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Modulation of DNA Conformations Through the Formation of Alternative High-order HU–DNA Complexes

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HU is an abundant, highly conserved protein associated with the bacterial chromosome. It belongs to a small class of proteins that includes the eukaryotic proteins TBP, SRY, HMG-I and LEF-I, which bind to DNA non-specifically at the minor groove. HU plays important roles as an accessory architectural factor in a variety of bacterial cellular processes such as DNA compaction, replication, transposition, recombination and gene regulation. In an attempt to unravel the role this protein plays in shaping nucleoid structure, we have carried out fluorescence resonance energy transfer measurements of HU–DNA oligonucleotide complexes, both at the ensemble and single-pair levels. Our results provide direct experimental evidence for concerted DNA bending by HU, and the abrogation of this effect at HU to DNA ratios above about one HU dimer per 10–12 bp. These findings support a model in which a number of HU molecules form an ordered helical scaffold with DNA lying in the periphery. The abrogation of these nucleosome-like structures for high HU to DNA ratios suggests a unique role for HU in the dynamic modulation of bacterial nucleoid structure.

Keywords: histone-like proteins; HU–DNA interactions; FRET; DNA bending; single molecule

Introduction

HU is a ubiquitous prokaryotic protein that plays important pleiotropic roles in DNA replication,[1] gene regulation,[2,3] translation,[4] DNA repair[5,6] and other processes.[7] In addition to these functional roles, HU has been implicated together with other so-called histone-like proteins such as IHF, H-NS and FIS in shaping the architecture of the bacterial nucleoid.[8] Its activity in mediating and maintaining supercoiling is well documented,[9,10] as well as its ability to induce DNA bending,[11,12] causing considerable compaction of DNA molecules *in vitro*, (B. Schnurr *et al*, unpublished results).[13] HU–DNA complexes were observed by electron microscopy to form nucleosome-like bead structures.[10] Nuclease digestion experiments and dichroism studies[9] were consistent with these observations. In contrast, atomic-force microscopy (AFM) studies have indicated that, under certain conditions, HU may counteract DNA compaction.[14] Some biochemical investigations have reported cooperative binding of HU to DNA,[15–17] while others have reached the opposite conclusion.[11,18] Moreover, it is not known whether the activity of HU in promoting DNA bending and supercoiling is induced by rapid binding and dissociation of single dimers, which would increase DNA flexibility, or *via* the formation of higher-ordered structures resulting in concerted DNA bending.

HU is a small (19 kDa) dimeric, basic protein, present in the cell in about 30,000 copies. It binds DNA non-specifically and is distributed throughout the bacterial chromosome.[19] The structure of HU of *Bacillus stearothermophilus* has been solved,[20–22] revealing a homodimer made of a hydrophobic core supporting two β-ribbon arms. The structure of the integration host factor IHF is

† D.S. & N.F. contributed equally to this work.

Abbreviations used: FRET, fluorescence resonance energy transfer; sp-FRET, single-pair FRET; dsDNA, double-stranded DNA; TAMRA, tetramethylrhodamine.

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similar to that of HU, and both proteins bend DNA by the intercalation of the conserved arm proline residues through the minor groove. This leads to specific DNA kinks separated by 9 bp.[23,24] Gel mobility-shift[25] and nuclease digestion[9] assays indicate that HU covers a site of 8–9 bp. In contrast to IHF, which shows a high level of specificity for DNA sites containing its consensus sequence, HU binds to DNA in a non-specific manner, although specific HU sites have been identified in higher-order complexes in the *gal* repressosome[26] and the Mu transpososome.[7] Recently, the structure of HU–DNA cocrystals has been determined for 21 bp long DNA.[23] While the structure reveals large bending angles, the DNA used in the cocrystal had both unpaired and mismatched bases.[23] On the basis of the structure of the dimer, it was hypothesized that HU dimers stack side-by-side cooperatively, forming a helical complex with DNA.[21]

In an effort to resolve the conflicting views on the formation of high-order structures of HU–DNA complexes, we have analyzed HU-induced DNA bending by fluorescence resonance energy transfer (FRET) experiments using double-stranded oligonucleotides labeled at both ends complexed with HU. FRET measurements permit the determination of the degree of DNA bending that brings the two ends of DNA into close proximity as a result of protein binding. FRET measurements offer the following advantages: first, complexes are probed in solution, away from surfaces and free from caging effects as in gels. Secondly, FRET allows for the direct measurement of structural characteristics of complexes in the 20–100 Å range. Third, measurements can be performed at the level of single complexes, providing a way to investigate the structural inhomogeneity of HU–DNA complexes in solution. In the present context, DNA bending was measured both by probing large ensembles of complexes simultaneously (ensemble-FRET), and at the level of single pairs (sp-FRET).[27] Surprisingly, our measurements show that the transfer efficiency of HU–DNA complexes increases cooperatively, peaks and then decreases as the HU to DNA ratio increases. This behavior contrasts with the non-cooperative and monotonic behavior shown by IHF–DNA complexes. Our observations suggest that HU molecules cooperatively form a scaffold[20] on which the DNA is wrapped, analogous to the eukaryotic nucleosome. Furthermore, the HU–DNA higher-order structures are modulated strongly by the formation of alternative HU–DNA complexes at high ratios of HU to DNA.

Results

DNA bending determination by ensemble-FRET measurements

Ensemble-FRET measurements of complexes formed by HU with 55 bp long double-stranded DNA (dsDNA) oligonucleotides having three different sequences are shown in Figure 1A. In all three cases, the 5′ end of one DNA strand was labeled with the donor fluorophore tetramethylrhodamine (TAMRA) and the complementary strand was 5′ end-labeled with a Cy5 acceptor. In solution, the distance between the donor and acceptor in the dsDNA is about 190 Å, a distance at which FRET is not observed. Note that the persistence length of DNA under the conditions of our experiments is ca 500 Å (150 bp), and hence the bare oligonucleotides can be thought of as nearly-straight rods.[28] The *55ibs* DNA contains a high-affinity IHF-binding site,[29] allowing us to compare DNA bending by each of the two proteins. The behavior of the HU–*55ibs* DNA complexes shown in Figure 1A is characterized by a number of salient features. First, the FRET transfer efficiency (*TE*) increases and peaks at $TE = 0.18$, which corresponds to an average donor–acceptor distance $R = 71(\pm 5)$ Å. Second, the increase in *TE* as a function of the concentration of HU is non-linear. We argue in the discussion below that these findings are suggestive of an ordered arrangement of the bound HU molecules, and cannot be explained as a result of binding by independent HU dimers, each one bending DNA in a random direction by a small angle. Thirdly, at concentrations above 400 nM, the *TE* displays a decrease with increasing concentrations of HU, reaching background levels (corresponding to donor–acceptor distances above 100 Å), indicating that excess HU can interfere with the formation of the ordered HU–DNA complexes. The non-linear increase in *TE* at low concentrations of HU implies that a number of HU molecules are needed to bend the DNA significantly. In contrast, a protein such as IHF, which binds with high affinity to a specific sequence (see below), induces a linear increase in *TE* as function of the concentration of protein.

To test for cooperative interactions between HU molecules, the following competition assay was carried out. First, a sample of HU–DNA complexes at a ratio of one HU dimer per 10–12 bp (90 nM DNA and 400 nM HU) was prepared, corresponding to the highest value of *TE* observed above. We then assayed for competition by the addition of identical non-labeled DNA. After each addition of non-labeled competitor, the value of *TE* was measured (Figure 1B). Indeed, the value of *TE* decreases as the added non-labeled competitor sequesters HU from labeled oligomers. However, the most important feature of this plot is that even below a 1 : 1 molar ratio of HU to the DNA oligonucleotide, the values of *TE* are significantly above background. In the absence of cooperativity, HU dimers would distribute themselves as homogeneously as possible among all DNA molecules. This would result in a large majority of DNA molecules having either one, or no HU molecules bound, and consequently no measurable *TE*, in contrast with the observed behavior. On the other

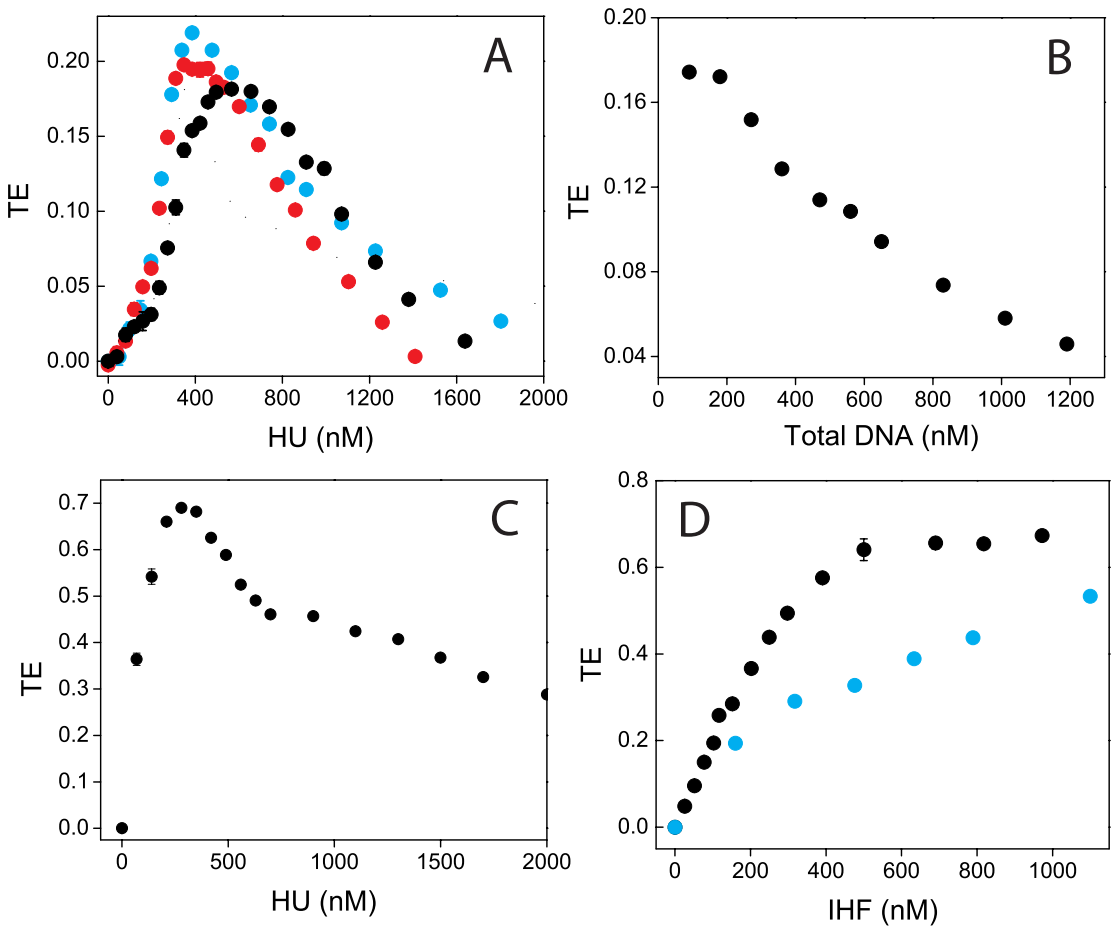

Figure 1. Ensemble-FRET measurements of the transfer efficiency (*TE*) of protein–DNA complexes. A, *TE* as a function of HU concentration for HU–*55ibs* DNA complexes (black), HU–*55rnd* DNA complexes (blue) and HU–*55mu* DNA complexes (red). B, *TE* as a function of the total concentration of DNA when adding unlabeled *55ibs* DNA competitor in steps to complexes formed originally with 400 nM HU and 90 nM labeled *55ibs* DNA. The concentration of HU is kept constant during the process. C, *TE* as a function of the concentration of HU for HU–DNA complexes formed from DNA 55-mers having two TT mismatches 9 bp apart. D, same as A, for IHF–*55ibs* DNA (black circles) and IHF–*55rnd* DNA (blue circles) complexes.

hand, cooperative interactions between HU molecules would result in an increased binding affinity of the complex to DNA, thus favoring complexes with several HU molecules. Therefore, cooperativity in binding to DNA implies that even at low ratios of HU to DNA, significant *TE* could be measured. To sum up, the ability of the HU complexes to maintain a significant *TE* in a competition assay provides strong evidence for cooperativity in binding of HU to the DNA. Taken together, these results suggest that DNA bending by HU is accomplished by the concerted cooperative interactions of a number of HU molecules. Excess HU somehow interferes with this process, possibly by destabilizing the concerted binding of HU to the DNA. Experiments with other DNA concentrations ranging from 9 nM to 450 nM yielded the same behavior: the transfer efficiency increases nonlinearly, peaks, and decays at high concentrations of HU (data not shown). Non-monotonic behavior has been reported in HU-induced supercoiling,[9] *in vitro* measurements of replication activity from OriC,[1] and in recent measurements of the elastic behavior of HU–DNA complexes conducted in our laboratory and by others.[30]

The behavior of HU–DNA complexes described above is sequence-independent. We studied the formation of complexes between HU and two other DNA sequences. One, *55rnd*, in which the sequence of the dsDNA *55ibs* was scrambled (see Materials and Methods) and the other, *55Mu* carries the HU high-affinity binding site of the phage Mu, located within the Mu transpososome.[7] We found that all three probes yield similar results: an increase in *TE* upon addition of HU, followed by a decrease in *TE* at high concentrations of HU (Figure 1A). The *TE* peak positions occur at nearly the same ratios of HU to DNA for the three DNA sequences. Thus the observed non-monotonic behavior is a property of HU–DNA complexes independent of specific DNA sequences.

Our results suggest that the *55Mu* site does not bind HU with high affinity when out of the

transposome context. To obtain a high-affinity site for HU we generated a dsDNA oligonucleotide carrying at its center two TT : TT mismatch bubbles 9 bp apart, which are expected to form a high-affinity binding site for HU.[6] FRET measurements of complexes of HU with this DNA (Figure 1C) show that *TE* can attain a value of ~0.7 (corresponding to an end-to-end distance of ~48 Å), which is considerably larger than the maximal value attained by complexes formed without mismatches. The large bending angle we calculate from these measurements, namely 148°, agrees favorably with the large angle of 105–140° observed in the HU–DNA co-crystal, in which a non-perfect dsDNA was used.[23] A high concentration of HU leads to a limited decrease in *TE*, probably due to non-specific binding.

To ensure that the non-monotonic behavior of HU–DNA complexes presented above is not due to any artifact related to our FRET measurements, we have carried out a number of control experiments. First, no change was observed in the emission spectra of the fluorophores in the presence of HU alone (not shown). Second, to eliminate the possibility that the FRET signal is due to the formation of HU–DNA aggregates rather than DNA bending, we measured the FRET efficiency of a mixture of dsDNA molecules, each labeled with a single fluorophore, some labeled only with the donor (TAMRA) and some only with the Cy5 acceptor. The maximal *TE* values inferred from these measurements were lower than ~0.05. These values are much lower than those inferred from the intramolecular FRET measurements and rule out the possibility that our *TE* measurements are due to aggregates of DNA molecules formed in the presence of HU. To demonstrate that both HU and the end-labeled DNA probe were intact after incubation at high concentrations of HU, we reactivated the HU-induced FRET signal by two independent procedures. Addition of non-labeled dsDNA thereby reducing the HU : DNA ratio; alternatively, dilution of the concentration of complexes formed at 1000 nM HU led to the complete recovery of the FRET signal (not shown).

The behavior of IHF–DNA complexes is rather

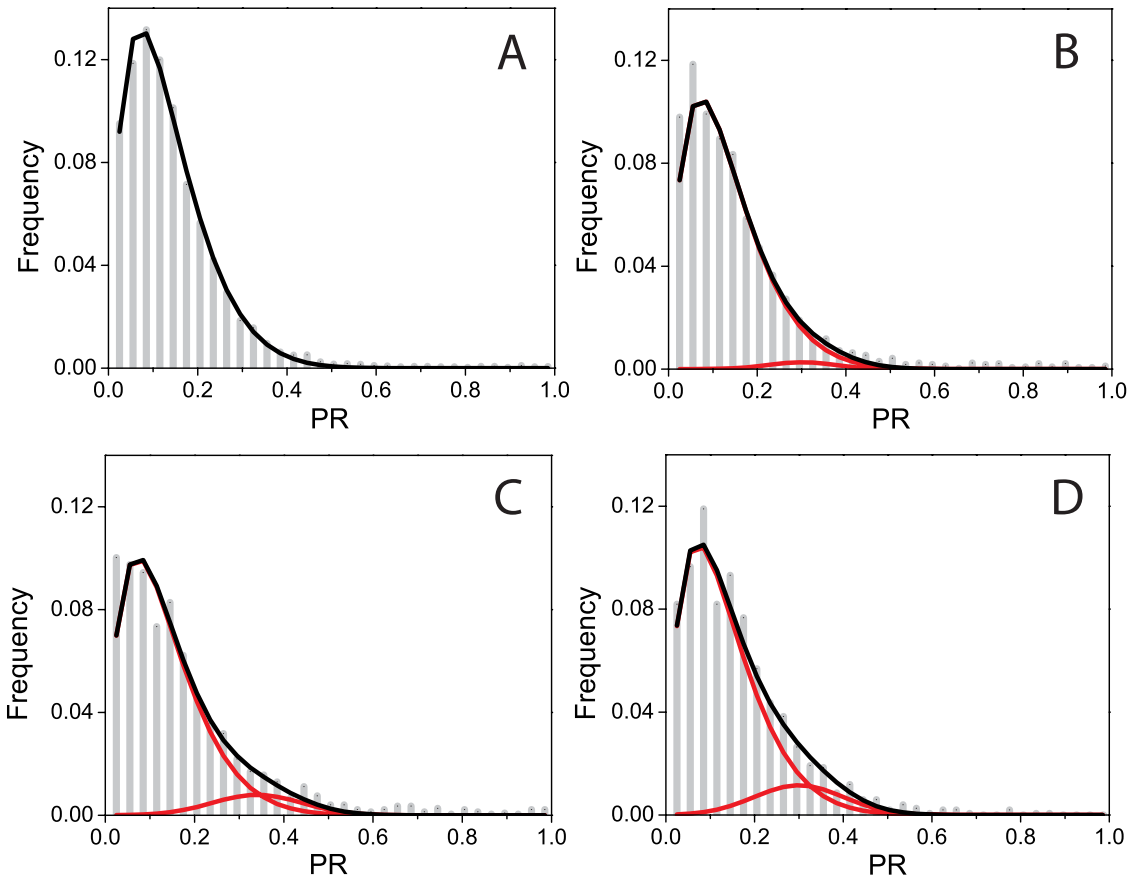

Figure 2. Histograms of proximity ratio (*PR*) of individual HU–*55ibs* DNA complexes for four different concentrations of HU: (A) 0; (B) 180 nM; (C) 310 nM; and (D) 520 nM. The corresponding HU to DNA ratio is 2.3 for B, 3.9 for C and 6.7 for D, respectively. All the histograms have been normalized by the total number of counts. Histograms have been fitted with a sum (black line) of a beta distribution[41] and a gaussian (red lines), the first of which is constrained so that its center and width coincide with a beta distribution fit to the histogram obtained at zero HU. This peak corresponds to complexes with either a large end-to-end distance, or DNA molecules in which the acceptor is either bleached or missing. We stress that this fitting procedure does not imply that there are only two subpopulations of complexes.

different. Measurements of DNA bending by IHF from *Escherichia coli* on the same DNA molecules (Figure 1D) show that *TE* increases linearly upon addition of IHF and reaches saturation, attaining a value of *TE* of ~0.6, which corresponds to $R = 50(\pm 5)$ Å. This distance is in agreement with that obtained previously on the same sequence but with a different donor–acceptor pair.[29] The results follow Michaelis–Menten behavior with an associated Hill coefficient of 1.1 ± 0.04. These findings are consistent with bending as a result of the binding of a single IHF molecule to the oligonucleotide.[29] We have measured the FRET induced by IHF on a DNA sequence without an IHF consensus site (*55rnd*, Figure 1D). As expected, IHF bends this sequence to a smaller extent ($R = 55(\pm 5)$ Å), and binds with a lower affinity than it does to the *55ibs* DNA. It has been shown that IHF compacts DNA by bending also at non-specific sites.[31] Furthermore, the ability of IHF to bend the *55rnd* DNA could be due, in part, to the presence of pseudo binding sites in the randomly generated sequence. We note that binding of IHF to *55Mu* DNA, whose sequence is unrelated to either *55ibs* or *55rnd*, resulted in no measurable *TE* signal.

DNA bending analysis by sp-FRET

Given that the HU–DNA interaction is non-specific and 55-mers can accommodate up to six HU dimers,[9,25] HU–DNA complexes may be highly heterogeneous structurally, and therefore individual complexes may differ considerably in their transfer efficiencies. The ensemble-FRET measurements described above, in which large numbers of complexes are interrogated simultaneously, yield only an average and do not provide information about individual HU–DNA complexes. In order to determine the end-to-end distance and test for structural heterogeneity, we have carried out experiments in which the proximity ratio *PR* of single complexes has been measured (for the relation between *PR* and *TE*, see Materials and Methods).[27] In these experiments, complexes in solution are monitored one-by-one, and *PR* is estimated for every individual complex. The distribution of *PR* values measured for single HU–DNA complexes is presented in Figure 2A–D, for four different concentrations of HU. As the concentration of HU increases, the proportion of complexes exhibiting large transfer efficiencies centered around $PR \sim 0.35$ grows gradually (see Table 1). This value of *PR* corresponds to a donor–acceptor distance of $55(\pm 5)$ Å (see Materials and Methods). We note that these relatively high values of *PR* were not observed in the ensemble-FRET experiments. This implies that there are complexes that are bent more strongly than the ensemble-FRET measurements may indicate.

Table 1. Area of the gaussian peak A_2, centered at $TE \sim 0.34$ divided by the sum of the areas of the beta distribution peak, A_1, and A_2, corresponding to the data shown in Figure 2

[HU] (nM)	0	180	310	520
$A_2/(A_1 + A_2)$	0	0.024	0.095	0.127

In contrast to the experiments described above, the high-affinity interactions of HU with DNA having two TT : TT mismatches 9 bp apart suggest smaller structural heterogeneities. As shown in Figure 3, the addition of a low concentration of HU (30 nM) leads to a bimodal population of complexes, most of which exhibit a strong *PR* centered at ~0.5, in contrast to the behavior of HU–DNA complexes formed with DNA without mismatches (Figure 2). The inferred donor–acceptor distance of $R \sim 48$ Å is in a very good agreement with that obtained from the ensemble-FRET measurements, indicating a high degree of homogeneity in the complex. These features are consistent with a high-affinity binding of HU to the site defined by the mismatches, leading to strong bending. As the concentration of HU is increased, the effects of the low-affinity, non-specific binding interfere, and the *PR* of highly bent complexes becomes gradually smaller (Figure 3C and D), in agreement with the behavior observed by ensemble-FRET.

In similar measurements performed on DNA–IHF complexes, a bent subpopulation with *PR* values centered at 0.47 was observed (Figure 3F), which corresponds to $R = 50(\pm 5)$ Å. This value agrees well with the corresponding value of *R* observed in the ensemble-FRET experiments. The fraction of this subpopulation increased and reached saturation with increasing concentration of IHF (data not shown). As expected and in contrast with the results obtained with HU, most (~85%) of the DNA molecules were found to generate a strong FRET signal, suggesting uniform bending of the DNA by IHF. The observation of small values of *PR* in the case of HU complexed with intact DNA, relative to those observed with either IHF or with HU complexed with DNA including mismatches, indicates that the bending angle per HU dimer is small. Otherwise, a subpopulation with high *PR* values would have been observed. Note that even when accommodating several HU molecules on intact DNA (as in Figure 2C and D), no subpopulation of complexes exhibits values of *PR* as high as those obtained in experiments with DNA including mismatches.

For comparison and calibration purposes (see Materials and Methods) the *PR* distribution corresponding to a 12 bp oligomer was analyzed as shown in Figure 3F. Here, the second gaussian peak is centered at $PR \sim 0.7$, corresponding to $R \sim 41$ Å in agreement with the actual length of the dsDNA oligonucleotide and with ensemble measurements (data not shown). We note that the width of the distributions observed in our experiments are in reasonable agreement with previous sp-FRET measurements of labeled short DNA

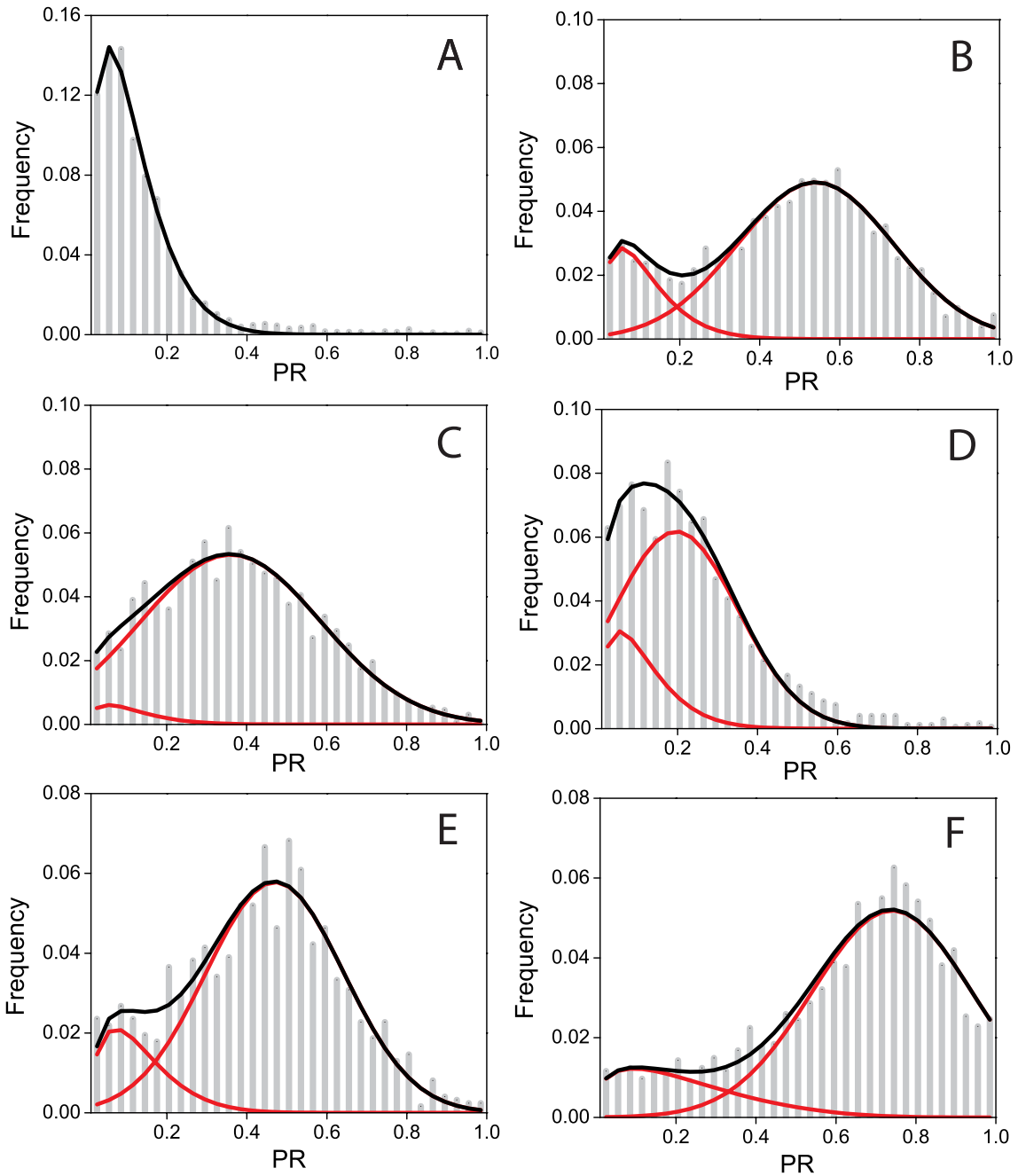

Figure 3. Histograms of proximity ratio (*PR*) of individual HU–DNA complexes formed with a 55-mer having two TT mismatches 9 bp apart. The normalized histograms correspond to four different concentrations of HU: (A) 0; (B) 30 nM; (C) 300 nM; and (D) 1000 nM. Histograms were fit as in Figure 2. Also shown are histograms of *PR* of individual IHF–*55ibs* DNA complexes at a concentration of IHF of 640 nM (E), and of 12 bp long DNA oligonucleotides (F).

oligomers, and with calculations which model the effects of shot-noise fluctuations.[27]

Discussion

Our results using both ensemble FRET and sp-FRET provide direct experimental evidence for concerted DNA bending by a number of HU molecules. The non-monotonic behavior of *TE* and the ability to observe significant efficiency despite the small bending angle of a single HU dimer, show that HU does not act solely by introducing bends in a random orientation. Indeed, the random-flight model[32] shows that for randomly distributed multiple DNA bends the average end-to-end distance R, can be estimated from:

$$\langle R^2 \rangle = N b_0^2 \frac{1+\cos\theta}{1-\cos\theta}$$

where N is 1 plus the number of bound HU dimers, each dimer introducing a single bend with an angle θ, and $b_0 = L/N$ is the average distance

between adjacent dimers (L being the total length of the DNA molecule). This expression yields an end-to-end distance that scales as $1/\sqrt{N}$ and hence decreases monotonously with HU concentration, as opposed to the non-monotonous behavior observed in Figure 1A. Furthermore, for five HU dimers ($N = 6$) and $\theta = 60°$,[33,34] the end-to-end distance one obtains is $R \sim 130$ Å. This distance corresponds to *TE* values well below our detection level.

These findings support basic features of a structural model of the HU–DNA complex proposed by Tanaka *et al*.[20] According to the model, HU–DNA complexes form a nucleosome-like structure, with DNA lying in the periphery around a scaffold formed by HU dimers. Note that in this geometry, with the HU dimers in near-register with the DNA helical pitch, the bending angles of the dimers add on, resulting in a large overall bend. The distance between cores of adjacent dimers is minimal, and hence favors protein–protein contacts. These contacts may be enhanced by changes in DNA pitch due to HU binding,[9] and by a reduction in the twisting modulus of DNA, as was observed in the elasticity of single supercoiled DNA substrates (B. Schnurr *et al*., unpublished results). Our measurements allow us to make a rough estimate of the bending angle per HU dimer. For a planar structure, the end-to-end distance d of an arc of radius r and contour length L is given by:

$$d = 2r \sin\left(\frac{L}{2r}\right)$$

If α denotes the bending angle per dimer, the following relation applies: $n\alpha r = L$, where n is the number of bound dimers. Our sp-FRET measurements yield for the donor–acceptor distance $d = 55(\pm 5)$ Å for the 55-mers. Assuming that these can accommodate five HU dimers, or one HU molecule per helical repeat of the DNA, we obtain $\alpha \sim 53°$. This value is consistent with previous estimates.[33,34] Preliminary measurements with DNA oligomers of different lengths give values consistent with this estimate. Note that the value of α is weakly dependent on the precise value of *TE*, given the strong dependence of this quantity on the donor–acceptor distance. For *TE* values between 0.2 and 0.5, α does not vary by more than 10%. Furthermore, α does not change by more than 15° if we assume an HU–DNA complex with four dimers instead of five. We point out that molecular computations based on the known structure of HU[21] indicate that, for a planar structure, there is steric hindrance between bound dimers in the assembly of the complex. Hence, the complex is most probably a superhelical generalization of the structure illustrated by Tanaka. The value of α does not change much if one assumes a complex of helical structure with a small enough pitch to give a measurable FRET signal.

We suspect that high concentrations of HU interfere with the ordered arrangement of HU along the DNA. This could result from the binding of HU molecules out of phase with the DNA helical pitch, preventing the formation of the concerted structure or by destabilization of the protein scaffold by protein–protein interactions. The increase in donor–acceptor distance observed above a threshold HU concentration is consistent with recent single-molecule elasticity studies of individual HU–DNA complexes, which indicate that above a threshold, HU-induced compaction gives way to rigidification, resulting in a complex with higher bending stiffness than bare DNA. Evidence for HU polymerization in a superhelical arrangement has been provided by atomic force microscopy observations.[30]

The high affinity and high degree of bending observed in complexes formed with DNA including mismatches is consistent with the large bending angles measured in the co-crystal.[23] It is possible that the high-affinity HU-binding sites, such as were observed in the *gal* repressosome[26] or the Mu transpososome,[7] are generated by similar DNA distortions. Thus, it is possible that the bacterial chromosome is decorated with HU molecules bound to specific loci. Our studies suggest that HU can modulate local DNA structure strongly by forming unique nucleosome-like structures. We propose that a portion of the HU molecules are participating in the formation of these "compactosomes",[35] providing for ordered compaction of the nucleoid. Compactosomes may play a role in the lumpy structure of the nucleoid at small scales, as revealed in recent atomic force microscopy studies.[36] Changes in superhelicity are known to occur during bacterial growth and under stress conditions. These changes may affect the occupancy of high-affinity HU-binding sites and modulate local DNA superhelicity. Thus we envisage that HU–DNA complexes play an important role in determining nucleoid structure and activity.

Materials and Methods

Protein purification

HU from *B. stearothermophilus* was purified after overproduction of the corresponding gene in *E. coli*.[37] The protein was of high purity, as analyzed by SDS-PAGE. IHF was prepared as described.[38,39]

Labeled DNA oligonucleotides

Single-stranded DNA oligonucleotides labeled at their 5′ ends with either tetramethylrhodamine (TAMRA) or a Cy5 fluorescent dye (Thermo Bioscience GmbH, Ulm, Germany), were HPLC and PAGE-purified. The TAMRA and Cy5-labeled complementary strands were mixed in a 1 : 1 ratio in 10 mM Hepes–KOH (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and were hybridized by cooling slowly from 90 °C to 20 °C over one hour. DNA sequences used in the experiment were: *55ibs*, a 55 bp long sequence, with the H′ IHF binding site near its

center (IHF consensus sequence underlined) 5′-TAGAC CTGTTCGTTGC*AACAAATTGAT*AAGCAATGCTTTTT TATAATGCAGTGAA-3′; *55rnd*, a 55 bp long sequence, which contains the same base composition as the *55ibs*, but in a random order 5′-TAGACCTGTTCGAAGTTTC GTATTGATTTGCTTATACAACAATGCATGCAGTGAA-3′; *55mu*, a 55 bp long sequence, which contains in its center the spacer region that separates the L1 and L2 transposase binding sites in Mu phage[7] 5′-TAGACCTGG GATCTGATGGGATTAGATCTGGTGGGGCTTGCAAG CCTGCAGTGAA-3′. At least five bases at each end were kept identical in all sequences, to reduce sequence-dependent changes in the physical properties of the fluorophores.

FRET measurements

Optical setup

Both ensemble and sp-FRET measurements were performed using confocal fluorescence detection. The illumination is provided by the 514 nm line of an argon ion laser. The laser light is collimated, and reflected with a dichroic mirror (Omega Optical, DRLP540) to fill the back aperture of an objective (Zeiss 100×, NA 1.4, oil immersion). The sample is placed inside a glass chamber, with a cover-glass window facing the objective. To detect freely diffusing molecules and reduce background fluorescence, the focal point is placed about 10 μm from the glass surface. Fluorescence is collected with the same objective, and focused on a 100 μm pinhole, after residual laser light is filtered with a 514 nm optical notch-filter (HNPF-514.5-1.0, Kaiser Optical Systems). Following the pinhole, the light is divided with a second dichroic mirror (Omega Optical, DRLP630), and focused onto two avalanche photodiodes (Perkin Elmer, SPCM AQR-14). Optical band-pass filters were used to increase signal-to-background ratio: (Omega Optical, 580DF30) in the donor channel, and (Omega Optical, 670DF40) in the acceptor channel. Detection is performed with a counter based on a PC counting board (National Instruments, DAQ 6602).

Ensemble-FRET

All FRET measurements were made in 10 mM Hepes–KOH (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$. For the ensemble measurements, a concentration of 90 nM DNA is used. To avoid photobleaching, the sample was illuminated with 14 μW laser power. Competition assays (Figure 1B) were performed by preparing first complexes with 400 nM HU and 90 nM *55ibs* labeled DNA oligonucleotides, yielding the highest value of *TE* (~0.17). Identical non-labeled DNA competitor was then added from a concentrated stock solution (at 45 μM), ensuring that the concentration of HU in the experiments remained essentially constant. The *TE* was measured after each addition of competitor.

Single-pair FRET

The sp-FRET experiments were performed with a laser power of 450 μW. A labeled DNA concentration of 90 pM was used, such that the single-molecule occupancy probability inside the detection volume is about 0.03. Under these conditions, the probability of detecting two molecules simultaneously is then $\sim 1 \times 10^{-3}$. Since the FRET results depend strongly on the protein to DNA molar ratio, 90 nM identical non-labeled DNA was added to the labeled DNA, such that the sp-FRET experiments could be compared directly with the ensemble results. An oxygen scavenger (1% (v/v) 2-mercaptoethanol, 25 mM glucose, 20 μg/ml of catalase, 100 μg/ml of glucose oxidase) was added in the sp-FRET measurements, to reduce photobleaching of the Cy5 acceptor. Counts from the detectors were binned in 1 ms intervals, which results in a typical background signal of five counts, whereas the typical number of bursts per DNA molecule is above 20.

FRET analysis

FRET efficiency

The emission intensity of the donor decreases in the presence of FRET and the intensity of the acceptor emission increases correspondingly. Therefore, we determined the transfer efficiency, *TE*, from the decay of the donor intensity in the presence of HU (or IHF), according to:

$$TE = \frac{(I_{D0} - I_D)}{I_{D0}}$$

where I_{D0} is the intensity of the donor when no protein is present, and I_D is the intensity of the donor in the presence of protein. Each protein concentration has its own I_D. We determined both values, I_{D0} and I_D, within one experimental set up by adding protein to the DNA solution and thus avoiding pipetting errors. Due to the large donor–acceptor distance, no energy transfer takes place in the labeled protein-free DNA, and we verified that the donor emission of the free DNA is equal to that of a donor-only sample. In addition, the transfer efficiency was determined by using both detectors. In that case, the actual measured number of counts from each detector unavoidably contains additional contributions due to spectral overlap between the two channels, direct excitation of the acceptor by the laser light, dependence of the intensity on the focus position and background counts. Thus, we subtracted the average background (measured with a similar sample, only with non-labeled DNA), and the average overlap of donor emission into the acceptor channel. Direct excitation of the acceptor was inferred from the free DNA acceptor emission (I_{A0}). The *TE* is then equal to $TE = (I_A - I_D I_{A0}/I_{D0})/(I_A + \gamma I_D)$, where $I_D(I_A)$ is the intensity of the donor (acceptor), and $I_{D0}(I_{A0})$ is the intensity of the donor (acceptor) of the free DNA. Note that $I_A(I_{A0})$ is the intensity after subtracting the spectral overlap between donor and acceptor. $\gamma = \eta_A \phi_A / \eta_D \phi_D = 0.25$ is a correction factor, which accounts for the quantum yields ($\phi_{A,D}$) and detection efficiencies ($\eta_{A,D}$) of the two dyes.

Ensemble-FRET measurements on the HU–*55ibs* DNA complex were repeated in a fluorometer (SLM-AMINCO 8100), where we used the reduction in donor emission to calculate FRET efficiency:[40]

$$TE = \frac{(I_{D0} - I_D)}{I_{D0}}$$

Here I_D is the measured emission from the donor (integrated in the range 565–595 nm) in the presence of acceptor, and I_{D0} is the donor emission when no energy transfer occurs. This quantity was measured on the same sample, before the addition of protein. The results obtained with the fluorometer were consistent with those of our confocal setup.

In the sp-FRET measurements, only events in which $I_D + 4I_A > 70$ counts were accounted for, where the

asymmetric criterion used reflects the factor γ discussed above. For every event that is above threshold, a proximity ratio, *PR*, was calculated as $PR = I_A/(I_A + I_D)$ where I_D and I_A are the number of counts of the donor and acceptor, respectively, after subtracting the average background and the spectral overlap between the channels. The large spectral separation between the fluorophores (~100 nm) results in a small direct excitation, so bursts on the acceptor channel result mainly from Cy5 emission caused by energy transfer. However, the sensitivity to noise of single-molecule measurements has prevented us from subtracting the residual direct excitation from the acceptor channel as well as using the factor γ when calculating the efficiency. As a result, the factor γ is used only for determining the threshold, and *PR* is an approximation to the *TE* described above.[27] Consequently, there is slight difference between ensemble FRET to sp-FRET measurements. In order to calculate the end-to-end distances in sp-FRET measurements we used the 1 : 1 correspondence between *TE* and *PR*.

Distance calibration

The FRET transfer efficiency *TE* is related to the distance between donor and acceptor, *R*, as:

$$TE = 1/(1 + (R/R_0)^6)$$

R_0 (the Förster distance) is a constant that accounts for the physical parameters that determine the transfer efficiency between the two dyes (spectral overlap, relative orientation, quantum yield of the donor and extinction coefficient of the acceptor). We measured the FRET efficiency obtained for six DNA "rulers" with lengths in the range 8–31 bp, and used the helical model for the inter-dye distance,[40] to fit the data. On the basis of this measurement, a value of $R_0 = 55$ Å was obtained, in good agreement with published data. When used with our data to evaluate the end-to-end distance of IHF–*55ibs*–DNA complex (~50 Å), very good agreement with previous measurements was obtained.[29]

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References

1. Dixon, N. E. & Kornberg, A. (1984). Protein–HU in the enzymatic replication of the chromosomal origin of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **81**, 424–428.
2. Flashner, Y. & Gralla, J. D. (1988). DNA dynamic flexibility and protein recognition-differential stimulation by bacterial histone-like protein Hu. *Cell*, **54**, 713–721.
3. Lewis, D. E. A., Geanacopoulos, M. & Adhya, S. (1999). Role of HU and DNA supercoiling in transcription repression: specialized nucleoprotein repression complex at gal promoters in *Escherichia coli*. *Mol. Microbiol.* **31**, 451–461.
4. Balandina, A., Claret, L., Hengge-Aronis, R. & Rouviere-Yaniv, J. (2001). The *Escherichia coli* histone-like protein HU regulates rpoS translation. *Mol. Microbiol.* **39**, 1069–1079.
5. Kamashev, D. & Rouviere-Yaniv, J. (2000). The histone-like protein HU binds specifically to DNA recombination and repair intermediates. *EMBO J.* **19**, 6527–6535.
6. Grove, A., Galeone, A., Mayol, L. & Geiduschek, E. P. (1996). Localized DNA flexibility contributes to target site selection by DNA-bending proteins. *J. Mol. Biol.* **260**, 120–125.
7. Lavoie, B. D., Shaw, G. S., Millner, A. & Chaconas, G. (1996). Anatomy of a flexer–DNA complex inside a higher-order transposition intermediate. *Cell*, **85**, 761–771.
8. Trun, N. J. & Marko, J. F. (1998). Architecture of a bacterial chromosome. *ASM News*, **64**, 276–283.
9. Broyles, S. S. & Pettijohn, D. E. (1986). Interaction of the *Escherichia coli* HU protein with DNA: evidence for formation of nucleosome-like structures with altered DNA helical pitch. *J. Mol. Biol.* **187**, 47–60.
10. Rouviere-Yaniv, J., Yaniv, M. & Germond, J. E. (1979). *E. coli* DNA binding protein HU forms nucleosome-like structure with circular double-stranded DNA. *Cell*, **17**, 265–274.
11. Wojtuszewski, K., Hawkins, M. E., Cole, J. L. & Mukerji, I. (2001). HU binding to DNA: evidence for multiple complex formation and DNA bending. *Biochemistry*, **40**, 2588–2598.
12. Hodges-Garcia, Y., Hagerman, P. J. & Pettijohn, D. E. (1989). DNA ring-closure mediated by protein HU. *J. Biol. Chem.* **264**, 14621–14623.
13. Endo, T., Sasaki, N., Tanaka, I. & Nakata, M. (2002). Compact form of DNA induced by DNA-binding protein HU. *Biochem. Biophys. Res. Commun.* **290**, 546–551.
14. Dame, R. T. & Goosen, N. (2002). HU: promoting or counteracting DNA compaction? *FEBS Letters*, **529**, 151–156.
15. Pinson, V., Takahashi, M. & Rouviere-Yaniv, J. (1999). Differential binding of the *Escherichia coli* HU, homodimeric forms and heterodimeric form to linear, gapped and cruciform DNA. *J. Mol. Biol.* **287**, 485–497.
16. Balandina, A., Kamashev, D. & Rouviere-Yaniv, J. (2002). The bacterial histone-like protein HU specifically recognizes similar structures in all nucleic acids. *J. Biol. Chem.* **277**, 27622–27628.
17. Esser, D., Rudolph, R., Jaenicke, R. & Bohm, G. (1999). The HU protein from *Thermotoga maritima*: recombinant expression, purification and physicochemical characterization of an extremely hyperthermophilic DNA-binding protein. *J. Mol. Biol.* **291**, 1135–1146.
18. Grove, A. & Lim, L. (2001). High-affinity DNA binding of HU protein from the hyperthermophile *Thermotoga maritima*. *J. Mol. Biol.* **311**, 491–502.
19. Wery, M., Woldringh, C. L. & Rouviere-Yaniv, J. (2001). HU-GFP and DAPI co-localize on the *Escherichia coli* nucleoid. *Biochimie*, **83**, 193–200.
20. Tanaka, I., Appelt, K., Dijk, J., White, S. W. & Wilson, K. S. (1984). 3–4 Å resolution structure of a protein with histone-like properties in prokaryotes. *Nature*, **310**, 376–381.
21. White, S. W., Wilson, K. S., Appelt, K. & Tanaka, I. (1999). The high-resolution structure of DNA-binding protein HU from *Bacillus stearothermophilus*. *Acta Crystallog. sect. D*, **55**, 801–809.

22. Vis, H., Mariani, M., Vorgias, C. E., Wilson, K. S., Kaptein, R. & Boelens, R. (1995). Solution structure of the HU protein from *Bacillus stearothermophilus*. *J. Mol. Biol.* **254**, 692–703.
23. Swinger, K. K., Lemberg, K. M., Zhang, Y. & Rice, P. A. (2003). Flexible DNA bending in HU–DNA cocrystal structures. *EMBO J.* **22**, 3749–3760.
24. Rice, P. A., Yang, S., Mizuuchi, K. & Nash, H. A. (1996). Crystal structure of an IHF–DNA complex: a protein-induced U-turn. *Cell*, **87**, 1295–1306.
25. Bonnefoy, E. & Rouviere-Yaniv, J. (1991). HU and IHF, two homologous histone-like proteins of *E. coli*, form different protein–DNA complexes with short DNA fragments. *EMBO J.* **10**, 687–696.
26. Aki, T. & Adhya, S. (1997). Repressor induced site-specific binding of HU for transcriptional regulation. *EMBO J.* **16**, 3666–3674.
27. Deniz, A. A., Dahan, M., Grunwell, J. R., Ha, T., Faulhaber, A. E., Chemla, D. S. *et al.* (1999). Single-pair flourescence resonance energy transfer on freely diffusing molecules: observation of Forster distance dependence and subpopulations. *Proc. Natl Acad. Sci. USA*, **96**, 3670–3675.
28. Bouchiat, C., Wang, M. D., Allemand, J.-F., Strick, T. R., Block, S. M. & Croquette, V. (1999). Estimating the persistence length of a worm-like chain molecule from force–extension measurements. *Biophys. J.* **76**, 409–413.
29. Lorenz, M., Hillisch, A., Goodman, S. D. & Diekmann, S. (1999). Global structure similarities of intact and nicked DNA complexed with IHF measured in solution by fluorescence resonance energy transfer. *Nucl. Acids Res.* **27**, 4619–4625.
30. van Noort, J., Verbrugge, S., Goosen, N., Dekker, C. & Dame, R. T. (2004). Dual architectural roles of HU: formation of flexible hinges and rigid filaments. *Proc. Natl Acad. Sci. USA*, **101**, 6969–6974.
31. Jaffar Ali, B. M., Amit, R., Braslavsky, I., Oppenheim, A. B., Gileadi, O. & Stavans, J. (2001). Compaction of single DNA molecules induced by binding of integration host factor (IHF). *Proc. Natl Acad. Sci. USA*, **98**, 10658–10663.
32. Doi, M. & Edwards, S. F. (1992). *The Theory of Polymer Dynamics* (Birman, J., Edwards, S. F., Llewellyn-Smith, C. H. & Rees, M., eds), Clarendon Press, Oxford.
33. Wojtuszewski, K. & Mukerji, I. (2003). HU binding to bent DNA: a fluorescence resonance energy transfer and anisotropy study. *Biochemistry*, **42**, 3096–3104.
34. Serban, D., Benevides, J. M. & Thomas, G. J. (2003). HU protein employs similar mechanisms of minor-groove recognition in binding to different B-DNA sites: demonstration by Raman spectroscopy. *Biochemistry*, **42**, 7390–7399.
35. Kellenberger, E. & Arnold-Schulz-Gahmen, B. (1992). Chromatins of low-protein content: special features of their compaction and condensation. *FEMS Microbiol. Letters*, **79**, 361–370.
36. Kim, J., Yoshimura, S. H., Hizume, K., Ohniwa, R. L., Ishihama, A. & Takeyasu, K. (2004). Fundamental structural units of the *Escherichia coli* nucleoid revealed by atomic force microscopy. *Nucl. Acids Res.* **32**, 1982–1992.
37. Padas, P. M., Wilson, K. S. & Vorgias, C. E. (1992). The DNA-binding protein HU from mesophilic and thermophilic bacilli-gene cloning, overproduction and purification. *Gene*, **117**, 39–44.
38. Nash, H. A., Robertson, C. A., Flamm, E., Weisberg, R. A. & Miller, H. I. (1987). Overproduction of *Escherichia coli* integration host factor, a protein with nonidentical subunits. *J. Bacteriol.* **169**, 4124–4127.
39. Mengeritsky, G., Goldenberg, D., Mendelson, I., Giladi, H. & Oppenheim, A. B. (1993). Genetic and biochemical-analysis of the integration host factor of *Escherichia coli*. *J. Mol. Biol.* **231**, 646–657.
40. Clegg, R. M., Murchei, A. I. H., Zechel, A. & Lilley, D. M. J. (1993). Observing the helical geometry of double-stranded DNA in solution by fluorescence resonance energy transfer. *Proc. Natl Acad. Sci. USA*, **90**, 2994–2998.
41. Deniz, A. A., Laurence, T. A., Dahan, M., Chemla, D. S., Schultz, P. G. & Weiss, S. (2001). Ratiometric single-molecule studies of freely diffusing biomolecules. *Annu. Rev. Phys. Chem.* **52**, 233–253.

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