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Single-Molecule Atomic Force Spectroscopy Reveals that DnaD Forms Scaffolds and Enhances Duplex Melting

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Abstract

The *Bacillus subtilis* DnaD is an essential DNA-binding protein implicated in replication and DNA remodeling. Using single-molecule atomic force spectroscopy, we have studied the interaction of DnaD and its domains with DNA. Our data reveal that binding of DnaD to immobilized single molecules of duplex DNA causes a marked reduction in the 'end-to-end' distance of the DNA in a concentration-dependent manner, consistent with previously reported DnaD-induced looping by scaffold formation. Native DnaD enhances partial melting of the DNA strands. The C-terminal domain (Cd) of DnaD binds to DNA and enhances partial duplex melting but does not cause DNA looping. The Cd-mediated melting is not as efficient as that caused by native DnaD. The N-terminal domain (Nd) does not affect significantly the DNA. A mixture of Nd and Cd fails to recreate the DNA looping effect of native DnaD but produces exactly the same effects as Cd on its own, consistent with the previously reported failure of the separated domains to form DNA-interacting scaffolds.

Keywords

DNA pulling; atomic force microscopy; protein–DNA interactions; DnaD; replication

Introduction

The development of DNA micromanipulation techniques has allowed us to investigate subtle effects of proteins bound to single molecules of DNA (Refs. ^{1–7} and references therein). Pioneering early experiments examined looping by the *lac* repressor⁸ and the force generated by RNA polymerase during transcription.⁹ Force measurements with single-molecule force spectroscopy using the atomic force microscope (AFM) have also provided invaluable insights into biomolecular interactions (Refs. ^{10–15} and references therein). Here, using this technique we examined the DnaD-mediated DNA looping and its significance in duplex melting at the single-molecule level. These DNA-remodeling effects are reportedly based upon the formation of DNA-interacting scaffolds by an N-terminal domain (Nd) and mediated by a direct interaction with the DNA *via* a distinct C-terminal domain (Cd).^{16–19}

The *Bacillus subtilis* DnaD is an essential protein implicated in replication and DNA remodeling.^{16–24} It interacts with DnaA,²⁵ PriA helicase²⁶ and DnaB,^{26,27} all of which are implicated in primosomal functions. Its precise role is not clear at present, but localized DnaD-induced DNA remodeling and interactions with other primosomal proteins are likely to be important functions in setting the stage for recruitment of the replicative helicase DnaC. DnaD exhibits single-stranded (ss) and double-stranded (ds) DNA-binding activities,²⁶ and a unique remodeling activity that opens up supercoiled plasmids in a concentration-dependent manner forming a central scaffold surrounded by plasmid DNA.^{16,24} Nd is responsible for scaffold formation in the absence of DNA, while Cd binds to DNA and oligomerizes.¹⁸ Both domains have been crystallized and structural determination is underway.²⁸ Topoisomerase I-based assays revealed that full-length DnaD or Cd binding to DNA induces untwisting of the duplex, thus converting it from plectonemic to paranemic.¹⁹ The significance of this duplex untwisting by DnaD is unknown but one speculative suggestion is that it may facilitate melting of the replication origin and replication restart sites in low-G+C content Gram-positive bacteria.^{16,17}

We have applied single-molecule force spectroscopy using the AFM to investigate whether DnaD forms scaffolds and whether duplex melting is facilitated by DnaD-mediated DNA duplex untwisting. Individual dsDNA molecules were covalently immobilized on a gold surface and an AFM tip, respectively, *via* 5'-thiol and 5'-biotin tags at opposite ends.²⁹ Extension and relaxation cycles were carried out using an AFM tip coated with streptavidin, and force measurements were taken in the presence and absence of DnaD or its domains. Our data indicate an effective shortening of the end-to-end length of the DNA in the presence of increasing concentrations of DnaD, consistent with DNA looping formed by reported DnaD scaffolds.^{16,17} However, in the presence of increasing concentrations of Cd, and also DnaD after the loops are broken, the overall end-to-end distance of the DNA increased, consistent with enhanced dsDNA melting. A comparison of the effects observed in the presence of Cd or ssDNA binding protein (SSB) suggests that Cd does not act simply as a ssDNA binding protein but it also enhances DNA melting by untwisting the DNA duplex. No significant hysteresis between extension and relaxation cycles was detected in the presence of Nd, indicating no significant interaction with the DNA, consistent with previous studies.^{18,19} However, the high sensitivity of the single-molecule atomic force spectroscopy method allowed us to detect a very weak interaction with the DNA at high concentrations of Nd that is unlikely to be a major contributor of DNA remodeling. In the presence of the Nd and Cd the data were similar to those obtained with Cd alone, indicating that the separated domains cannot recreate the same DNA-remodeling effects as the native DnaD. Both native DnaD and Cd binding to DNA appeared to enhance melting of the double helix. The biological significance of DnaD-mediated duplex melting is discussed.

Results and Discussion

DnaD mediates bending of DNA, formation of scaffolds and duplex melting

DNA molecules (2532 bp) were immobilized onto a gold surface *via* the 5'-thiol tags of one strand, and single molecules were subsequently extended in the presence and absence of DnaD, using a streptavidin-coated AFM tip *via* the 5'-biotin tag located at the opposite end of the complementary strand (see Methods and Supplementary Fig. 1s). In control experiments with naked DNA, typical extension/relaxation curves were obtained (Supplementary Fig. 1sB). Force curves indicated the characteristic overstretching (also referred to as the B–S transition) force plateau^{10,13} as single DNA molecules were extended to their contour lengths, stretched to approximately halfway along the plateau and then relaxed. The extension and relaxation curves were identical and no hysteresis was observed (Supplementary Fig. 1sB, blue and red curves, respectively). Once such traces were obtained, the same DNA molecule was retained between the tip and the surface and

subsequently manipulated in the presence of increasing concentrations (0 to 8.5 μM) of DnaD (Fig. 1). The same experiments were performed on different DNA molecules and similar characteristic responses were always observed. Upon the addition of DnaD, clear hysteresis between the extension and relaxation curves consistently became apparent, suggesting an interaction with the DNA, with the profile of the relaxation trace indicating considerable ssDNA character (Fig. 1a and b, Ref. ²⁹). In the extension trace, a number of early multiple peaks in a 'sawtooth-like' pattern were also observed after which the final force plateau was shifted to the right, relative to the naked DNA traces, as the concentration of DnaD increased further (Fig. 1a and b).

We believe that the early force peaks are not caused by the detachment of other DNA molecules that might have become attached to the one being extended, because first, the density of the immobilized DNA molecules on the gold surface was kept low by co-immobilization with 1 mM mercaptohexanol (see Methods), and second, this pattern was specific to native DnaD and was not observed with the Cd, despite the fact that it also binds to DNA (see below and Ref. ¹⁸). It is also unlikely that these 'jumps in force' are due to the release of DNA-bound protein or to some other artifact due to a high protein concentration, because again the Cd does not produce these effects on its own at the same concentrations (see below). Instead, these early force peaks suggest an overall condensed end-to-end distance of the immobilized DNA molecule, in agreement with the previously reported DnaD-mediated scaffold formation and DNA looping (Fig. 1c and Refs. ^{16,17}). The fact that the magnitude of the peaks does not increase with extension suggests that a structure is being pulled apart sequentially. These peaks are consistent with the progressive incremental breakdown of the intramolecular forces within a DnaD scaffold. However, this technique does not provide specific information on the path of the DNA between the surface and the tip. Therefore, it is difficult to distinguish unequivocally between a single scaffold producing a single DNA loop or multiple mini-scaffolds (mini-aggregates) that bridge the DNA *via* multiple mini-loops. The fact that DnaD has been observed by AFM imaging to form single distinct scaffolds with individual circular and linear DNA molecules,¹⁷ however, suggests that the observed effects here are more likely to be due to the incremental breakdown of a single scaffold. It is also difficult to determine precisely the force required to break down a DnaD scaffold, as its magnitude will depend on both the speed of the measurement and the number of DnaD molecules involved in the scaffold. Although it is possible to control the speed of the measurement, the number of DnaD molecules involved in a scaffold is indeed variable.¹⁷ For the DNA molecule shown in Fig. 1b an average force of 40 pN (with a stretching speed of 1.92 $\mu\text{m/s}$) was, however, sufficient to cause localized disintegration of the scaffold.

The shift of the plateau to the right as the concentration increases from 5 to 8.5 μM may be explained by two possibilities. One possibility is that as the DnaD molecules bind to DNA, the duplex is gradually untwisted, thus causing an apparent increase in its end-to-end length before the scaffold is fully assembled (Fig. 1a and c). This is consistent with previous reports that DnaD untwists the DNA duplex, thus converting it from a plectonemic to a paranemic form.^{17,19} A second possibility is that as the DNA is extended and converted from a B to an overstretched form (sometimes referred to as S-DNA), some single-stranded regions of the DNA are exposed and DnaD may be acting simply as an ssDNA binding protein to interact with such regions and enhance its melting. However, from the appearance of the early force peaks much earlier than the onset of the B-S transition, it is reasonable to assume that at least at the beginning of a stretching phase, DnaD molecules will be bound to dsDNA rather than to ssDNA. SsDNA regions are unlikely to be present at this early stage of the stretching phase. Therefore one could argue that the first possibility described above is more likely to be the case. Once the scaffold has been assembled, the overall end-to-end length of the DNA is decreased dramatically, as explained above.

We propose that as the DNA is extended, initial DnaD-mediated untwisting facilitates partial melting of the duplex at forces that are normally not sufficient to completely melt dsDNA (compare the relaxation curves in Supplementary Fig. 1sB and Fig. 1a and b). Indeed, on relaxation the DNA exhibits ssDNA characteristics, as demonstrated by the observed hysteresis between the extension and relaxation trace. However, the fact that on subsequent extensions of the same molecule the double-stranded nature of the DNA as well as reassembly of the scaffold were observed implies that DnaD molecules must remain bound to the partially melted DNA structure, bridging the two strands and thus indirectly preventing fast reannealing into the plectonemic dsDNA form but allowing reannealing and reassembly of the scaffold on a slower time scale before the next stretching/relaxation cycle (Fig. 1c). Indeed, DnaD has been shown to bind ssDNA and dsDNA,^{16, 18, 26} which would be required for this to occur. Overall, these data suggest that DnaD binding to plectonemic dsDNA results in looping mediated by scaffold formation and enhances melting of the double helix by untwisting the duplex.

Cd enhances melting of the DNA duplex while Nd has no effect

The DNA-binding activity resides on the Cd and can be uncoupled from scaffold formation that is mediated exclusively by the Nd.¹⁸ Since Cd can also untwist the DNA duplex without forming a scaffold,¹⁹ we examined the effects of Cd on the extension/relaxation force curves in order to establish unequivocally that the effects observed are the result of duplex untwisting by DnaD. Extension/relaxation experiments were carried out with increasing concentrations of Cd, 0 to 8.5 μ M (Fig. 2). The data revealed two characteristic features. Firstly, as the concentration of Cd increased, this time, the onset of the force plateau shifted to the right, and secondly, the relaxation curve exhibited strong hysteresis (Fig. 2a and b). Also, compared to the force curves obtained with the full-length DnaD no early jumps in force were apparent. This is significant because it implies that despite the fact that Cd binds to DNA and has a DNA-induced oligomerisation activity,¹⁸ the Cd oligomers that form on the DNA have a different effect than the oligomers formed by the native DnaD. Therefore, the early jumps in force observed with native DnaD are not the result of protein release from the DNA or some other artifact. The simplest explanation is that because of the presence of the scaffold-forming Nd,¹⁸ the native DnaD can form a scaffold that loops the DNA, hence the appearance of the early jumps in force upon extension produced by the incremental breakdown of the scaffold. The Cd does not form a scaffold-mediated loop and therefore does not exhibit these early jumps in force upon extension.

The shift of the onset of the plateau to the right is consistent with an apparent increase in the end-to-end length of the DNA, while the observed hysteresis between the extension and relaxation traces is consistent with DNA melting. Although the Cd does not form scaffolds, it still binds to the immobilized DNA molecule and causes significant melting of the duplex, thus increasing its apparent end-to-end length during extension (Fig. 2c). The fact that the relaxation curve indicates significant ssDNA character suggests that Cd binding to DNA can also facilitate melting of the duplex during extension (Fig. 2c). However, these data cannot distinguish between melting resulting from duplex untwisting or simple binding to single-stranded regions that might have been exposed in the overstretched DNA (see below). Indeed, this is entirely consistent with the properties of Cd. It binds to both ssDNA and dsDNA and possesses a DNA-dependent oligomerisation activity that is separate from scaffold activity that resides on the Nd.¹⁸ In a manner similar to the native DnaD experiments, after relaxation and subsequent stretches of the same molecule the double-stranded nature of the DNA was re-established. Therefore, Cd molecules must bind to segments along the partially melted DNA molecule, thus preventing fast reannealing, but allowing slower reannealing between successive stretching and relaxation cycles (Fig. 2c).

By comparison, similar experiments with Nd revealed no significant hysteresis in the force curves, indicating no significant interaction with the DNA (Fig. 3a). This is consistent with the failure to detect any significant DNA-binding activity on the Nd by gel shifts, AFM imaging or topoisomerase I-based untwisting assays.^{18,19} However, as the concentration of Nd increased the force plateau shifted slightly below the plateau of the naked DNA ($F_1 > F_2$, F_3 in Fig. 3a), suggesting that a non-specific weak interaction of the Nd with the DNA at high concentrations might cause a slight destabilization of the duplex. The sensitivity of single-molecule atomic force spectroscopy has allowed us to detect this weak interaction despite the fact that previous gel shifts, AFM imaging and topoisomerase I-based assays had indicated previously that Nd does not bind significantly to DNA.^{18,19} To verify this, we examined binding of Cd and Nd to dsDNA in comparative band shift assays. Under our experimental conditions, Cd bound to a 261-bp dsDNA fragment at 10 μ M and above, but Nd did not even at 50 μ M (Fig. 3b). The subtle Nd-mediated effect observed by atomic force spectroscopy was masked in the native DnaD and the Cd+Nd experiments, presumably because of the dominant effects of the native DnaD and Cd proteins that bind the DNA much stronger than Nd.¹⁸ The biological significance of this effect is not clear at present but, given its weak nature, it is unlikely to be a major contributor towards the DnaD-mediated enhancement of duplex melting. Overall, these data reveal that Nd does not cause a significant effect on the DNA, but at high concentrations it may have a weak destabilizing effect.

In the presence of equimolar concentrations of Cd and Nd (0–8.5 μ M) the force curves appeared identical to the ones obtained with Cd alone (data not shown), indicating that the two domains are unable to recreate the same overall DNA-remodeling effects as the native DnaD. This is consistent with previous data showing that the two domains do not interact with each other, suggesting that the scaffold-forming activity of Nd and the DNA-binding activity of Cd are distinct and must be linked in the same polypeptide for complete DNA remodeling to take place.¹⁸

Comparison of native DnaD, Cd and Cd+Nd effects on DNA melting

Comparison of typical curves obtained at the same concentration (8.5 μ M) of native DnaD and its domains reveals subtle details of the DnaD-mediated DNA remodeling. With native DnaD the apparent end-to-end length of the dsDNA initially appeared shorter ($L_1 < L_2$), we propose due to scaffold formation, and then once the scaffold was disrupted, the end-to-end length of the DNA appeared longer due to DnaD-mediated untwisting of the duplex (Fig. 4). By comparison, dsDNA exhibited only apparent end-to-end lengthening effects ($L_3 > L_2$) in the presence of Cd and Cd+Nd due to duplex untwisting. Another subtle difference is the onset and the length of the plateaus in the presence of native DnaD or Cd and Cd+Nd. In the presence of native DnaD the onset of the plateau was shifted to the right ($L_4 > L_3$) and the length of the plateau was decreased compared to Cd and Cd+Nd (compare the plateaus in Figs. 1a, 2a and 4). This suggests that the end-to-end distance of the dsDNA molecule in the presence of native DnaD appears to be longer and the duplex nature of the DNA appears to be less compared to Cd or Cd+Nd. Therefore, native DnaD must facilitate melting of the dsDNA more efficiently than Cd or Cd+Nd. This is in agreement with previous data showing that native DnaD stimulates the topoisomerase I activity more effectively than Cd.¹⁹ The implication here is that when Nd is covalently linked to Cd in the native DnaD, it must play an important role not only in forming the scaffold but also, indirectly through scaffold formation, in facilitating melting of the DNA. Therefore, the connecting link between the two domains is not just a flexible connector but must act as a ‘molecular switch’ transmitting the effects of the scaffold formation by the Nd to the DNA-binding site that resides on the Cd.

The hysteresis observed with Cd is stronger than that observed with SSB

Although the existence of the overstretching force plateau in DNA force extension experiments is well established, the actual nature of the overstretched form of the DNA (or S-DNA) remains a subject of debate.^{30,31} Some models assume that the bases remain paired during overstretching, whereas others have proposed that the base pairs that hold the duplex together partially break as the DNA is stretched.^{32,33} Whatever the nature of the overstretched form of DNA, an important question to answer is whether single-stranded regions are exposed and available for a protein to bind. With our data above, we would not be able to distinguish unequivocally whether Cd interacts exclusively with the DNA to actively melt the duplex by untwisting or, alternatively, binds to single-stranded regions along the S-DNA, thus enhancing melting indirectly during stretching.

In order to investigate whether stretching of the DNA under our conditions results in exposed single-stranded regions, we carried out the same experiments in the presence of the ssDNA binding protein SSB. Since SSB binds to ssDNA, with 600-fold higher affinity than Cd and DnaD (compare gel shifts in Fig. 5a), but does not bind to dsDNA (Fig. 5), we argued that if single-stranded regions are exposed during stretching, then SSB should bind to them and prevent rapid reannealing during relaxation, which would be manifested as hysteresis that would be similar to that observed in the Cd experiments. Our data showed that in the presence of SSB hysteresis was indeed observed (Fig. 6), suggesting that in our DNA stretching experiments and under our experimental conditions there are stretch-induced single-stranded regions exposed and accessible to SSB along the S-DNA. However, a qualitative comparison between the relaxation curves in the Cd experiments (Fig. 2a) and the relaxation curves in the SSB experiments (Fig. 6), reveals that even at high 7.4 μ M SSB concentration the hysteresis observed is much less than that observed at a lower 5 μ M concentration of Cd, despite the fact that Cd binds to ssDNA with a 600-fold lower affinity than SSB. This suggests that the Cd-bound DNA appears to exhibit increased melting compared to SSB-bound DNA. Therefore, we conclude that although the overstretched DNA structure exhibits single-stranded regions that are accessible to an ssDNA binding protein such as SSB, binding of Cd induces more melting that would be attributed simply to binding at ssDNA regions along the S-DNA. We argue that this enhanced melting is facilitated by the reported Cd-mediated duplex un-twisting activity.¹⁹

In addition, as mentioned above, the appearance of early force peaks much earlier than the onset B–S transition in our DnaD experiments suggests that, at least at the initial stages of stretching, DnaD binds to dsDNA rather than to ssDNA regions, because such regions are unlikely to be present while the DNA is still in its B conformation at this early stage of the stretching/relaxation cycle.

What is the biological significance of DnaD-mediated DNA remodeling?

Mutations in the *Staphylococcus aureus dnaD* cause defects in DNA replication and repair.²³ Interactions of DnaD with DnaA²⁵ and PriA²⁶ also support a role in chromosomal and plasmid replication initiation.^{20,22} Despite the direct association of DnaD and replication initiation, its precise functional role remains unclear. The recent discovery of a DnaD-mediated remodeling activity^{16,17} has raised the important question—how to reconcile this activity with initiation of DNA replication. DnaD may participate directly with DnaA in localized DNA remodeling at *oriC* to enhance melting of the origin and facilitate loading of the helicase DnaC (Supplementary Fig. 2sA). At restart sites away from the *oriC*, DnaA will be absent and DnaD may facilitate local duplex melting either on its own or *via* an interaction with PriA. Histone-like DNA bending proteins such as IHF and FIS are emerging as integral members of initiator complexes (Ref. ³⁴ and references therein). In *Escherichia coli*, HU β suppresses the thermosensitive replication initiation phenotype of the *dna46ts*

mutant,³⁵ while in the absence of H-NS, overreplication is inhibited in the *dnaA*(Cs) replication initiation mutant.³⁶ DnaD may also participate in global remodeling of the nucleoid to present the *oriC* or other restart sites to a membrane-attachment site defined by membrane-bound DnaB (Refs. ^{17,24} and Supplementary Fig. 2sB). There is considerable evidence for coupling replication initiation and membrane attachment (Ref. ³⁷ and references therein). Finally, global DnaD-mediated remodeling of the bacterial nucleoid may affect topologically distinct domains, thus causing pleiotropic effects on transcription, repair and replication (Supplementary Fig. 2sC). Histone-like bacterial proteins have been shown to affect global transcription.³⁸ The reported abundance of DnaD in the cell, estimated at 3000–5000 molecules per cell,²⁷ implies that DnaD may be involved in other functions in addition to replication initiation. Indeed, it will be interesting to examine whether diverse pleiotropic functions are mediated by this fascinating protein.

Methods

Protein purifications

Native DnaD protein and its C-terminally His-tagged Nd and Cd were purified as described before.^{16,18} Briefly, native DnaD was purified by a three-column procedure using the SourceQ, Heparin and Superdex S75 chromatography columns (Amersham Biosciences) and the two domains were purified by a two-column procedure using an affinity Ni²⁺-chelating HiTrap column followed by gel filtration with a Superdex S75 column (Amersham Biosciences).

The *B. subtilis* SSB was purified using the same three-column purification procedure of Resource Q, Heparin and Superdex S75, as described elsewhere.³⁹

Preparation of the DNA substrate and immobilization on a surface

The dual-labeled (5'-thiol, 5'-biotin) p53 dsDNA substrate (2532 bp) was prepared by PCR and immobilized on a gold surface, as described before.²⁹ Immobilization of DNA molecules on the gold surface was carried out in the presence of 1 mM mercaptohexanol in order to reduce the density of the immobilized DNA molecules on the surface and to enhance the manipulation of single molecules. The primers with the thiol or biotin labels were purchased commercially (Eurogentec) and the DNA was purified exactly as described in Ref. ²⁹.

AFM tip modification

The AFM tip was coated with streptavidin using a procedure described in detail elsewhere.²⁹

AFM single-molecule force spectroscopy of DNA

Single-molecule spectroscopy and DNA pulling experiments were carried out using a 1D Molecular Force Probe (MFP-1D) from Asylum Research, as described before.²⁹ Briefly, DNA molecules were attached *via* their 5' ends between an AFM tip (*via* the biotin–streptavidin interaction) and gold substrate (*via* gold–thiol chemistry). Individual molecules were extended to approximately the halfway point of their overstretching force plateaus¹⁰ by careful control of the *z*-scan range and then relaxed to avoid force-induced DNA melting¹³ in the presence or absence of DnaD (0–8.5 μ M), Cd (0–8.5 μ M), Nd (0–8.5 μ M) or equimolar mixtures of Cd+Nd (total concentrations, 5 and 8.5 μ M). The Cd and Nd were premixed before being added to the immobilized DNA. All DNA pulling experiments were done at a stretching speed of 2.0 μ m/s unless otherwise stated. The naked DNA controls were also extended in this manner to ensure that no hysteresis was apparent on relaxation due to force-induced DNA melting.

Band shift assays

Band shift assays were carried out in 50 mM Tris, pH 7.5, 0.1 mM ethylenediaminetetraacetic acid, 200 mM NaCl, 4 mM MgCl₂, 1 mM DTT, 2.5 nM 5'-³²P-labeled 34mer oligonucleotide (5'-TTACACAGTAACTCATATGCTTCGCTATTACGCC-3') or a 261-bp dsDNA fragment in the presence of varying concentrations (0.05, 0.1, 1, 2 and 5 μM) of DnaD or SSB. Binding reactions were incubated at room temperature for 15 min and resolved through an 6% (w/v) non-denaturing polyacrylamide gel made in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Electrophoresis was carried out in 0.5×TBE. The 261-bp dsDNA fragment was prepared by PCR from the pCERoriD plasmid³⁹ using the primers 5'-TTCATCTCATCATCCATGGCGTATGTTGTGTGGA-3' and 5'-TTACACAGTAACTCATATGCTTCGCTATTACGCC-3'. Both the single-stranded oligonucleotide and the dsDNA fragment were radiolabelled using [γ -³²P]ATP and T4 polynucleotide kinase (NEB) and separated from unincorporated [γ -³²P]ATP using a Microspin™ S-200 HR spin column (Amersham Biosciences), according to the manufacturer's instructions.

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Abbreviations used

AFM	atomic force microscope
Nd	N-terminal domain
Cd	C-terminal domain
ssDNA	single-stranded DNA
dsDNA	double-stranded DNA
SSB	ssDNA binding protein

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Fig. 1.

The effects of native DnaD on the nanomechanical properties of DNA. (a) Representative extension/relaxation curves of DNA in the presence of increasing concentrations of DnaD (0 to 8.5 μM), as indicated. (b) Repeated manipulation of the same DNA molecule in the presence of 8.5 μM DnaD. The stretching curves (blue) show a sawtooth pattern; the relaxation curves are shown in red. The histogram shows the statistical analysis of the force distributions of the sawtooth-like early peaks. (c) A schematic model to explain the effects of DnaD on the double helix of the DNA. DnaD binds to DNA and untwists the duplex to a paranemic form (I to II). As the concentration increases it forms a scaffold (II to III). The changes in the end-to-end length relative to the naked duplex are indicated. As the DNA is pulled the scaffold is disrupted (IV), the duplex melts (V), while the protein remains bound to DNA as it is relaxed (VI). For clarity the force curves in (a) and (b) have been arrayed on top of each other along the y -axis. The apparent 'shift' of the bottom curves with naked DNA in (a) is due to the relative point where the pulling has started. The naked DNA has been pulled even further along the x -axis to ensure that no hysteresis due to pulling was apparent. Scale bars along the y - and x -axes indicate the absolute force (piconewtons) and length (nanometers), respectively.

Fig. 2.

The effects of Cd on the nanomechanical properties of DNA. (a) Representative extension (blue) and relaxation (red) curves of DNA in the presence of increasing concentrations of Cd (0 to 8.5 μM), as indicated. (b) Superposition of typical stretching (dark colours) and equivalent relaxation (light colours) curves of the same DNA in the presence of different concentrations of Cd (0, 5, 8.5 μM). The increase of the contour length ($L_2 > L_1 > L_0$) and the hysteresis between the stretching and equivalent relaxation curves in the presence of increasing concentrations of Cd indicate the untwisting effects of Cd on the DNA duplex. (c) A schematic model to explain the effects of Cd on the DNA double helix. Cd binds to DNA and untwists the duplex, converting it from plectonemic to paranemic (I to II). The change in the apparent end-to-end length relative to naked DNA is indicated. As the DNA is extended, melting of the duplex takes place (III) and the protein remains attached to the DNA as it is relaxed (IV). For clarity, the force curves in (a) and (b) have been arrayed on top of each other along the y -axis. Scale bars along the y - and x -axes indicate the absolute force (piconewtons) and length (nanometers), respectively.

Fig. 3.

(a) The effects of Nd on the nanomechanical properties of DNA. Representative extension (blue) and relaxation (red) curves obtained in the presence of increasing concentrations of Nd, as indicated. No clear hysteresis was apparent, indicating no significant interaction with the DNA. In the presence of high concentrations of Nd (7 and 8.5 μM) the force plateau shifts slightly below the plateau of the naked DNA ($F_1 > F_2, F_3$). This is attributed to a weak interaction of Nd with the DNA, as explained in the text. For clarity the force curves have been arrayed on top of each other along the y -axis. Scale bars along the y - and x -axes indicate the absolute force (piconewtons) and length (nanometers), respectively. (b) Band shift assays of Cd and Nd with dsDNA. A ^{32}P -radiolabelled 261-bp dsDNA fragment was used to compare binding of Cd and Nd to DNA. Binding reactions were carried out in the presence of increasing concentrations of Nd (lanes 1–7: 0.1, 0.5, 1.0, 10, 20, 30, 50 μM , respectively) and Cd (lanes 1–6: 0.1, 0.5, 1.0, 10, 20, 30 μM , respectively) and resolved by non-denaturing polyacrylamide gel electrophoresis, as indicated. Only Cd exhibited binding to dsDNA.

Fig. 4.

Comparison of the effects of native DnaD and its domains on the nanomechanical properties of DNA. All the force curves were normalized and superposed for direct comparison. *L1–L4* indicate the relative end-to-end length of dsDNA in the presence (*L1*, *L3*, *L4*) or absence (*L2*) of native DnaD and its separate domains. *L1* indicates the apparent end-to-end length of dsDNA due to scaffold formation in the presence of native DnaD, while *L4* indicates the end-to-end length due to duplex untwisting by native DnaD. *L3* indicates the relative end-to-end length of dsDNA in the presence of Cd (or Cd + Nd).

Fig. 5.

Band shift assays of DnaD, Cd and SSB. (a) Comparative gel shift assays with a radioactively labelled 34mer single-stranded oligonucleotide and increasing concentrations of SSB, DnaD (lanes 1–6 correspond to 0.05, 0.1, 0.5, 1, 2 and 5 μM , respectively) or Cd (lanes 1–6 correspond to 0.1, 0.5, 1.0, 10, 20 and 30 μM , respectively), as indicated. (b) Comparative gel shift assays with a radioactively labelled 261-bp double-stranded fragment of DNA and increasing concentrations of SSB and DnaD (lanes 1–7 correspond to 0.01, 0.05, 0.1, 0.5, 1, 2 and 5 μM , respectively), as indicated. Lanes labelled C show controls with the labelled DNA substrates in the absence of protein. SSB has a higher affinity for ssDNA than DnaD. The main lower shifted SSB band corresponds to a tetramer bound to DNA with a higher shifted band appearing at higher [SSB] corresponding to two SSB tetramers bound to the DNA. By contrast, DnaD binds to dsDNA whilst SSB does not. Cd has much lower affinity for ssDNA than DnaD.

Fig. 6.

The effects of SSB on the nanomechanical properties of DNA. Representative extension (red) and relaxation (blue) curves at 7.4 μM SSB. Hysteresis is apparent, suggesting that with our DNA molecule and under our experimental conditions single-strand regions along the S-DNA are exposed to SSB. However, a qualitative comparison with similar curves in the presence of Cd shown in Fig. 2a indicate that hysteresis with SSB is weaker than hysteresis with Cd. Scale bars along the y - and x -axes indicate the absolute force (piconewtons) and length (nanometers), respectively.