Multiple Site-Specific Binding of Fis Protein to *Escherichia coli nuoA-N* Promoter DNA and its Impact on DNA Topology Visualised by Means of Scanning Force Microscopy

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The control of bacterial DNA topology is one of the general means to coordinating gene activity as a function of environmental changes. Among the few "histone-like" proteins, which are involved in architectural changes of prokaryotic DNA, Fis (*factor for inversion stimulation*), a small protein (98 aa/12 kD), acts on DNA in a growth phase-dependent manner.[1] The regulatory response of Fis is associated with a substantial increase in protein concentration during the exponential growth phase of *E. coli*. The Fis protein is involved in different biological processes in *E. coli* that enhance site-specific recombination, control DNA replication and regulate transcription of a number of genes.[2–4] For instance, the proton- or Na^+-pumping NADH dehydrogenase I of *E. coli* is encoded by the *nuoA-N* operon, which comprises a regulatory region with two promoters, P1 and P2, located upstream of the coding sequence.[5] Under respiratory growth conditions, *nuo* expression is stimulated in the early exponential growth phase and to a smaller extent in the stationary growth phase. The stimulation in the early exponential phase is affected mainly by the Fis protein. Motivated by these findings, the effect of relevant transcriptional regulator (Fis) on *nuoA-N* expression has been investigated by using the respective regulatory mutants of previous work.[6] By DNAseI footprinting assays, three Fis binding sites were identified.

The observation that Fis can change the overall shape of supercoiled DNA molecules provides an important clue to the possible role of Fis in controlling the architecture of prokaryotic chromosomal DNA in exponentially growing bacterial cells.[7] To achieve a deeper understanding of the structural organisation and the interactions between the *nuo* promoter and the site-specific binding protein, Fis, we focused on the direct visualisation of Fis–DNA complexes by scanning force microscopy (SFM). Scanning force microscopy is particularly well suited since, as well as unambiguous identification of the location of the proteins by direct visualisation of the protein–DNA complex, the determination of the oligomeric state of the protein is possible by estimating the volume of the protein aggregate and the topology of the DNA. SFM has already been proven to be a powerful tool for the structural analysis of different protein–DNA complexes at high resolution without the need of external contrast enhancement.[8–12] Hence, SFM analysis of DNA–Fis complexes would provide direct evidence that the protein has a strong impact on the general conformation of DNA.[14–16]

Our objective was twofold. On the one hand, we wanted to investigate whether binding is specific and if up to three Fis dimers bind to the proposed three binding sites in the region of the *nuo* promoter. On the other hand, our goal was to investigate the impact of Fis on the architecture of DNA. Analysis of DNA scission of Fis–DNA by a collection of Fis conjugates to 1,10-phenanthroline copper combined with comparative gel electrophoresis provides evidence that a bending angle between 50° and 90° occurs due to flanked DNA wrapping around the bound Fis molecules.[13]

It is possible to determine the degree of compaction due to wrapping of the DNA around the protein complexes. For this purpose, we compared the contour length of the DNA in the absence and presence of Fis. Figure 1 displays the 1242 bp dsDNA fragment that was used in this study and contains the complete intergenic region upstream of *nuo* with the three binding sites for Fis (*fis1*, *fis2*, *fis3*) located near one end.

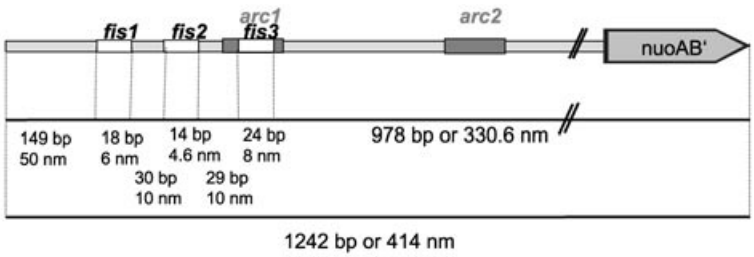

Figure 1. *The scheme shows the proposed location of the binding sites for Fis (*fis1, fis2, fis3*) and for ArcA (*arc1, arc2*) in the* nuo *promoter region on the DNA fragments. The whole length of the 1242 bp fragment is 414 nm. The DNA contour length was calculated from the number of base pairs (0.338 nm per bp).*

From gel-retardation assays it is known that the Fis concentration required for half-maximal binding to *fis1*, *fis2* and *fis3*, is about 20, 40 or 100 nM, respectively.[6] In order to form protein–DNA complexes with all three binding sites occupied, DNA was incubated with 100 nM of Fis protein in solution. The protein–DNA complexes were then immobilised on mica and subsequently analysed by SFM.

Figure 2A shows SFM images obtained in intermittent-contact mode of DNA molecules on a mica surface in the absence of Fis protein (control), while Figure 2B displays protein–DNA complexes after incubating the DNA with Fis protein prior to

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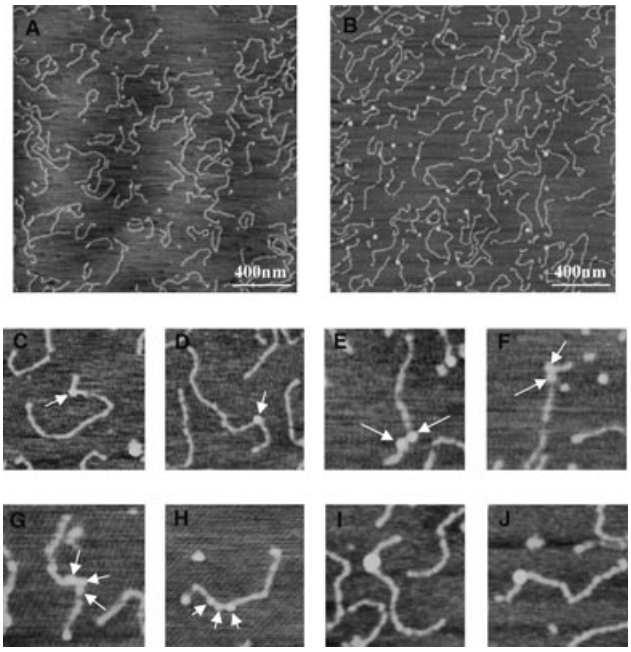

Figure 2. *SFM images of pure DNA and Fis–DNA complexes deposited onto mica. A) Typical height image of the DNA fragment. B) Typical height image of Fis–DNA complexes. The bright spots are Fis proteins occupying the binding sites. C)–H) Magnifications of Fis–DNA complexes occupying one, two or three binding sites. Image size is 300×300 nm with a z-range of 4 nm. I) and J) Magnifications of Fis aggregates binding to specific positions of the DNA strand. Image size is 300×300 nm with a z-range of 6 nm.*

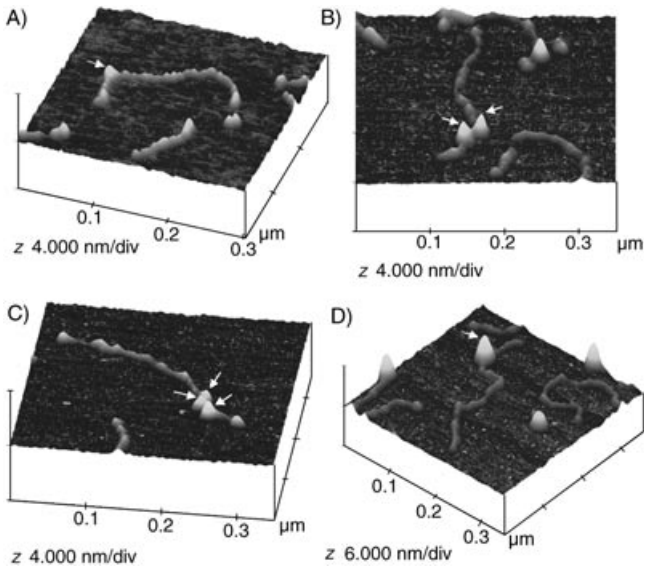

Figure 3. *Three-dimensional representation of SFM images of Fis–DNA complexes. The white arrows indicate the fine structures of specific complexes with one, two or three sites occupied by Fis dimers or alternatively one large aggregate consisting of many single Fis proteins. A)–C) Fis proteins bind to one, two or three binding sites, respectively; z-range is 4 nm. D) One big aggregate of Fis proteins occupying presumably all three binding sites; z-range is 6 nm.*

adsorption. It is evident that Fis is bound to the *nuo* promoter DNA. Different numbers of protein–DNA complexes are discernable as shown in Figure 2C–J, which were taken at higher magnifications. Figure 2C–H show the distinctive binding of one (C, D), two (E, F) or three (G, H) Fis proteins to the linear DNA strand. Individual Fis dimers can clearly be resolved, and the observed dimensions correspond well to the expected size of a dimer of proteins (expected height of 1–2 nm from separate experiments imaging Fis dimers (25 000 g mol^{-1}) in the absence of DNA).

Besides the clearly resolved individual Fis dimers bound to DNA, larger protein aggregates (oligomers) were also found to occupy the Fis-binding region. As a consequence, the large aggregates obscure the individual binding of Fis dimers as shown in Figures 1I and J. The large protein aggregates exhibit an average height of 3.4 nm, while the dimers (Figures 1C–H) only show a mean height of 1.8 nm. Hence, the aggregates must consist of at least three Fis tetramers or even larger oligomers (up to 40 Fis dimers as determined from measuring the volume of the protein aggregates). Since, in some images, protein aggregates were found on the mica surface without being attached to the DNA, it is conceivable that the oligomerisation occurs already in solution.

The differences in height and volume between individual Fis dimers and Fis aggregates are revealed most clearly by three-dimensional images of the protein–DNA complexes. Figure 3A–C shows the binding of one, two and three Fis dimers, respectively, while Figure 3D shows the interaction of large oligomers with the DNA. Evidently, the separated Fis–DNA complexes composed of individual Fis dimers are much lower and less broadened than the oligomeric aggregates. Single Fis dimer–DNA complexes are sometimes difficult to distinguish from each other because, firstly, the height of the protein is similar to that of the DNA corrugations and, secondly, the binding sites are located close together. The formation of large protein aggregates at the Fis-binding sites of the DNA strand might either abolish further binding of dimers to the remaining binding sites by steric hindrance or alternatively occupy all three binding sites in an indiscernable fashion. The formation of Fis aggregates could not be diminished by reducing the Fis concentration to 80 nM.

The specificity of Fis binding can be elucidated by the location of bound protein on the DNA strand. If the Fis proteins bind to the specific binding sites (see Figure 1), individual dimers should be centred at 53, 69 and 85 nm away from one end of the DNA strand. To obtain the exact location of Fis binding, we measured the distribution of the distance between the occurrence of proteins and the end of the DNA strand nearest to the protein. If more than one protein bound to one DNA strand, we listed all positions. Figure 4 shows the histogram of Fis position relative to the DNA strand obtained from several SFM images. Evidently, the binding of Fis proteins to the *nuo* promoter region observed by SFM is consistent with our previously published results from gel-shift experiments on the same DNA.[6] The broad distribution of the Fis position results partly from the three adjacent binding sites and the tip sample convolution, but also from nonspecific binding of Fis to other locations of the DNA. Assuming that specific binding is identified by finding Fis in the region of 30 to 110 nm away

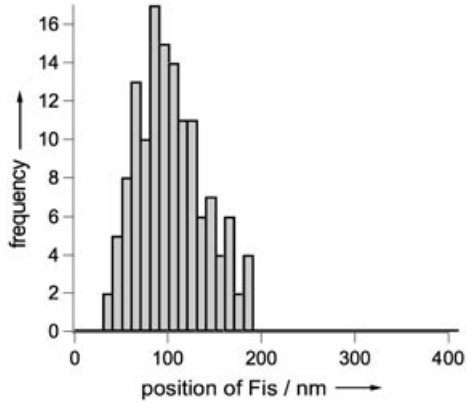

Figure 4. *Histogram of the distance between the location of Fis on the DNA and the shortest way to one end of the DNA strand.*

from one end of the DNA strand, in accordance with the width of the proteins measured by SFM, a selectivity of 62% is obtained. Thus, we conclude that Fis does not bind exclusively to the designated binding sites but also in part nonspecifically to DNA in the nanomolar range.

Figure 5 shows the impact of Fis binding on the contour length of the DNA. In the presence of Fis (80 nM), a considerable reduction of the contour length of 80 nm can be observed.

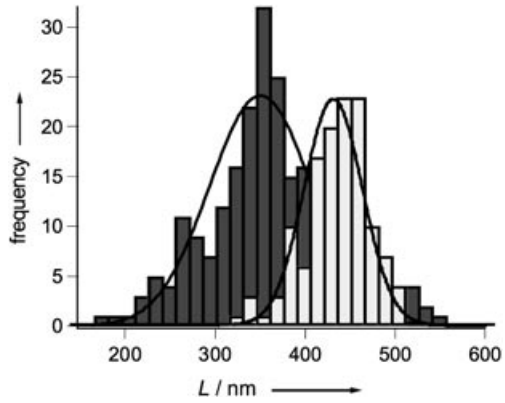

Figure 5. *Histogram of the contour length of DNA in the absence (light grey bars) and presence (dark grey bars) of Fis (80 nM). The fit of the Gaussian distribution resulted in a mean contour length of (350 ± 3) nm for the DNA with bound Fis (σ = 6 nm) and (431 ± 1) nm for DNA in the absence of protein (σ = 5 nm).*

A t-test (assuming Gaussian distribution) and a Wilcoxon rank-sum test ($P < 0.0001$) confirm that the shift in contour length is highly significant. The SFM images did not resolve whether wrapping around the proteins occurs. The presence of large structures (see Figure 2), however, indicates that more complex architectures are conceivable.

In many of the observed complexes, the DNA seems to be bent at the position where the protein is bound, that is, the two flanking DNA arms are pointing from the complex at a relatively sharp angle (see Figure 2C, F, G). The Fis–DNA complexes exhibit a broad Gaussian distribution of bending angles or—since wrapping around the protein complexes occurs—the angle between the entering and exiting DNA strand centred at (87 ± 5)° (Figure 6A). In the absence of Fis proteins, a distribution of bending angles at the binding site centred at

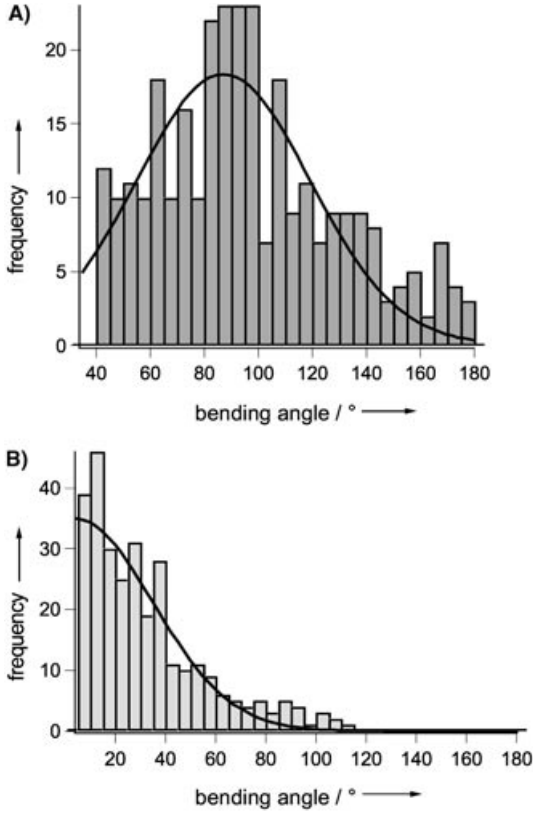

Figure 6. *A) Histogram of the bending angles for Fis proteins bound to the DNA strand. The mean value is (87 ± 5)° as obtained from fitting a Gaussian distribution function to the histogram data.* B*) Histogram of the bending angles for pure DNA in the absence of protein.*

around 0° was found, as expected, for DNA without spontaneous curvature (Figure 6B). The finding that the average bending angle or angle between entering and exiting of the DNA at the position of the protein is around 90° is in good agreement with results obtained from gel electrophoresis providing values between 40 and 90°.[13–16]

From the SFM images alone it is not possible to infer the exact architecture of the protein–DNA complex. Implications of bending and/or wrapping of DNA with respect to the possible biological significance of Fis might comprise the regulation of the activity of the promoters or an increase in the accessibility of other proteins, such as DNA-binding proteins.

In conclusion, we were able to visualise for the first time the effect of Fis dimers on the structural organisation of DNA on a molecular level. We investigated the specificity of the interaction of Fis dimers with specific binding sites in the *nuo* promoter region and the substantial apparent bending of DNA at the position of the bound protein. In fact, a strong tendency towards compaction of the DNA in the presence of Fis has

been observed. These results pave the way for further studies on the formation of complexes of the *nuo* promoter DNA with the protein ArcA and other regulatory proteins involved in *nuoA-N* expression.

Experimental Section

Expression and isolation of Fis: For overexpression of Fis, a 324 bp fragment of chromosomal DNA (from *E. coli* AN387) containing the coding sequence of the *fis* gene was amplified by using PCR with primers fis3NdeI (GACAGACATATGTTCGAACAACGC) and fis4BamHI (GCATTTAGGGATCCTGAATTAGTTC). After restriction, the fragment was cloned into the vector pET28a (Novagen); this yielded pMW279 coding for Fis with a His_6-tag and a linker with a thrombin cleavage site close to the N terminus of Fis. For the isolation of Fis, *E. coli* BL21DE3 pMW279 (pET28a.fis) was grown in LB medium (0.8 L) and induced, and the cells were broken in a French Press cell. After the removal of debris and of the membranes by centrifugation, the supernatant was applied to a Ni^{2+}-NTA column (3 mL) equilibrated with buffer A (50 mM Na-K-phosphate, pH 7, 200 mM NaCl, 10 mM imidazole). After being washed with buffer B (30 mL, buffer A with 20 mM imidazole), the Fis protein was eluted with buffer C (30 mL, buffer A with 500 mM imidazole). The eluted protein was stored at 4 °C in buffer with 40% glycerol.

Sample preparation: The intergenic region in front of *nuoA* was amplified by PCR with oligonucleotide primers nuo2 (CCGGAAGGGGAGAATTCATTGTTGATTG) and nuoB′EcoRI (ACGGATCCCCGAATTCTTGCTCC) from pMW6 and purified with the Qiaquick PCR Purification Kit (Qiagen). The binding reactions were performed essentially as described previously.[6] Isolated Fis protein was incubated with *nuo* promoter DNA (20 ng) in buffer (20 μL, 4 mM HEPES, pH 7.4, 10 mM NaCl, 2 mM $MgCl_2$) for 15 min at 4 °C. Mg^{2+} in the binding buffer is necessary to ensure binding of DNA to the mica surface.[10] After incubation, 5–10 μL of the reaction mixture was deposited onto freshly cleaved mica. DNA without Fis was deposited under the same buffer conditions. After approximately 1 min, the 1–1.5 cm^2 mica disc was gently rinsed with ultrapure deionised water (2–3 mL, Millipore, Germany). Excess water was removed with highly absorbent tissue paper, and the disc was subsequently dried under a gentle flow of nitrogen gas.

Scanning force microscopy: Images were acquired with a Multimode scanning force microscope equipped with a Nanoscope IIIa controller (Digital Instruments Inc., Santa Barbara, CA, USA), operating in TappingMode™ in air with an E-scanner. Rectangular silicon cantilevers (Nanosensors, 125×30×4 μm) with an integrated tip, a nominal spring constant of 42 Nm^{-1} and a resonance frequency of 330 kHz were used. To control and enhance the range of the attractive interaction regime, the instrument was equipped with a special active feedback circuit, called Q-control (Nanoanalytics, Germany).[17]

Image processing: Raw SFM images were processed only for background removal (flattening) by using the NanoScope Image software (Veeco Instruments Inc., Santa Barbara, CA, USA). DNA molecule lengths and bending angles induced by Fis protein were determined manually from the SFM images by employing a graphic tablet. The bending angle was measured by using the tangents method by drawing lines from the centre of the Fis protein to the entry and exit points of the DNA. The deviation from linearity of one tangent from the other corresponds to the bending angle. Data workup was performed with the angle-measurement tool of the software package from Veeco Instruments.

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Keywords: DNA · *E. coli* · Fis · *nuo* promoter · proteins · scanning probe microscopy

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