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Single-Molecule Unzipping Force Analysis of HU–DNA Complexes

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The genome of bacteria is organized and compacted by the action of nucleoid-associated proteins. These proteins are often present in tens of thousands of copies and bind with low specificity along the genome. DNA-bound proteins thus potentially act as roadblocks to the progression of machinery that moves along the DNA. In this study, we have investigated the effect of histone-like protein from strain U93 (HU), one of the key proteins involved in shaping the bacterial nucleoid, on DNA helix stability by mechanically unzipping single dsDNA molecules. Our study demonstrates that individually bound HU proteins have no observable effect on DNA helix stability, whereas HU proteins bound side-by-side within filaments increase DNA helix stability. As the stabilizing effect is small compared to the power of DNA-based motor enzymes, our results suggest that HU alone does not provide substantial hindrance to the motor's progression in vivo.

Introduction

The dimensions of an unrestrained bacterial genome largely exceed those of the bacterial cell itself. Therefore the genomic DNA of bacteria needs to be compacted and functionally organized by architectural proteins.[1–3] In *E. coli*, approximately ten different nucleoid-associated proteins (NAPs) are likely involved in this process. Two main types of architectural properties can be noted: bridging of two sites/regions along DNA (e.g., H-NS) and bending of DNA (e.g., histone-like protein from strain U93 (HU), IHF).[1–3] Under certain experimental conditions, a DNA-stiffening mode is observed in addition to one of these two modes. In the case of H-NS-like proteins, switching between bridging and stiffening appears to depend on magnesium ions[4] and might correspond to a switch between binding *in trans* and *in cis*.[5] The HU protein bends DNA at low protein concentrations, thus promoting compaction, and stiffens DNA at high concentrations (Figure S1 in the Supporting Information).[6–8] The affinity of HU for short regular B-DNA substrates is in the micromolar range, whereas affinities in the nanomolar range have been reported for binding to predistorted DNA substrates.[9–11]

DNA transactions, such as transcription, replication and repair, all take place on a genome decorated with many different proteins, a large fraction of which are NAPs.[12] The speed and efficiency of the motor enzymes RNA polymerase and DNA polymerase in translocating along DNA can be affected by the presence of DNA-bound proteins. Transcription and replication can come to a halt if a protein's off rate is so low that it acts as a roadblock.[13] The effect of nucleoid-associated proteins on transcription elongation and replication has not been investigated directly. We earlier argued that, based on force measurements, DNA–DNA bridges mediated by H-NS do not interfere with transcription.[14] In this study, we have investigated the effects of HU on DNA helix stability, which has implications for both the aforementioned processes, by applying single-molecule DNA-unzipping force analysis to single HU–DNA complexes. This study provides insight into the strength of binding of the HU protein in its two binding modes, and dissects the role of DNA sequence composition.

Results and Discussion

Unzipping of single DNA molecules yields a characteristic sequence-dependent unzipping force landscape, with GC-rich regions requiring higher forces for unzipping than AT-rich regions.[15] Binding of a protein to a specific site along the DNA results in specific changes in this landscape.[16,17] For instance, force-induced dissociation of a eukaryotic nucleosome from a specific positioning sequence is manifested in a complex force landscape that reflects the disruption of specific nucleosomal protein–DNA interactions.[18,19] The force required for the dissociation of nucleosomes is of the order of several tens of pNs. In addition to yielding information on dissociation pathways and kinetics under force, unzipping experiments also permit the mapping of protein binding locations along DNA.

In this study, we investigated the generic effect of the binding of the nucleoid-associated protein HU on the force required for DNA unzipping and specific effects related to DNA sequence composition. DNA-unzipping experiments can pro-

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vide detailed insight into the binding patterns of HU (and their stability against applied force) along DNA, thus deepening and extending observations on global mechanical effects, as seen in DNA-stretching experiments[20] (Figure S2). In control force-extension experiments, we first reproduced the bimodal binding behaviour seen in our previous magnetic-tweezers studies[7] (data not shown). Up to a HU concentration of 400 nM, the apparent persistence length is reduced, and the DNA compacts. This behaviour is attributed to the binding of individual HU proteins. At higher concentrations (400–1600 nM), the apparent persistence length increases. This corresponds to the "DNA stiffening" mode, in which HU proteins are bound closely side-by-side.[7] Next, we constructed 750 bp DNA substrates suitable for DNA unzipping[16,18,19] and carried out DNA-unzipping experiments over a range of concentrations (from 0–1600 nM HU) under the same conditions as used in our previous studies.[7] The speed of unzipping (~75 bp s^{-1}) is of the order of the progression rate found in vivo for RNA polymerase,[21] but an order of magnitude lower than the rate of replication.[22]

Under these conditions, DNA unzipping curves characteristic of bare DNA are obtained in the absence of protein (Figure 1 A); the local force required for unzipping ranges between 11 and 15 pN depending on AT content. Regions of low-AT/high-GC content require higher unzipping forces than regions of high-AT/low-GC content. The average unzipping curves do not change substantially following addition of 100, 200 or 400 nM HU; however, when the amount of HU is increased beyond 400 nM, corresponding to the DNA stiffening regime, the average force required for unzipping increases by ~2 pN.

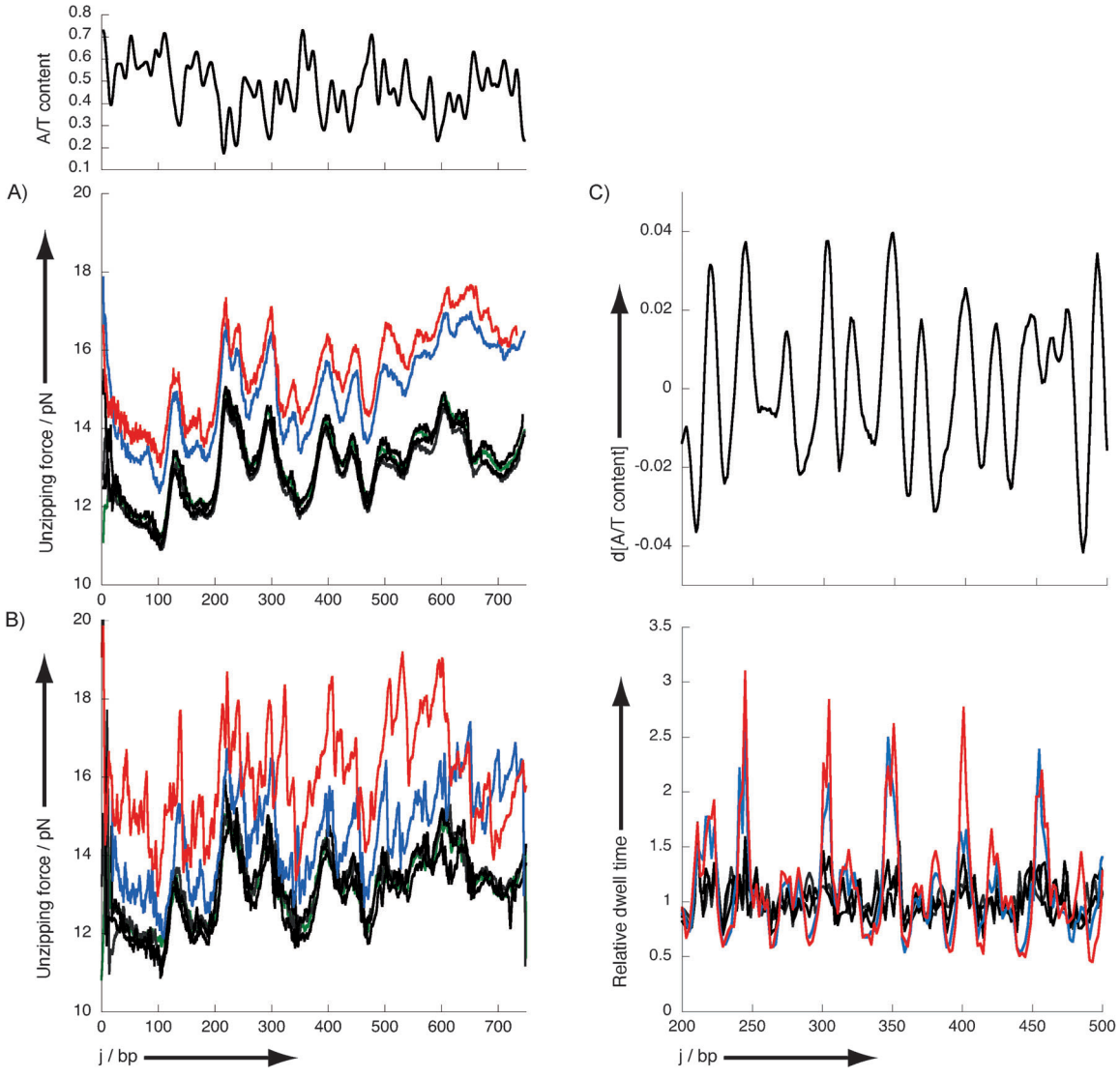

Figure 1. Unzipping of DNA and HU–DNA complexes. Unzipping traces for HU DNA complexes. A) Average unzipping force landscape obtained at different HU concentrations. Note that the unzipping forces at 800 (blue) and 1600 nM (red) HU increase by ~2 pN compared to bare DNA (green) and at HU concentrations of 100, 200 and 400 nM (black). B) Typical unzipping force landscape obtained at each HU concentration. Note that additional peaks are observed in the traces obtained at 800 and 1600 nM. The A/T content along the unzipped DNA fragment is shown at the top. C) Relative dwell time along the unzipped DNA fragment indicating how much longer a certain position is maintained within an HU–DNA complex compared to bare DNA; colours as in (A). The derivative of A/T content along the unzipped fragment is shown at the top.

If we next focus on individual unzipping traces rather than on the averages (Figure 1B), it is evident that the traces obtained at concentrations up to 400 nM are essentially identical to the bare DNA traces. This indicates that individual HU molecules sparsely bound along the DNA are dissociated very easily. However, distinct features are present along the unzipping trace (peaks superimposed on the average raised DNA baseline) in the concentration regime in which filaments are formed (i.e., HU concentrations higher than 400 nM). The occurrence of these features is correlated with regions of high AT content directly following regions of low AT content (characterized by high unzipping force). These features are expected to translate into long dwell times at the locations of the maxima in a plot of the derivative of AT content. Indeed, sites with long dwell times perfectly match the location of such maxima (Figure 1C). This suggests that HU release from such regions requires more energy than elsewhere along the DNA, in line with the reported in vivo propensity of HU to bind AT-rich DNA.[23] The large drop in force after rupture suggests that gaps are present in the HU–DNA filaments following AT-rich regions. It is tempting to speculate that HU filament formation initiates at AT-rich regions and that the observed irregularities within the filament arise from improper phasing between independently nucleated filaments. Note that, under these conditions, no individual proteins can be seen to dissociate from the filament extremity due to their close packing.

The average difference in unzipping force between bare DNA and the HU–DNA filament at the unzipping rate used is about 2 pN. As the dissociation rate of the individual bound proteins at lower concentrations is too fast to be measured, this value reflects the energy required to disrupt cooperative HU–HU interactions within HU–DNA filaments.[7,24] In individual traces, high-force peaks (up to 5 pN above the reference trace) corresponding to rupture events are observed. Our studies did not permit the determination of a consensus sequence in the DNA related to these events. We speculate that structural features such as sequence-dependent stiffness promote the formation of stable filaments, so that a number of subunits held together through cooperative interactions dissociate at once.

What are the implications of our observations for in vivo collisions between HU and the transcription and replication machinery? The applied rate of unzipping of 75 bp s^{-1} is in the range that is physiologically relevant for both types of processes. The high off-rate of HU (leading to undetectable events in our experiments) suggests that neither of the two types of machinery is affected by the sparse binding of individual HU molecules along the genome. Assuming a binding site of 9 bp, a maximum of eight HU proteins is forced to dissociate from the DNA by the applied force per second. This condition is only encountered in the filament and results in a 2 pN increase in unzipping force along the DNA. At low DNA coverage, no force peaks are observed along the DNA, thus suggesting an off rate $\gg 8\ s^{-1}$. Although local filament formation might occur in vivo in regions of high AT content, sparse binding is physiologically likely to be the most relevant. Nevertheless, in our experiments, even such filaments have only a minor effect on DNA duplex stability and thus on the progression of enzymes moving along DNA. Transcription has even been seen to occur through eukaryotic nucleosomes,[25,26] which have much higher characteristic rupture forces.[18,19] Also, for bacteria, it has previously been suggested that DNA regions bridged by H-NS are not a barrier to the progression of RNA polymerase.[14] However, HU filaments, as well as H-NS bridged regions, probably do act as barriers to the diffusion of proteins involved in target search; this stresses the importance of target localisation through multiple association/dissociation events. One also needs to keep in mind that the situation in vivo is more complex and involves cooperative action by different nucleoid-associated proteins. This suggests that, locally, more stable complexes composed of different NAPs can be formed that affect the progression of DNA-based motor enzymes, and thus NAPs might play direct regulatory roles.

Experimental Section

Biological materials: The DNA construct used in unzipping experiments was designed as two separate segments, as described.[16,18,19] A 1.1 kb anchoring segment was prepared by PCR from plasmid pRL574[27] by using a digoxigenin (dig)-labelled primer and then digested with BstXI (NEB) to produce a ligatable overhang. The 0.8 kb unzipping fragment was prepared by PCR from plasmid 601[28] by using a biotin-labelled primer and then digested with BstXI and dephosphorylated by using CIP (NEB) to introduce a nick into the final DNA template. A 2.2 kb DNA construct was prepared by PCR from plasmid pRL574 by using primers with a biotin label and a dig label for force-extension measurements. HU was purified as described before.[7]

Single-molecule sample preparation: Sample preparation for the optical trapping experiments was essentially the same as that previously described.[16] HU was diluted to concentrations from 50–1600 nM in HEPES (20 mM, pH 7.9), KCl (60 mM) and dithiothreitol (1 mM).[7] The glass surface of the sample chamber was functionalized with antidigoxigenin, then passivated with Blotting Grade Blocker (Biorad). The digoxigenin end-labeled DNA construct was introduced into the sample chamber, followed by binding of streptavidin-coated polystyrene microspheres (0.48 μm) to the biotinylated extremity of the DNA construct. Finally, the HU solutions of different concentration were flowed into the sample chamber, and the chamber was incubated for 30 min before data acquisition. Unzipping experiments were carried out in HU solution.

Single-molecule data collection: Data were collected in a single-beam optical trap as described previously.[16] Experiments were conducted at constant loading rate ((8.2 ± 1.1) pN s^{-1}) that was achieved through computer-controlled feedback. Data were low-pass filtered to 5 kHz, digitized at ~15 kHz, and then further averaged to 100 Hz. The tether length used to calculate base pair position was Gaussian filtered with $\sigma = 0.01$. Experiments were conducted at a room temperature of 23 °C. The acquired data signals were converted into force and number of base pairs unzipped, as previously described.[16]

Data analysis: The unzipping landscape for each of the individual traces collected at 0 nM HU was aligned to a theoretical unzipping landscape for the used DNA sequence by using a SIMPLEX procedure; this improved positional accuracy and precision to a few base pair.[19] The energies used to create the theoretical unzipping curve that best fitted our experimental data were 1.02 and 2.9 kT for AT and GC pairs, respectively. Average AT content was calculat-

ed by using a 9 bp moving window. Traces collected in the presence of HU were aligned with the average of 37 aligned experimental reference traces. Dwell times for all positions *j* along the unzipped DNA fragment were determined by using a 2 bp window. The calculated dwell times were averaged over all obtained unzipping traces at each HU concentration. Relative dwell times were calculated by using the bare DNA unzipping trace as reference. Average data were obtained by averaging multiple unzipping traces/dwell time plots (0 nM, $N=57$; 100 nM, $N=76$; 200 nM, $N=43$; 400 nM, $N=87$, 800 nM, $N=130$; 1600 nM, $N=54$). The apparent persistence length for DNA and HU–DNA complexes was estimated by fitting to the WLC model.[29]

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