that SSB proteins use a conserved domain called an oligonucleotide/oligosaccharide-binding (OB) fold to bind ssDNA (Murzin, 1993). OB domains bind ssDNA in a cleft formed primarily by β-strands, by using aromatic residues that stack against nucleotide bases, and positively charged residues that form ionic interactions with the DNA backbone (Shamoo *et al*., 1995; Bochkarev *et al*., 1997; Raghunathan *et al*., 2000; Matsumoto *et al*., 2000). The second common feature of SSB proteins is obligate oligomerization that brings together four DNA-binding OB folds.

### Homotetrameric SSBs

Most prokaryotic SSBs, along with mitochondrial SSBs, form homotetrameric structures. SSB from *E. coli* (*Eco*SSB) is one of the first discovered SSBs and it has been studied the most extensively (Sigal *et al*., 1972). The *Eco*SSB monomer consists of 177 amino acids and has a molecular mass of about 19 kDa (Weiner *et al.*, 1975); it comprises two parts: the N-terminal fragment (about 120 amino acids), rich in α-helices and β-sheets, and the less structured C-terminal one (Sancar *et al.*, 1981). The N-terminal fragment contains the DNA-binding domain (OB fold). The function of the C-terminal fragment has not been characterized yet. However, we know that this fragment is neither essential in DNA binding nor in tetramer formation. At the end of the C-terminus there is an acidic region of ten amino acids, containing four aspartate residues, highly conserved among prokaryotic SSB proteins (Williams *et al*., 1983). The negative charges of these last ten amino acids weaken the binding of *Eco*SSB to DNA. However, this acidic region is essential for *in vivo* function — it is probably responsible for interactions with other proteins. Probably the region between the DNA-binding domain and the acidic region functions only as a spacer, keeping the negative charges away from the DNA bound to SSB (Curth *et al.*, 1996). Many other prokaryotic homotetrameric SSBs, such as *Mtu*SSB from *Mycobacterium tuberculosis* (Purnaparte & Varshney, 1999) and SSB from *Pseudomonas aeruginosa* (Genschel *et al.*, 1996) have been discovered recently. A single OB fold per monomer, with the active form of the protein as a homotetramer with four OB folds, appears to define a structural paradigm for bacterial SSB family proteins because all but three of the >250 currently identified bacterial *ssb* genes encode proteins with a single OB fold.

It has been noticed that *Eco*SSB can form different types of complexes with single-stranded nucleic acids, depending on the length of DNA bound by the protein. The ssDNA or RNA site size bound by *Eco*SSB basically depends on NaCl concentration. Poly(dT) forms two types of complexes with *Eco*SSB. $(SSB)_{33}$ complex is formed below 10 mM NaCl and $(SSB)_{65}$ complex — above 0.2 M NaCl (up to 5 M). Between 10 mM and 0.2 M NaCl the site size expands continuously with the concentration of NaCl. In the $(SSB)_{33}$ complex the nucleic acid interacts with two protomers of the tetramer whilst in the $(SSB)_{65}$ complex the nucleic acid interacts with all four protomers. Probably SSB may use both binding modes for one of its functions (DNA replication, recombination and repair). *In vivo* changes in the ionic strength may play an important role in regulation of some of these processes (Lohman & Overman, 1985). There are at least two other types of *Eco*SSB complexes with single-stranded nucleic acids — $(SSB)_{40}$ and $(SSB)_{56}$ (Lohman & Bujalowski, 1994). The type of SSB complex with the nucleic acid, besides the concentration of NaCl, depends also on other factors such as the concentration of other monovalent salts (both cations and anions), bivalent cation concentration, pH, temperature, and concentration of the SSB (Bujalowski *et al*., 1988).

Like most prokaryotic SSBs, also SSBs from mitochondria form stable homotetramers — human mitochondrial SSB (*Hs*mtSSB) can be an example. In spite of the relatively low sequence identity with *Eco*SSB (36%) *Hs*mtSSB has a high degree of structural similarity (Webster *et al.*, 1997).

Crenarchaeal SSB proteins, such as SSB from *Sulfolobus solfataricus*, are monomers in solution and multimers (probably tetramers) in complexes with DNA. The *Sulfolobus* OB-fold domain responsible for DNA binding is clearly related most closely to other archaeal SSB domains, the C-terminal fragment has similarities to eubacterial SSBs, playing probably a role in protein–protein interactions (Richard *et al*., 2004). *Sulfolobus* SSB lack also the zinc finger motif found in the eukaryal and euryarchaeal proteins. DNA binding is not highly cooperative (Wadsworth & White, 2001).

**Heterotrimeric SSBs**

In eukaryotes, replication protein A (RPA) acts as an SSB protein. RPA family members are heterotrimeric and contain six OB folds, four of which bind DNA. In terms of domain organization, archaeal SSB proteins are divided into two groups: those that resemble bacterial SSBs (found in crenarchaea) and those that resemble RPA (found in euryarchaea). Exceptions to the general four-OB-fold rule exist outside of cellular SSB family members, including bacteriophage and viral SSB proteins.

The human RPA (*hs*RPA) has been identified as the first eukaryotic replication protein A (Wold *et al*., 1997). This protein consists of three different subunits of molecular masses of 70, 32 and 14 kDa. The largest subunit, RPA70, consists of an N-terminal fragment, which interacts with other proteins, a central fragment which binds to ssDNA (residues 181–422), and a C-terminal fragment, which binds with subunits RPA32 and RPA14 and contains an evolutionarily conserved zinc finger motif (Pfuetzner *et al*., 1997; Lin *et al*., 1998). The ssDNA-binding domain consists of two structurally homologous subdomains oriented in tandem. The ssDNA lies in a channel extending from one subdomain to the other (Bochkarev *et al*., 1997). The ssDNA-binding site size comes to 6, 12 or 17–19 deoxynucleotides. Two alternative kinds of complexes of RPA with ssDNA are formed, probably depending on the salts' concentration (Pfuetzner *et al*., 1997).

The RPA32 subunit carries the third functional ssDNA-binding site, which is phosphorylated in a cell-cycle-dependent manner (Henricksen *et al*., 1996; Bochkareva *et al*., 1998). RPA14 has an additional, putative ssDNA-binding motif. However, there is no direct evidence for DNA binding by this subunit. Those four ssDNA-binding motifs and the motifs in SSBs share a significant degree of sequence homology.

Euryarchaeal SSBs form also heterotrimeric structures. SSB from *Methanococcus jannaschii* (*Mja*SSB) can be an example. This protein has a significant amino-acid sequence similarity to the eukaryotic RPAs but not to *Eco*SSB. It contains four tandem repeats of the conserved core ssDNA-binding domain. On the basis of sequence comparisons it has been suggested that *Mja*SSB may contain four core ssDNA-binding domains distributed over three subunits (Kelly *et al*., 1998). It also contains, like other euryarchaeal SSBs, a putative zinc finger motif near the C-terminus (Chedin *et al*., 1998). Euryarchaeal SSBs can also exist as monomers and heterodimers (Chedin *et al*., 1998).

**Homodimeric SSBs**

Although virtually all bacterial SSB family members act as homotetramers, recent discoveries have shown that SSB proteins from the *Deinococcus-Thermus* genera of bacteria adopt a different architecture. Bacteria from this group thrive in extreme environments that would kill most cells (desiccation, severe DNA-damaging and/or high-temperature conditions) by using mechanisms that are presently unclear. One distinguishing DNA-metabolic feature of *Deinococcus-Thermus* bacteria is that their SSB proteins are homodimeric, with each SSB monomer encoding two OB folds linked by a conserved spacer sequence (Dąbrowski *et al*., 2002a; Eggington *et al*., 2004; Bernstein *et al*., 2004). *T. thermophilus* and *T. aquaticus* SSBs were the first homodimeric SSBs identified and characterized (Dąbrowski *et al*., 2002a; 2002b). These proteins (*Tth*SSB and *Taq*SSB) and their counterpart from *D. radiodurans* (*Drad*SSB) are the largest bacterial SSBs and consist of 263, 264 and 301 amino-acid residues, with molecular masses of about 29.9, 30 and 32.6 kDa, respectively (Dąbrowski *et al*., 2002a; 2002b; Eggington *et al*., 2004; Witte *et al*., 2005). The monomers of these proteins have two putative ssDNA-binding sequences: N-terminal (in *Tth*SSB and *Taq*SSB located in the region from amino acids 1 to 123) and C-terminal (located between amino acids 124 and 263 or 264 in *Tth*SSB and *Taq*SSB, respectively) (Dąbrowski *et al*., 2002a; 2002b). The structure of *Drad*SSB is known and it indicates that the two ssDNA-binding domains are connected by a β-harpin linker (Bernstein *et al*., 2004). The C-terminal domain has nearly all of the amino-acid residues that would be predicted to bind ssDNA based on the *Eco*SSB–ssDNA complex, but the N-terminal domain does not retain a number of potentially important ssDNA-binding residues (Bernstein *et al*., 2004). The differences between these two ssDNA-binding domains impose an asymmetry that is likely to affect the DNA binding properties and other functions of each domain (Bernstein *et al*., 2004). Homodimeric SSBs, contrary to tetrameric SSB proteins, possess only two C-terminal tails in each active form. Reducing the number of C-terminal tails by half could dramatically affect the function of the two-OB-fold-containing SSB proteins *in vivo*. Other acidic areas of the *D. radiodurans* protein could act in place of the tails to make these interactions, or the protein

could form the necessary complexes by using only two acidic tails as mediators (Dabrowski *et al*., 2002; Bernstein *et al*., 2004). These SSBs may represent an evolutionary convergence between homotetrameric bacterial/crenarcheal SSB and eukariotic/euryarcheal RPA family members (Bernstein *et al*., 2004).

Probably the fusion of two OB folds linked by a conserved spacer sequence is an adaptation of the *Deinococcus-Thermus* genera to extreme conditions (Dąbrowski *et al*., 2002a; Bernstein *et al*., 2004).

**Other SSBs**

The product of T4 gene 32 (gp32) was discovered as the first protein from the SSB family (Alberts & Frey, 1970). The molecular mass of the native protein is 35 kDa. It binds cooperatively to ssDNA and one protein molecule binds about ten single-stranded DNA nucleotides. gp32 exists mainly as a dimer or higher aggregates at concentrations above 0.1 mg/ml even in the absence of DNA (Williams & Koningsberg, 1978). Probably there are two kinds of gp32 dimers: one — heterologous intermediate in the indefinite aggregation process which should bind oligonucleotides and the other — an isologous self-limited dimer with very little affinity for oligonucleotides. Only one of those conformations is suitable for cooperative protein–protein interactions between neighboring gp32 molecules (Kelly & von Hippel, 1976).

Recently it was discovered that the *Bacillus subtilis* genome contains two paralogous single-stranded DNA-binding protein genes, *ssb* and *ywpH* (Lindner *et al*., 2004). The main *ssb* gene is strongly expressed during exponential growth and is flanked by the *rpsF* and *rpsR* genes, coding for the ribosomal protein S6 and S18, respectively, that coregulate the *ssb* gene (Lindner *et al*., 2004). The gene organization *rpsF-ssb-rpsR*, as found in *B. subtilis*, is also present in many other bacteria, but not in *E. coli* (Lindner *et al*., 2004). The *ssb* gene is necessary for cell viability, and its expression is elevated during SOS response (Lindner *et al*., 2004). However, the paralogous *ywpH* gene has its own promoter, and the product of this gene is expressed during stationary phase in minimal medium only (Lindner *et al*., 2004). In view of that discovery bacteria can be divided into four groups based on *ssb* gene organization: group I contains bacteria with the same *ssb* organisation as *B. subtilis* (*rpsF-ssb-rpsR*, multiple SSB paralogues); group II contains bacteria with the same *ssb* gene organization as group I but without multiple SSB paralogues; group III contains bacteria with the same *ssb* gene organization as *E. coli* (*uvrA-ssb*, only one *ssb* gene); group IV contains bacteria with *ssb* neither placed between *rpsF* and *rpsR* nor divergently located to *uvrA* (Lindner *et al*., 2004).

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