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Escherichia coli single-stranded DNA-binding protein: nanoESI-MS studies of salt-modulated subunit exchange and DNA binding transactions

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Abstract

Single-stranded DNA-binding proteins (SSBs) are ubiquitous oligomeric proteins that bind with very high affinity to single-stranded DNA and have a variety of essential roles in DNA metabolism. Nanoelectrospray ionization mass spectrometry (nanoESI-MS) was used to monitor subunit exchange in full-length and truncated forms of the homotetrameric SSB from *Escherichia coli*. Subunit exchange in the native protein was found to occur slowly over a period of hours, but was significantly more rapid in a truncated variant of SSB from which the eight C-terminal residues were deleted. This effect is proposed to result from C-terminus mediated stabilization of the SSB tetramer, in which the C-termini interact with the DNA-binding cores of adjacent subunits. NanoESI-MS was also used to examine DNA binding to the SSB tetramer. Binding of single-stranded oligonucleotides [one molecule of (dT)₇₀, one molecule of (dT)₃₅, or two molecules of (dT)₃₅] was found to prevent SSB subunit exchange. Transfer of SSB tetramers between discrete oligonucleotides was also observed and is consistent with predictions from solution-phase studies, suggesting that SSB-DNA complexes can be reliably analyzed by ESI mass spectrometry.

Keywords

exchange, subunit, modulated, salt, studies, ms, nanoesi, protein, transactions, binding, escherichia, dna, stranded, single, coli, CMMB

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***Escherichia coli* Single-Stranded DNA-Binding Protein: NanoESI-MS Studies of Salt-Modulated Subunit Exchange and DNA Binding Transactions**

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Running Title: ESI-MS of Single-Stranded DNA-Binding Protein

Abstract

Single-stranded DNA-binding proteins (SSBs) are ubiquitous oligomeric proteins that bind with very high affinity to single-stranded DNA and have a variety of essential roles in DNA metabolism. Nanoelectrospray ionization mass spectrometry (nanoESI-MS) was used to monitor subunit exchange in full-length and truncated forms of the homotetrameric SSB from *Escherichia coli*. Subunit exchange in the native protein was found to occur slowly over a period of hours, but was significantly more rapid in a truncated variant of SSB from which the eight C-terminal residues were deleted. This effect is proposed to result from C-terminus mediated stabilization of the SSB tetramer, in which the C-termini interact with the DNA-binding cores of adjacent subunits. NanoESI-MS was also used to examine DNA binding to the SSB tetramer. Binding of single-stranded oligonucleotides [one molecule of (dT)₇₀, one molecule of (dT)₃₅ or two molecules of (dT)₃₅] was found to prevent SSB subunit exchange. Transfer of SSB tetramers between discrete oligonucleotides was also observed and is consistent with predictions from solution-phase studies, suggesting that SSB-DNA complexes can be reliably analyzed by ESI mass spectrometry.

Key words: nanoelectrospray ionization mass spectrometry; subunit exchange; single-stranded DNA-binding protein (SSB); DNA-protein complex; DNA replication

Introduction

Single-stranded DNA binding (SSB) proteins are essential in organisms from all domains of life [1-5]. They provide protection against nucleases and prevent reannealing and formation of secondary structures in the single-stranded DNA (ssDNA) formed transiently during DNA metabolism, including replication, recombination and repair [1]. Binding of SSBs to exposed ssDNA occurs with very high affinity and without sequence specificity [6,7]. In addition to providing a protective coating for ssDNA, SSBs also recruit other proteins to their sites of action on ssDNA *via* an extensive network of protein–protein interactions, highlighting their central role as coordinators of DNA metabolism [8]. SSB from *Escherichia coli* (*EcoSSB*), a model SSB protein and the focus of this study, interacts directly with at least 14 other proteins involved in DNA replication, recombination and repair [8].

Most bacterial SSB proteins, including *EcoSSB*, form stable asymmetric homotetramers [1]. Within each 18843 Da subunit of the *EcoSSB* tetramer, the N-terminal portion (115 amino acids of 177) is highly structured, forming an oligonucleotide-binding (OB) fold [9,10] (Figure 1b, top) responsible for binding ssDNA as well as mediating tetramerization [10,11]. The crystal structure of the SSB tetramer reveals its asymmetric dimer-of-dimers arrangement (Figure 1a) – two distinct dimer interfaces are present, and the tetramer possesses overall D_2 symmetry [10,11]. The association of monomers into dimers is mediated primarily by hydrogen bonding between β -strands of adjacent monomers, and the interaction of His55 with Asn6 and Leu83 in a neighboring protomer is particularly important in stabilizing this interaction [10]. The dimer-dimer interface (the horizontal interface in Figure 1a) consists of two 6-stranded β -sheets, one from each dimer [10]. This interface is hydrophobic in its core, but also contains extensive H-bonding contacts among lysine, tyrosine, glutamine and glutamic acid residues [10].

As a result of its multiple OB-folds, the *Eco*SSB tetramer is able to interact with ssDNA in different binding modes. *In vitro*, these are salt and protein concentration dependent [6,12-15]. The two best characterized of these are the (SSB)₃₅ mode, in which ~35 nucleotides of ssDNA bind to SSB, interacting with two of the four subunits [16], and the (SSB)₆₅ mode, in which ~65 nucleotides wrap completely around the tetramer, binding to all four subunits [6,11,15]. The (SSB)₃₅ mode, which possesses a high degree of positive inter-tetramer cooperativity and forms a continuous coating on DNA [16], tends to be favored at low salt concentrations and high SSB:ssDNA ratios [14,16]. The (SSB)₆₅ mode forms more readily in high-salt solutions, or at low SSB concentrations, and possesses limited cooperativity between tetramers [14]. A crystal structure of the core DNA-binding region of SSB bound to two molecules of (dC)₃₅ has allowed construction of models of the DNA topology of each of these binding modes [11] (Figure 1b, bottom).

Beyond the core OB-fold, the lack of electron density for the C-terminal portion of SSB in its crystal structures suggests that this region is intrinsically disordered and remains so when the protein is bound to ssDNA [11,17]. Nevertheless, the amphipathic C-terminal tip (of sequence -DFDDDIPF in *Eco*SSB; Figure 1b, top) is highly conserved among bacterial SSBs [18] and is absolutely required for SSB function [19]. Characterization of many of the SSB-protein interactions has shown in each case that the C-terminus is the site of interaction with its protein binding partners, which include the χ subunit of the replicative clamp loader complex, the replication restart helicase PriA, and exonuclease I [8]. Structural information about several of these interactions suggests that SSB-protein interactions share a conserved binding mechanism that relies on a mixture of hydrophobic and electrostatic contacts [18,20,21].

Although the C-terminus of SSB is clearly a mediator of protein-protein interactions, evidence is accumulating that it also plays a role in regulating the ssDNA affinity and binding

modes of SSB. Deletion of the eight C-terminal residues enhances the affinity of SSB for ssDNA at moderate salt concentrations [22], and it was proposed that in the absence of ssDNA the predominantly acidic C-terminal residues interact with the ssDNA binding sites of the SSB tetramer [22]. Such a mechanism could act to regulate both the ssDNA binding and protein-protein interaction activities of SSB; binding of ssDNA to SSB would displace the C-terminus from the ssDNA binding site, freeing it for interaction with binding partners, which in turn would sequester the regulatory effect of the C-terminus upon DNA binding. This is also consistent with early work that showed that cooperative binding of SSB to ssDNA increased the susceptibility of its C-terminus to proteolysis [23]. Direct evidence for an interaction between the C-terminus and the OB-fold region of SSB is yet to be obtained, but similar interactions have been proposed to occur in the SSB proteins from bacteriophages T4 and T7 [24-26], and in the case of the dimeric T7 gp 2.5, the C-terminus appears to play a role in stabilizing the dimer [25,27].

All of its roles in DNA metabolism require SSB to be highly mobile upon ssDNA. For example, during replication, SSB needs to be rapidly displaced as the lagging strand replicative polymerase progresses at ~1000 nucleotides/second [28], and to bind to lagging strand ssDNA generated by helicase action. Exactly how the strong ssDNA–SSB complex is able to achieve such dynamics remains unclear, although *in vitro* experiments have suggested some possibilities. Evidence for direct transfer of SSB tetramers between ssDNA strands when SSB is in the (SSB)₃₅ binding mode has been provided by filter-binding assays [29] and stopped-flow fluorescence quenching [30]. Some studies have suggested that SSB may be able to ‘roll’ along ssDNA by making continuous binding readjustments [31,32], and evidence for a sliding mechanism [33] that allows it to diffuse along ssDNA [34] has been presented recently. The relevance of these proposed transfer mechanisms *in vivo* is unknown, and SSB may utilize these (or other) mechanisms selectively for its different cellular roles.

In this work, we used ESI-MS to examine some of the dynamic aspects of SSB, both within the tetramer and in its interactions with ssDNA. Subunit exchange in oligomeric proteins has been widely studied using FRET [35,36]. However, that approach to monitoring subunit exchange has limitations that include the need for fluorescent labeling of subunits and the inability to directly detect individual oligomeric species and abundances. The ability of ESI-MS to preserve noncovalent interactions in the gas phase is well established, and it has been used extensively to probe the stoichiometry and architecture of multisubunit and multiprotein assemblies [37,38]. ESI-MS offers several advantages for the study of subunit exchange: it does not require chemical labeling, allows direct determination of subunit stoichiometries (providing there is a sufficient mass difference, species differing in composition by a single subunit can be resolved), and importantly, reveals the relative abundances of multiple exchange products [37,38]. Additionally, the rapid analysis possible with ESI-MS makes it convenient for following subunit exchange in real time [38]. Despite these benefits, only a small number of studies applying ESI-MS to multimeric protein subunit exchange have been reported [39-48].

In the current study, nanoESI-MS was used to follow subunit exchange in SSB over time, at different salt concentrations, by uniformly labeling SSB with ^{15}N and monitoring the appearance of heterotetramers in the spectra. Differences were observed in the nature of subunit exchange at high and low salt concentrations. Further, a study of subunit exchange in a C-terminally truncated SSB suggests that the basis of these differences is the behavior of the C-terminal tail. As might be anticipated, the binding of SSB to short ssDNAs has a substantial inhibitory effect on subunit exchange. Finally, ESI-MS was used to examine the direct and rapid transfer of intact SSB tetramers between separate ssDNA strands.

Experimental

Protein Expression and Purification

Plasmid construction and purification procedures for SSB proteins are described in full in the Supplementary Information. Briefly, SSB was overproduced in *E. coli* strain BL21(λ DE3)*recA* [49] containing plasmid pND73 [50], with the *ssb* gene under the control of thermoinducible bacteriophage λ p_R and p_L promoters. Uniformly ^{15}N -labeled SSB was overproduced in M9 minimal media containing $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotope Laboratories, Andover, MA) as the sole source of nitrogen. The C-terminally truncated proteins SSB Δ C8 and ^{15}N -SSB Δ C8, which are missing the final eight residues of the SSB sequence, were produced similarly using the mutated plasmid pAL1379. For nanoESI-MS analysis under denaturing conditions, proteins were dialyzed into 0.1% formic acid, and then diluted to 3 μM with 50% (v/v) acetonitrile in 0.1% formic acid. For all other mass spectrometry experiments, proteins were exchanged into either 10 mM or 1 M ammonium acetate, NH_4OAc (pH 7.2) by extensive dialysis at 4°C immediately prior to use. The concentrations of SSB and ^{15}N -SSB after dialysis were determined spectrophotometrically at 280 nm, using ϵ_{280} (tetramer) = $1.13 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ [51], and are reported as concentrations of tetramer throughout. MS of unlabeled and ^{15}N -labeled SSB under denaturing conditions (0.1% formic acid, 50% acetonitrile) gave masses of 18840 and 19085 Da respectively, consistent with the sequence-based mass of SSB (18843 Da [1]), and a labeling efficiency of approximately 98.8% for ^{15}N -SSB (SSB contains 245 N atoms).

Oligonucleotides

The deoxyoligonucleotides (dT)₃₅ and (dT)₇₀, along with their 5'-biotinylated counterparts, were obtained in HPLC-purified form from Geneworks (Adelaide, Australia), and dialyzed into either 10 mM or 1 M NH_4OAc (pH 7.2), and further diluted in the same buffers prior to

use. Oligo-dT concentrations were determined spectrophotometrically at 260 nm, using $\epsilon_{260} = 8100 \text{ M}^{-1}\text{cm}^{-1}$ [52].

Subunit Exchange and DNA Transfer Reactions

To monitor subunit exchange, equimolar quantities of either SSB and ^{15}N -SSB or SSB Δ C8 and ^{15}N -SSB Δ C8 were mixed (to give final concentrations of $\sim 0.5 \mu\text{M}$ in 1 M NH_4OAc or $\sim 3 \mu\text{M}$ in 10 mM NH_4OAc) and placed at 30°C . Aliquots ($3 \mu\text{L}$) were withdrawn immediately after mixing, then at regular time points up to 10 h, and subjected to nanoESI-MS analysis. The time between sampling and analysis was <1 min. Subunit exchange in DNA-bound SSB was monitored in the same way, except that SSB and ^{15}N -SSB were first bound separately to oligonucleotides by mixing the required amounts of protein and ssDNA. The complexes were analyzed by nanoESI-MS to confirm their identity and stoichiometry before the unlabeled and labeled complexes were mixed. Each time course experiment was repeated in either duplicate or triplicate to obtain reproducible results (see Supplementary Figure S1). To monitor exchange of SSB tetramers between DNA strands, SSB–ssDNA complexes (as specified in the text) were formed by mixing appropriate ratios of SSB and oligonucleotides, and then analysed by nanoESI-MS. These complexes were challenged with an equimolar quantity of a second oligonucleotide, then immediately analyzed again. If transfer onto the added strand was not observed, it was added in excess and the mixture set aside at 4°C for several hours before being re-analyzed.

Mass Spectrometry

All MS experiments were carried out in positive ion mode on an extended mass range (m/z 32000) Q-Tof *Ultima* instrument (Waters, Manchester, UK) equipped with a nanoelectrospray ionization source. Cesium iodide (10 mg/mL in 70% v/v isopropanol) was used as an external calibrant. Spectra were processed using MassLynx version 4.1 software

(Waters). Typically, 50-70 acquisitions were combined to produce representative spectra. Conditions for nanoESI-MS were optimized such that the noncovalent interactions within the SSB tetramer were maintained, and typical parameters were capillary voltage: 1.5 kV, sample cone voltage: 120 V, source temperature: 50°C, RF lens 1: 120 V, collision energy: 4 V. Under optimized conditions, exclusively tetrameric SSB was observed in spectra. These conditions also allowed for observation of noncovalent SSB–ssDNA interactions.

Analysis of Subunit Exchange Rates

As expected, SSB and ^{15}N -SSB had identical ionization efficiencies within the mass spectrometer, as determined by mixing equal quantities of each. It was therefore assumed that heterotetramers arising from subunit exchange would also have identical ionization efficiencies, and the intensities of all species could be compared directly. The relative abundance of each homo- or heterotetramer was calculated from the intensities (peak heights) of its most abundant ions (15^+ and 16^+ for spectra acquired from 1 M NH_4OAc , or 14^+ , 15^+ and 16^+ for spectra acquired from 10 mM NH_4OAc) and expressed as a percentage of the sum of the intensities for those ions. Exchange data (%) were plotted vs. time, and fit to a single-exponential model (for exchange in 1 M NH_4OAc) or the sum of two exponentials (for exchange in 10 mM NH_4OAc) using Prism v.5.0 (GraphPad Software, La Jolla, CA). The final equilibrium values were constrained to statistical values of 12.5% (for summed homotetramers), 50% (for the sum of 3:1 and 1:3 heterotetramers) and 37.5% (for 2:2 heterotetramers).

Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments to examine direct transfer of SSB between DNA strands were conducted using a Biacore T100 instrument (GE Healthcare, UK) equipped with an SA (streptavidin-coated) sensor chip. All experiments were carried out at

20°C with a flow rate of 5 μ l/min. The chip was prepared by injecting 70% (v/v) glycerol for 1 min, followed by three 1 min injections of 1 M NaCl, 50 mM NaOH. All experiments were carried out in Biacore buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% v/v surfactant P20). 5'-Biotinylated (dT)₇₀ and (dT)₃₅ were diluted to 5 nM in Biacore buffer and immobilized onto separate flow cells to levels of ~200 and ~250 RU (response units), respectively. SSB was dialyzed into Biacore buffer and diluted to 90 nM before being injected over the immobilized oligonucleotides for 5 min. After allowing the bound SSB to stabilize, (dT)₇₀ (1 μ M) was injected for 15 min. Once all SSB was removed (as judged by a return of the signal to the baseline), SSB (90 nM) was again injected for 5 min, allowed to stabilize, then (dT)₃₅ (1 μ M) was injected for 10 min. An unmodified flow cell was used as a reference surface and its signal subtracted from those on other flow cells.

Results and Discussion

Full-length SSB Tetramers Undergo Slow but Complete Subunit Exchange

In this work, SSB was uniformly ¹⁵N-labeled to provide a mass difference from unlabeled SSB, allowing subunit exchange between the ‘heavy’ and ‘light’ tetramers to be monitored. Subunit exchange between the two tetramers (SSB)₄ and (¹⁵N-SSB)₄ has the potential to form three different heterotetramers, each with a unique mass: (SSB)₃:(¹⁵N-SSB)₁, (SSB)₂:(¹⁵N-SSB)₂, and (SSB)₁:(¹⁵N-SSB)₃. For brevity, these will be referred to as the 3:1, 2:2 and 1:3 heterotetramers, respectively, and SSB and ¹⁵N-SSB as the 4:0 and 0:4 homotetramers, respectively. When solution conditions for preserving the native quaternary structures of the proteins were used (optimally 1 M NH₄OAc, pH 7.2), spectra of exclusively tetrameric species were obtained (Figures S2a, b). Given that SSB is known to remain tetrameric in a wide range of solution conditions at concentrations as low as 30 nM [6,53,54], these data reflect the species expected to be present in solution under these conditions. The behavior of

^{15}N -SSB with regard to ionization efficiency and charge state distribution was indistinguishable from that of unlabeled SSB (Figures S2a, b).

The well-documented importance of salt concentration for several aspects of SSB behavior made it desirable to carry out subunit exchange experiments under both low and high salt solution conditions. To do this in a manner compatible with ESI-MS, the NH_4OAc concentration was varied in the range 10-1500 mM. SSB proved to be highly sensitive to variations in buffer concentration, and was largely insoluble under many solution conditions. Eventually, it was found that sufficient solubility and spectral quality were afforded by using either 10 mM or 1 M NH_4OAc at pH 7.2, although in 1 M NH_4OAc only low SSB concentrations ($<0.5\ \mu\text{M}$) could be achieved.

In an initial experiment, in 1 M NH_4OAc at 4°C , subunit exchange was found to be so slow that heterotetrameric species were barely detectable by nanoESI-MS 10 h after mixing (not shown). By increasing the temperature to 30°C , the rate of subunit exchange was increased so that it could be monitored over a period of hours. Subunit exchange at 30°C was followed by acquisition of nanoESI mass spectra immediately after mixing equimolar SSB and ^{15}N -SSB, and then periodically over 10 h, in both 10 mM (Figure 2a) and 1 M NH_4OAc (Figure 2b). Immediately after mixing, the spectra consisted solely of homotetrameric ions. With increasing time, the abundance of both homotetramers slowly decayed, and simultaneous increases in the abundances of the three hybrid tetramers were observed. After 9 h of exchange, the dominant species in the spectra was the 2:2 heterotetramer, and the final distribution of intensities of all tetrameric species (4:0, 3:1, 2:2, 1:3, 0:4) approached the binomial 1:4:6:4:1 distribution expected at equilibrium for a statistical exchange of subunits. Also notable in all spectra was the absence of any monomeric or dimeric species, suggesting that the rate of exchange is governed by tetramer dissociation; *i.e.* reassociation of exchange intermediates occurs much faster than tetramer dissociation, which is thus rate limiting. Such

a situation is to be expected for an oligomer with a driving force for assembly as strong as is present in the SSB tetramer. To examine this further, exchange was followed over a range of initial SSB concentrations. The low SSB concentrations achievable in 1 M NH_4OAc precluded further dilution, but in 10 mM NH_4OAc it was possible to observe subunit exchange over a modest SSB concentration range. The rates of loss of homotetramer over 10 h with initial concentrations of 0.75 μM – 3 μM were indistinguishable (Figure S3), so it is tetramer dissociation that determines the rate of exchange.

Closer examination of the exchange kinetics in 1 M and 10 mM NH_4OAc revealed differences between rates. In 1 M NH_4OAc , the loss of homotetrameric ions (SSB and ^{15}N -SSB combined), and the simultaneous formation of the three heterotetramers are well described by first-order decay (homotetramers) or association (heterotetramers) equations, with a rate constant (k_{obs}) for the disappearance of homotetramers of $(3.44 \pm 0.05) \times 10^{-3} \text{ min}^{-1}$ (Figure 3a and Table 1), corresponding to a half-time of ~ 200 min. However, in 10 mM NH_4OAc , the decrease in homotetramer intensity was not well described by a single exponential (Figure 3b). It could, however, be fit well to the sum of two independent exponential decay processes, with rate constants of $(13 \pm 2) \times 10^{-3}$ and $(2.2 \pm 0.2) \times 10^{-3} \text{ min}^{-1}$ (Table 1). Direct comparison of the plots shows that the overall rates of exchange in 1 M and 10 mM NH_4OAc were not substantially different; however, the more complex exchange kinetics in 10 mM NH_4OAc indicate that there may be underlying differences in the exchange process at high and low salt concentrations. Two (or more) independent first-order processes suggests that there are two different populations of SSB tetramers in solution that (i) do not interconvert rapidly and (ii) dissociate at different rates in low-salt conditions. At high salt, these species would then need either: (i) to be able to interconvert or (ii) to dissociate at similar rates.

We considered the potential contribution of the C-terminal tail of SSB to subunit exchange to provide an explanation for this behavior. As described earlier, recent evidence has suggested that the acidic C-terminus is capable of occupying the DNA-binding site [22,55]. Such an interaction can be visualized in (at least) two ways. Within the tetramer, the C-termini of each subunit could fold back and occupy the OB-fold of the same subunit (interaction in *cis*) or could bind in *trans* to the DNA-binding site of an adjacent subunit. The latter configuration is expected to contribute additional stability to the tetramer, and the long intervening flexible sequence (~53 residues) between the structured core of SSB and its C-terminal tip makes it plausible that such distances could easily be spanned. Therefore, the two distinct subunit exchange rates in 10 mM NH₄OAc could arise from two (or more) different populations of tetramers, each possessing a distinct stability arising from the configuration of its C-terminus–OB-fold interaction. In 1 M NH₄OAc, such a predominantly ionic interaction should be more easily disrupted, allowing the different forms of SSB to interconvert.

The similarity of rate constants for exchange in 1 M and 10 mM NH₄OAc seem to be at odds with this explanation. However, hydrophobic interactions, which are strengthened at high salt concentrations [56,57], make a significant contribution to the SSB tetramer interfaces [10]. Stabilization of these interactions in 1 M relative to 10 mM NH₄OAc could therefore compensate for weakening of electrostatic contacts to account for the similar exchange rates. Note that the hydrophobic interaction between the ϵ and θ subunits of *E. coli* DNA polymerase III is stabilized by high NH₄OAc concentrations as judged by ESI-MS [58].

Subunit Exchange in a C-Terminally Truncated SSB Suggests a Stabilizing Role for the C-Terminus

The existence of *cis* and *trans* interactions within SSB tetramers in 10 mM NH₄OAc, defined by the locations of their C-terminal tails, would suggest that truncation of the C-terminus

might give rise to a single population, with exchange kinetics similar to the more rapidly exchanging full-length population. The truncated SSB mutant SSB Δ C8, missing the eight amino acid residues from the C-terminus of SSB, was used to test this hypothesis. SSB Δ C8, along with its ^{15}N -labeled counterpart, were exclusively tetrameric when subjected to nanoESI-MS under the same conditions as used for the full-length proteins, and also displayed identical ionization behaviour (Figure S2c, d). Subunit exchange between SSB Δ C8 and ^{15}N -SSB Δ C8 was followed as for the full-length proteins; selected spectra are shown in Figures 2c (10 mM NH_4OAc) and 2d (1 M NH_4OAc). While subunit exchange between the truncated proteins in 1 M NH_4OAc appears to proceed at a similar rate to that observed for the full-length SSBs, exchange in the lower salt condition proceeds much more rapidly, and appears to be almost complete after just 2 h.

Further analysis of the SSB Δ C8 exchange rates revealed some interesting results. Although the time dependence of exchange in 1 M NH_4OAc showed little overall difference to that of its full-length counterpart (Figure 3a), the rate was no longer well described by a single exponential decay (Table 1), suggesting that the interpretation of rapid interconversion between populations giving rise to a first-order exchange process represents an oversimplification. Moreover, exchange in 10 mM NH_4OAc was also described as the sum of two exponential processes even in the absence of the C-terminus, with fast and slow rate constants of $(26 \pm 1) \times 10^{-3} \text{ min}^{-1}$ and $(3.5 \pm 0.3) \times 10^{-3} \text{ min}^{-1}$ (Figure 3b and Table 1). The faster rate constant is approximately two-fold higher than the corresponding value for the full-length proteins, and also makes a much larger contribution to the overall rate (78% vs. 43%; Table 1). Thus, although the C-terminal tail of SSB certainly stabilizes the tetramer with respect to exchange at low salt (Figure 3b), the persistence of complex kinetics in its absence suggests that the mechanism by which this occurs is more complex than suggested by the simple models considered above. It may be that residues in the unstructured C-

terminal region of SSB other than the last eight amino acids make contacts in *trans* that stabilize the tetramer, at least under low ionic strength conditions.

The range of potential *cis* and *trans* OB-fold–tail interactions possible within the tetramer makes it likely that several ‘modes’ of binding contribute to the observed stabilization; our data do not allow us to resolve these multiple possibilities. The non-equivalence of the two dimer interfaces in SSB (Figure 1a) adds further complexity; stabilizing contacts made by the C-terminus may occur preferentially across one interface, changing their relative probability of dissociation. Nonetheless, when taken together with the recent indirect evidence that the C-terminus is able to interact with the OB-fold domain of SSB, this salt-dependent modulation of the subunit exchange rate by the C-terminal residues of SSB provides strong evidence that the C-terminus, *via* electrostatic interactions with the more positively charged SSB core, exerts a moderately stabilizing effect on the SSB tetramer. A similar mechanistic scenario has been proposed for the dimeric gp 2.5 SSB protein from phage T7; the C-terminus, which like that of *E. coli* SSB is highly acidic, competes with ssDNA for the DNA-binding surface of the protein [26], and this interaction has been proposed to occur *in trans* between subunits to stabilize the dimer [25].

Binding to Single-Stranded Oligonucleotides Abolishes SSB Subunit Exchange

Having established that SSB tetramers undergo slow subunit exchange, it was desirable to examine the effect of DNA binding upon this exchange. As expected, DNA binding has been observed to dramatically affect the subunit exchange properties of several DNA-binding proteins. The bacterial nucleoid-associated protein, Fis [59], the DNA-binding/bending protein TF1 [60] and the transcription factor C/EBP [61] are dimers, and all three exhibit rapid subunit exchange that is dramatically slowed by binding to DNA.

Given the complexity with which SSB binds to DNA, it was first necessary to establish that it was possible by nanoESI-MS to observe SSB–DNA complexes that were consistent with those expected from solution studies. Fluorescence quenching studies have shown that a maximum of four molecules of (dT)₁₆, two of (dT)₃₅, or one of (dT)₇₀ are able to bind to a single SSB tetramer [62,63]. These stoichiometries are consistent with the models of the (SSB)₃₅ and (SSB)₆₅ DNA binding modes, whereby ~65 nucleotides wrap around the SSB tetramer in the (SSB)₆₅ mode, and ~35 nucleotides are bound to two subunits in the (SSB)₃₅ mode [11] (Figure 1b). We were able to observe 2:1 and 1:1 DNA:protein stoichiometries by nanoESI-MS when SSB was mixed with an excess of (dT)₃₅ or (dT)₇₀, respectively, in either 1 M or 10 mM NH₄OAc (not shown). By carefully controlling the ratios of SSB and ssDNA, preformed complexes of SSB or ¹⁵N-SSB with one (dT)₇₀ strand (SSB:dT₇₀), one (dT)₃₅ strand (SSB:dT₃₅) or with two (dT)₃₅ strands (SSB:2dT₃₅) could be prepared, such that the complexes were the only species present in the mass spectra (no free proteins or ssDNA). That the SSB:dT₃₅ complex could be prepared, uncontaminated by either free SSB or the SSB:2dT₃₅ complex, confirms the high degree of negative cooperativity of DNA binding in the (SSB)₃₅ mode, where it is much more difficult to occupy a second pair of binding sites once the first pair is filled [62,63].

To analyze SSB subunit exchange in the presence of ssDNA, SSB and ¹⁵N-SSB were premixed (separately) with either (dT)₇₀ or (dT)₃₅ to form complexes, which were then mixed in equimolar amounts and treated at 30°C, as for the protein-only experiments. The mixture was sampled and analyzed by nanoESI-MS for evidence of subunit exchange (Figure S4). A shorter total treatment time of 6 h was used, as it was difficult to maintain spectral quality after longer periods in some cases. All three complexes, (SSB/¹⁵N-SSB:dT₇₀), (SSB/¹⁵N-SSB:dT₃₅), and (SSB/¹⁵N-SSB:2dT₃₅) were examined; the 1:1 complexes of SSB with (dT)₇₀ and (dT)₃₅ have commonly been used as models for the (SSB)₆₅ and (SSB)₃₅ binding modes,

respectively [15,30,55,62,63]. In each case, before mixing the SSB and ^{15}N -SSB complexes, they were analyzed separately by nanoESI-MS to confirm their stoichiometry and the absence of free protein.

Upon mixing equimolar quantities of the SSB:dT₇₀ and ^{15}N -SSB:dT₇₀ complexes in 10 mM NH₄OAc, both could be clearly observed in the spectrum (Figure S4a, bottom), and the spectrum after 6 h at 30°C remained essentially unchanged (Figure S4a, top). The lack of subunit exchange was in direct contrast with the extensive exchange between the free proteins after a comparable period (Figure 2a). The extensive wrapping of SSB by (dT)₇₀ in the (SSB)₆₅ binding mode provides an intuitive explanation for inhibition of subunit exchange by (dT)₇₀ binding. The high affinity of the SSB-(dT)₇₀ interaction could be predicted to prevent dissociation of the complex as a prerequisite for subunit exchange. However, a similar absence of subunit exchange was observed with both 1:1 (Figure S4b) and 2:1 (Figure S4c) complexes of SSB and ^{15}N -SSB with (dT)₃₅. In the 1:1 SSB:dT₃₅ complex, which is presumed to utilize the (SSB)₃₅ binding mode, only two subunits of the tetramer are expected to be occupied by DNA, and the tetramer might be more easily able to undergo subunit exchange than when it is fully wrapped by (dT)₇₀. This was not observed on the time scale of these experiments, confirming that all of the DNA complexes are quite stable to dissociation.

The stabilizing effect of ssDNA-binding on the SSB tetramer is much greater than that provided by the C-terminus, reflecting the relative weakness of the proposed C-terminus–OB-fold interaction compared to that of the SSB-ssDNA interaction. The model in Figure 1b shows that the path of the ssDNA in the (SSB)₃₅ mode is predicted to traverse the ‘dimer-dimer’ (rather than the monomer-monomer) interface of the tetramer. The fact that binding of a single (dT)₃₅ molecule is sufficient to completely abolish exchange suggests that the mechanism of SSB subunit exchange very likely proceeds *via* dissociation to dimers across this interface as a first step, with further dissociations only possible after this has occurred.

Intact SSB Tetramers Exchange Rapidly Between ssDNA Strands

The dynamic nature of processes that involve SSB means that its tetramers need to be progressively removed from ssDNA to allow access by other proteins, but must then bind to new regions of ssDNA as they are formed. It has long been suggested that SSB tetramers are able to be recycled between sites, but given the very high affinities and slow dissociation times of SSB–ssDNA complexes [64], it is not obvious how SSB recycling can occur with sufficient speed. Recent single-molecule FRET studies have shown that SSB is able to diffuse along ssDNA [34] by reptation, a sliding mechanism in which small ssDNA bulges are propagated around the SSB tetramer [33]. A second mechanism, whereby SSB tetramers bound in the (SSB)₃₅ mode can rapidly transfer directly between distinct ssDNA sites without passing through a free SSB intermediate, has also been proposed as a prospective mechanism for SSB recycling [29,30]. Given the very slow rate of dissociation of SSB from SSB–ssDNA complexes, such a mechanism could provide a means of increasing the speed of SSB displacement. Kozlov and Lohman [30] have used stopped-flow fluorescence quenching to characterize the kinetics of this transfer mechanism in some detail. The mechanism of transfer between discrete oligonucleotides was shown to occur *via* a transient ternary complex consisting of two ssDNA molecules bound to a single SSB tetramer, followed by dissociation of the ‘donor’ ssDNA strand [30]. The identity of the initial SSB–ssDNA complex was shown to be crucial: the rate of transfer was highly dependent on whether or not there were unoccupied binding sites available on the SSB tetramer to accept the second strand [30]. Having established that SSB–oligonucleotide complexes could be successfully observed by nanoESI-MS, we carried out experiments to demonstrate direct transfer of SSB tetramers between oligonucleotides by challenging preformed SSB–oligonucleotide complexes with ‘acceptor’ DNAs.

Complexes of SSB with various oligonucleotides were formed as described above, and challenged with an equimolar quantity of either an oligonucleotide of a different length, or an oligonucleotide of the same length that had been 5'-biotinylated to provide a mass difference. NanoESI-MS of the mixture immediately after addition of the second oligonucleotide revealed whether the spectrum (i) remained unchanged, with SSB still bound to the original oligonucleotide; (ii) contained only complexes of SSB with the added oligonucleotide, indicating complete transfer onto the challenging ssDNA, or (iii) contained ions corresponding to complexes of both oligonucleotides with SSB, demonstrating a partial transfer, or partitioning of SSB between the two ssDNA strands. Owing to the highly salt-dependent nature of direct transfer [30], both low (10 mM NH_4OAc) and high (1 M NH_4OAc) salt concentrations were used.

When a 1:1 complex of SSB with $(\text{dT})_{70}$ in 10 mM NH_4OAc was challenged by addition of equimolar $(\text{dT})_{35}$, no transfer onto the added $(\text{dT})_{35}$ was observed: the spectrum was unchanged (Figure 4a). This was also the case in 1 M NH_4OAc (Figure S5d), as well as when greater excesses of $(\text{dT})_{35}$ were added. When the same complex was challenged with 5'-biotinylated $(\text{dT})_{70}$ [Bio- $(\text{dT})_{70}$] in 10 mM NH_4OAc , no transfer onto Bio- $(\text{dT})_{70}$ was observed (Figure S5a). However, when the complex was formed in 1 M NH_4OAc , challenge by Bio- $(\text{dT})_{70}$ resulted in 1:1 complexes of SSB with both DNA strands being observed in the spectra (Figure S5e), indicating partial transfer of SSB onto Bio- $(\text{dT})_{70}$. By mixing a two-fold excess of SSB with $(\text{dT})_{70}$ in 10 mM NH_4OAc , a complex consisting of two SSB tetramers cooperatively bound to a single $(\text{dT})_{70}$ strand [2SSB: dT_{70}] was formed (Figure S5b). This complex was only formed at low salt concentrations; increasing the salt concentration resulted in its conversion back to the 1:1 SSB: dT_{70} complex (not shown). Upon addition of equimolar Bio- $(\text{dT})_{70}$ to the 2SSB: dT_{70} complex, 1:1 complexes of SSB with both $(\text{dT})_{70}$ and

Bio-(dT)₇₀ in approximately equal amounts were immediately observed in the mass spectrum (Figure S5b).

These results are consistent with expectations based on the existence of (SSB)₃₅ and (SSB)₆₅ binding modes and their utilization at high and low salt concentrations. The 1:1 SSB:dT₇₀ complex, assumed to adopt the fully-wrapped (SSB)₆₅ binding mode, lacks accessible binding sites for the challenging oligonucleotide, making direct transfer infeasible (Figures 4a, S5a). In contrast, to accommodate two SSB tetramers on one strand of (dT)₇₀, the 2SSB:dT₇₀ complex needs to be in the (SSB)₃₅ binding mode, leaving two unoccupied binding sites on each tetramer. This permits binding of Bio-(dT)₇₀, and because there is no longer an excess of SSB over DNA, 1:1 complexes in the (SSB)₆₅ mode are formed (Figure S5b). This transition has been shown to occur *via* a ternary intermediate in which both strands bind to the two SSB tetramers, followed by a rapid rearrangement to form the 1:1 complexes [30]. The ability of SSB to partially transfer from the 1:1 SSB:dT₇₀ complex to Bio-(dT)₇₀ at higher ionic strength (Figure S5e) is probably due to transient partial unwrapping of the (dT)₇₀ from around the tetramer [30,31], resulting in a small population of tetramers with unoccupied binding sites that enable binding of a second DNA strand. The lack of transfer from (dT)₇₀ to (dT)₃₅ is also consistent with expectations. Presumably, binding of (dT)₃₅ to the unoccupied sites to form a ternary dT₇₀:SSB:dT₃₅ complex is able to occur, but because of the higher affinity of SSB for (dT)₇₀, (dT)₃₅ dissociates preferentially to return to the original 1:1 SSB:dT₇₀ complex (Figure 4a; Figure S5b).

Challenge of the 1:1 complex of SSB with (dT)₃₅ by addition of (dT)₇₀ resulted in complete transfer of SSB onto (dT)₇₀, with none of the original SSB-dT₃₅ complex remaining in the spectrum (Figure 4b; Figure S5f). This is consistent with the (SSB)₃₅ binding mode providing the necessary ‘free’ binding sites for (dT)₇₀ to be able to form an intermediate ternary complex required for direct transfer. The higher affinity of SSB for (dT)₇₀ than for (dT)₃₅

presumably drives the transfer to completion. Similarly, even when a 1:2 SSB:2dT₃₅ complex (with all four binding sites occupied by two separate (dT)₃₅ strands) was challenged with Bio-(dT)₇₀, complete transfer of SSB to Bio-(dT)₇₀ was observed (Figure S5c), despite the lack of free binding sites in the original complex. This result can be explained by the high degree of negative cooperativity which exists among the subunits of the SSB tetramer [62,63]. Binding of a second (dT)₃₅ molecule to an SSB tetramer is much more difficult than binding of the initial (dT)₃₅, with the consequence that the second strand remains weakly bound, enabling it to be displaced by the longer, higher affinity (dT)₇₀ strand.

Surface plasmon resonance was also used to examine the direct transfer of SSB tetramers between ssDNA molecules. When Bio-(dT)₇₀ was immobilized on the surface of a streptavidin-coated sensor chip and SSB made to flow over it, SSB binding could be detected as a rapid increase in response (Figure 4c), which was maintained stably for long periods. The magnitude of the response was consistent with an SSB:(dT)₇₀ stoichiometry of approximately 1:1. Flowing free (unbiotinylated) (dT)₇₀ over this complex resulted in an immediate and rapid drop in response (Figure 4c), corresponding to transfer of SSB from the immobilized to the free (dT)₇₀. After re-binding SSB to the immobilised (dT)₇₀, the complex was again challenged by flowing unbiotinylated (dT)₃₅ over the surface. A minor decrease in response was observed upon injection of (dT)₃₅ (Figure 4c), but this decrease was very small in comparison to that seen with the previous injection of (dT)₇₀.

These SPR results, which were carried out in 150 mM NaCl, are consistent with those obtained by mass spectrometry. The relatively complete transfer from Bio-(dT)₇₀ to free (dT)₇₀ (compared to the partial transfer observed by ESI-MS) can be attributed to the continuous excess of free (dT)₇₀ supplied by injection over the SPR sensor surface. The lack of transfer from (dT)₇₀ to (dT)₃₅, even with a large excess of (dT)₃₅, is as expected; (dT)₃₅ is unable to insinuate into the fully wrapped complex. The small decrease in response is

possibly due to a small percentage of SSB tetramers bound in the (SSB)₃₅ mode to the immobilized Bio-(dT)₇₀ (two tetramers per strand), which could allow some transfer onto (dT)₃₅.

With Bio-(dT)₃₅ immobilized on the SPR sensor chip, SSB was also able to bind and form a stable complex on the surface (Figure 4d). Upon injection of either free (dT)₇₀ or (dT)₃₅, the response dropped rapidly to the level observed before SSB binding, indicating complete transfer of SSB onto both lengths of free oligonucleotide. Examination of the sensorgrams in Figures 4c and d shows that, with SSB bound to immobilized Bio-(dT)₃₅, the transfer rate onto both lengths of ssDNA was faster, with a steeper decrease in signal upon injection of free oligonucleotide, than the rate of transfer onto free (dT)₇₀ from immobilized Bio-(dT)₇₀. This again reflects that transfer is able to occur more readily when binding sites are available, as in the (SSB)₃₅ binding mode.

The consistency of the results obtained here using nanoESI-MS with those obtained using fluorescence quenching [30] shows that MS provides an effective method for directly examining SSB-ssDNA complexes. Taken together with the previous evidence, our results support the superior ability of the (SSB)₃₅ mode, which has been proposed to function in DNA replication [16] to facilitate such a direct transfer mechanism. Whether such a mode of transfer is relevant *in vivo* awaits further investigation.

Conclusions

This work has demonstrated that the C-terminus of SSB exerts a modest, salt-dependent effect on the stability of the *E. coli* SSB tetramer, suggesting for the first time that the C-terminus may play a role in SSB tetramer formation. NanoESI-MS was used as a sensitive and direct method to follow subunit exchange in the SSB tetramer in real time under high and low salt solution conditions. The rate of exchange under low salt conditions was dependent

on the presence of the C-terminal eight residues of SSB; removal of the C-terminus increased the rate of exchange, indicating some destabilization of the tetramer. Previously, the SSB Δ C8 tetramer was thought to form equally as efficiently as the tetramer comprised of the full-length protein, and the C-terminus was assumed to be extraneous to tetramer stability. This work therefore shows how examination of subunit exchange processes can expose differences between very stable oligomeric proteins that otherwise appear to be equivalent. We propose that the increased stability provided to the SSB tetramer by its C-terminal tails results from intersubunit interactions between these tails and the OB-folds of adjacent subunits. Understanding the behavior of the SSB C-terminus is of critical importance, as its interactions with a variety of binding partners are central to the cellular role of SSB. Further work is required to probe the precise nature of the behavior of the C-terminus and of any such intersubunit contacts. This work also represents the first MS observation of the DNA-binding modes of SSB. Binding of oligonucleotides to the SSB tetramer was shown to dramatically inhibit subunit exchange such that it was not observable within the timescale examined. Here, we have also shown that the behaviour of SSB and SSB-ssDNA complexes in dilute (10 mM) and concentrated (1 M) ammonium acetate solutions reflects that observed in previous solution studies using traditional buffer conditions and salts, and that the complexes can be preserved in the gas phase. Transfer of intact SSB tetramers between discrete oligonucleotides was shown to depend, as expected, on the identity of the initial complex, with complexes in the (SSB)₃₅ mode undergoing transfer much more readily. Ultimately, a combination of techniques is likely to shed light on the detailed mechanisms by which SSB achieves its dynamics on ssDNA, but this work demonstrates that mass spectrometry is a valuable method for illuminating the complexities of the SSB tetramer.

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Tables

Table 1. Rate constants for subunit exchange between full-length SSB and ^{15}N -SSB and between SSB ΔC8 and ^{15}N -SSB ΔC8 in either 1 M or 10 mM NH_4OAc . Rate constants were calculated from the exponential fits shown in Figure 3. Percentages in brackets refer to the contribution made by the fast or slow rate constant to the fit.

	Full-length SSB		SSB ΔC8	
Conditions	$10^3 \cdot k_{\text{fast}} (\text{min}^{-1})$	$10^3 \cdot k_{\text{slow}} (\text{min}^{-1})$	$10^3 \cdot k_{\text{fast}} (\text{min}^{-1})$	$10^3 \cdot k_{\text{slow}} (\text{min}^{-1})$
1 M NH_4OAc	3.44 ± 0.05 (100%)	—	6.7 ± 0.2 (70%)	1.3 ± 0.1 (30%)
10 mM NH_4OAc	13 ± 2 (44%)	2.2 ± 0.2 (56%)	26 ± 1 (78%)	3.5 ± 0.3 (22%)

Figure Legends

Figure 1. SSB structure and domain organization. **(a)** The crystal structure of the SSB tetramer is a dimer-of-dimers consisting of four identical subunits. The first 113 residues of each subunit, corresponding to the structured OB-fold domain, are visible in the crystal structure. The horizontal dimer-dimer interface consists of a mixture of hydrophobic and hydrogen bonding interactions. The vertical monomer-monomer interfacial interactions are mediated by a number of hydrogen bonds. Figure generated using PyMOL (www.pymol.org) and PDB 1SRU [17]. **(b)** (Top) Schematic organization of the SSB monomer. Residues 1-115 form the DNA-binding OB-fold domain. The extreme C-terminus (residues 170-177) is highly conserved, and contains a large number of acidic and hydrophobic residues. The intervening sequence (residues 116-169) is rich in glycine and proline and thought to be flexible. (Bottom) Models showing the proposed topology of the (SSB)₆₅ and (SSB)₃₅ DNA-binding modes, based on the crystal structure of SSB in complex with two (dC)₃₅ molecules (right; PDB 1EYG [11]). The ssDNA is shown as a tube and a line in the structure and models respectively. In the (SSB)₆₅ mode, ~65 nucleotides completely wrap the SSB tetramer, interacting extensively with all four subunits. In the (SSB)₃₅ mode, ~35 nucleotides interact with an average of two subunits, although the DNA may make some contacts with all subunits.

Figure 2. Nano-ESI mass spectra showing subunit exchange between **(a)** SSB and ¹⁵N-SSB in 10 mM NH₄OAc, pH 7.2, **(b)** SSB and ¹⁵N-SSB in 1 M NH₄OAc, pH 7.2, **(c)** SSBΔC8 and ¹⁵N-SSBΔC8 in 10 mM NH₄OAc, pH 7.2 and **(d)** SSBΔC8 and ¹⁵N-SSBΔC8 in 1 M NH₄OAc, pH 7.2. Equimolar amounts of the two proteins were mixed and placed at 30°C, with aliquots withdrawn and analyzed by nanoESI-MS at the times indicated. Total protein concentrations were ~3 μM in reactions performed in 10 mM NH₄OAc and ~0.5 μM in

reactions performed in 1 M NH₄OAc. Dotted lines through the highest intensity charge state show the identity of each tetramer. The other charge states shown contain analogous distributions.

Figure 3. Time courses of subunit exchange **(a)** between SSB and ¹⁵N-SSB (squares) or SSBΔC8 and ¹⁵N-SSBΔC8 (circles), in 1 M NH₄OAc, **(b)** between SSB and ¹⁵N-SSB (squares) or SSBΔC8 and ¹⁵N-SSBΔC8 (circles) in 10 mM NH₄OAc. The relative homotetramer abundance (%) was calculated from nanoESI-MS spectra at each time point by dividing the total abundance arising from homotetrameric ions by the total abundance of all ions (for the most abundant charge states). Each time course was fit to one or two independent exponentials as described in the text with the initial and final values constrained to 100% and 12.5%, respectively. Rate constants are shown in Table 1.

Figure 4. Transfer of intact SSB tetramers between single-stranded oligonucleotides. In **(a)** and **(b)**, the top spectra represent initial complexes formed by mixing SSB and the specified oligonucleotide in 10 mM NH₄OAc, pH 7.2, followed by nanoESI-MS analysis. Complexes were challenged by addition of an equimolar amount of a second oligonucleotide as indicated, then analyzed again by nanoESI-MS (bottom spectra). Comparison of the top and bottom spectra allowed assessment of whether protein transfer had occurred. **(c)** and **(d)** Transfer of SSB tetramers between oligonucleotides detected by SPR. Either Bio-(dT)₇₀ **(c)** or Bio-(dT)₃₅ **(d)** were immobilized on the surface of a streptavidin-coated sensor chip prior to the injection of SSB and nonbiotinylated oligonucleotides over the surface. Increases and decreases in response reflect binding to and dissociation from the surface, respectively.

Figures

Figure 1

Figure 2

Figure 3 (above)

Figure 4 (above)

SUPPLEMENTARY INFORMATION

***Escherichia coli* Single-Stranded DNA-Binding Protein: NanoESI-MS Studies of Subunit Exchange and DNA Binding Transactions**

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Supplementary Methods

Construction of Plasmids

Oligonucleotides were purchased from GeneWorks (Adelaide, Australia). Sequences of all PCR-generated inserts used in plasmid construction were confirmed by nucleotide sequence determination, using vector primers 9 and 10 [1].

pND72 and pND73 (SSB): Plasmid pRLM55 [2], a gift from Dr Roger McMacken, contains the *E. coli ssb* gene centrally located in a 2.25 kb *KpnI* restriction fragment. This DNA fragment, after isolation from an agarose gel, was treated with sufficient exonuclease *Bal31* to remove ~750 bp from each end. Product fragments (600–800 bp) were gel purified, treated with the large fragment of DNA polymerase I and dNTPs to repair frayed ends, and ligated into the *SmaI* site of the temperature-inducible phage λ -promoter vector pCE30 [1]. Transformants of *E. coli* strain AN1459 [3] were initially screened for poor growth at 41°C (due to high-level overproduction of SSB), then plasmids were restriction mapped with endonuclease *HinfI* to identify those with deletion endpoints just preceding the ribosome-binding site of *ssb*. Finally, plasmids that directed the highest level of overproduction of SSB protein on treatment of cells containing them at 42°C were identified, and the precise endpoints of *Bal31* deletion determined by nucleotide sequence determination. Two plasmids (pND72 and pND73) that had different-sized *ssb*⁺ inserts were used interchangeably for overproduction of SSB; pND72 contains a 741 bp fragment commencing 47 bp before the *ssb* start codon, whereas pND73 contains a 660 bp fragment commencing at position –49.

pAL1379 (SSB Δ C8): Plasmid pND72 was used as template with KOD polymerase (Novagen) for PCR amplification of the *ssb* Δ C8 gene, missing nucleotides coding for the eight C-terminal amino acids (-DFDDDIPF). Site-directed mutagenesis according to the Quik-Change method (Stratagene) used complementary PCR primers 178 (5'-AACGAGCCGCCGATGTAATTT GATGATGACATT) and 179 (5'-AATGTCATCATCAAATTACATCGGCGGCTCGTT) to introduce a TAA stop codon (italicized) just following the ATG codon for Met169 (underlined). This plasmid directs temperature-inducible production of the SSB Δ C8 protein.

Protein Expression, labeling and Purification

Buffers were: T₅₀: 50 mM Tris-HCl, pH 8.0, 1 mM EDTA; T₇₀: 70 mM Tris-HCl, pH 8.0, 1 mM EDTA; P: 20 mM sodium phosphate, pH 6.9, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol; SSB storage buffer: 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 300 mM NaCl, 10% (v/v) glycerol; SSB Δ C8 storage buffer, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 500 mM NaCl, 30% (v/v) glycerol. The (NH₄)₂SO₄ solution used to precipitate SSB was made by adding 100 g of ammonium sulfate to 200 mL of Buffer T₅₀. All protein purification steps were carried out at 2–6°C.

SSB: Strain BL21(λ DE3)*recA* [4] containing pND72 or pND73 was grown at 30°C in LB medium containing 100 μ g/mL ampicillin to an A₆₀₀ of ~0.7. The temperature was rapidly shifted to 42°C to induce SSB overproduction, and incubation was continued for a further 3 h. Cells were collected by centrifugation (11000 $\times$ g; 7 min) and stored at –80°C. After thawing, cells (1.55 g from 1 L of culture) were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol, 2 M NaCl, 20 mM spermidine, 10% (w/v) sucrose; 23.2 mL) and lysed by passage twice through a French press at 12000 psi. Cell debris was removed by centrifugation (38000 $\times$ g; 40 min). Streptomycin sulfate solution (10% w/v) was added dropwise to the soluble fraction to 1.3%, and the mixture stirred for 30 min before being centrifuged (34000 $\times$ g, 80 min). (NH₄)₂SO₄ solution (1.265 volumes, as above) was added to the supernatant. After stirring for 30 min, precipitated proteins were collected by centrifugation (38000 $\times$ g, 30 min) and resuspended in Buffer T₅₀ + 300 mM NaCl (10 mL), before being dialysed against two changes of 3 L of Buffer T₇₀. Wild-type SSB is only slightly soluble in 70 mM Tris-HCl and its selective precipitation during this dialysis step affords significant purification. The dialysed sample, containing insoluble SSB, was centrifuged at 38000 $\times$ g for 30 min, and the pellet resuspended in buffer T₅₀ + 300 mM NaCl

(5 mL). The suspension was clarified by centrifugation ($36000 \times g$; 20 min) and the supernatant was added dropwise to 60 mL of Toyopearl DEAE-650M resin that had been equilibrated in Buffer T₅₀ + 30 mM NaCl. The resin was stirred for 90 min, then packed into a column (2.5 cm I.D.) before attachment to an ÄKTA FPLC system. The column was washed with 120 mL of Buffer T₅₀ + 30 mM NaCl (1 mL/min), then a linear gradient of NaCl (470 mL; 30–600 mM) in Buffer T₅₀ was applied. Fractions containing pure SSB, which eluted in a single peak at ~165 mM NaCl, were pooled and dialyzed against two changes of 3 L of SSB storage buffer. Typical yields were 50–150 mg.

SSBΔC8: This SSB mutant protein (missing the C-terminal 8 residues) was overproduced under direction of plasmid pAL1379 in a 3-L culture, essentially as described for wild-type SSB, above, and purified in the same way through the ammonium sulfate precipitation step. The pellets were resuspended with 30 mL of Buffer T₅₀ + 300 mM NaCl, and dialyzed against Buffer T₇₀ (5 L) overnight. Wild-type SSB precipitates in this buffer. However, only a slight precipitate of SSBΔC8 was observed. The dialysate (35 mL) was clarified by centrifugation ($40,000 \times g$, 40 min) and loaded at 1 mL/min onto a column (2.5 × 14 cm) of Toyopearl DEAE-650M resin that had been equilibrated with Buffer T₅₀. SSBΔC8 eluted in a peak at ~440 mM NaCl in a gradient (300 mL) of 0–1 M NaCl in Buffer T₅₀; fractions were pooled and dialyzed against two changes of 2 L of Buffer T₅₀. The dialyzed fraction (48 mL) was loaded at 1 mL/min onto a column (2.5 × 3 cm) of HiTrap™ Heparin HP resin (GE Healthcare) that had been equilibrated with Buffer T₅₀. SSBΔC8 eluted in a peak at ~230 mM NaCl in a linear gradient (200 mL) of 0–500 mM NaCl in Buffer T₅₀. Proteins in pooled fractions (28 mL) were precipitated with ammonium sulfate (0.313 g/mL) and the pellet resuspended in <3 mL of Buffer T₅₀ + 100 mM NaCl for gel filtration on a HiLoad 26/600 Superdex 75 prep grade column (GE Healthcare). Purified SSBΔC8 (~5 mg) was dialyzed into SSBΔC8 storage buffer and stored frozen at –80°C.

Supplementary References

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Supplementary Figures

(a)

(b)

Supplementary Figure S1

Supplementary Figure S2

Supplementary Figure S3

Supplementary Figure S4

Supplementary Figure S5