An Atomic Force Microscopy Assay of Intercalation Binding, Unwinding, and Elongation of DNA, Using a Water-Soluble Psoralen Derivative as a Covalent Binding Probe Molecule

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A single-molecule strategy using atomic force microscopy has simply yet robustly probed an intercalation binding related specific structural relaxation of covalently closed circular pBR322 DNA as well as double-strand elongation in its linear form by taking advantage of a new psoralen derivative, *N*,*N*,*N*-trimethyl-1-(2,5,9-trimethyl-7-oxo-7*H*-furo[3,2-*g*]chromen-3-yl)methanaminium chloride, that covalently binds to DNA through a photo-crosslinking reaction.

The linear isomers of the furocoumarin family, known as psoralens, are a class of intercalator and photoreact with nucleic acids when they are irradiated with long wavelength ultraviolet light (365 nm).[1] This reaction leads to mono- or di-adducts on the pyrimidine bases and in particular, later results in a covalent interstrand cross-link that freezes nucleic acid double strands. Therefore, they are important for investigations of nucleic acid superstructure. On the other hand, intercalation binding has been considered as essential for clinically important drugs to exert their primary biological action.[2] This particular mode of DNA binding, in which the drug molecule becomes inserted between adjacent base pairs, necessarily involves the local unwinding of the double helix. Since regulatory protein binding is sensitive to specific changes in helical geometry, intercalation binding can mislead a trigger protein to inhibit DNA transcription. Thus, the specific structural changes caused by intercalation have long been the subject of research, mainly involving viscosity and sedimentation measurements.[2] Later, the DNA topology issue was wonderfully developed by Keller by taking advantage of topoisomerase enzymic reaction,[3] and was further followed by X-ray crystallographic studies.[4]

Early on high-resolution microscopy, including electron microscopy, also was used to characterize drug–DNA interactions by observing individual complex molecules.[5] Recently, efforts have been revitalized by taking advantage of the biological interaction operations that atomic force microscopy (AFM) can provide.[6–8] These studies indicated that DNA intercalation could be examined by a single-molecule strategy. However, most intercalator molecules are characterized by the reversible nature of their binding reactions. Under the typical conditions of microscopy experiments, DNA–intercalator complex molecules that are individually attached to a substrate will necessarily dissociate because the specific binding equilibrium proceeds in reverse to produce the coexisting free species. This suggests that intercalators that irreversibly bind to DNA overcome the difficulty. In the present study, we have achieved more robust measurements using a new type of intercalator compound, a water-soluble psoralen derivative (Figure 1), which can bind covalently to double-helical DNA.

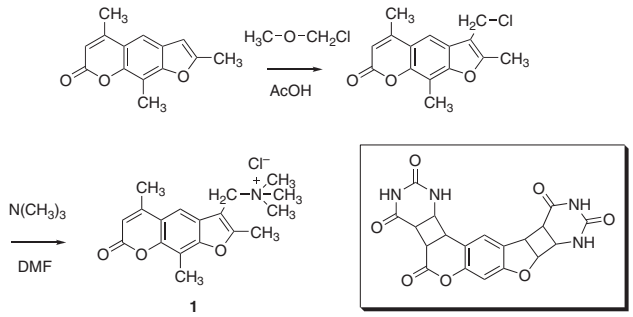

Figure 1. Synthetic route of quaternary ammonium psoralen derivative and the structure of the photoadduct formed.

A water-soluble psoralen derivative **1** was synthesized by reacting 3-chloromethyl-2,5,9-trimethylfuro[3,2-*g*]coumarin with trimethylamine in DMF. The analytical data indicated that the reactions proceed as intended. Gel retardation analysis showed the covalent nature of the DNA binding. Additionally, we found that the new molecular entry was characterized by moderate solubility (ca. 0.1 M) that was superior to amine-functionalized trioxsalen (34 mM).[9]

Coury and co-workers reported an AFM-based assay for the binding reaction of ethidium bromide (EtBr) and daunomycin by simply evaluating the specific lengths of the individual host DNA molecules.[6] Pope and his assistants described the structural transition of plasmid DNA upon intercalation of EtBr[7] and later, Utsuno et al. extended this to liquid-phase measurements.[8] However, some disagreement with parameters obtained from homogeneous, bulk analysis[10] make further analysis of the helix parameters almost impossible.

Tapping mode AFM (Nanoscope IIIa, Veeco Metrology Inc., Santa Barbara, CA, USA) experiments for native pBR322 DNA gave a full-length portrait of the closed-circular structure, which further develops plectonemic supercoils (Figure 2). The cross sectional profiles have revealed that the particular structure had a specific height of 1.1 ± 0.11 nm ($n = 50$) while it was 0.72 ± 0.12 nm for the simple double-strands ($n = 50$). Thus, we can confirm that the plectonemic conformer almost doubles its steric bulk. On the other hand, changes in the image width corresponding to conformational transitions were unclear: plots of the image width vs. the image height only gave a linear relationship due to tip-apex destruction.

As generally recognized, intercalator binding can transform the topology for a closed-circular DNA from negatively supercoiled structures to a totally relaxed state (a flat circle).

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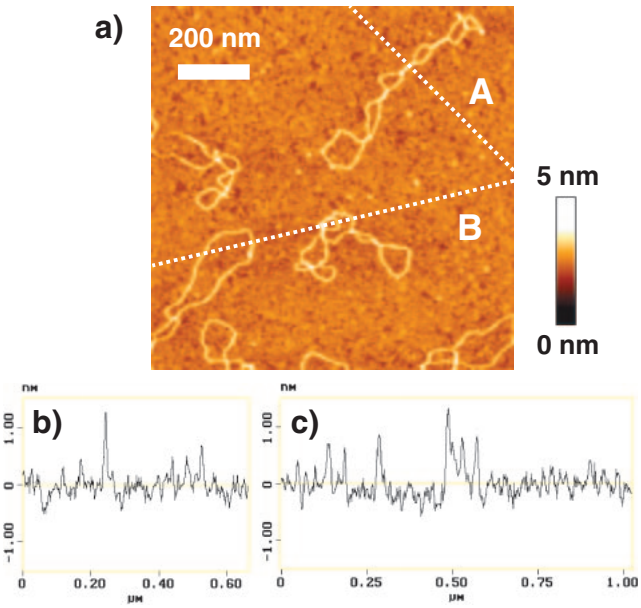

Figure 2. A representative AFM image of native pBR322 DNA on mica a). In panels b) and c) are shown the cross-section profiles obtained along with the lines A and B, respectively.

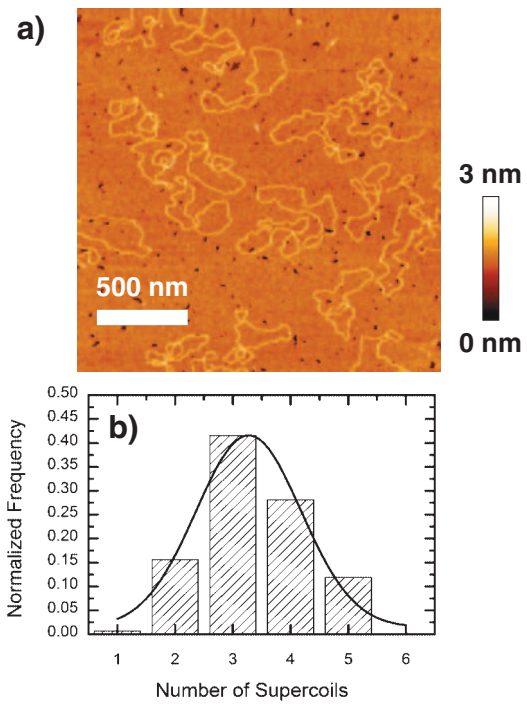

Figure 3. A representative AFM image of pBR322 a) at an intercalator-to-nucleotide ratio of 0.1. In panel b) are shown the distribution of the supercoil numbers determined for the 135-numbers of the conjugate and the result of nonlinear curve fitting (solid line).

We found that AFM imaging can uncover the superhelical transition, in part, in a single plasmid level. As shown in Figure 3, the tightly formed supercoiled structures can relax to a considerable extent: at 10% binding, most of the plasmid was converted to flexible, flat circular conformations. One may

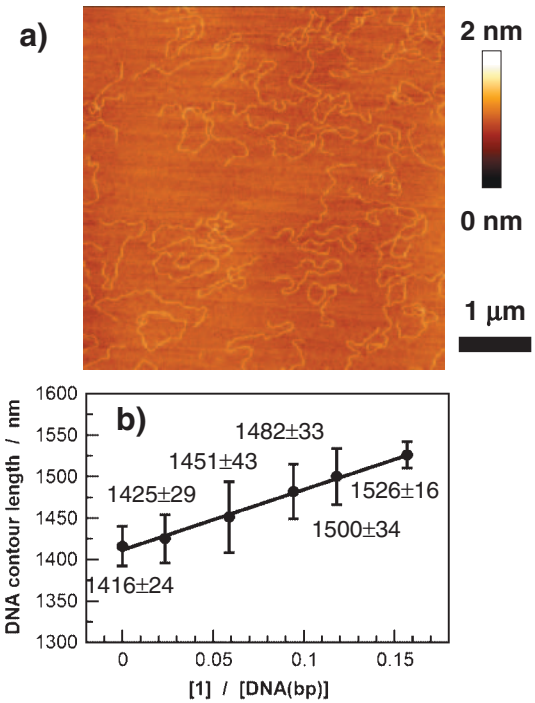

Figure 4. A representative AFM image of linear pBR322 (a). In panel (b) are shown the plots of the mean molecular length vs. concentration of **1**.

notice that the linear conformer unexpectedly exists: the sample preparation procedure including pipetting may cause DNA scission. Furthermore, DNA nicking also affects the outcome seriously since it can relax the supercoiled structure. However, we have experienced that both incidents were noted only occasionally all through the experiments.

In a study of the linking number change due to specific nutrient condition shifts in *Escherichia coli*, Balke and Gralla reported that pBR322 normally contains 28 supercoils.[11] If one takes the established data for the double-helical unwinding angle, $18 \pm 8°$, into account,[2] one can find that a specific DNA base pair to ligand ratio of 0.12 should completely eliminate the original superhelical turns. Our AFM results deem to follow the simple binding–unwinding stoichiometry. Meanwhile, Keller reported that with the topoisomerase treatment, SV40 DNA consisted of a group of DNA molecules differing in their number of superhelical turns (τ) around a mean value in a Gaussian-like distribution.[3] It should be apparent that for the individual pBR322 DNA molecules, τ is easily determined from the AFM image with the naked eye. By examining 135 plasmid molecules, we have obtained specific distribution of τ, which was further analyzed by nonlinear curve fitting assuming a Gaussian-distribution function (eq 1) determining the mean value (x_0) and the relative standard deviation ($w/2$) to be 3.3 and 0.9, respectively.

$$y = y_0 + \frac{A}{w \cdot \sqrt{\frac{\pi}{2}}} \exp\left[-\frac{2(x - x_0)^2}{w^2}\right] \quad (1)$$

Figure 4 shows a representative image for native pBR322 in linear form. As seen in the figure, most DNA molecules possess a similar topology, which makes single-molecule-

directed contour length measurement practical. A phase diagram relating conformational changes of DNA with varying humidity revealed that the B form DNA partly converts to A-DNA under moderate humidity conditions.[12] The average length of pBR322 as determined (1416 ± 24 nm, $n = 21$) gives a base pair increase of 0.32 nm, showing that the molecules were predominantly in the B form double-helix (81%). The average height (0.62 ± 0.08 nm, $n = 15$) data was essentially consistent with that of the closed-circular DNA. Moreover, AFM imaging confirmed that pBR322 samples lengthened with the photoreaction with **1** maintaining their entire double-helical structure; the length of the DNA conjugate was clearly greater than that of native, intercalator-free DNA. Indeed, the DNA length gave a linear relationship ($y = 1412 + 732x$, $r = 0.997$) over the concentration range investigated. Here, all contour length data presented were verified by duplicate measurements, including different sample preparations. Typically, ten AFM samples, each of which allowed contour length measurements on 5–6 pairs of different DNA molecules, were used to determine the specific molecular length of DNA conjugates. Repeated measurements on a single DNA molecule confirmed that the experimental error of the measurement was within 3%. Previous AFM studies revealed that the contour length measurements on a series of DNA images with varying EtBr to DNA concentration ratios can follow the equilibrium to give a specific binding isotherm.[6,7] However, for detailed discussion, the reversible nature of the reaction inherently requires a much more cumbersome analysis of binding isotherms displaying the characteristics of saturation binding. Therefore, by using this type of approach, the helical unwinding angle, which is an indispensable parameter, still seems elusive. On the contrary, the covalent binding that is unique to **1**, allows us a simple yet robust determination of the helices parameter. The fraction of occupied binding sites should simply be equivalent to [**1**]/[DNA(bp)], and thus from the concentration dependence one can conclude that every individual intercalator binding with DNA produces an unwinding of 19°, using a base pair increase of 0.32 nm and a base pair rotation of $2\pi/10$. Few studies have been quantitatively analyzed for unwinding upon psoralen binding. In early work, Wiesehahn and Hearst reported an unwinding angle of $28 \pm 4°$ upon intercalation binding[13a] and this has been confirmed in recent years by establishing the molecular structure using two-dimensional ^{1}H NMR spectroscopy.[13b] However, the data are somewhat larger than that which has been acknowledged. It is tempting to speculate that the optimum unwinding of DNA is approximately 18° in the absence of other strong influences that might change the interaction between the intercalated ligand and the two adjacent base pairs. The optimum could perhaps be modified by ±8° if the modification enhances other interactions such as specific ligand–DNA hydrogen bonding, e.g., phenanthridines, or weaken them, e.g., anthracyclines.[2] Our result correctly follows the empirically determined trend.

In conclusion, we have synthesized a new, water-soluble psoralen derivative. Using this derivative enabled determination of DNA lengthening and unwinding, based on a single-molecule strategy that associates with AFM imaging. The helices unwinding angle was found to fall approximately within the range of previously reported values which were determined by bulk analysis. As far as we know, this method of determining the helix parameters has not previously been reported. Recently, certain types of psoralen derivatives have been finding applications in nanobio material development involving separation of DNA-binding substances,[14a] targeting specific DNAs,[14b] and also photoactive DNA immobilization.[14c] We believe that the results obtained here should also be informative in such applications.

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Supporting Information

The synthesis procedure of **1** including physical data of elemental analysis, NMR spectrum, mass spectrum, and HPLC separation is described at full length. Results of brief studies on the photo-crosslinking reaction are compactly represented by UV–vis spectral measurements and gel retardation analysis. This material is available free of charge on the Web at http://www.csj.jp/journals/bcsj/.

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