doi:10.1016/S0022-2836(03)00153-0 J. Mol. Biol. (2003) 327, 571–578

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Kinetic Regulation of Single DNA Molecule Denaturation by T4 Gene 32 Protein Structural Domains

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Bacteriophage T4 gene 32 protein (gp32) specifically binds single-stranded DNA, a property essential for its role in DNA replication, recombination, and repair. Although on a thermodynamic basis, single-stranded DNA binding proteins should lower the thermal melting temperature of double-stranded DNA (dsDNA), gp32 does not. Using single molecule force spectroscopy, we show for the first time that gp32 is capable of slowly destabilizing natural dsDNA. Direct measurements of single DNA molecule denaturation and renaturation kinetics in the presence of gp32 and its proteolytic fragments reveal three types of kinetic behavior, attributable to specific protein structural domains, which regulate gp32's helix-destabilizing capabilities. Whereas the full-length protein exhibits very slow denaturation kinetics, a truncate lacking the acidic C-domain exhibits much faster kinetics. This may reflect a steric blockage of the DNA binding site and/or a conformational change associated with this domain. Additional removal of the N-domain, which is needed for binding cooperativity, further increases the DNA denaturation rate, suggesting that both of these domains are critical to the regulation of gp32's helix-destabilization capabilities. This regulation is potentially biologically significant because uncontrolled helix-destabilization would be lethal to the cell. We also obtain equilibrium measurements of the helix-coil transition free energy in the presence of these proteins for the first time.

Keywords: single-stranded binding protein; DNA melting; single molecule; force spectroscopy; DNA replication

Introduction

Single-stranded DNA binding proteins (SSBs) bind selectively to single-stranded DNA (ssDNA) and play important roles in DNA replication, recombination and repair.[1] Although the details of the functioning of SSBs in these processes are not fully understood, they likely interact with ssDNA and prevent the formation of secondary structure.[2] As a consequence of their binding selectivity, SSBs are expected to be helix-destabilizing proteins and should therefore lower the melting temperature of natural DNA.[3] However, bacteriophage T4 gene 32 protein (gp32)[2,4,5] and *Escherichia coli* SSB,[6] do not. This disagreement between thermodynamic predictions and the behavior of SSBs suggests that DNA helix-destabilization by these proteins is kinetically regulated.[5] Here we use single molecule force spectroscopy to clearly demonstrate by direct measurement that DNA helix-destabilization by gp32 is indeed kinetically regulated. We identify the specific structural domains that accomplish this regulation, and quantify the effects of these domains on the kinetics of DNA denaturation. Because single-stranded DNA binding proteins are found in viruses and all three domains of life[7] and are essential for virtually all DNA functions,[8] it is important to understand how helix-destabilization by SSBs is regulated.

In this work, we utilize single molecule force spectroscopy[9] to monitor DNA denaturation and renaturation in the presence of intact and truncated forms of gp32. In each experiment, a single double-stranded DNA (dsDNA) molecule is stretched by pulling on one end of the DNA while the resulting

Abbreviations used: gp32, T4 gene 32 protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

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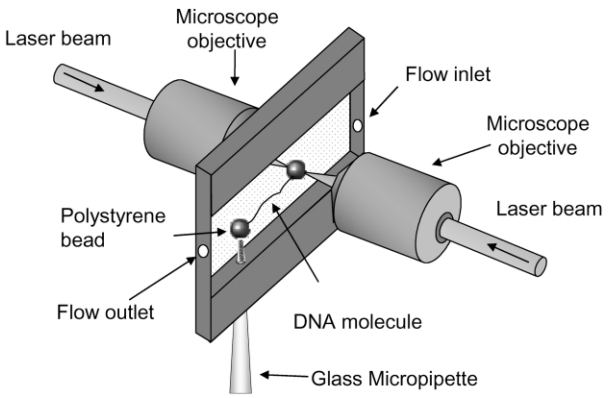

Figure 1. Schematic drawing (not to scale) of an optical tweezers experiment in which a single DNA molecule is stretched between two polystyrene beads in a fluid flow cell. One bead is held on the end of a glass micropipette (attached to the cell) by suction, while another bead is held in an optical trap. Two counter-propagating laser beams focused to a common point form the optical trap. Force–extension curves are obtained by moving the micropipette and measuring the resulting force on the bead in the optical trap.

force on the other end of the molecule is measured by means of an optical trap, as shown in Figure 1. In previous single-molecule studies, we have shown that a model of the DNA overstretching transition as force-induced melting accurately describes its dependence on pH, temperature, and ionic strength.[10] Thus, this technique allows us to study the DNA helix-coil transition at high resolution and at room temperature.[10] The latter capability is particularly useful for studying protein–nucleic acid interactions,[11,12] since protein thermal lability does not complicate the analysis.

Truncation of the 301-residue T4 gene 32 protein, originally achieved by selective proteolysis (Figure 2), alters the nucleic acid and protein-interactive properties of the protein. The first 21 amino acid residues (N-domain) are responsible for binding cooperativity; the central or core domain (residues 22–253) contains the nucleic acid binding surface,[13,14] and the acidic C-domain (254–301) is involved in binding other T4 replication, repair, and recombination proteins. In addition, when the C-domain is removed, with or without the retention of the N-domain, the resulting truncates, *I and *III, respectively, possess helix-destabilizing activity, as measured in thermal melting experiments.[15,16] *I, which retains binding cooperativity, lowers the t_m of dsDNA by about 55 deg.C in 50 mM NaCl, consistent with the thermodynamic prediction based on its preferential binding to ssDNA.[15,16]

The helix-destabilizing activities of *I and *III may depend on their ability to bind to transient single-stranded regions generated by thermal fluctuations, causing dsDNA to melt at a much lower temperature. For full-length gp32, the presence of the acidic C-terminal domain inhibits helix-destabilizing activity. Conceivably, this domain must be displaced in order for the protein to bind single strands, a process that may occur too slowly relative to the lifetimes of the fluctuation-induced melted regions, resulting in a "kinetic block" to helix-destabilization. It is possible that the kinetic block is removed at higher temperatures, where the transient single-stranded regions are likely to be larger and longer-lived, but this has not been observed because gp32 denatures above 50 °C. The use of stretching methods overcomes this limitation, and provides new insights into the unusual effects of the protein's structural domains on DNA denaturation and renaturation.

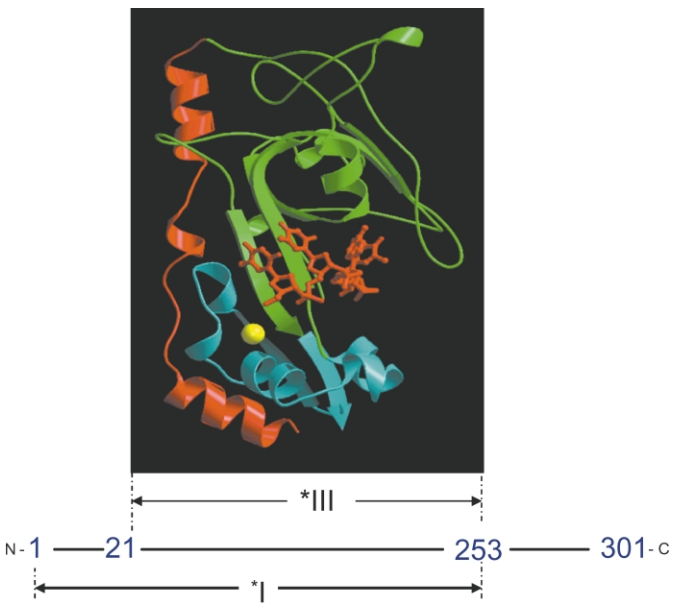

Figure 2. Proteolytic fragments of gp32. *I can be obtained by trypsin cleavage of full-length gp32 at residue 253, while *III results from cleavage at residues 21 and 253. A MOLSCRIPT[35] representation of a *III-oligonucleotide complex is shown at its location within the protein sequence. The protein is pictured in ribbon mode, with the major lobe green, the minor (Zn-containing) lobe blue, and the residues 198–239 flap red. The bound oligonucleotide, in sticks mode, is red, and the coordinated Zn^{2+}, in space-filling mode, is yellow. The position of the oligodeoxynucleotide, pTTAT, is approximate; it was modeled by Shamoo *et al.* to maximally overlap excess electron density in the trough.[13] The Protein Data Bank entry for core domain (without the oligonucleotide) is 1gpc.pdb.

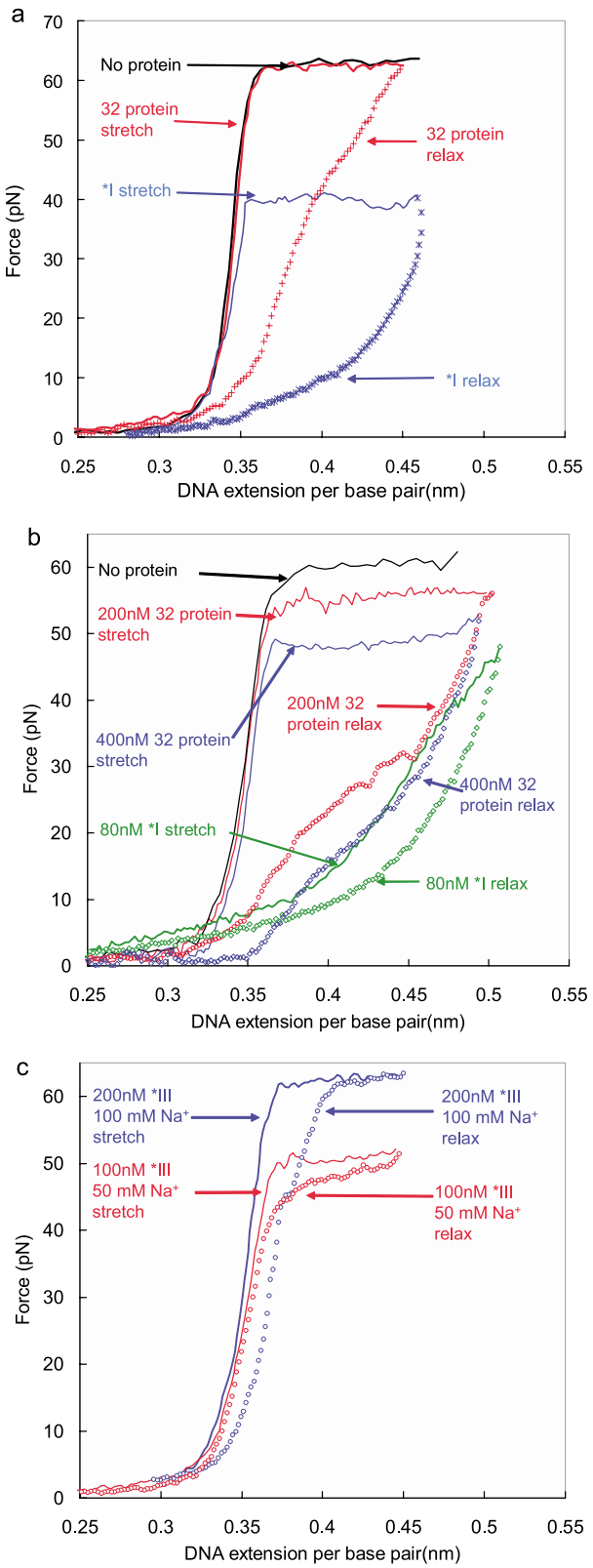

Figure 3. a, Stretching (continuous line) and relaxation (symbols) curves for λ-DNA in 10 mM Hepes (pH 7.5), 100 mM [Na^+] (95 mM NaCl and 5 mM NaOH) in the absence of protein (black) and in the presence of 200 nM gp32 (red) and *I (blue). b, Stretching and relaxation curves for λ-DNA in 10 mM Hepes (pH 7.5), 50 mM [Na^+] in the absence of protein (black) and in the presence of 200 nM (red) gp32, 400 nM (blue) gp32, and 80 nM (green) *I. c, Stretching and relaxation curves for λ-DNA in 10 mM Hepes (pH 7.5), 100 mM [Na^+] (95 mM NaCl and 5 mM NaOH) in the presence of 200 nM *III (blue) and in 10 mM Hepes (pH 7.5), 50 mM [Na^+] in the presence of 100 nM *III (red). In contrast to gp32 and *I, *III shows little hysteresis even under strong binding conditions in 50 mM [Na^+].

Results and Discussion

Force spectroscopy of single DNA molecules

To investigate the helix-destabilization capabilities of gp32 and its proteolytic fragments *I and *III, we measured the force–extension curve of λ-DNA over a range of salt and protein concentrations. The results of our measurements in 100 mM [Na^+] and 200 nM gp32 and *I are shown in Figure 3a. Within the one minute duration of the experiment, intact protein had no effect on the DNA overstretching force, in contrast to *I, which significantly lowered this force. These results suggest that *I destabilizes DNA under these conditions, while gp32 does not, consistent with the results of thermal melting experiments.[16] When the DNA was relaxed, the relaxation curves for both *I and gp32 did not match the stretching curves at any point during the overstretching transition. Thus, these proteins bind to exposed regions of ssDNA created when the DNA is stretched, but do not dissociate within the one minute time-scale of the relaxation experiment.

The failure of full-length protein to alter the denaturation of dsDNA in 100 mM [Na^+] is consistent with previous studies, and likely reflects the kinetic barrier to DNA melting by intact gp32. However, at lower [Na^+], 50 mM, gp32 significantly lowers the overstretching force at 200 nM and 400 nM protein concentration (Figure 3b). This result is the first demonstration of full-length gp32-induced denaturation of natural DNA (the t_m of polyd(A-T), which is capable of forming alternative palindromic structures[17] is lowered by gp32[5]). The stretching results differ from thermal melting observations, where even after an eight hour incubation with gp32 at a temperature above the thermodynamically determined t_m, the DNA remained double-stranded.[5] The relaxation plots in Figure 3a and b show considerable hysteresis, and indicate that in the presence of gp32 and *I the melted DNA does not re-anneal until the DNA molecule is completely relaxed. In the presence of 80 nM *I in 50 mM [Na^+], we observe that the single λ-DNA molecules are almost completely melted at room temperature and the stretch curve thus resembles that of ssDNA (Figure 3b).

Lacking the N-domain, *III binds ssDNA non-cooperatively, resulting in an affinity for ssDNA three to four orders of magnitude below that of intact protein or *I.[18] In 100 mM [Na^+], *III does not alter the DNA overstretching force (Figure 3c). These curves also show little hysteresis, but *III may not be bound to the DNA under these

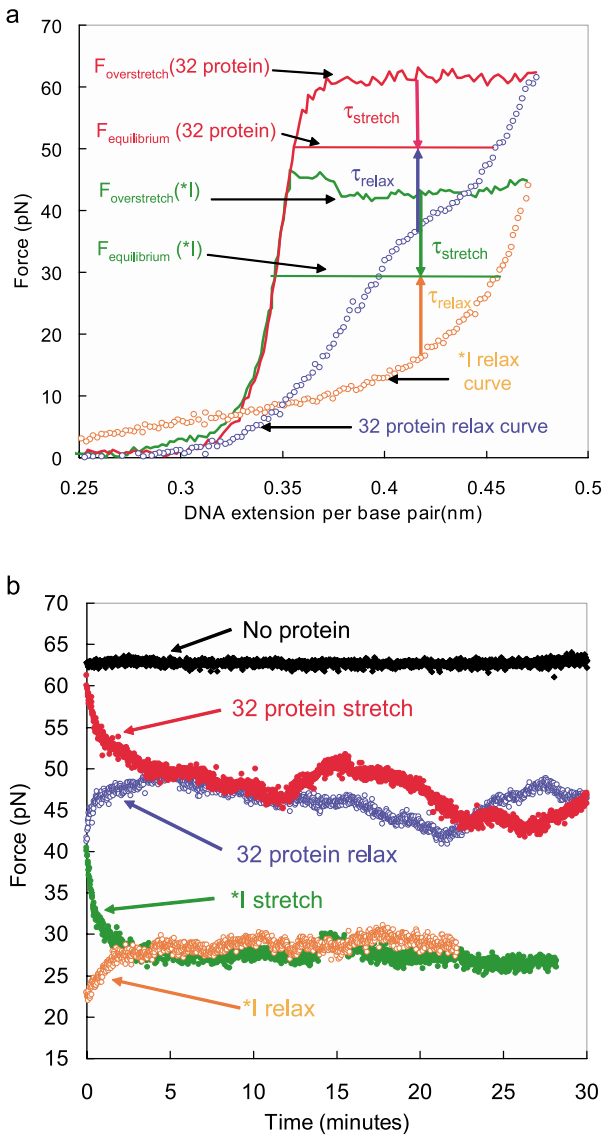

Figure 4. a, Stretching (continuous line) and relaxation (symbols) curves for λ-DNA in 10 mM Hepes (pH 7.5), 100 mM [Na^+] in the presence of 200 nM gp32 (stretch, red; relax, blue) and *I (stretch, green; relax, orange). The arrows are a schematic representation of the time dependence measurements shown in b. To measure the denaturation rate, the DNA is overstretched to 0.42 nm/bp and the force is measured as a function of time. The force decreases initially in this measurement. In a second type of experiment to measure DNA renaturation, the DNA is stretched to the full length shown on the graph, then relaxed to 0.42 nm/bp. In this case, the force increases with time. For each protein the force approaches an equilibrium force at long times that is the same for denaturation and renaturation, shown as a straight line. b, Representative curves for time dependence of DNA stretching force at constant position in the absence of protein and in the presence of gp32 (red) and *I (green). Also shown is the time dependence of DNA that has been overstretched and relaxed back to the same position in the presence of gp32 (blue) and *I (orange).

conditions.[18] At a lower [Na^+] of 50 mM, where the affinity for ssDNA is greater than at 100 mM [Na^+], *III is now observed to significantly reduce the overstretching force (Figure 3c). Under these conditions, there was also very little hysteresis, indicating that this measurement represents the equilibrium overstretching force. Therefore, *III binds to exposed regions of ssDNA created by DNA force-induced melting, but in contrast to *I and intact gene 32 protein, *III quickly unbinds ssDNA upon relaxation, allowing the DNA strands to re-anneal in the short time-scale of our experiment.

Kinetics of DNA helix-destabilization by T4 gp32

The strong hysteresis observed in the stretching and relaxation curves shown in Figure 3 for gp32 and *I suggests that DNA denaturation and renaturation and corresponding protein association and dissociation are much slower than the one minute duration of our experiment. To directly measure the rate of base-pair melting and re-annealing, we monitored the change in force in the presence of each protein as a function of time. In these experiments, single dsDNA molecules and force-denatured DNA molecules were each brought to a fixed position relative to the center of the optical trap (0.42 nm/bp, where approximately one-third of the DNA is melted during the initial stretch), and the force was measured in the presence of each protein as a function of time. The red and green arrows originating from dsDNA stretching curves in Figure 4a represent measurements of the rate of DNA helix-destabilization (and protein binding) for gp32 and *I, respectively. The blue (gp32) and orange (*I) arrows in Figure 4a represent measurements of the rate of DNA re-annealing and protein dissociation for these proteins (obtained after first rapidly stretching to 0.48 nm/bp and then relaxing to 0.42 nm/bp).

Typical results from measurements of the time dependence of the overstretching force at constant position in 100 mM [Na^+] buffer are given in Figure 4b. The initial decrease, which represents DNA melting due to protein binding, can be fit to an exponential decay:

$$F(t)_{\text{stretch}} = F_{\text{equilibrium}} + (F_{\text{overstretch}} - F_{\text{equilibrium}}) \times \exp\left(-\frac{t}{\tau_{\text{overstretch}}}\right) \quad (1)$$

where $F_{\text{overstretch}}$ is the initial force observed upon DNA overstretching and $F_{\text{equilibrium}}$ is the final force observed at long times. Measurements of the rate of DNA re-annealing and protein dissociation can be fit to a similar function:

$$F(t)_{\text{relax}} = F_{\text{equilibrium}} + (F_{\text{relax}} - F_{\text{equilibrium}}) \times \exp\left(-\frac{t}{\tau_{\text{relax}}}\right) \quad (2)$$

Table 1. Results of measurements of the thermodynamics and kinetics of DNA melting and re-annealing in the presence of T4 gp32 and its proteolytic fragments *I and *III

Protein	$\tau_{overstretch}$ (s)	τ_{relax} (s)	Melting (bp/second)	Re-annealing (bp/second)	$F_{overstretch}$ (pN)	$F_{equilibrium}$ (pN)	ΔG^a (k_BT/bp)
None		<1		>800	62.6 ± 0.7[b]	62.6 ± 0.7[b]	2.2 ± 0.1
gp32	118 ± 13	37 ± 4	18 ± 3	53 ± 11	62.5 ± 1.0	52.5 ± 1.1	1.5 ± 0.1
*I	38 ± 2.5	80 ± 4	107 ± 10	70 ± 11	41.3 ± 2.5	27.7 ± 1.2	0.5 ± 0.1
*III		<1		>800	62.1 ± 1.0	62.1 ± 1.0	2.0 ± 0.1

All measurements were performed in 10 mM Hepes (pH 7.5), 100 mM [Na^+], and 200 nM protein concentration. Data are reported as mean ± standard error for at least three time-dependent curves. The helix-coil transition free energy ΔG is reported in units of k_BT per base-pair (bp), where k_B is the Boltzmann constant and $T = 293$ K is room temperature ($1k_BT = 581$ cal/mol). Melting and re-annealing parameters were calculated from equation (1).

[a] Helix-coil transition free energy is calculated assuming a constant equilibrium transition force $F_{equilibrium}$ obtained from time measurements and measuring the area between dsDNA and ssDNA stretching curves below that force. A stretching curve for single-stranded DNA in the presence of *I was used for all results except in the absence of protein, where the ssDNA stretching curve from Smith *et al.*[36] was used.

[b] Data are from Wenner *et al.*[37]

where F_{relax} is the initial force observed after rapidly stretching the DNA to 0.48 nm/bp, relaxing to 0.42 nm/bp, and then measuring the force as a function of time. $F_{equilibrium}$ is the final force observed at long times. Fits of the time dependence of the DNA stretching force to equations (1) and (2) allow us to obtain the time constants $\tau_{overstretch}$ for DNA melting and τ_{relax} for DNA re-annealing, respectively. We also obtain an initial force ($F_{overstretch}$) and a final equilibrium force ($F_{equilibrium}$), as shown in Table 1. To obtain the results of Table 1, time dependence curves for each solution condition were obtained. These curves were separately fit to equation (1) or (2) and the average results are presented in the Table.

The large fluctuations in force about $F_{equilibrium}$ (Figure 4b) are reproducible and most likely represent cooperative binding and unbinding of clusters of gp32 and *I. (*III does not show these fluctuations). While fluctuations of such high amplitude are initially surprising, strong fluctuations are expected in measurements of interactions with single molecules.[19] If an ensemble of DNA molecules were simultaneously probed in a bulk measurement, these fluctuations would be masked in the average signal obtained. This is illustrated in Figure 5, where we show the force signals for three single DNA molecules in the presence of gp32 (Figure 5a) and *I (Figure 5b). These individual curves show strong fluctuations, while an average over several measurements shows a smooth exponential decay in each case. The fluctuations appear to be of a larger amplitude for gp32 than for *I, although both proteins bind ssDNA cooperatively. This suggests that both the N and C-terminal domains of gp32 are responsible for the observed fluctuations. Since the fluctuations represent sections of DNA melting and re-annealing, the cooperatively bound and unbound clusters that induce this melting and re-annealing may be larger with gp32 than *I.

The parameters resulting from the fitting procedure allow us to calculate an initial rate of base-pair melting and re-annealing for each protein (Table 1). To do this, we calculate the equilibrium fraction of melted base-pairs Θ at any force by fitting our DNA stretching curves to the Zimm–Bragg model for the helix-coil transition.[11,20] We then use the resulting values for $\Theta(F)$ to calculate the melting rate:

$$\frac{\partial N_m}{\partial t} = N_{total}\frac{\partial \Theta}{\partial t} = N_{total}\frac{\partial \Theta}{\partial F}\frac{\partial F}{\partial t} \qquad (3)$$

where N_m is the number of melted base-pairs, N_{total} is the total number of base-pairs in the DNA molecule (48,500), and $\partial F/\partial t$ is calculated from the function $F(t)$ determined from the fitting parameters in Table 1 for each condition. We report the initial melting rate in Table 1, given by:

$$\left.\frac{\partial F(t)_{stretch}}{\partial t}\right|_{t=0} = -\frac{(F_{overstretch} - F_{equilibrium})}{\tau_{stretch}} \qquad (4)$$

The melting rate for gp32 of 18 bp/second is very slow, well below the rate of helix opening at the DNA replication fork *in vivo*.[21] Assuming a binding site size of seven nucleotide residues,[22] this is equivalent to a binding rate of only five proteins/second, or more than an order of magnitude slower than the rate of DNA helicase-catalyzed fork opening. If the protein must undergo a conformational rearrangement associated with the C-terminal domain in order to bind DNA,[16,22–24] one would expect that the melting rate for *I, which lacks this domain, should be much greater. While the melting rate for *I of 107 bp/second (or 31 proteins bound per second) is indeed significantly greater than the rate for gp32, it is still much slower than for *III, which was too rapid to measure. It thus appears that establishing the protein–protein interactions responsible for cooperativity slows the overall rate of DNA melting. We also note that only in the presence of full-length gp32 is the DNA renaturation rate faster than the rate of denaturation. This unusual property may reflect the complex role of gp32 in T4 recombination-dependent DNA replication and repair processes.[25] In previous bulk solution measurements it has been shown that both gp32[26] and *I[16] induce rapid DNA renaturation under certain conditions, although

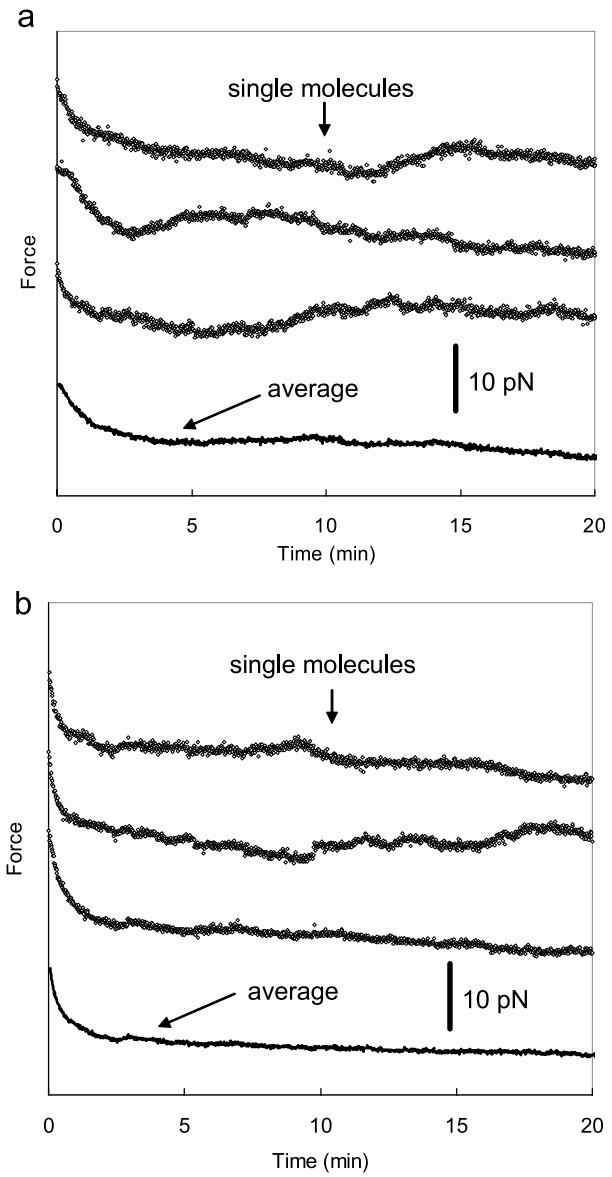

Figure 5. a, Time dependence of DNA stretching force at constant position (0.42 nm/bp) for λ-DNA in 10 mM Hepes (pH 7.5), 100 mM [Na^+] in the presence of 200 nM gp32. The top three data sets represent stretching curves for individual DNA molecules, showing large fluctuations in force. The bottom curve represents an average of all data sets obtained under these conditions, revealing a monotonic exponential decay that would be expected for a bulk measurement. b, Time dependence of DNA stretching force at constant position in the presence of 200 nM *I. Similar to gp32, the top three individual stretching curves show significant fluctuations in force, while the average of all data sets show a monotonic exponential decay.

the effects of these two proteins on DNA denaturation are quite different. This is consistent with the results shown in Table 1, in which intact gp32 denatures dsDNA much more slowly than *I, but the renaturation rates for both proteins are similar.

Thus, we observe a hierarchy of kinetic behavior associated with the three structural domains of gp32. *III, which constitutes the nucleic acid-binding "core" domain, binds non-cooperatively and relatively rapidly with transiently melted regions (bubbles) of dsDNA because it lacks the N-terminal cooperative binding domain and the acidic C-terminal domain. In contrast, *I, which contains the N-domain but not the C-domain, binds cooperatively and therefore more strongly, but its interaction with transiently melted DNA is much slower than *III. Finally, full-length gp32 binds and destabilizes DNA very slowly, most likely a consequence of a rate-limiting conformational change associated with the acidic C-terminal domain. This may be related to the repetition of the "*LAST*" sequence, Lys-Arg-Lys-$(Ser/Thr)_2$, found in the N-domain (where it is critical for binding cooperativity) as well as at the nucleic acid binding surface of the core domain.[16,23,27] Both *I and gp32 appear to bind readily to the large regions of ssDNA exposed upon DNA overstretching. This is supported by the significant hysteresis in our stretching curves for *I and gp32. Thus, the DNA tends to remain single-stranded even upon relaxation because the relatively slow unbinding of protein slows re-annealing. Protein binding to denatured regions must happen relatively quickly (on the time-scale of seconds) because the DNA remains single-stranded even when the DNA is rapidly stretched and relaxed in less than one minute. This is consistent with previous measurements of the (rapid) binding kinetics of gp32 to ssDNA.[28]

Measurements of $F_{\text{equilibrium}}$ from our time dependence data can be used to obtain the helix-coil transition free energy, assuming a constant equilibrium transition force (Table 1). In accord with the results discussed above, and the relative affinities for ssDNA,[18] the transition free energy is only slightly reduced, if at all, by *III ($2.0(\pm 0.1)k_BT$), and is lowered to $1.5(\pm 0.1)k_BT$ for intact protein and $0.5(\pm 0.1)k_BT$ for *I. The fact that both *I and gp32 significantly destabilize dsDNA is consistent with their binding preference and significant affinity for single *versus* double-stranded DNA. However, reaching this equilibrium state required stretching of the single dsDNA molecule until a significant portion of the molecule was denatured. Under these conditions, additional domains of denatured DNA created by thermal fluctuations are larger[29] and longer lived[30] than those created at low forces and temperatures. Therefore, under solution conditions where DNA is not subjected to stretching forces, the equilibrium state measured here is never reached. An additional potential factor in the regulation of helix-destabilizing activity is the effect of interaction of gp32 with other proteins involved in DNA replication, recombination or repair, such as T4 DNA polymerase or UvsX recombinase. These proteins are known to bind to a recombinant fragment of gp32 corresponding to residues 213–301, which includes the proteolytically defined C-domain.[24,31] An intriguing and biologically significant notion, now

testable, is that such binding might modulate the helix-destabilizing activity of gp32.

Conclusions

The results presented herein are the first demonstration of natural DNA destabilization induced by intact gp32, and the first measurements of the kinetics of this process for gp32 and its proteolytic fragments. The process of stretching DNA to forces sufficient for denaturation creates larger and longer-lived transient single-stranded regions than are present in thermal melting experiments below the t_m, allowing us to directly measure the kinetics of DNA helix-destabilization that are not observed in thermal melting experiments. Because uncontrolled DNA melting would be lethal to the cell,[17] the helix-destabilizing activity of the single-stranded DNA binding protein must be regulated. Clearly, control of this process can be achieved kinetically, as we have demonstrated, although thermodynamic considerations are also important.[32]

Materials and Methods

The dual-beam optical tweezers instrument used here consists of two counter-propagating diode lasers focused to a small spot inside a liquid flow-cell (Figure 1). One 5.6 μm diameter streptavidin-coated polystyrene bead (Bangs Labs, Fisher, IN) was held in the optical trap formed by the laser beams. Another streptavidin-coated bead was held on the end of a glass micropipette. To obtain force–extension measurements, a single double-stranded DNA molecule that had been labeled on the 3′ end of opposite strands with biotin was captured between the two beads.[33] The DNA molecule was then stretched by moving the pipette and measuring the resulting calibrated force on the bead in the trap, as described.[33,34] The buffer used here for capturing DNA was 10 mM Hepes (pH 7.5) with 95 mM NaCl and 5 mM NaOH (100 mM [Na^+]) or 10 mM Hepes with 45 mM NaCl and 5 mM NaOH (50 mM [Na^+]).

The absolute extension of the molecule was estimated by measuring the distance between the centers of the two beads using an image captured with a CCD camera. The change in position of the pipette was measured using a feedback-compensated piezoelectric translation stage that is accurate to 5 nm (Melles Griot, Irvine, CA). The position measurement was converted to a measurement of the molecular extension by correcting for the trap stiffness, which was 52(±3) pN/μm. For the force–extension measurements reported here, the pipette was moved in 100 nm steps at a rate of ~1 step/second and after each step the force was measured 100 times and averaged, thus averaging out contributions of thermal motion to the force measurement. To obtain measurements of DNA helix-destabilization kinetics, the DNA was stretched in 100 nm steps and then held at a constant position while the resulting force as a function of time was measured.

T4 gp32 and truncated forms *I and *III used in these experiments were prepared as described.[16] After capturing a single DNA molecule in the tethering buffer, the molecule was stretched to verify that the usual force–extension curve was obtained. To measure the effect of the protein on this transition, four to five cell volumes of a buffer solution containing a fixed amount of protein was added to the experimental cell until the buffer surrounding the captured DNA molecule was completely exchanged.

Acknowledgements

We thank Ms Carmen Silvers for preparation of biotin-labeled λ-DNA, Elizabeth Flynn and Min Wu for assistance with protein purification, and Michael Zawrotny for generation of the MOLSCRIPT representation in Figure 2. We also thank Dr Karin Musier-Forsyth for discussions and for a critical reading of the manuscript and Dr Ioulia Rouzina for valuable discussions. Funding for this project was provided by NIH (GM 52049, to R.L.K.) and Northeastern University (to M.C.W.) as well as the Designated Research Initiative Fund at UMBC (to R.L.K.).

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Edited by D. E. Draper

(Received 26 November 2002; received in revised form 28 January 2003; accepted 28 January 2003)