Probing *E. coli* SSB Protein-DNA topology by reversing DNA backbone polarity

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Abstract

E. coli single strand (ss) DNA binding protein (SSB) is an essential protein that binds ssDNA intermediates formed during genome maintenance. SSB homotetramers bind ssDNA in two major modes differing in occluded site size and cooperativity. The (SSB)₃₅ mode in which ssDNA wraps on average around two subunits is favored at low [NaCl] and high SSB to DNA ratios and displays high "unlimited", nearest-neighbor cooperativity forming long protein clusters. The (SSB)₆₅ mode, in which ssDNA wraps completely around four subunits of the tetramer, is favored at higher [NaCl] (> 200 mM) and displays "limited" low cooperativity. Crystal structures of *E. coli* SSB and *P. falciparum* SSB show ssDNA bound to the SSB subunits (OB-folds) with opposite polarities of the sugar phosphate backbones. To investigate whether SSB subunits show a polarity preference for binding ssDNA, we examined *Ec*SSB and *Pf*SSB binding to a series of (dT)₇₀ constructs in which the backbone polarity was switched in the middle of the DNA by incorporating a reverse polarity (RP) phosphodiester linkage, either 3'-3' or 5'-5'. We find only minor effects on the DNA binding properties for these RP constructs, although (dT)₇₀ with a 3'-3' polarity switch shows decreased affinity for *Ec*SSB in the (SSB)₆₅ mode and lower cooperativity in the (SSB)₃₅ mode. However, (dT)₇₀ in which every phosphodiester linkage is reversed, does not form a completely wrapped (SSB)₆₅ mode, but rather binds *Ec*SSB in the (SSB)₃₅ mode, with little cooperativity. In contrast, *Pf*SSB, which binds ssDNA only in an (SSB)₆₅ mode and with opposite backbone polarity and different topology, shows little effect of backbone polarity on its DNA binding properties. We present structural models suggesting that strict

backbone polarity can be maintained for ssDNA binding to the individual OB-folds if there is a change in ssDNA wrapping topology of the RP ssDNA.

Keywords: reverse polarity ssDNA, SSB-ssDNA interactions; cooperativity; binding mode transitions; DNA topology

Statement of Significance

Single stranded (ss) DNA binding (SSB) proteins are essential for genome maintenance. Usually homo-tetrameric, bacterial SSBs bind ssDNA in multiple modes, one of which involves wrapping 65 nucleotides of ssDNA around all four subunits. Crystal structures of *E. coli* and *P. falciparum* SSB-ssDNA complexes show ssDNA bound with different backbone polarity orientations raising the question of whether these SSBs maintain strict backbone polarity in binding ssDNA. We show that both *E. coli* and *P. falciparum* SSBs can still form high affinity fully wrapped complexes with non-natural DNA containing internal reversals of the backbone polarity. These results suggest that both proteins maintain a strict backbone polarity preference, but adopt an alternate ssDNA wrapping topology.

Introduction

Single-stranded DNA binding proteins (SSBs) are essential in all kingdoms of life and function by selectively binding to the single-stranded DNA (ssDNA) intermediates that are formed during genome maintenance protecting them from degradation and inhibiting DNA secondary structures (1-4). *Escherichia coli* SSB (*EcSSB*) also serves as a central hub for interaction with numerous metabolic proteins (SSB interacting proteins – SIPs) involved in replication, recombination and repair (5).

EcSSB functions as a homo-tetramer (Fig. 1B) (3, 6), with each subunit (177 amino acids) composed of two domains (Fig. 1A): a structured N-terminal DNA binding domain (DBD) (residues 1-112), and a C-terminal domain (residues 113-177) composed of a flexible, intrinsically disordered linker (IDL) [56 aa (Fig.1A)] and a nine residue “acidic tip”. This acidic tip is conserved among many bacterial SSBs and is the primary site of interaction with the SIPs (5, 7-13). *EcSSB* binds ssDNA in two major modes referred to as (SSB)₃₅ and (SSB)₆₅, where the subscripts denote the average number of ssDNA nucleotides occluded (14, 15). The relative stabilities of these modes depend primarily on salt concentration and type, and protein to DNA ratio (binding density) (14, 16-24), as well as applied force (25-28).

In the (SSB)₆₅ mode, favored at [NaCl]>0.20 M or [Mg²⁺]>10 mM at 25°C, the ssDNA wraps around all four subunits of the tetramer (6) (see Fig. 1B), with a ~65 nucleotide occluded site size. The topology of ssDNA wrapping in the (SSB)₆₅ binding mode follows the seams on a baseball such that ssDNA enters and exits the

tetramer in close proximity. On long ssDNA, the (SSB)₆₅ mode displays "limited" cooperativity between adjacent tetramers (16, 24, 29) as depicted in Fig. 1B. In this mode SSB can diffuse along ssDNA transiently destabilizing DNA hairpins and promoting RecA filament formation (25, 30). Single stranded DNA translocases are able to actively push SSB tetramers bound in the (SSB)₆₅ mode along ssDNA providing a potential mechanism for reorganization and clearance of tightly bound SSBs from ssDNA (31). In the (SSB)₆₅ mode, non-nearest neighbor SSB tetramers can interact cooperatively, possibly through IDLs (23, 32). These interactions result in condensation of nucleoprotein complexes and require presence of IDL and are promoted by glutamate and acetate salts (23, 28, 32).

In the (SSB)₃₅ mode, favored at [NaCl]<10 mM (Fig. 1C) or [MgCl₂]<1 mM, and high SSB to DNA ratios (14, 15, 18), ssDNA wraps around only two subunits on average with a ~35 nucleotide occluded site size. In this mode SSB binds ssDNA with unlimited nearest-neighbor cooperativity favoring formation of long protein clusters (17, 18, 20, 22, 24, 32, 33) as depicted in Fig. 1C. Structural models for the (SSB)₃₅ binding mode have been proposed, suggesting direct interactions of adjacent tetramers through the L₄₅ loops within the tetrameric core of the protein (6) (Fig. 1C). In this mode SSB can diffuse along ssDNA (25, 26) and undergo direct or intersegment transfer between separate ssDNA molecules (34) or between distant sites on the same DNA molecule (35). The ability to undergo direct transfer appears to play a role in SSB recycling during replication (34, 36). Nearest neighbor cooperativity can also be displayed between the different SSB binding modes (24). A recent study (37) identified residues Y22 and K73 as important for cooperative

interactions between adjacent tetramers. Both the linker and the conserved tip of C-terminus are also important for cooperativity (23, 24, 32).

Most bacterial SSBs are homo-tetramers (38). Although the dimeric *Deinococcus radiodurans* SSB (DrSSB) is an exception (39, 40), it still contains 4 OB folds (2 OB folds per monomer). In addition to *E. coli*, several other homo-tetrameric SSB-ssDNA crystal structures have been reported, including *Plasmodium falciparum* (PfSSB) (41), *Deinococcus radiodurans* (DrSSB (40), *Helicobacter Pylori* (HpSSB) (42), *Bacillus subtilis* (BsSSB-B (43) and BsSSB-A (37)), and *Pseudomonas aeruginosa* SSB (PaSSB)(44). All but one of these structures shows DNA bound to the OB-folds with the same phosphodiester backbone polarity orientation (following the path across the β -sheet in the direction from 2-3 loop towards 1-2 loop). This is exemplified by PfSSB and HpSSB in Fig. 1D and 1E. The one exception is the EcSSB-DNA structure that displays the opposite 5'-3' polarity to each OB-fold (Fig. 1B). It is interesting to note that the predicted topology of ssDNA wrapping in the (SSB)₆₅ binding mode also differs for EcSSB when compared to PfSSB and HpSSB. A crystal structure of EcSSB tetramer bound with two (dC)₃₅ molecules (6) predicts a ssDNA binding topology that wraps around the OB folds of the upper AA' dimer (first (dC)₃₅ molecule), then crosses the dimer-dimer interface in the direction of the B subunit α -helix and finally wraps around the OB folds of the BB' dimer (second (dC)₃₅ molecule) as shown in Fig. 1B (suggested path is AA'BB'). In a PfSSB structure (41), the first and the second molecule of (dT)₃₅ are wrapped around the upper and lower dimer, similarly to EcSSB (though with different polarity). However, crossing of the dimer-dimer interface occurs in the direction of the B'

subunit following a path under the α -helix of the A' subunit as shown in Fig. 1D (suggested path is AA'B'B). Essentially, the same path is predicted for the (SSB)₆₅ mode of *Hp*SSB (42)(Fig. 1E) and *Bs*SSB-B (43). Interestingly, a recent structure of *Pa*SSB co-crystallized with (dT)₂₅ suggests that individual subunits could accommodate ssDNA with opposite polarity (e.g. (dT)₂₅ shown in yellow in Fig. 1F binds to subunit A with 5'-3' polarity, whereas it binds to subunit B' with 3'-5' polarity).

Based on available SSB-DNA structural data it is reasonable to expect that polarity of ssDNA binding might affect the SSB wrapping topology and the binding properties. In the current study we examined the effects of ssDNA backbone polarity for *Ec*SSB and *Pf*SSB for two reasons. First, crystallographic studies suggest that they bind ssDNA with opposite polarities and slightly different topologies. Second, their ssDNA binding properties have been extensively studied in solution. In particular, it was established that *Pf*SSB forms a (SSB)₆₅ binding mode similar to that of *Ec*SSB, yet *Pf*SSB does not form a stable (SSB)₃₅ binding mode (45). Yet, both proteins can diffuse on ssDNA in their fully wrapped modes, and effectively melt out short hairpins (25, 30, 45). Here we examine the binding of these proteins to a variety of oligodeoxythymidylates (dT₇₀) that contain a reversal of the phosphodiester backbone polarity in the middle of the DNA molecule (after residue 35) either through a 3'-3' or 5'-5' linkage. We also examine the binding of ap-(dT)₇₀ and ap-(dT)₃₅ DNA in which the polarity alternates after every residue.

Materials and Methods

Reagents and buffers

Buffers were prepared with reagent grade chemicals and distilled water treated with a Milli Q (Millipore, Bedford, MA) water purification system. Buffer T is 10 mM Tris, pH 8.1, 0.1 mM Na₃EDTA; buffer H is 10 mM Hepes, pH 8.1, 0.1 mM Na₃EDTA.

SSB proteins and ssDNA.

E. coli SSB protein (SSB) was expressed and purified as described (32). The SSB protein is a stable tetramer under all solution conditions used in this study as determined by sedimentation velocity (32). Protein concentration was determined spectrophotometrically (14) (buffer T, 0.20 M NaCl) using $\epsilon_{280}=1.13 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (tetramer). *Plasmodium falciparum* SSB (PfSSB) was expressed and purified as described (41) and its concentration was determined using $\epsilon_{280}=9.58 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (tetramer) (41, 45)

Oligodeoxythymidylates possessing 70 and 35 nucleotides were used in this study ((dT)₇₀ and (dT)₃₅, respectively). Along with DNA possessing normal 5' to 3' polarity, we also examined two variations of (dT)₇₀ (Fig. 1A(iii)) in which the backbone polarity was reversed in the middle of the sequence: 5'-(dT)₃₅-3'-3'-(dT)₃₅-5' (abbreviated as 5'-(dT)₇₀-5') and 3'-(dT)₃₅-5'-5'-(dT)₃₅-3' (abbreviated as 3'-(dT)₇₀-3'). Two DNA molecules, termed ap-(dT)₇₀ and ap-(dT)₃₅ were synthesized in which the backbone polarity is reversed after each residue. A similar set of (dT)₇₀ molecules were synthesized that were doubly labeled at the ends with Cy5 and Cy3 fluorescence dyes: (5'-Cy5-(dT)₇₀-Cy3-3', 5'-Cy5-(dT)₃₅-3'-3'-(dT)₃₅-Cy3-5', 3'-Cy5-

(dT)₃₅-5'-5'-(dT)₃₅-Cy3-3' and 5'-Cy5-ap-(dT)₇₀-Cy3-3'). All oligonucleotides were synthesized and purified to >99% homogeneity as described (20, 46). DNA concentrations were determined spectrophotometrically in buffer T + 0.10 M NaCl using $\epsilon_{260} = 8.1 \times 10^3 \text{ M}^{-1} \text{ (nucleotide) cm}^{-1}$ for all not labeled (dT)₇₀ and (dT)₃₅ constructs (47) ($70 \times \epsilon_{260}$ and $35 \times \epsilon_{260}$, molecule). For all Cy5-(dT)₇₀-Cy3 the extinction coefficient $\epsilon_{260} = 5.82 \times 10^5 \text{ M}^{-1} \text{ (molecule)}$ was used (24, 46).

Fluorescence equilibrium titrations.

Fluorescence titrations were performed in buffers T and H, 25°C, at the NaCl and NaBr concentrations indicated in the text and Figure legends, using a QM-4 spectrofluorometer (Photon Technology International/Horiba Scientific, Edison, NJ). Reverse titrations of SSB (0.3 μM) with (dT)₇₀ constructs (Fig. 2,4 and 6) and normal titration of SSB (0.3 μM) with ap-(dT)₃₅ (Fig.S5) were performed by monitoring quenching of the intrinsic SSB tryptophan fluorescence ($\lambda_{\text{exc}}=296 \text{ nm}$ and $\lambda_{\text{em}}=345 \text{ nm}$) and analyzed as described (24, 32, 48). Normal titrations of Cy5-(dT)₇₀ Cy3 constructs (0.1 μM) with SSB (Fig. 2,3,6 and Fig.S2) were performed by exciting Cy3 donor (515 nm) while monitoring sensitized emission from Cy5 acceptor at 665 nm and analyzed as described (21, 24, 48).

Binding isotherms in Fig. 4 for (dT)₇₀ (X) binding to SSB (M) to determine equilibrium binding constants (K) were analyzed using a single site binding model described in Eq.1 (48),

$$Q = Q_{\text{max}} \frac{KX}{1+KX} \quad (1)$$

where $Q=(F_0-F_{obs})/F_0$ is normalized Trp fluorescence quenching with F_0 representing the fluorescence intensity of SSB alone and F_{obs} the Trp fluorescence measured at each point in the titration; and $Q_{max}=(F_0-F_{max})/F_0$ is the normalized fluorescent quenching at saturation. The concentration of the free DNA (X) in Eq.1 was obtained by solving mass conservation Eq. (2):

$$X_{tot} = X + X_{bound} = X + \frac{KX}{1+KX} M_{tot} \quad (2),$$

where X_{tot} and M_{tot} represent total concentrations of (dT)₇₀ and SSB, respectively. Non-linear least squares (NNLS) fitting of the isotherms to Eqs. 1-2 was performed as described (48) using SCIENTIST (Micromath, St. Louis, MO)

Isothermal titration calorimetry.

ITC titrations were performed in buffer H (25°C, 20 mM NaCl) using VP-ITC microcalorimeter (Malvern Panalytical Inc., Wesborough, MA). In these experiments the SSB (1μM) was titrated with (dT)₇₀ DNA constructs with stock solutions of reagents thoroughly dialyzed prior use. The data were analyzed using software provided by manufacturer as described (49)

Stopped-Flow kinetics.

Fluorescence stopped-flow experiments were performed as described (46) in Buffer H, 25°C, in the presence of 2M NaBr using an Applied Photophysics SX.18MV instrument (Applied Photophysics Ltd., Leatherhead, U.K.). In experiments shown in Fig.S4 the SSB at concentration 50 nM (final) was mixed with increasing

concentrations of (dT)₇₀ constructs and intrinsic Trp fluorescence ($\lambda_{\text{exc}}=296$ nm) signal change was monitored at ($\lambda>340$ nm) using long-pass filter (Oriel, catalog no. 51258). The observed rates, r_{obs} (s^{-1}), obtained fitting signal change to one-exponential decay function were then plotted as a function of DNA concentration (Fig.S4). Linear fit of these data produced k_{on} and k_{off} as slope and intercept, respectively.

Analytical sedimentation

Sedimentation velocity experiments were performed as described (23, 24, 32) with an Optima XL-A analytical ultracentrifuge and An50Ti rotor (Beckman Instruments, Fullerton, CA) at 42000 rpm in buffer H, 25°C, with the salt concentrations and type indicated in the text and Fig. S1 and S3 legends. Constant concentrations of (dT)₇₀ constructs (0.5 μM) were used while adding SSB to make solutions with different protein to DNA ratios ($r=[\text{SSB}]_{\text{tot}}/[(\text{dT})_{70}]_{\text{tot}}$). The absorbance was monitored at 260 nm, reflecting predominantly the contribution from ssDNA. The contribution of SSB to the absorbance at 260 nm is small compared to the DNA at protein/DNA ratios, $r\leq 2$, used in this study. Data were analyzed using SEDFIT (www.analyticalultrasentrifugation.com) to obtain $c(s)$ distributions and estimate molecular weights for the species described by single-peaks (50). The $c(s)$ refers to the continuous sedimentation distribution that results from a SEDFIT analysis of the absorbance traces obtained in a sedimentation velocity AUC experiment using the Lamm equation (50). $C(s)$ represents the concentration (in absorbance units in this case) of species with a given sedimentation coefficient rang.

The densities and viscosities at 25°C were calculated using SEDNTERP for NaCl T-buffer solutions. Partial specific volumes for protein-DNA complexes were calculated using formula: $v = (v_{DNA}M_{DNA} + n v_P M_P) / (M_{DNA} + n M_P)$, where v_{DNA} and M_{DNA} are the partial specific volume and molecular weight of DNA; v_P and M_P are the partial specific volume and molecular weight of the protein and n is the number of SSB tetramers bound to DNA. In all our experiments the absorbance estimated from integrated $c(s)$ profiles was the same as the absorbance of loaded solutions indicative that no precipitation/aggregation occurred during the experimental runs.

Results

Effect of a reversal of backbone ssDNA polarity on *Ec*SSB and *Pf*SSB binding mode formation and cooperativity.

Depending on solution conditions one (dN)₇₀ is able to bind either one SSB tetramer in its fully wrapped (SSB)₆₅ binding mode (65 nts occluded site size) (Fig, 1B) or two SSB tetramers in its (SSB)₃₅ binding mode with high cooperativity (Fig, 1C) (20, 24, 51). The proposed structure of an (SSB)₆₅ complex in Fig. 1B (6) shows that ~35 nucleotides first wrap around the upper two SSB subunits then DNA crosses the dimer-dimer interface continuing to wrap another 35 nts around the other two subunits following the same path as for the upper subunits. If the SSB tetramer binds ssDNA in a manner that maintains a strict 3' to 5' backbone polarity, then a reversal of the backbone polarity in the middle of a (dN)₇₀ might weaken or even preclude formation of the fully wrapped (SSB)₆₅ binding mode. The ssDNA wrapping topology proposed for the (SSB)₃₅ mode, although more speculative (6) (Fig. 1C),

also suggests that a reversal of the backbone polarity in the middle of a (dN)₇₀ might also affect the relative orientation of two tetramers bound in the (SSB)₃₅ mode and affect any cooperative interactions. In order to test these concepts, we designed three non-natural (dT)₇₀ variants (see Materials and Methods). In two variants the backbone polarity was reversed in the middle of the DNA by incorporating a 3'-3' or 5'-5' phosphodiester linkage; these are denoted as 5'-(dT)₇₀-5' and 3'-(dT)₇₀-3', respectively. The third variant alternated a 3'-3' and 5'-5' phosphodiester linkage after each nucleotide and is denoted ap-(dT)₇₀. DNA molecules containing backbone polarity reversals have been used to probe directional movement of DNA translocases and helicases (31, 52-57). Binding of these unlabeled DNA molecules was monitored by the quenching of the intrinsic Trp fluorescence of SSB (24, 32, 48). Other versions of these (dT)₇₀ variants were made in which the ends of each DNA were labeled with the fluorophores, Cy3 and Cy5, which can undergo Förster resonance energy transfer (FRET). Use of these labeled (dT)₇₀ molecules enabled us to monitor formation of the different SSB binding modes since a higher FRET efficiency is observed in the (SSB)₆₅ mode due to the closer proximity of the two ends of (dT)₇₀ in this mode as demonstrated previously (21, 24).

The results of equilibrium titrations of SSB with (dT)₇₀, 5'-(dT)₇₀-5' and 3'-(dT)₇₀-3' monitoring SSB Trp fluorescence quenching are shown in Fig. 2A and 2B in Buffer T at 25.0°C at 10mM and 0.30 M [NaCl]. At 10 mM NaCl, both the (SSB)₆₅ and the (SSB)₃₅ binding modes can form as determined by the SSB/DNA ratio, whereas at 0.30 M NaCl, only the (SSB)₆₅ binding mode is formed (14, 18, 19, 24, 51). At 10 mM NaCl (Fig. 2A) for all (dT)₇₀ constructs the normalized quenching

increases linearly with addition of DNA indicative of stoichiometric binding (all added DNA binds to SSB). The inflection point at a DNA to protein ratio of 0.5 ($r = [\text{SSB}]_{\text{tot}}/[(\text{dT})_{70}]_{\text{tot}} = 2$) reflects the transition from the $(\text{SSB})_{35}$ mode to the $(\text{SSB})_{65}$ mode, the latter becoming solely populated at $r=1$ as shown previously for $(\text{dT})_{70}$ (24, 51). However, we note that both RP DNA constructs show a higher extent of Trp quenching in the $(\text{SSB})_{35}$ mode ($Q_{35} \sim 0.6$ for green and orange isotherms in Fig. 2A at $[(\text{dT})_{70}]_{\text{tot}}/[\text{SSB}]_{\text{tot}} = 0.5$) compared to $(\text{dT})_{70}$ (cyan), for which $Q_{35} \sim 0.5$ is expected (24, 51). This might reflect a slightly different positioning of the two SSB tetramers on the RP DNAs. However, independent of polarity, no difference in quenching ($Q_{65} = 0.9$) is observed for the 1:1 $(\text{SSB})_{65}$ complexes that form at $[(\text{dT})_{70}]_{\text{tot}}/[\text{SSB}]_{\text{tot}} > 1$. At the higher salt concentration of 0.30 M NaCl (Fig. 2B) all three titrations are identical, showing a linear increase in Trp quenching up to $r=1$ indicative of stoichiometric binding exclusively in the $(\text{SSB})_{65}$ mode (24, 51).

We next performed titrations of the Cy3/Cy5 fluorescently labeled $(\text{dT})_{70}$ constructs with SSB at the same two [NaCl]. The results are consistent with the Trp fluorescence titrations in Fig. 2A and 2B. At 10 mM NaCl (Fig. 2C), the Cy5 FRET signal increases linearly up to $r=1$ reflecting formation of the fully wrapped $(\text{SSB})_{65}$ complex, and then decreases linearly up to $r=2$ reflecting a transition to a 2:1 $(\text{SSB})_{35}$ complex with increasing [SSB]. Interestingly, the Cy5 FRET signal is similar for all 1:1 complexes ($F_{\text{Cy5},65} \sim 3.5 \times 10^5$ at $r=1$), whereas the 2:1 complexes ($r>2$) show different Cy5 FRET signals. SSB bound to 5'-($\text{dT})_{70}$ -5' shows a higher Cy5 fluorescence compared to 3'-($\text{dT})_{70}$ -3' and $(\text{dT})_{70}$. This suggests some difference in the DNA wrapping in the $(\text{SSB})_{35}$ mode with 5'-($\text{dT})_{70}$ -5'. However, the sharpness of

the transitions between binding modes at $r=1$ argues that SSB still binds to both RP DNA constructs with a high nearest neighbor cooperativity (24). However, all three titrations at 0.30 M NaCl (Fig. 2D) are identical indicating that they can all form a fully wrapped 1:1 (SSB)₆₅ complex in these conditions consistent with the results in Fig. 2B.

To obtain an independent check on our conclusion that both 1:1 and 2:1 SSB complexes can be formed on both RP (dT)₇₀ constructs at low [NaCl], we performed sedimentation velocity experiments at total concentration ratios of $r=1$ and 2 SSB/DNA. Fig. S1A shows the results plotted as $c(s)$ distributions (24, 50). All three DNA molecules sediment identically in the absence of SSB. At each SSB/DNA ratio, only a single $c(s)$ peak is observed ($s_{20,w}=5.1S$ at $r=1$ and $s_{20,w}=6.7S$ at $r=2$). The sedimentation coefficients of the $r=1$ and $r=2$ complexes also are the same for each DNA-SSB complex indicating that the change in RP does not lead to major differences in hydrodynamic properties. At the higher 0.30 M [NaCl] (Fig. S1B) only one peak at $s_{20,w}=5.3S$ is observed at both $r=1$ and $r=2$ indicating that only a 1:1 (SSB)₆₅ complex forms at both SSB ratios.

We showed previously that the SSB from *Plasmodium falciparum*, PfSSB, binds ssDNA only in an (SSB)₆₅ binding mode independent of [NaCl] with a DNA topology similar to EcSSB, but with a DNA backbone polarity opposite to that observed in the *E. coli* SSB-DNA structure(41, 45). Using equilibrium titrations (Fig. S2) and sedimentation velocity (Fig. S3) we demonstrate that the backbone polarity of (dT)₇₀ has no effect on the formation of the 1:1 fully wrapped complexes, which form stoichiometrically both at low (10 mM) and moderate (0.30 M) NaCl

concentrations ($s_{20,w}=4.8S$ and $s_{20,w}=5.4S$, respectively, Fig. S3). However, a somewhat lower Cy5 fluorescence (FRET) characterizes formation of the *Pf*SSB-3'-(dT)₇₀-3' complex compared to the 5'-(dT)₇₀-5' complex and (dT)₇₀ (Fig. S2). This suggests a slight difference in DNA wrapping in the *Pf*SSB-3'-(dT)₇₀-3' (SSB)₆₅ binding mode.

So far we investigated the binding mode formation at NaCl concentrations of salt, where either the (SSB)₃₅ ([NaCl]≤0.01 M) or the (SSB)₆₅ ([NaCl]≥0.3 M) modes are formed predominantly. However, at intermediate [NaCl] both modes are expected to be populated (14, 15). To monitor the transition between binding modes we performed titrations of DNA constructs with *Ec*SSB at 40, 80 and 100 mM NaCl (Fig. 3) and found that, while the binding of the first SSB tetramer (formation of 1:1 (SSB)₆₅ complex) is stoichiometric for all DNAs, the transition to the (SSB)₃₅ mode upon binding the second SSB tetramer is less favorable for 5'-(dT)₇₀-5' (green circles), whereas the isotherms for binding 3'-(dT)₇₀-3' and (dT)₇₀ are very similar. This difference can be due to either a higher affinity (K_{65}) of *Ec*SSB to 5'-(dT)₇₀-5' in the (SSB)₆₅ binding mode or a lower nearest-neighbor cooperativity (ω_{35}) in the (SSB)₃₅ binding mode (24).

To examine this further, we performed equilibrium titrations of *Ec*SSB with the RP (dT)₇₀ DNA constructs (Fig. 4A) in the presence of 2.0 M NaBr, which lowers the binding affinity of *Ec*SSB to (dT)₇₀ into a range where it can be measured accurately(34, 51, 58). We find that K_{65} for SSB binding to (dT)₇₀ ($1.8\pm0.1\times10^7 M^{-1}$) and 3'-(dT)₇₀-3' ($3.3\pm0.1\times10^7 M^{-1}$) are similar under these conditions, whereas binding to 5'-(dT)₇₀-5' ($2.4\pm0.1\times10^6 M^{-1}$) is much lower. Additional stopped-flow

experiments (Fig. S4A) established that the decrease in affinity for 5'-(dT)₇₀-5' is due to a more than 13-fold increase in the dissociation rate constant ($k_{-5'5'}=13.1\pm1.9\text{ s}^{-1}$ vs $k_{-3'3'}=0.1\pm0.7\text{ s}^{-1}$, assuming an upper bound value for the latter), whereas the same bimolecular association rate constant of $\sim 4\times10^7\text{ M}^{-1}\text{s}^{-1}$ is measured for both DNA constructs. The lower value of K_{65} for the 5'-(dT)₇₀-5' construct in 2.0 M NaBr suggests that the preference for the (SSB)₆₅ binding mode for the 5'-(dT)₇₀-5' construct (see Fig. 2) results from a significantly decreased cooperativity in the (SSB)₃₅ mode, (ω_{35}).

In contrast, equilibrium binding of *Pf*SSB under the same conditions (see Fig. 4B) shows little difference in affinity for (dT)₇₀ ($K_{65}=(3.2\pm0.2)\times10^7\text{ M}^{-1}$) and the two RP DNAs ($K_{65, 5'5'}=(1.7\pm0.1)\times10^7\text{ M}^{-1}$ vs. $K_{65, 3'3'}=(4.0\pm0.4)\times10^7\text{ M}^{-1}$). Furthermore, *Pf*SSB (Fig. S4B) shows only a ~ 3 fold difference in dissociation rate constants, $k_{-5'5'}=3.5\pm0.5\text{ s}^{-1}$ and $k_{-3'3'}=1.1\pm0.7\text{ s}^{-1}$ and similar association rate constants ($\sim 3\times10^7\text{ M}^{-1}\text{s}^{-1}$). We note that affinities obtained from these rate constants ($K=k_{\text{on}}/k_{\text{off}}$) agree well with the affinities determined from our equilibrium titration experiments (Fig. 4).

In spite of the fact that the affinity in the (SSB)₆₅ binding mode might vary for the RP constructs, the most interesting result is that both *Ec*SSB and *Pf*SSB are still able to form fully wrapped complexes with the RP DNA variants under all conditions examined. Furthermore, these RP constructs are also able to form highly cooperative (SSB)₃₅ complexes. However, we note that quantification of the nearest neighbor cooperativity parameters for the different DNA constructs is difficult due to the strong correlation of the binding parameters (K_{65} , K_{35} and ω_{35}). We have recently performed such an analysis for wt *Ec*SSB binding to (dT)₇₀, but this required having

reasonable estimates of either K_{65} or K_{35} (24). Such an analysis cannot be performed at this time since estimates of K_{35} and K_{65} are not available for SSB binding to 5'-(dT)₇₀-5' and 3'-(dT)₇₀-3'.

We next performed isothermal titration calorimetry (ITC) experiments in order to probe the thermodynamics of the *Ec*SSB and *Pf*SSB binding to the RP-(dT)₇₀ DNA in more detail. Fig. 5 shows the results of titrations of *Ec*SSB (1 μ M tetramer) with 5'-(dT)₇₀-5', 3'-(dT)₇₀-3' and (dT)₇₀ at low [NaCl] (buffer H, 20 mM NaCl). All *Ec*SSB titrations (Fig. 5A and 5B) show biphasic character, reflecting the initial formation of a 2:1 (SSB)₃₅ complex in the range of DNA to protein ratios ≤ 0.5 , which then transforms to a 1:1 (SSB)₆₅ complex for DNA to protein ratios from 0.5 – 1.0. Since binding is stoichiometric for each DNA under these conditions, the normalized heats in the flat portions of the isotherms can be related to the enthalpies for formation of the binding modes as given in Eqs. 3 (59),

$$Q_{\text{norm},0-0.5} = \Delta H_{2 \times 35, \text{tot}} \quad (3a)$$

$$Q_{\text{norm},0.5-1.0} = 2\Delta H_{65} - \Delta H_{2 \times 35, \text{tot}} \quad (3b)$$

where $\Delta H_{2 \times 35, \text{tot}} = 2\Delta H_{35} + \Delta H_{\omega 35}$ represents overall enthalpy of (SSB)₃₅ binding mode formation (including an enthalpic contribution from cooperativity) and ΔH_{65} is the enthalpy of SSB binding in (SSB)₆₅ binding mode. Inspection of Fig. 5A and 5B indicates that *Ec*SSB binding to 3'-(dT)₇₀-3' is very similar to its binding to (dT)₇₀ (Fig.5B) and slightly more enthalpically favorable compared to binding to 5'-(dT)₇₀-5' (Fig. 5A). Solution of the system of equations (Eq.3) enables us to quantify the differences in the binding enthalpies for the different binding modes formed on the

different DNAs. For 5'-(dT)₇₀-5', $\Delta H_{2 \times 35, \text{tot}} = -142.3 \pm 0.4$ kcal/mol and $\Delta H_{65} = -148.4 \pm 1.6$ kcal/mol, whereas for 3'-(dT)₇₀-3' and (dT)₇₀ $\Delta H_{2 \times 35, \text{tot}} = -152.3 \pm 1.9$ kcal/mol and $\Delta H_{65} = -160.0 \pm 2.4$ kcal/mol, Hence, the interaction of *Ec*SSB with 5'-(dT)₇₀-5' is ~ 10 and 12 kcal/mol less enthalpically favorable in the (SSB)₃₅ and (SSB)₆₅ binding modes, respectively. This somewhat less favorable binding to 5'-(dT)₇₀-5' is consistent with the equilibrium titrations and stopped-flow results discussed above.

In contrast, the interaction of *Pf*SSB with both RP (dT)₇₀ constructs at these low salt conditions (Fig. 5C) leads to formation of only the 1:1 (SSB)₆₅ complex, consistent with previous studies showing that *Pf*SSB does not form an (SSB)₃₅ complex (45). *Pf*SSB binds to DNA molecules with similar enthalpies of $\Delta H_{65, 5'5'} = -152.3 \pm 2.3$ kcal/mol and $\Delta H_{65, 3'3'} = -156.2 \pm 1.4$ kcal/mol.

SSB binding to oligodeoxythymidylates with alternating polarity (ap-(dT)₇₀ and ap-(dT)₃₅)

We next determined whether SSB is able to bind to ssDNA in which the backbone polarity is reversed after each nucleotide. We refer to these as ap-(dT)₇₀ (ap- for alternating polarity), ap-(dT)₃₅ and Cy5-ap-(dT)₇₀-Cy3 DNA (See Materials and Methods). The results are presented in Fig. 6A and 6B, at [NaCl] in the range from 10 mM to 0.30 M. Titrations at 10, 30 and 100 mM NaCl show transition points at DNA to protein ratios of 0.5 and 1.0 indicating that both 2:1 (SSB)₃₅ and 1:1 (SSB)₆₅ modes can form. Whereas, only a 1:1 (SSB)₆₅ complex is formed in 0.30 M NaCl. However, we note that the normalized Trp quenching only reaches values in the range $Q_{\text{obs}}=0.62-0.70$ ($[\text{DNA}]_{\text{tot}}/[\text{SSB}]_{\text{tot}} > 1.0$), much lower than the value of $Q_{65}=0.9$ observed for the (SSB)₆₅ mode on (dT)₇₀ (24, 51) (see also Fig. 2B). This

suggests that ap-(dT)₇₀ is unable to form a fully wrapped (SSB)₆₅ binding mode. This conclusion is also supported by the results of titrations with the C3/Cy5 end-labeled ap-(dT)₇₀ shown in Fig. 6B. The 1:1 (SSB)₆₅ complexes formed in 0.03 M, 0.1 M and 0.3 M NaCl at r=1 have Cy5 intensities in the range (2.5-2.8)×10⁵, while the Cy5 signal intensity for normal (dT)₇₀-SSB complex is ~ 3.5×10⁵ (see Fig.2C and 2D and Fig.3).

The ap-(dT)₇₀ data are also consistent with the results for *Ec*SSB binding to ap-(dT)₃₅. Two molecules of normal polarity (dT)₃₅ can bind to an SSB tetramer, although with negative cooperativity that depends on salt concentration and type (51, 60). All titrations of SSB with ap-(dT)₃₅ presented in Fig.S5 show that only one ap-(dT)₃₅ molecule binds to an SSB tetramer at all [NaCl]. Binding is stoichiometric in the 0.01-0.1 M NaCl concentration range (panel A) and affinity is measurable ($k_{1,35}=1.7\pm0.2\times10^8$ M⁻¹) at 0.3 M NaCl (panel B), whereas binding of the first molecule of normal (dT)₃₅ is always stoichiometric ($k_{1,35}$ affinity is too high to measure) even at much higher [NaCl] (51, 58). The results shown in Fig. S5B also indicate that even at a ~16 fold molar excess of ap-(dT)₃₅ only a small fraction of a 2:1 complex can form ($k_{2,35}=6.8\pm0.6\times10^4$ M⁻¹ is very low). This inability to bind a second molecule of ap-(dT)₃₅ is consistent with inability of ap-(dT)₇₀ to form a fully wrapped complex. However, it is surprising that *Ec* SSB still can bind the ap-(dT)₃₅ with substantial affinity.

Discussion

The two tetrameric SSB proteins from *E.coli* and *P.falciparum* have similar subunit organization: an N-terminal DNA binding core and an intrinsically disordered C-terminal tail (Fig. 1A). While the tetrameric DNA binding cores share 39% amino acid identity, and 66% homology (41), their intrinsically disordered C-terminal domains differ greatly. In *EcSSB* the conserved acidic tip (9 aa) of the C-terminal tail, which serves as a binding site for numerous metabolic SIPs (SSB interaction proteins) (5, 8, 13), is connected to the DNA binding core via a 56 aa linker containing few charged residues (Fig.1A). In contrast, *PfSSB* possesses a different acidic tip that lacks the conserved IPF residues of the *EcSSB* tip, and is connected to the DNA binding core by a longer (82 aa) more highly charged linker. This difference in the C-terminal tails has a dramatic effect on the DNA binding properties, including binding mode formation and cooperative binding (23, 24, 32). In particular the *PfSSB* is unable to form a low site size, (SSB)₃₅ equivalent binding mode (45) and shows no evidence of highly cooperative binding to ssDNA (32) in spite of the fact that the DNA binding cores of *EcSSB* and *PfSSB* are very similar.

Our focus in this study was whether *EcSSB* and *PfSSB* proteins bind ssDNA with strict polarity. This was motivated by the observation that crystallographic structures show that *PfSSB* and *EcSSB* bind ssDNA with opposite polarity and slightly different topology in their fully wrapped (SSB)₆₅ binding modes (compare Fig. 1B and 1D). In fact, of the SSB-ssDNA crystal structures that have been reported, only the *EcSSB* OB-fold binds ssDNA with a different polarity.

To examine this we designed two types of (dT)₇₀ variants with modified phosphodiester backbone polarities. In the first variant, a reversal of the backbone

polarity, was introduced in the middle of the sequence, either via a 3'-3' or 5'-5' linkage. In a second variant, every phosphodiester backbone in the sequence was reversed, i.e., a 3'-3' linkage alternated with a 5'-5' linkage, designated ap-(dT)₇₀ and ap-(dT)₃₅. If either SSB binds ssDNA with strict polarity, we hypothesized that a fully wrapped (SSB)₆₅ binding mode would be inhibited. Surprisingly, we found that both 3'-(dT)₇₀-3' and 5'-(dT)₇₀-5' bind the same as normal (dT)₇₀ to *Pf*SSB. Only 1:1 fully wrapped complexes with the same hydrodynamic characteristics are formed stoichiometrically at both high (0.30 M) and low (10 mM) [NaCl] (Fig. S2 and S3). Moreover, no differences are observed in the thermodynamics and kinetics of complex formation (Figs. 4B, 5C and S4B).

However, the binding of *Ec*SSB to these variants shows more differences. Both (SSB)₃₅ and (SSB)₆₅ complexes can form stoichiometrically on the RP (dT)₇₀ similar to normal (dT)₇₀ at low (10 mM) and high (0.30 M) [NaCl] (Fig. 2 and Fig. S1). However, the relative stabilities of the two modes are affected. At intermediate [NaCl] (40-100 mM) the (SSB)₆₅ mode is favored with the 5'-(dT)₇₀-5' RP construct, whereas (dT)₇₀ and 3'-(dT)₇₀-3' show little difference (Fig. 3). This could result from increased affinity (K_{65}) in the (SSB)₆₅ mode or decreased affinity (K_{35}) or cooperativity (ω_{35}) in the (SSB)₃₅ binding mode (24). However, equilibrium titrations (Fig. 4A) and kinetic data (Fig. S4A) in buffer containing 2.0 M NaBr indicate that actually the affinity in the (SSB)₆₅ mode (K_{65}) is ~10-fold lower for the 5'-(dT)₇₀-5' construct compared with (dT)₇₀ and 3'-(dT)₇₀-3'. This suggests that the differences are due to weakening of the interactions (either K_{35} and/or ω_{35}) of SSB with 5'-(dT)₇₀-5' in the (SSB)₃₅ binding mode. This is corroborated by ITC measurements (Fig. 5A

and 5B) demonstrating that the total enthalpies for forming both binding modes on 5'-(dT)₇₀-5' are 10-12 kcal/mol less favorable compared with (dT)₇₀ and 3'-(dT)₇₀-3'. However, even for *Ec*SSB, the effects of the backbone polarity reversal on DNA binding is relatively small.

Why would a backbone polarity reversal in the middle of (dT)₇₀ have no effect on formation of the *Pf*SSB (SSB)₆₅ binding mode, and show only small effects for *Ec*SSB and only with 5'-(dT)₇₀-5'. We hypothesize that this is related to some structural differences of the DNA binding domains (tetrameric cores) of these proteins, which dictate slightly altered pathways of ssDNA wrapping in the two (SSB)₆₅ binding modes. In *Ec*SSB the ssDNA wraps around the upper AA' dimer and then crosses the dimer-dimer interface towards the β helix of the B subunit, finally wrapping around the B'B dimer in “*trans*” (AA'BB' topology, Fig.1 B). Whereas in *Pf*SSB, as well as in *Hp*SSB (Fig. 1D and 1E, respectively) ssDNA wraps around the upper and lower dimers similarly to *Ec*SSB (although with opposite polarity), but crosses the dimer-dimer interface under the β helix of the A' subunit in “*cis*” following an different AA'B'B topology. The *Ec*SSB “*trans*” topology appears to be dictated by the presence of 5-6 rigid loops (6, 61)(Fig.1B), whereas a different orientation of these loops in *Pf*SSB (41) (Fig. 1D) and their absence in *Hp*SSB (42) (Fig. 1E) promotes wrapping of ssDNA in “*cis*”. These different crossings of DNA at the dimer-dimer interface could explain the differences that we observe for *Ec*SSB and *Pf*SSB. As a matter of fact for the “*cis*” wrapping pathway that is displayed in the *Pf*SSB structure (Fig. 7Ai) could accommodate fully wrapped 3'-(dT)₇₀-3' and 5'-(dT)₇₀-5' constructs in the (SSB)₆₅ binding mode, with no change in the polarity of ssDNA

binding to the individual OB-folds, as shown in Fig. 7Aii and Fig. 7Aiii, respectively.

The situation differs for *Ec*SSB where DNA follows the “trans” topology (Fig. 7Bi). In this case the DNA strand crossing the dimer-dimer interface (in blue) is further removed from the DNA strand wrapping around the opposite B’ subunit. Assuming that binding polarity to the individual OB-folds is maintained, we propose the wrapping topologies for the 3’-(dT)₇₀-3’ and 5’-(dT)₇₀-5’ DNA constructs shown in Fig. 7Bii and Fig. 7Biii, respectively. Interestingly, in the proposed topology for the 3’-(dT)₇₀-3’ DNA (Fig. 7Bii), the DNA crosses the dimer-dimer interface in the same region as proposed for normal polarity (dT)₇₀. However, in the proposed topology for the 5’-(dT)₇₀-5’ DNA (Fig. 7Biii), the dimer-dimer crossing occurs in the region of the 5-6 loops, which would appear to be sterically unfavorable. This difference might explain the lower affinity and faster dissociation of 5’-(dT)₇₀-5’ from *Ec*SSB, whereas 3’-(dT)₇₀-3’ and (dT)₇₀ show similar binding properties.

The ability of *Ec*SSB to form a 2:1 (SSB)₃₅ complex on both (dT)₇₀ RP variants is not as surprising since in that binding mode only two subunits of each bound tetramer interact with the DNA. However, due to different orientation of those tetramers on the RP constructs, a decrease in the nearest-neighbor cooperativity might be expected due to the loss of some contacts required for such interactions. Yet, we still observe significant cooperativity for the 3’-(dT)₇₀-3’ DNA, which is comparable to (dT)₇₀, whereas cooperativity is decreased with the 5’-(dT)₇₀-5’ DNA. Based on a proposed model for the (SSB)₃₅ binding mode presented in Fig.1C, a 2:1 complex should be possible with both RP (dT)₇₀ constructs. A simple 180° rotation of the upper tetramer within the x-y plane relative to the lower tetramer

creates a 5'-(dT)₇₀-5' complex, while a similar rotation performed on the lower tetramer creates a 3'-(dT)₇₀-3' complex. However, in both cases the interactions between the 4-5 loops that have been proposed to be important for nearest neighbor cooperativity (6) would be lost. We speculate that alternative orientations of the two tetramers in 3D space (e.g. around the Y axes) could restore such nearest-neighbor cooperative contacts and this might be easier for the 3'-(dT)₇₀-3' construct. We note here, that the model for the (SSB)₃₅ binding mode in Fig. 1C is only speculative and awaits experimental testing. So alternative wrapping pathways in this mode, which might explain the differences observed for the RP (dT)₇₀ constructs cannot be excluded. We also know that nearest neighbor cooperativity in the (SSB)₃₅ mode is also dependent on the intrinsically disordered linkers (IDL) in the C-terminal tails (24) that are not observed in the crystal structures. If cooperativity requires direct interactions between the IDLs of adjacent tetramers, such interactions should be possible on (dT)₇₀ and both RP variants.

Interestingly, we find that *Ec*SSB still binds stoichiometrically to the fully alternating ap-(dT)₇₀ in both binding modes (Fig. 6) over a broad NaCl concentration range. However, the affinity in a fully wrapped (SSB)₆₅ mode is weakened significantly. This is indicated by the fact that *Ec*SSB is not able to bind more than one molecule of ap-(dT)₃₅ under any solution condition (Fig. S5). Yet, it is somewhat surprising that it can still bind to *Ec*SSB with substantial affinity. E.g. ap-(dT)₃₅ binds to *Ec*SSB in 0.30 M NaCl with $k_{35,1}=1.7 \times 10^8 \text{ M}^{-1}$ (Fig. S5B), whereas a lower limit for normal (dT)₃₅ is $\sim 2 \times 10^9 \text{ M}^{-1}$ (51).

The results reported here demonstrate that both tetrameric *Ec*SSB and *Pf*SSB can accommodate stretches of ssDNA with reverse polarity and still bind with high affinity. *Ec*SSB also retains the ability to form its different binding modes and even retains high nearest neighbor cooperativity. Only severe backbone polarity changes, such as for the ap-ssDNA constructs, show a large effect on affinity. We have proposed structural models that can explain this behavior while still retaining strict backbone polarity for binding to the individual OB-folds. Hence, our results are consistent with the different ssDNA polarities observed in the crystal structures of ssDNA complexes with *Ec*SSB and *Pf*SSB. This adaptability may play important roles in the metabolic functions of SSB.

Author Contributions

TML and AGK designed the research; AGK performed and analyzed the experiments; AGK and TML wrote the manuscript

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References

1. Chase, J. W., and K. R. Williams. 1986. Single-stranded DNA binding proteins required for DNA replication. *Annu.Rev.Biochem.* 55:103-136.
2. Meyer, R. R., and P. S. Laine. 1990. The single-stranded DNA-binding protein of *Escherichia coli*. *Microbiological Reviews* 54:342-380.

3. Lohman, T. M., and M. E. Ferrari. 1994. Escherichia coli single-stranded DNA-binding protein: multiple DNA-binding modes and cooperativities. *Annu Rev Biochem* 63:527-570.
4. Wold, M. S. 1997. Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annu Rev Biochem* 66:61-92.
5. Shereda, R. D., A. G. Kozlov, T. M. Lohman, M. M. Cox, and J. L. Keck. 2008. SSB as an organizer/mobilizer of genome maintenance complexes. *Crit Rev Biochem Mol Biol* 43(5):289-318.
6. Raghunathan, S., A. G. Kozlov, T. M. Lohman, and G. Waksman. 2000. Structure of the DNA binding domain of E-coli SSB bound to ssDNA. *Nature Structural Biology* 7(8):648-652.
7. Genschel, J., U. Curth, and C. Urbanke. 2000. Interaction of E-coli single-stranded DNA binding protein (SSB) with exonuclease I. The carboxy-terminus of SSB is the recognition site for the nuclease. *Biological Chemistry* 381(3):183-192.
8. Kozlov, A. G., M. J. Jezewska, W. Bujalowski, and T. M. Lohman. 2010. Binding specificity of Escherichia coli single-stranded DNA binding protein for the chi subunit of DNA pol III holoenzyme and PriA helicase. *Biochemistry* 49(17):3555-3566.
9. Marceau, A. H., S. Bahng, S. C. Massoni, N. P. George, S. J. Sandler, K. J. Marians, and J. L. Keck. 2011. Structure of the SSB-DNA polymerase III interface and its role in DNA replication. *EMBO J* 30(20):4236-4247.
10. Shereda, R. D., N. J. Reiter, S. E. Butcher, and J. L. Keck. 2009. Identification of the SSB binding site on E. coli RecQ reveals a conserved surface for binding SSB's C terminus. *J Mol Biol* 386(3):612-625.
11. Ryzhikov, M., and S. Korolev. 2012. Structural studies of SSB interaction with RecO. *Methods Mol Biol* 922:123-131.
12. Antony, E., E. Weiland, Q. Yuan, C. M. Manhart, B. Nguyen, A. G. Kozlov, C. S. McHenry, and T. M. Lohman. 2013. Multiple C-Terminal Tails within a Single E. coli SSB Homotetramer Coordinate DNA Replication and Repair. *J Mol Biol* 425(23):4802-4819.
13. Shinn, M. K., A. G. Kozlov, B. Nguyen, W. M. Bujalowski, and T. M. Lohman. 2019. Are the intrinsically disordered linkers involved in SSB binding to accessory proteins? *Nucleic Acids Res* 47(16):8581-8594.
14. Lohman, T. M., and L. B. Overman. 1985. Two binding modes in Escherichia coli single strand binding protein-single stranded DNA complexes. Modulation by NaCl concentration. *J Biol Chem* 260(6):3594-3603.
15. Bujalowski, W., and T. M. Lohman. 1986. Escherichia coli single-strand binding protein forms multiple, distinct complexes with single-stranded DNA. *Biochemistry* 25(24):7799-7802.
16. Chrysogelos, S., and J. Griffith. 1982. Escherichia coli single-strand binding protein organizes single-stranded DNA in nucleosome-like units. *Proc Natl Acad Sci U S A* 79(19):5803-5807.

17. Griffith, J. D., L. D. Harris, and J. Register, 3rd. 1984. Visualization of SSB-ssDNA complexes active in the assembly of stable RecA-DNA filaments. *Cold Spring Harb Symp Quant Biol* 49:553-559.
18. Lohman, T. M., L. B. Overman, and S. Datta. 1986. Salt-dependent changes in the DNA binding co-operativity of *Escherichia coli* single strand binding protein. *J Mol Biol* 187(4):603-615.
19. Bujalowski, W., L. B. Overman, and T. M. Lohman. 1988. Binding mode transitions of *Escherichia coli* single strand binding protein-single-stranded DNA complexes. Cation, anion, pH, and binding density effects. *J Biol Chem* 263(10):4629-4640.
20. Ferrari, M. E., W. Bujalowski, and T. M. Lohman. 1994. Co-operative binding of *Escherichia coli* SSB tetramers to single-stranded DNA in the (SSB)₃₅ binding mode. *J Mol Biol* 236(1):106-123.
21. Roy, R., A. G. Kozlov, T. M. Lohman, and T. Ha. 2007. Dynamic Structural Rearrangements Between DNA Binding Modes of *E. coli* SSB Protein. *J Mol Biol* 369(5):1244-1257.
22. Hamon, L., D. Pastre, P. Dupaigne, C. Le Breton, E. Le Cam, and O. Pietrement. 2007. High-resolution AFM imaging of single-stranded DNA-binding (SSB) protein--DNA complexes. *Nucleic Acids Res* 35(8):e58.
23. Kozlov, A. G., M. K. Shinn, E. A. Weiland, and T. M. Lohman. 2017. Glutamate promotes SSB protein-protein Interactions via intrinsically disordered regions. *J Mol Biol* 429(18):2790-2801.
24. Kozlov, A. G., M. K. Shinn, and T. M. Lohman. 2019. Regulation of Nearest-Neighbor Cooperative Binding of *E. coli* SSB Protein to DNA. *Biophys J* 117(11):2120-2140.
25. Zhou, R., A. G. Kozlov, R. Roy, J. Zhang, S. Korolev, T. M. Lohman, and T. Ha. 2011. SSB functions as a sliding platform that migrates on DNA via reptation. *Cell* 146(2):222-232.
26. Suksoombat, S., R. Khafizov, A. G. Kozlov, T. M. Lohman, and Y. R. Chemla. 2015. Structural dynamics of *E. coli* single-stranded DNA binding protein reveal DNA wrapping and unwrapping pathways. *Elife* 4.
27. Hatch, K., C. Danilowicz, V. Coljee, and M. Prentiss. 2008. Measurement of the salt-dependent stabilization of partially open DNA by *Escherichia coli* SSB protein. *Nucleic Acids Res* 36(1):294-299.
28. Bell, J. C., B. Liu, and S. C. Kowalczykowski. 2015. Imaging and energetics of single SSB-ssDNA molecules reveal intramolecular condensation and insight into RecOR function. *Elife* 4:e08646.
29. Bujalowski, W., and T. M. Lohman. 1987. Limited co-operativity in protein-nucleic acid interactions. A thermodynamic model for the interactions of *Escherichia coli* single strand binding protein with single-stranded nucleic acids in the "beaded", (SSB)₆₅ mode. *J Mol Biol* 195(4):897-907.
30. Roy, R., A. G. Kozlov, T. M. Lohman, and T. Ha. 2009. SSB protein diffusion on single-stranded DNA stimulates RecA filament formation. *Nature* 461(7267):1092-1097.

31. Sokoloski, J. E., A. G. Kozlov, R. Galletto, and T. M. Lohman. 2016. Chemo-mechanical pushing of proteins along single-stranded DNA. *Proc Natl Acad Sci U S A* 113(22):6194-6199.
32. Kozlov, A. G., E. Weiland, A. Mittal, V. Waldman, E. Antony, N. Fazio, R. V. Pappu, and T. M. Lohman. 2015. Intrinsically Disordered C-terminal Tails of *E. coli* Single Stranded DNA Binding Protein Regulate Cooperative Binding to Single Stranded DNA. *Journal of Molecular Biology* 427:763-774.
33. Sigal, N., H. Delius, T. Kornberg, M. L. Gefter, and B. Alberts. 1972. A DNA-unwinding protein isolated from *Escherichia coli*: its interaction with DNA and with DNA polymerases. *Proc Natl Acad Sci U S A* 69(12):3537-3541.
34. Kozlov, A. G., and T. M. Lohman. 2002. Kinetic mechanism of direct transfer of *Escherichia coli* SSB tetramers between single-stranded DNA molecules. *Biochemistry* 41(39):11611-11627.
35. Lee, K. S., A. B. Marciel, A. G. Kozlov, C. M. Schroeder, T. M. Lohman, and T. Ha. 2014. Ultrafast Redistribution of *E. coli* SSB along Long Single-Stranded DNA via Intersegment Transfer. *J Mol Biol* 426(13):2413-2421.
36. Spenkelink, L. M., J. S. Lewis, S. Jergic, Z. Q. Xu, A. Robinson, N. E. Dixon, and A. M. van Oijen. 2019. Recycling of single-stranded DNA-binding protein by the bacterial replisome. *Nucleic Acids Res*.
37. Dubiel, K., A. R. Myers, A. G. Kozlov, O. Yang, J. Zhang, T. Ha, T. M. Lohman, and J. L. Keck. 2019. Structural Mechanisms of Cooperative DNA Binding by Bacterial Single-Stranded DNA-Binding Proteins. *J Mol Biol* 431(2):178-195.
38. Arif, S. M., and M. Vijayan. 2012. Structural diversity based on variability in quaternary association. A case study involving eubacterial and related SSBs. *Methods Mol Biol* 922:23-35.
39. Bernstein, D. A., J. M. Eggington, M. P. Killoran, A. M. Misic, M. M. Cox, and J. L. Keck. 2004. Crystal structure of the *Deinococcus radiodurans* single-stranded DNA-binding protein suggests a mechanism for coping with DNA damage. *Proc Natl Acad Sci U S A* 101(23):8575-8580.
40. George, N. P., K. V. Ngo, S. Chitteni-Pattu, C. A. Norais, J. R. Battista, M. M. Cox, and J. L. Keck. 2012. Structure and cellular dynamics of *Deinococcus radiodurans* single-stranded DNA (ssDNA)-binding protein (SSB)-DNA complexes. *J Biol Chem* 287(26):22123-22132.
41. Antony, E., E. A. Weiland, S. Korolev, and T. M. Lohman. 2012. *Plasmodium falciparum* SSB tetramer wraps single-stranded DNA with similar topology but opposite polarity to *E. coli* SSB. *J Mol Biol* 420(4-5):269-283.
42. Chan, K. W., Y. J. Lee, C. H. Wang, H. Huang, and Y. J. Sun. 2009. Single-stranded DNA-binding protein complex from *Helicobacter pylori* suggests an ssDNA-binding surface. *J Mol Biol* 388(3):508-519.
43. Yadav, T., B. Carrasco, A. R. Myers, N. P. George, J. L. Keck, and J. C. Alonso. 2012. Genetic recombination in *Bacillus subtilis*: a division of labor between two single-strand DNA-binding proteins. *Nucleic Acids Res* 40(12):5546-5559.

44. Huang, Y. H., I. C. Chen, and C. Y. Huang. 2019. Characterization of an SSB-dT25 complex: structural insights into the S-shaped ssDNA binding conformation. *Rsc Adv* 9(69):40388-40396.
45. Antony, E., A. G. Kozlov, B. Nguyen, and T. M. Lohman. 2012. Plasmodium falciparum SSB tetramer binds single-stranded DNA only in a fully wrapped mode. *J Mol Biol* 420(4-5):284-295.
46. Kozlov, A. G., and T. M. Lohman. 2002. Stopped-flow studies of the kinetics of single-stranded DNA binding and wrapping around the Escherichia coli SSB tetramer. *Biochemistry* 41(19):6032-6044.
47. Kowalczykowski, S. C., N. Lonberg, J. W. Newport, and P. H. von Hippel. 1981. Interactions of bacteriophage T4-coded gene 32 protein with nucleic acids. I. Characterization of the binding interactions. *J.Mol.Biol.* 145:75-104.
48. Kozlov, A. G., R. Galletto, and T. M. Lohman. 2012. SSB-DNA binding monitored by fluorescence intensity and anisotropy. *Methods Mol Biol* 922:55-83.
49. Kozlov, A. G., and T. M. Lohman. 2012. SSB binding to ssDNA using isothermal titration calorimetry. *Methods Mol Biol* 922:37-54.
50. Dam, J., and P. Schuck. 2004. Calculating sedimentation coefficient distributions by direct modeling of sedimentation velocity concentration profiles. *Methods Enzymol* 384:185-212.
51. Bujalowski, W., and T. M. Lohman. 1989. Negative co-operativity in Escherichia coli single strand binding protein-oligonucleotide interactions. II. Salt, temperature and oligonucleotide length effects. *J Mol Biol* 207(1):269-288.
52. Amaratunga, M., and T. M. Lohman. 1993. *Escherichia coli* Rep helicase unwinds DNA by an active mechanism. *Biochemistry* 32:6815-6820.
53. Wu, C. G., C. Bradford, and T. M. Lohman. 2010. Escherichia coli RecBC helicase has two translocase activities controlled by a single ATPase motor. *Nat Struct Mol Biol* 17(10):1210-1217.
54. Xie, F., C. G. Wu, E. Weiland, and T. M. Lohman. 2013. Asymmetric regulation of bipolar single-stranded DNA translocation by the two motors within Escherichia coli RecBCD helicase. *J Biol Chem* 288(2):1055-1064. Research Support, N.I.H., Extramural.
55. Simon, M. J., J. E. Sokoloski, L. Hao, E. Weiland, and T. M. Lohman. 2016. Processive DNA Unwinding by RecBCD Helicase in the Absence of Canonical Motor Translocation. *J Mol Biol* 428(15):2997-3012.
56. Nguyen, B., J. Sokoloski, R. Galletto, E. L. Elson, M. S. Wold, and T. M. Lohman. 2014. Diffusion of human replication protein A along single-stranded DNA. *J Mol Biol* 426(19):3246-3261.
57. Nguyen, B., M. A. Ciuba, A. G. Kozlov, M. Levitus, and T. M. Lohman. 2019. Protein Environment and DNA Orientation Affect Protein-Induced Cy3 Fluorescence Enhancement. *Biophys J* 117(1):66-73.
58. Kozlov, A. G., and T. M. Lohman. 1998. Calorimetric studies of E. coli SSB protein-single-stranded DNA interactions. Effects of monovalent salts on binding enthalpy. *J Mol Biol* 278(5):999-1014.

59. Wiseman, T., S. Williston, J. F. Brandts, and L. N. Lin. 1989. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal.Biochem.* 179:131-137.
60. Bujalowski, W., and T. M. Lohman. 1989. Negative co-operativity in Escherichia coli single strand binding protein-oligonucleotide interactions. I. Evidence and a quantitative model. *J Mol Biol* 207(1):249-268.
61. Raghunathan, S., C. S. Ricard, T. M. Lohman, and G. Waksman. 1997. Crystal structure of the homo-tetrameric DNA binding domain of Escherichia coli single-stranded DNA-binding protein determined by multiwavelength x-ray diffraction on the selenomethionyl protein at 2.9-A resolution. *Proc Natl Acad Sci U S A* 94(13):6652-6657.

Figures

Figure 1. *E. coli* SSB-ssDNA structure and binding modes and its comparison with other SSB-DNA structures. **(A)** – *Ec*SSB (i) subunit (177 aa) and *Pf*SSB (ii) subunit (210 aa) are composed of an N-terminal DNA binding domain (OB fold) (112 aa and 119 aa, respectively), intrinsically disordered linkers (IDL), 56aa and 82aa long, respectively, and 9aa acidic tips, with different aa sequences. (iii) – RP (reverse polarity) (dT)₇₀ constructs used in the study in which the polarity of phosphodiester backbone was reversed in the middle of the DNA (abbreviated as 5'-(dT)₇₀-5' (green) and 3'-(dT)₇₀-3' (dark orange)). **(B)** - cartoon of *Ec*SSB-ssDNA complex in its (SSB)₆₅ binding mode (6), with 65 nts of DNA (orange ribbon) wrapped around the tetramer, showing the polarity of DNA wrapping. The identical subunits are marked as AA'BB'. Note that the IDL and acidic tip are not visible in this structure. **(C)** - cartoon of a proposed model for *Ec*SSB-ssDNA binding to (dT)₇₀ (orange tube) in the (SSB)₃₅ binding mode using an average of only two subunits per tetramer. **(D)** - cartoon of a *Pf*SSB in complex with two molecules of (dT)₃₅ (orange tubes) showing a polarity for ssDNA binding that is opposite to *Ec*SSB (41). **(E)** - cartoon of *Hp*SSB-ssDNA complex in the (SSB)₆₅ binding mode (42). **(F)** - cartoon of *Pa*SSB in complex with two molecules of (dT)₂₅ (44).

Figure 2. *Ec*SSB can form (SSB)₃₅ and (SSB)₆₅ modes on reverse polarity (dT)₇₀.

(A) and **(B)** - results of equilibrium titrations of *Ec*SSB (0.30 μ M) with (dT)₇₀ (cyan), 3'-(dT)₇₀-3' (orange) and 5'-(dT)₇₀-5' (green) (ex: 296 nm, em: 350 nm) in 10 mM NaCl and 0.30 M NaCl (buffer-T, 25°C), plotted as fraction of Trp fluorescence quenching versus the ratio of total DNA to total SSB tetramer concentrations. The cartoons depict the (SSB)₃₅ and (SSB)₆₅ binding modes that form during the course of the titrations.

(C) and **(D)** - results of equilibrium titrations of Cy3/Cy5 labeled (dT)₇₀ (cyan), 3'-(dT)₇₀-3' (orange) and 5'-(dT)₇₀-5' (green) (0.1 μ M each) (see Figure 1A(iii)) with *Ec*SSB monitoring Cy5 fluorescence enhancement (exc: 515 nm, em: 665 nm) in 10 mM NaCl and 0.30 M

NaCl (buffer-T, 25°C). The cartoons depict the (SSB)₃₅ and (SSB)₆₅ binding modes and where they form during the course of the titrations.

Figure 3. *EcSSB* binding to reverse polarity (dT)₇₀ at intermediate [NaCl].

Results of equilibrium titrations of Cy3/Cy5-labeled (dT)₇₀ (cyan), 3'-(dT)₇₀-3' (orange) and 5'-(dT)₇₀-5' (green) (0.1 μ M each) with *EcSSB* monitoring Cy5 fluorescence enhancement (exc: 515 nm, em: 665 nm) in 40 mM NaCl (**A**), 80 mM NaCl (**B**) and 100 mM NaCl (**C**) (buffer-T, 25°C), plotted as Cy5 fluorescence signal versus the ratio of concentrations of total SSB tetramer to total DNA.

Figure 4. Reverse polarity (dT)₇₀ bind to *EcSSB* with different affinities, but with the same affinity to *PfSSB*

Results of equilibrium titrations of *EcSSB* (**A**) and *PfSSB* (**B**) (0.30 μM each) with (dT)₇₀ (cyan), 3'-(dT)₇₀-3' (orange) and 5'-(dT)₇₀-5' (green) monitoring intrinsic Trp fluorescence quenching (ex: 296 nm, em: 350 nm) in 2.0 M NaBr (buffer-T, 25°C). Solid lines are simulated isotherms based on the best fit parameters shown that were obtained from a NLLS fit to a single site binding model (Eqs 1-2 in Materials and Methods).

Figure 5. Enthalpic contributions to binding of *EcSSB* and *PfSSB* to reverse polarity (dT)₇₀.

Results of ITC titrations of **(A)-** *EcSSB* with 5'-(dT)₇₀-5' (green), **(B)-** *EcSSB* with (dT)₇₀ and 3'-(dT)₇₀-3' (cyan and orange), and **(C)-** *PfSSB* with 3'-(dT)₇₀-3' and 5'-(dT)₇₀-5' (orange and green) in 20 mM NaCl (buffer-H, 25°C) plotted as heats per injection normalized per amount of injected DNA (Q_{norm} , kcal/mole) versus the ratio of total DNA to total SSB tetramer concentration. The cartoons depict the (SSB)₃₅ and (SSB)₆₅ complexes and where they form during the course of the titrations.

Figure 6. *EcSSB* binding to alternating polarity (ap)-(dT)₇₀.

(A) - results of equilibrium titrations of *EcSSB* (0.30 μ M) with ap-(dT)₇₀ in buffer-T (25°C) in 10 mM NaCl (green), 30 mM NaCl (orange), 100 mM NaCl (cyan) and 300 mM NaCl (dark green), plotted as fractional Trp fluorescence quenching versus the ratio of total DNA to SSB tetramer concentrations. The cartoons depict the (SSB)₃₅ and (SSB)₆₅ modes and expected transitions during the titrations.

(B) - results of equilibrium titrations of Cy3/Cy5 labeled ap-(dT)₇₀ (0.1 μ M each) with *EcSSB* in 10 mM NaCl (green), 30 mM NaCl (orange), 100 mM NaCl (cyan) and 300 mM NaCl (dark green) in buffer-T (25°C), plotted as Cy5 fluorescence versus the ratio of concentrations of total SSB tetramer to total DNA. The cartoons depict the (SSB)₃₅ and (SSB)₆₅ modes and expected transitions during the titrations.

A *Pf*SSB

B *Ec*SSB

Figure 7. Proposed alternative DNA wrapping topologies in the (SSB)₆₅ binding modes for *Ec*SSB and *Pf*SSB formed with the RP (dT)₇₀.

Proposed topology of ssDNA binding to *Pf*SSB (**A**) and *Ec*SSB (**B**) in an (SSB)₆₅ binding mode for (dT)₇₀ with normal polarity (**i**), 3'-(dT)₇₀-3' (**ii**) and 5'-(dT)₇₀-5' (**iii**). In each case the ends of the DNA entering and exiting the tetramer are designated by red arrows with the indicated polarity. The blue arrows crossing the dimer-dimer (AA'/BB') interface in each particular case represent the middle regions of the DNA showing the different

wrapping topologies needed to maintain the polarity of the DNA backbone bound to each individual subunit (OB-fold) (3'-5' for *Pf*SSB and 5'-3' for *Ec*SSB).