Direct measurements of the stabilization of single-stranded DNA under tension by single-stranded binding proteins

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(Received 18 April 2007; revised manuscript received 5 July 2007; published 16 August 2007)

The unzipping and rezipping of a double-stranded DNA molecule is carried out in the presence of two single-stranded binding proteins T4 gp32 and E. Coli SSB protein to determine the effect of the proteins on the stability of single- and double-stranded DNA. The proteins do not have a significant effect on unzipping, indicating that the two proteins do not destabilize the double-stranded DNA; however, both proteins inhibit rezipping. At protein concentrations where the rezipping force response is saturated, E. Coli SSB protein reduces the rezipping force to 5.5 ± 1.5 pN, while T4 gp32 completely blocks rezipping on the time scale of the experiment.

DOI: 10.1103/PhysRevE.76.021916 PACS number(s): 87.14.Gg, 82.37.Rs, 87.14.Ee

I. INTRODUCTION

The opening and maintenance of single-stranded DNA (ssDNA) within a double-stranded DNA (dsDNA) molecule is necessary for cellular processes such as transcription, replication, and recombination. Nearly all organisms produce ssDNA-binding (SSB) proteins, which have a high affinity for ssDNA and are expected to aid in the maintenance of open ssDNA; therefore, it is important to understand the effectiveness of the proteins in maintaining partially open DNA and to determine whether the proteins alone are sufficient, or whether additional stabilization must be provided in order to maintain the open DNA. In most cases, the partially open DNA must eventually reclose, so it is also important to determine the conditions under which the complementary DNA will displace the protein, allowing the molecule to reclose.

The binding behavior of SSB proteins to ssDNA has been studied extensively [1]. Bulk assays provide vast information about the binding of SSB proteins, but are unable to probe their effectiveness in sustaining ssDNA within dsDNA. The ability of the SSB proteins to stabilize partially open dsDNA has not yet been probed, nor has there been an investigation of the circumstances under which the DNA will close despite the presence of the bound proteins. As the proteins must compete against complementary DNA in stabilizing single-stranded DNA, it cannot be assumed that the stabilization effect will be dependent on binding in a simple fashion [2]. Furthermore, previous binding assays cannot provide information about the extent of the stabilizing effect of the proteins.

Recently, single molecule DNA overstretching experiments have provided an alternative approach to probing the stabilizing effects of SSB proteins [3–5]. When dsDNA is overstretched, some models suggest that the overstretched state is a mixture of both dsDNA and ssDNA [6,7], and the overstretching transition can be modeled as force-induced melting. Others have suggested that the transition may create alternative states of dsDNA rather than creating large regions of ssDNA available to interact with the protein [8–12]. In this work, we create ssDNA through single molecule DNA unzipping, where the ssDNA is readily accessible to the protein and the zipped dsDNA is in a relaxed state. We present a study of the effectiveness of the proteins in maintaining partially open DNA that includes the complementary single strand and a fork that connects the two matching open single strands to a double-stranded region, a situation that much more closely resembles the conditions *in vivo* under which transcription and replication occur. The concentrations of SSB proteins that are observed to inhibit rezipping in these experiments are an order of magnitude lower than those that are reported to affect overstretching transitions, suggesting a significant difference in dynamics [3,5].

Single molecule techniques have studied the process of opening and closing of the base pairs in dsDNA and RNA *in vitro* under an applied force [13–17]. It has been shown that natural DNA opens, or "unzips," when a constant force of 18–20 pN is applied. The heterogeneity of the dsDNA sequence prevents reannealing, or "rezipping," at this same force, rather the force must be lowered to 12–15 pN for rezipping to occur [18]. Thus natural ssDNA can be stably maintained over a force range of 5–6 pN in the absence of any proteins. We find that the presence of SSB proteins increases the range over which ssDNA is stable, and we determine whether the proteins are sufficient to maintain ssDNA or if additional stabilization is required. The rezipping force as a function of concentration is well described by a dose response curve and correlates well to previously measured values of net binding affinity and the binding cooperativity, if one associates the EC50 point for the rezipping force with the EC50 point for the binding.

E. Coli SSB protein and bacteriophage T4 gene 32 protein (gp32) are two well-known SSB proteins. Both proteins are vital to replication, recombination, and repair of DNA [1]. The preference of SSB proteins for ssDNA would suggest that they lower the melting temperature of dsDNA; however, in the case of natural DNA, this has not been observed [1]. It is proposed that this disagreement between thermodynamic prediction and experiment is due to a "kinetic block," such that the SSB protein binding rate is slower than the rate at which ssDNA bubbles close [3,4,19]. While the function of these proteins is not fully understood, it is expected that their high affinity for ssDNA destabilizes the double helix and prevents secondary structure formation in ssDNA such as

hairpins. Although both proteins serve a similar purpose, earlier work has shown they have very different binding behaviors. T4 gp32 exhibits a high amount of cooperativity in binding with unlimited aggregation of proteins on ssDNA and a unitless cooperativity parameter ω on the order of 10^3 [20]. E. Coli SSB forms a stable tetramer in solution and, at salt concentrations of 0.2 M NaCl or greater, exhibits cooperativity limited only to the formation of tetramer dimers with ω on the order of 10^2 [21–23]. We find that in addition to different binding behaviors, the two proteins exhibit different ssDNA stabilization behaviors as well.

II. MATERIALS AND METHODS

The DNA construct used for unzipping measurements has been described previously [13]. Briefly, it consists of a linker λ-DNA (New England Biolabs) which is hybridized and ligated to one end of the λ-DNA strand which is to be opened. The second strand of the λ-DNA to be opened is hybridized and ligated to a biotinylated oligonucleotide. The other end is closed with a hairpin loop to prevent complete separation of the construct in an unzipping event. The linker λ-DNA is tagged with a digoxigenin-labeled oligonucleotide. It is attached to a glass capillary coated with an antidixogenin antibody. The λ-DNA strand to be opened is bound to a 2.8 μm streptavidin-coated magnetic bead (Dynabeads) via the biotinylated oligonucleotide. The λ-DNA and beads are stored at 4 °C after preparation and incubated with either protein, T4 gp32 (New England Biolabs), or E. Coli SSB protein (Epicentre), at room temperature for 15 min. prior to the experiment. The unzipping experiments are done in a PBS buffer (pH 7.4, 10 mM phosphate, 137 mM NaCl, 2.3 mM KCl) at room temperature.

Mechanical unzipping of dsDNA is carried out by a magnetic tweezer apparatus [24]. A stack of magnets exerts a force F on the magnetic beads by $F=m\nabla B$, where m is the magnetization of the bead and B is the magnetic field. The force on the beads is controlled and determined by the distance of the magnet from the beads, with a force range of 3–30 pN. Spread in magnetization of the beads leads to a standard deviation of ~30% in the force measurements. To reduce the effect of variation in bead magnetization in presenting data, the forces are normalized by the unzipping force of the molecule.

III. RESULTS

A. Rezipping in presence of E. Coli SSB proteins

A single λ-phage DNA molecule is unzipped and rezipped in the presence of E. Coli SSB protein, shown in Fig. 1. The constant applied force is incrementally increased by 0.5 pN every 2 s until complete unzipping occurs. Subsequently, the constant force is incrementally decreased by 0.5 pN every 2 s until complete rezipping occurs. The cycle is then repeated. In the absence of stabilizing proteins, DNA rezips on the rate of 1000 base pairs (bp), thus we have chosen a timescale on the order of seconds. Furthermore, within a cell replication forks move at a rate of 500–1500 bp/s, again suggesting that this time scale is an appropriate regime to work within. Figure 1 shows the extension of the DNA molecule as a function of force, normalized by the force at which complete unzipping occurs F_u. In the case of low protein concentration, 1 pM (shown in blue crosses), rezipping occurs rapidly, with a rezipping force F_z of $0.6–0.7F_u$, where F_z is defined as the force at which the extension of the molecule returns to zero. At 70 nM (shown in red circles), rezipping occurs more slowly, indicating an increased stabilization of ssDNA due to the E. Coli SSB protein, with complete rezipping occurring at $0.25–0.35F_u$.

The presence of the E. Coli SSB protein has little effect on F_u, as shown in Fig. 2, where each data point represents the measurement from a single molecule. The spread of forces measured is due to the spread of magnetization in the beads. The average unzipping force is 17.4±2 pN, with no concentration dependence over a range of 1 pM to 1 μM. This would be expected in the case of a kinetic block, where proteins cannot bind to ssDNA bubbles within dsDNA to destabilize the double helix.

The rezipping force is strongly affected by protein concentration, as can be seen from Fig. 1. At the higher concentrations, the rezipping occurs more slowly and at lower forces. Figure 2 shows the ratio of F_z/F_u as function of protein concentration. At concentrations less than 1 pM, $F_z/F_u = 0.6–0.7$, which is similar to that measured in the absence of proteins. Within this concentration range, the proteins have no stabilizing effect on the ssDNA.

For concentrations ranging from 1 pM–0.1 μM, the ratio F_z/F_u depends on concentration in a logarithmic fashion. This behavior saturates at 0.1 μM, beyond which F_z/F_u is independent of concentration and maintains a value of 0.35±0.06, corresponding to a rezipping force of 5.5±1.5 pN. *In vivo*, approximately 300 tetramer proteins are found per cell [1], resulting in an approximate concentration of 0.1–1 μM, which is similar to the concentration at which saturation occurs. Thus the E. Coli SSB protein stabilizes the ssDNA enough to significantly reduce the force necessary to maintain open ssDNA regions within the dsDNA, but cannot prevent reannealing of dsDNA if tension is reduced below 5.5±1.5 pN. The applied tension of 5.5 pN effectively reduces the stability of the dsDNA molecule. The reduction in free energy due to the tension is $0.2k_BT$ per base pair [25,26]. Within the cell, such a reduction in free energy may be realized by tension, torsion, or other competitive binders.

As binding assays are done in the absence of tension, the lowering of ssDNA tension alone cannot remove the proteins. Furthermore, the dissociation rate of E. Coli SSB protein from ssDNA has been measured as approximately 1 molecule/s or 65 bp/s [1,27], a rate far slower than the rezipping rate >1000 bp/s observed. Thus it must be concluded that either the DNA rezips around the bound proteins or that the complementary strand of ssDNA removes the protein.

As the molecule unzips, there is a "fork" or boundary between the ssDNA and dsDNA. Once complete unzipping occurs, this fork is lost, as the molecule is now completely ssDNA. For rezipping to occur, the molecule must reestablish a fork at the correct location in the sequence. It is possible that a blocked formation of a new fork by the SSB proteins may be the cause of the decreased rezipping force

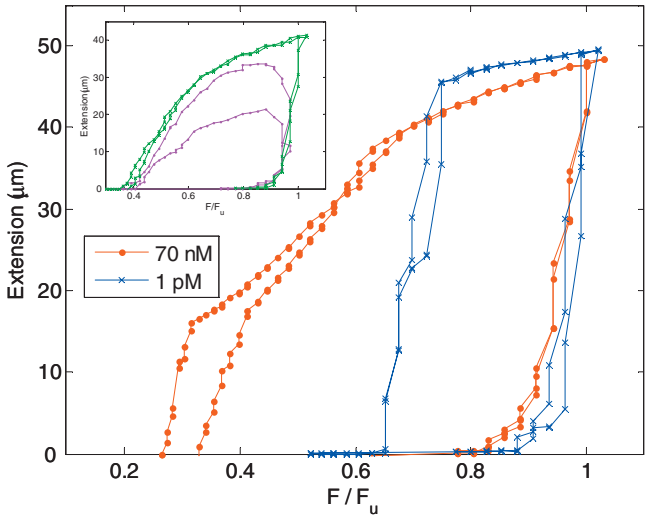

FIG. 1. (Color) The complete unzipping and subsequent rezipping of λ-DNA in the presence of 1 pM (blue crosses) and 70 nM (red circles) E. Coli SSB proteins. Inset: complete (green crosses) and partial (purple circles) unzipping of λ-DNA in the presence of 140 nM E. Coli SSB protein.

observed. To determine if SSB protein can stabilize ssDNA when a rezipping fork is already present, partial unzipping and rezipping of the molecule was carried out, as shown in the inset of Fig. 1. A molecule which has not lost its unzipping fork behaves in a similar manner to a completely unzipped molecule. Thus this stabilizing behavior occurs for both completely and partially open molecules.

The rezipping response to protein concentration can be fit by a dose response equation

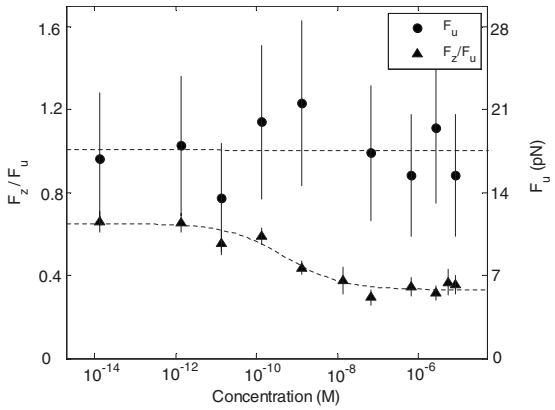

FIG. 2. Unzipping force measured in the presence of E. Coli SSB proteins as a function of concentration (circles). The range of measured forces is due to a spread in the magnetic beads. The dashed line through the circles represents a best-fit line to the data. The triangles represent the ratio of rezipping force to unzipping force as a function of E. Coli SSB protein concentration. The dashed line through the triangles represents a best-fit to the data from Eq. (1) with $C^{-1}=2\times10^{9}$ M^{-1} and a slope factor of 0.6. The right y axis corresponds to the unzipping force, while the left y axis corresponds to the ratio of rezipping force to unzipping force.

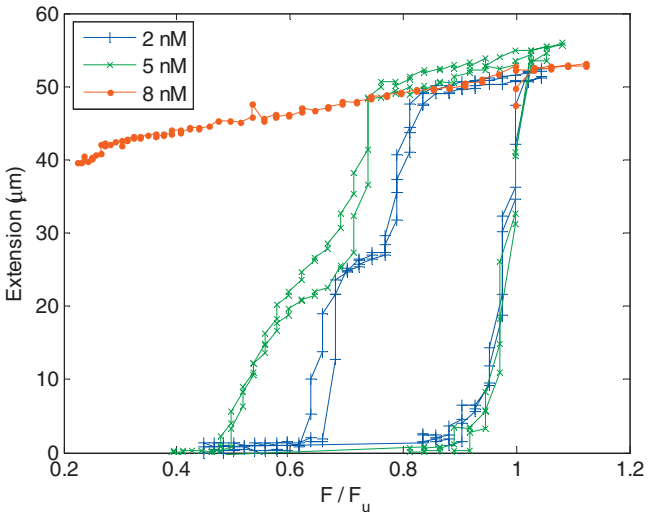

FIG. 3. (Color) Complete unzipping and rezipping in the presence of 2 nM (blue plusses), 5 nM (green crosses), and 8 nM (red circles) T4 gp32.

$$F_z(X) = \frac{F_z(0) - F_z(\infty)}{1 + \left(\frac{X}{C}\right)^b} + F_z(\infty), \tag{1}$$

where X is the protein concentration, $F_z(0)$ is the rezipping force in the absence of proteins, and $F_z(\infty)$ is the rezipping force at saturation. C is the EC_{50}, or the concentration of protein required for the rezipping force to be halfway between $F_z(\infty)$ and $F_z(0)$, and b is a "slope factor" determined by the cooperativity of the binding and is the mathematical equivalent of the Hill coefficient [28]. By fitting the measured rezipping force to this equation, we can extract b and C. If one assumes the rezipping force to be a direct reflection of protein binding, then the binding affinity could be defined as C^{-1}. A best-fit to the data results in a binding affinity (C^{-1}) of 2×10^{9} M^{-1}, compared to previous results of 10^{8}–10^{10} M^{-1} [1,29,30], and a slope factor of 0.6, shown in Fig. 2. Previously published values of ω, the cooperativity parameter, were determined by fitting to titration assay data using the McGhee–von Hippel equation, which is dependent on the binding affinity, cooperativity and binding site size [20,22,31]. Comparison of these parameters to the McGhee–von Hippel equation, with a binding site size of 30–60 base pairs [29] and a net binding affinity of 2×10^{9} M^{-1}, yields a range of 5–30 for ω. Experiments that measure the binding rather than the rezipping force have yielded a value of 50 ± 10 [23].

B. Rezipping in the presence of T4 gp32

The unzipping and rezipping of a λ-phage DNA molecule in the presence of T4 gp32 is shown in Fig. 3. It can be seen that at concentrations of 2 and 5 nM, rezipping is still observed to occur, although at 5 nM, rezipping occurs at a lower force. At 8 nM, however, complete rezipping does not occur in the time allotted, indicating that the proteins have effectively prevented double-helix reformation. The concen-

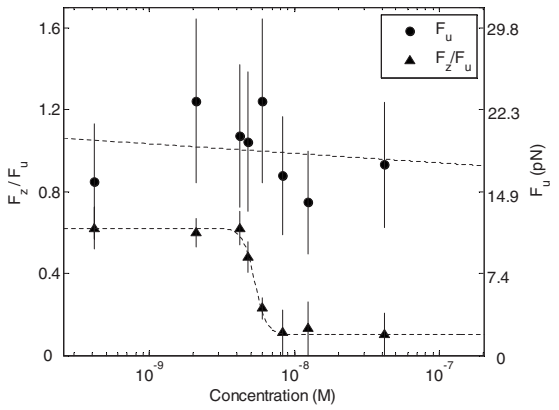

FIG. 4. The unzipping force measured in the presence of T4 gp32 as a function of protein concentration (circles). The dashed line through the circles represents a best-fit line to the data. No significant concentration trend is observed within the resolution of the experiment. The triangles represent the ratio of rezipping force to unzipping force in the presence of T4 gp32 as a function of concentration. The large error bars at the higher concentrations (8 nM and greater) are indicative of the difficulty in measuring a rezipping force when little to no rezipping occurs. The dashed line through the triangles represents a best fit to the data from Eq. (1) with $C^{-1}=2\times10^{8}$ M^{-1} and a slope factor of 10.3. The right *y* axis corresponds to the unzipping force, while the left *y* axis corresponds to ratio of rezipping force to unzipping force.

tration at which this saturation occurs, 8 nM, is much lower than concentrations found *in vivo* of 2–3 μM [1,32].

As found with E. Coli SSB protein, F_u is not significantly affected by the presence of T4 gp32, shown in Fig. 4. However, in contrast with E. Coli SSB protein, high concentrations of T4 gp32 are capable of maintaining ssDNA at very low tensions. In this concentration regime, complete rezipping of the dsDNA does not occur at forces greater than 0.2 F_u in the 2 s allotted. Rezipping does still occur at this force, but at a greatly decreased rate, on the order of 1 μm/min, or approximately 20 base pairs per second. Thus gp32 can maintain large regions of ssDNA at tensions on the order of 1 pN for several minutes.

The rate at which gp32 dissociates from ssDNA in the absence of a competitor at 150 mM NaCl is approximately 10–100 molecule/s [33,4], or 80–800 bp/s. The similarity in the dissociation rates and rezipping rates suggests that dissociation rates may be similar in the presence or absence of a rezipping fork.

The concentration-dependent behavior of T4 gp32 can be seen in Fig. 4, where the ratio F_z/F_u is plotted as a function of protein concentration. At concentrations less than 2 nM, F_z/F_u is independent of concentration with a value of 0.6–0.7, similar to that observed in the absence of proteins. In the small range of 2–8 nM, F_z/F_u decreases with increasing concentration. At concentrations greater than 8 nM, rezipping is effectively blocked by gp32. In this region, no rezipping force can be measured, which is indicated by the large error bars. This strongly nonlinear behavior is expected, due to the highly cooperative binding mechanism of T4 gp32. Using Eq. (1) a binding affinity constant (C^{-1}) of 2×10^{8} M^{-1} is extracted, compared to previous values of $10^{7}-10^{9}$ M^{-1} [1,20], with a slope factor of 10.3. This corresponds to a cooperativity parameter ω of $1-2\times10^{3}$ when a binding site of 8 base pairs [1,34] and net binding affinity of 2×10^{8} M^{-1} are assumed.

In vivo, the stability of DNA, will be affected by a number of factors beyond the presence of SSB proteins. Variations in buffer content and temperature may affect the DNA itself, as well as the binding behavior of the proteins. The limited volume in which the DNA occupies, the amount of over- and under-twisting of the DNA, as well as the presence of helicases and other proteins, will also greatly affect the stability of partially open DNA.

As a control, the unzipping and rezipping of dsDNA was carried out in the presence of a nonspecific protein, bovine serum albumin (BSA). At concentrations up to 2 μM, BSA does not exhibit any effect on the unzipping or rezipping forces of dsDNA. Thus the mere presence of proteins does not inhibit rezipping.

IV. CONCLUSIONS

We examined the stability of dsDNA and partially open DNA in the presence of E. Coli SSB proteins and T4 gp32 by measuring the forces required for unzipping and rezipping dsDNA in buffers containing various concentrations of the binding proteins. At the concentrations studied, neither protein had a significant effect on the unzipping force, suggesting that they do not destabilize the double helix on the 2 s time scale of the experiment. This result is consistent with the kinetic blocking of the binding of the proteins to the dsDNA that was observed in earlier work. The rezipping force, however, is strongly reduced by the presence of the SSB proteins, demonstrating that the protein significantly increases the stability of ssDNA. For both the E. Coli SSB protein and the T4 gp32, the measured rezipping force as a function of protein concentration is well described by a dose response curve; however, our results show a strong difference in the behavior of the two proteins. T4 gp32 exhibits strong nonlinear behavior, with no effect on rezipping at concentrations below 2 nM, and complete blocking at concentrations greater than 8 nM. At concentrations greater than 8 nM, the protein alone completely maintains the ssDNA, with no rezipping observed in the 2 s allotted; however, rezipping does occur on longer timescales at a rate of approximately 20 bp/s. In contrast, E. Coli SSB proteins exhibit a gradual ssDNA-stabilizing effect over concentrations from 1 pM to 0.1 μM. From 0.1–10 μM, no concentration dependent changes are observed, indicating a saturation of the effect of the proteins. In this saturation regime, ssDNA cannot be maintained at forces less than 5.5 ± 1.5 pN. If one assumes that the EC50 point for rezipping correlates to the EC50 point for binding, the net binding affinity can be extracted from this technique and results indicate a value of 2×10^{9} M^{-1} for E. Coli SSB, compared to previous results of $10^{8}-10^{10}$ M^{-1} [1,29,30] and 2×10^{8} M^{-1} for T4 gp32, compared to previous values of $10^{7}-10^{9}$ M^{-1} [1,20]. Thus, this single molecule technique demonstrates the correlation between ssDNA binding affinity and ssDNA stabilization. This

technique of unzipping dsDNA to probe ssDNA-protein interactions provides direct measurements of the protein's stabilizing effect on ssDNA under tension, allowing us to determine the conditions under which the protein alone can stabilize the partially open DNA and to determine the tension required to maintain the partially open DNA when the proteins alone do not provide sufficient stability. Finally, we have shown that there are significant differences between the stabilizing properties of the two proteins.

ACKNOWLEDGMENTS

We acknowledge useful conversations with Nancy Kleckner, Guido Guidotti, and Charles Limouse. This material is based upon work supported by NSF and funded by Grant No. ONR DARPA N00014-01-1-0782; Materials Research Science and Engineering Center (MRSEC) Grant No. NSF DMR 0213805, and Army Research Office Grant No. W911NF-04-1-0170.

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