Single-Stranded DNA Binding Protein in DNA Metabolism

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Single-stranded DNA-binding proteins (SSBs) play vital roles in DNA metabolism. Proteins of the SSB family exclusively and transiently bind to ssDNA, preventing the DNA double helix from re-annealing and maintaining genome integrity. In the meantime, they interact and coordinate with various proteins vital for DNA replication, recombination, and repair. Although SSB is essential for DNA metabolism, proteins of the SSB family have been long described as accessory players, primarily due to their unclear dynamics and mechanistic interaction with DNA and its partners. Recently-developed single-molecule tools, together with biochemical ensemble techniques and structural methods, have enhanced our understanding of the different coordination roles that SSB plays during DNA metabolism.

single-stranded DNA-binding proteins | single-molecule technique | DNA replication | DNA repair | DNA recombination

1. Introduction

Central to many genome-maintenance machineries are single-stranded DNA binding proteins (SSBs). These SSB proteins play a vital role in the maintenance of genomes by binding exclusively and transiently to ssDNA intermediates during DNA replication, recombination, and repair. By further interacting with different proteins crucial to all aspects of genome maintenance and recruiting them to their targets on DNA, the SSB protein plays a prominent role in bridging genome maintenance pathways and modulating their activity. Due to their interaction with DNA, they influence many other downstream processes, which include all the possible protein-mediated biological functions. Biochemical studies have demonstrated that SSB plays an essential role in DNA metabolism. However, the real-time interaction dynamics between SSB with DNA and its partner proteins have proven elusive owing to the limited averaged population and time resolution. The recent development of single-molecule assays, in combination with robust ensemble biochemical techniques and structural methods, have contributed significantly to our understanding of the molecular mechanisms of SSB, interaction dynamics with other protein partners, and the mechanistic interactions with partner proteins.

2. Classification of SSB

2.1. Properties of SSB

As suggested by the name, single-stranded DNA binding proteins bind to ssDNA. While this is mostly understood in the context of preventing re-annealing during lagging strand synthesis or during DNA damage repair, SSB proteins are vital in many other processes. All proteins of this broad class interact with single-stranded DNA, and some also interact with dsDNA [1][2][3][4][5][6][7].

2.2. Classification of SSB

As far as is known, all classified organisms with available genomes encode SSBs, suggesting that the role they play is essential to life processes at a fundamental level [8]. The role of SSBs is most saliently communicated as their role in replication, to prevent the re-annealing of single-stranded DNA, so that the template strand can be copied. The SSBs differ significantly from one another, and variations between different kingdoms of life trump intra-kingdom differences [9].

3. Single-Molecule Toolbox to Study SSB

3.1. Single-Molecule Force Studies of SSB–ssDNA Interactions

Generally, single-molecule techniques fall into two classes, namely those that measure force, displacement, and torque, and those that detect fluorescence. The first category, called single-molecule force spectroscopy, has become increasingly important for understanding the tensions, motions, and torques associated with biological molecules and their enzymatic activity. Studies have been conducted using single-molecule force spectroscopy to determine the interaction between different SSBs with dsDNA, ssDNA, or both [1][2][3][4][5][6][7], attempting to examine, for example, whether SSBs can destabilize duplex DNA.

With optical tweezers, DNA molecules have been manipulated to investigate the kinetics and thermodynamics of the binding of T7 SSB (gp2.5) and T4 SSB (gp32) to dsDNA and ssDNA [4][5][6][7]. An optical tweezer assay involves attaching one end of a DNA molecule to an optically trapped bead. On the other end, one of the following methods is employed: a micropipette ([10]), the surface of a microfluidic device ([11][12][13]), or a second optically trapped bead (as referenced in [14][15][16][17]) which is commonly referred to as a dual-trap optical tweezers setup. To study the effect of SSB on DNA molecules, double-stranded DNA is usually melted by force to obtain ssDNA [18]. Alternatively, SSB can be directly observed destabilizing duplex DNA. In the latter experiments, the dsDNA melting force was monitored in relation to the SSB concentration and pulling rate measured by elongation of end-to-end distance for the trapped DNA per time unit; Additionally, models were created to calculate the size of the SSB binding sites, referred to as the “footprint size”, as well as the association rates and equilibrium dissociation constants (K_D) of SSB proteins binding to both single-stranded and double-stranded DNA [19][20][21].

Another commonly used assay to study SSB at the single-molecule level is the magnetic tweezer. In a magnetic tweezer assay, a biomolecule is tethered to a micron-sized superparamagnetic bead and a microchannel surface through antigen–antibody interactions. The corresponding force applied to biomolecules can be calibrated by analyzing the Brownian motion of the beads obtained through the bright-field images [22][23][24][25]. The relevant

distance between the magnetic bead and the surface is determined by measuring the change in the diffraction pattern of the bead with respect to the magnet height [22][23][24][25]. By varying the magnet strength and the experimental design, forces of between 0.001 and 100 pN can typically be achieved [23][24][26]. When compared with optical tweezers to study SSB, which is often limited by its lower throughput, magnetic tweezers allow many single DNA molecules to be tethered with separate beads and probed in parallel, thus, achieving high throughput of data collection. Example studies using magnetic tweezers were to determine whether the gp32 and *E. coli* SSB proteins could prevent DNA strand rezipping [2][3].

3.2. Image Measurement of SSB-ssDNA Complex

Another significant category of single-molecule tools is based on the detection of fluorescence. Several single-molecule fluorescence approaches have proven to be particularly useful for studying the SSB–DNA complex, for which smFRET has provided a high-resolution dynamic picture of how SSB interacts with ssDNA. The smFRET technique involves the use of two fluorescent dyes, which are covalently attached to specific locations within the DNA molecule or its interacting protein. The smFRET assay can be performed using either confocal microscopy of freely diffusing molecules or TIRF microscopy of molecules attached to surfaces [27]. The smFRET measurements are frequently combined with other stretching techniques to provide a more comprehensive understanding of the DNA-protein interactions. When the distance between the two fluorophores is short (usually less than 10 nanometers), the donor transfers energy without radiation to the acceptor, resulting in the emission of fluorescence by the acceptor instead of the donor [28][29]. The FRET efficiency, defined as the efficiency of energy transfer from the donor to the acceptor, depends upon the proximity between the two fluorophores; therefore, it can be used to measure shifts in the distance up to ~10 nm. This tool is excellent for tracking real-time conformational and relative position changes within single biological molecules. The generated data, by measuring the dynamic states in a molecular system, can be quite different depending on the investigated system. Data fitting in smFRET data analysis is critical in understanding molecular dynamics and, thus, should be adopted based on the research question. Classic examples of smFRET to study the SSB–ssDNA complex are direct demonstrations of *E. coli* SSB in its $(SSB)_{65}$ binding mode diffusing along ssDNA [30], which is consistent with early ensemble studies [31][32][33][34].

3.3. Hybrid Single-Molecule Tools

Besides the independent use of single-molecule force spectroscopy and fluorescence microscopy, combined force manipulation and fluorescence visualization have been extensively exploited to probe the binding dynamics of SSB to ssDNA and its interaction with other protein partners. These combined approaches are instrumental in understanding, for example, DNA–binding protein interactions that are sequence-dependent [15], for monitoring protein translocation along DNA [35][36][37], and for examining the relationship between protein binding and the mechanical properties of DNA [7][38][39].

3.4. Example Output of Single-Molecule Studies

From single-molecule experiments, several parameters can be extracted. For basic properties, there are the binding properties, which include the binding rate constants k_{on} and k_{off}, corresponding to the on rates and off

rates, respectively. In addition to binding properties, one can determine stoichiometries to gain insights into the binding footprint of an individual SSB binding event. Information on the kinetics of binding can also be obtained, such as the presence of one, two, or multistage binding. As well as measuring these parameters, one may also measure their dependence on experimental conditions, such as ionic concentration, template tension, temperature, and pH.

Once bound, an important value is the diffusion constant, which can determine if the SSB is stationary or diffusive (such as *E. coli* SSB [30]). It can also test cooperativity through the concentration-dependent binding kinetics. Lastly, single-molecule techniques also allow for the direct observation of interactions between SSBs and other proteins, such as the role of T7 gp2.5 in replisome coordination. These different parameters come together to describe the system of SSB interactions with DNA and with other proteins.

Additionally, it can calculate maximal coating densities, which can be used to calculate the binding footprint. These can be determined by finding an association between DNA length shortening and fluorescence intensity. This is typically linear, as one SSB will induce a near-constant contour length change by bending nucleotides in its OB fold or wrapping the DNA around a tetramer in the case of *E. coli* [40]. The shortening of the DNA will be directly correlated to the fluorescence intensity, which serves as a proxy for the number of SSB bound. When the system is saturated, one can calculate the total contour length change and divide it by the number of SSB bound (found via fluorescence intensity). This allows one to calculate the length change per bound SSB, which is an important quantity in understanding the binding mechanism.

Single-molecule experiments provide insights into the binding footprint of SSB (single-stranded DNA binding) proteins by analyzing the maximal occupancy, which is the point where an increase in fluorescent intensity stops, even with an increase in concentration along the bound DNA. By determining the number of SSB proteins, the average linear occlusion of DNA per bound SSB can be calculated by dividing the number of nucleotides by the number of SSBs. However, it should be noted that the average linear occlusion is not equivalent to the binding footprint, as some SSBs bind disorderedly. Interestingly, the SSB of phi 29 binds in a consistent manner, forming a “unit cell” with a nearly constant spacing of 3.4 ± 0.3 nucleotides per phi 29 SSB monomer [41].

Thermodynamic aspects of binding can be determined through bulk methods, such as isothermal titration calorimetry (ITC) or melting experiments, but they can also be probed by the single-molecule techniques. At a rough level, it is possible to determine the binding and unbinding as a function of force. Most proteins will be evicted from DNA held at high tension. Another glimpse into the thermodynamics and binding mode is by measuring the saturation dependence of certain binding parameters. Since salts shield the negative charges along the DNA backbone, along with certain amino acid sequences, such as the C-terminal tail of gp2.5 [42] as well as *E. coli* [43], information about the electrostatics of DNA–protein interactions can be garnered by varying the salt concentration of monovalent, bivalent, and polyvalent anions.

Monovalent anions are much less effective at shielding, even when normalized per unit charge than bi- or polyvalent anions. Bivalent anions pack double the charge in a more compact volume [44], allowing it to come close

to the DNA or protein to screen the negative charge [45][46][47]. The change in binding properties as a function of mono-, bi-, and polyvalent anions may possibly be analyzed to determine the relevant length scales of the electrostatic interaction and the allosteric exclusion emerging from the close contact of the protein with DNA.

4. Examine the Interaction between ssDNA with SSB

4.1. General Binding Dynamics of SSB

SSB proteins play a critical role in binding ssDNA. The mode of SSB binding can vary, with some forming multimers, exhibiting cooperativity or exhibiting strong periodicity (as seen in phi29 [48]). Single-molecule experiments offer a deeper understanding of SSB behaviour, allowing the determination of binding and unbinding constants and the exploration of factors such as sequence dependence, DNA-conformation dependence, and conditions such as salt concentrations, temperature, pH, crowding agents, protein concentration, and the presence of co-factors. Additionally, single-molecule methods enable the investigation of multiple binding modes, which can be challenging to study in ensemble assays. Stoichiometries can provide insight into the binding footprint of SSBs and further our understanding of the structure of these proteins.

When bound, one can measure the diffusion and lifetime of the SSB. Diffusion can be characterized by the diffusion constant, but it is also helpful to determine if there is a directional bias to SSB motion. Interactions with other proteins can also be studied, such as colocalization, assisted binding, and the potential impact on the function of other enzymes.

4.2. Binding Dynamics of SSB to ssDNA under Tension

In addition to the general binding properties of SSB proteins, which can be probed with both bulk assay and single-molecule studies, the binding dynamics of SSBs to ssDNA under tension can be studied exquisitely with the single-molecule tools, such as by using optical tweezers [38][49] and magnetic tweezers [50]. The force-dependence of binding often depends on the binding mode, which varies between SSBs, from the monomeric binding in an OB-fold by T7 gp2.5 SSB [51] to the wrapping of DNA by *E. coli* SSB [40]. As *E. coli* SSB is highly sensitive to force, the unwrapping of the DNA from *E. coli* SSB begins at tensions as low as 1 pN, and complete dissociation occurs between 7 and 12 pN [52].

The binding dynamics for T7 gp2.5 have been investigated at different pulling timescales to investigate the prevention of secondary structure formation and the impact of T7 gp2.5 binding on the energetics of DNA stretching. The experiment observed a clear shortening with the addition of T7 gp2.5, and by varying the speed, it could limit the number of SSBs binding [6]. It was determined that under the fast-pulling regime, fewer SSB bind, and the force relationship was similar to that of naked DNA.

The real-time dynamics of SSB binding have also been investigated via high-speed AFM imaging [53]. The *E. coli* SSB binds, diffuses, and dissociates, and this is shown in real-time with AFM imaging. In the emerging high-speed

AFM technique, high-resolution images of the sample can be obtained in a fully hydrated state, thus, allowing millisecond-scale visualization of the nanoscale dynamics of the system. The buffer conditions, such as cation types, concentration, and pH, as well as the length of the substrate, can be varied in order to gain a better understanding of how environmental factors affect binding dynamics.

4.3. Movement of SSB on ssDNA Probed with Single-Molecule Approaches

The distinction between diffusive and non-diffusive proteins is important. It is possible that diffusive proteins can cover a larger effective footprint (i.e., preventing secondary structure formation in this linear region of DNA). The SSB diffusion is passive and is thought to be driven largely by thermal motions. The diffusion of *E. coli* SSB has been observed in experiments using smFRET [30]. The DNA was labelled with donor and acceptor fluorophores located 69 nt apart, such that when the SSB was bound, there was a fluorescent signal produced. It was found that there is free diffusion of the SSB along the DNA. It is possible to 'lock' the *E. coli* SSB by forming a duplex structure with the bases at the 3′ and 5′ ends of the SSB–DNA complex.

Additional experiments were conducted to test two distinct diffusion modes of *E. coli* SSB, which differ by the relative motions of the SSB with the DNA. The first mode, rolling, involves the DNA at the 5′ or 3′ ends lengthening or shortening by moving around the SSB tetramer. In this case, the relative position of a given DNA base and a given spot on the SSB tetramer do not move in relation to one another. The other diffusion model is that of sliding, where the ssDNA moves in relation to a fixed spot on the SSB tetramer. Experimental results support the sliding mechanism, as the site of the DNA FRET tag does not alter the FRET intensity pattern.

4.4. Sequence-Dependent Properties of SSB

For a nucleic acid binding protein, it is assumed that its interaction with the template is largely non-specific and that sections of the template are largely interchangeable and homogenous, except in the case of specific binding sequences, for example, such as Kozak sequences for translation initiation in eukaryotes [54]. While the assumption of a largely homogenous polymer may be useful in some applications, bases are often processed differently in important ways. Hairpins of GC-rich DNA require more force to unfold, but also have faster kinetics than AT-rich DNA [55]. GC-rich regions form a more stable secondary structure than AT-rich regions and more quickly. T4 gp32 and *E. coli* SSB proteins both act through the inhibition of refolding [2], so sequence manifests itself due to the different timescales of folding of GC-rich versus AT-rich hairpins. In the related case of mRNA translation, hairpins often slow and stall the ribosome, as the ribosome must resolve the secondary structure before proceeding [56], as the entry tunnel only allows ssRNA [57].

5. Coordination Role of SSB in DNA Metabolism

5.1. Overview of Single-Molecule Studies on SSB Interacting with Helicase

Helicases carry out many essential genome maintenance processes within the cell, such as replication, recombination, and repair [58][59][60][61][62]. It has been reported that the same helicase can carry out several of

these functions [63][64][65]. Helicase activity must, therefore, undergo strict regulation. While there is no clear evidence regarding how this regulation occurs, growing evidence indicates that interactions with protein partners may be one of the mechanisms involved [61][63][64][65]. On the other hand, DNA helicases function to unwind dsDNA into ssDNA intermediate or to translocate along ssDNA, suggesting a frequent encounter with protein binding to ssDNA, such as SSBs, during various DNA processing events. Single-strand binding proteins have been shown to improve the unwinding efficiency of many helicases [66][67][68][69].

5.1.1. Interplay with Replicative Helicase CMG Complex

Replicative DNA helicases play an essential role in duplicating the genome in every cell cycle. Replicative DNA helicases are usually protein complexes with multi-subunit structures, such as the replicative helicase of eukaryotes, which is composed of 11 subunits and requires 2 subcomplexes and 1 protein to function. This heterohexameric helicase, the Cdc45-Mcm2-7-GINS (CMG) complex, is initiated through the formation of a complex with Cdc45 and the heterotetrameric GINS complex [70]. This CMG complex translocates in the direction of 3′–5′ along the leading-strand template and unwinds DNA at the replication fork powered by ATP hydrolysis [70][71]. In vitro single-molecule studies reveal that translocation on ssDNA of the yeast CMG helicase shows a rate at 5–10 bp s^{-1} [72], while the observed dsDNA unwinding rate to be 0.1–0.5 bp s^{-1}, possibly slowed by a frequent long-lived pausing state [73][74].

5.1.2. Interplay with Recombinational Repair Helicase XPD

Xeroderma pigmentosum group D (XPD) helicase belongs to subfamily 2B of helicases, including yeast Rad3 and human FANCJ, CHLR1, and RTEL [61][65][75][76][77], and is involved in a variety of DNA repair pathways. The XPD helicase mutation can affect nucleotide excision repair (NER) [78]. Human XPD is also associated with the transcription factor IIH and plays a significant role in the repair of nucleotide excisions [79][80][81][82]. Further evidence indicates that it also plays a role in chromosome segregation [83] and defence against retroviral infection [84]. While functioning on single-stranded DNA, XPD is likely to come into contact with other proteins, such as cognate SSB replication protein A (RPA). When the XPD encounters a bound RPA, it can bypass the RPA without dislodging it or facilitating its dissociation.

5.1.3. Interplay with Recombinational Repair Helicase RecQ

In the case of recombinational repair, another well-studied example of helicase is RecQ. During recombinational repair, RecQ plays a role in repairing ssDNA gaps and dsDNA breaks in *E. coli* after the primary repair pathway, RecBCD, is inactivated [85]. It also has been demonstrated that RecQ is responsible for suppressing the production of illegitimate recombinants [86][87], resolving replication fork stalled events [88] and stimulating the SOS response in Escherichia coli. [89][90][91][92]. Additionally, *E. coli* defective in RecQ is susceptible to ultraviolet light, resulting in a decrease in the frequency of recombination that leads to impaired cell growth and death [90][93]. Single-molecule approaches have been used, complementary to bulk biochemical and structural tools, to analyze the interactions of single molecules with high spatial and temporal resolutions and reveal dynamic heterogeneities missing in ensemble experiments due to population averaging [94][95][96][97][98][99][100][101].

5.1.4. Interplay with Replication Restart Helicase PriA

The DNA replication protein complex can be dissociated before replication is completed by collisions with damaged DNA or immovable protein barriers [102][103][104][105][106]. Cells can resolve this potentially lethal problem by reloading the replisome by activating “replication restart” reactions [106]. In bacteria, the PriA DNA helicase orchestrates this vital activity by binding to structure-specific DNA and interacting with replication-associated SSBs [107][108][109].

5.2. SSB Interacting with Replicative DNA Polymerase during Primer Extension

Bulk studies have shown that the presence of SSB significantly enhances DNA replication in vitro [110][111][112][113][114][115]. This enhancement may be attributed to the multiple roles that SSBs perform [40][110][116], including the prevention of degradation of ssDNA, the removal of secondary structures, the increase in recognition and initiation of primers, a decrease in non-specific DNA polymerase binding to the template, and an increase in DNA polymerase’s activity in displacing strands and extending primers [40][110][111][112][113][114][115][116]. However, it remains unclear whether the seemingly conflicting roles of polymerase and SSB on ssDNA can be coordinated during the lagged strand replication.

5.3. Single-Molecule Studies on SSB with DNA Polymerase during Strand Exchange

The effect of SSB on strand displacement DNA replication has also been examined using single-molecule tools [117][118]. It was previously demonstrated that strand displacement DNA synthesis, such as by Poly, accomplishes replication by utilizing stable secondary structures [119][120], ensuring that the D-loop DNA structure is maintained at the origin of heavy strands [121], and removing primers through the coordination of primer processing factors [122][123][124]. However, the efficiency of Poly is limited to a few nucleotides [123][125][126][127][128], in accordance with other DNA polymerases involved in strand displacement synthesis [117][129][130].

5.4. Single-Molecule Studies of SSB Interplay with Recombinase

During DNA metabolism, replication forks can stall or collapse, resulting in extensive single-strand gaps [131][132][133][134]. Consequently, the SSB protein binds to the ssDNA in these gaps, preventing other proteins from accessing the ssDNA. The RecA protein from *E. coli* is essential to repair broken DNA and maintain genomic integrity through homologous recombination. In order to function, RecA filaments are required to nucleate and grow on single-stranded DNA concurrently with SSB, which sequesters ssDNA continuously and thereby causes it to compete with and prevent RecA assembly [135][136]. Because of the complexity resulting from dynamic competition with SSB during self-assembly on ssDNA lattices, our knowledge of RecA filament assembly and its role in DNA recombination has been compromised. Despite extensive and varied efforts, ensemble measurements based on an averaged population are not able to distinguish between nucleation and growth in a reliable manner [135][136][137][138].

5.5. Chemo-Mechanical Pushing of *E. coli* SSB by a Translocating Protein Partner

Both bulk and single-molecule assays have shown that SSB binds exclusively to ssDNA with very high (pM to fM) affinities [34][38][52][139]; however, these tightly bound complexes must be displaced, bypassed, or redistributed along ssDNA to complete replication, recombination, and repair. As discussed in Section 5.1.2 [140], a pre-bound RPA on ssDNA can be dislodged by a translocating helicase XPD or bypassed without dissociation. The PriA binding to *E. coli* SSB can modulate the binding mode from SSB_{65}-to-SSB_{35} to expose more ssDNA for DNA replication restart. Considering the diffusive property of *E. coli* SSB, one other potential mechanism for reorganization can be pushed along ssDNA by a translocating protein.

The DNA translocases are motor proteins capable of translocating ssDNA at high rates powered by the hydrolysis of ATP [63][141][142]. To delve into the impact of a directional translocase encountering an *E. coli* SSB tetramer bound to single-stranded DNA, a smFRET assay was utilized to detect such pushing events [143]. A fluctuating FRET signal is observed when Cy5-labeled *E. coli* SSB is bound to surface-immobilized 3′-Cy3–labelled ssDNA, indicating that SSB is randomly diffusing on ssDNA. When adding Saccharomyces cerevisiae Pif1, a translocase for ssDNA 5′ to 3′, irregular-spaced saw-tooth FRET spikes are observed with ATP.

6. Conclusions

As transient and exclusive binders to ssDNA intermediates, SSBs are crucial for genome maintenance. Further interacting with various proteins vital for DNA maintenance, the SSB protein bridges genome maintenance pathways and modulates their activity by recruiting them to their DNA sites of action. Although it has been demonstrated that SSB is essential for DNA metabolism, the dynamics of SSB interaction with DNA and its partners remain unclear. Recent developed single-molecule assays have provided essential insights into the molecular mechanisms of SSB and interacting dynamics with other protein partners. By combining robust ensemble biochemical techniques and structural methods, a more comprehensive understanding of these SSBs has been gained.

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