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Detection of Abasic Sites on Individual DNA Molecules Using Atomic Force Microscopy

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We have developed an atomic force microscopy-based method for detecting abasic sites (AP sites) on individual DNA molecules. By using uracil and uracil DNA glycosylase, we first prepared a 250-bp DNA template consisting of two AP sites at specific locations. We then detected the AP sites by marking them with biotinylated aldehyde-reactive probes and monomeric avidin. We demonstrate here that (i) the location of monomeric avidin bound on a single DNA molecule was detectable by atomic force microscopy; (ii) the observed location of avidin was in good agreement to the predicted AP sites at a few nanometer resolution; and (iii) by end-labeling the 5′-terminus of one DNA strand, the AP sites were determined without directional ambiguity. The technique described here will provide a sensitive way of locating AP sites and contribute to screen DNA damages from individual molecules.

DNA is a complex biological molecule of finite chemical stability. Its sugar−phosphate backbone can be broken by oxidative stress or ionizing radiation, and its bases can be altered enzymatically or by mutagens.[1−3] One of the most prevalent DNA lesions is an abasic site, also called an apurinic/apyrimidinic (AP) site, where a DNA base such as A, C, G, or T residue is removed.[4,5] These nonfunctional sites can be derived from spontaneous depurination or deamination followed by a removal of the deaminated base, generating a potential cause of DNA alterations and cellular death as well as carcinogenesis.[6] Therefore, detecting AP sites and examining the genetic integrity is of prime importance in DNA diagnosis. Since DNA damages are different from one DNA molecule to another, a sensitive and reliable method to detect AP sites on individual DNA molecules would be useful. Although several methods are presently available for quantifying a level of AP sites from a population of chromosomes or cells, no method allows us to assay a physical position or a distribution of AP sites from individual DNA molecules.[7−9]

In identifying DNA damages such as AP sites on single DNA molecules, atomic force microscopy (AFM) can provide a powerful visualization tool.[10−12] We previously developed the AFM-based method for detecting base-pair mismatches, another form of DNA damage, where a noncomplementary pair of bases are positioned on complementary double strands.[13] We used MutS protein as a biological sensor of DNA base-pair mismatches and identified mismatches at the MutS binding sites on the uncoiled DNA molecules. Likewise, using a DNA-binding agent specific to AP sites an AFM-based visualization method should be developed to physically locate abasic sites.

In order for AFM to identify AP sites on individual DNA molecules, we used a biotinylated aldehyde-reactive probe (bio-ARP, 445 Da) and monomeric avidin (∼16 kDa). The predominant reactive group at the AP sites was an open-chain aldehyde derived from DNA's ribose, and bio-ARP served as an initial marker of the aldehyde group on the AP sites.[14,15] The location of bio-ARPs on DNA was then visualized by AFM via monomeric avidin bound to bio-ARP. In preparing the DNA samples consisting of AP sites at known locations, we employed uracil DNA glycosylase. DNA glycosylase can remove damaged or mutated bases, and uracil DNA glycosylase removes uracil or 5-hydroxyuracil.[16,17] After incorporating two uracil residues into 250-bp DNA templates, the AP sites were created by removing uracils using uracil DNA glycosylase. To determine the location of AP sites without directional ambiguity, one 5′-end of a single DNA strand was biotinylated and marked with avidin. DNA templates were gently uncoiled and immobilized on a flat mica surface, and the location of avidin-bound AP sites was identified by AFM at a few-nanometer resolution.

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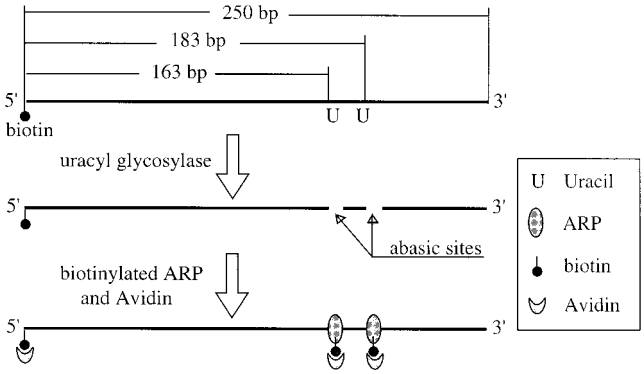

Figure 1. DNA template used in this study. The DNA template was biotinylated at the 5′-end, and the two AP sites were formed by uracyl *N*-glycosylase. The AP sites were reacted with biotinylated aldehyde-reactive probe (bio-ARP) and subsequently monomeric avidin, ~16 kDa in mass.

MATERIALS AND METHODS

Preparation of DNA Templates. The double-stranded DNA template, 250 bp in length, was constructed including two uracils at 163 and 183 bp away from the biotinylated 5′-end on the upper DNA strand (Figure 1). Two uracil residues were then removed from the DNA templates using thermolabile uracil *N*-glycosylase (HU59100, Epicenter Technology). Briefly, 100 ng of DNA molecules was incubated for 30 min at room temperature in 10 μL of the buffer containing 50 mM Tris-HCl (pH 9.0), 20 mM ammonium sulfate, and 1 unit of uracil N-glycosylase.

The glycosylated DNA templates consisting of the two AP sites were labeled with 5 mM *N*-aminooxyacetyl-*N*′-D-biotynoylhydrazine (bio-ARP, Dojindo Laboratories) in a 10-μL volume of the buffer consisting of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 50 mM KCl for 30 min at room temperature. The DNA templates were separated from uracil *N*-glycosylase and the unreacted bio-ARP by using a phenol−chloroform extraction and ethanol precipitation procedure. Finally, the DNA sample was reacted with monomeric avidin, ~16 kDa in mass (A2036, Sigma), in a buffer containing 50 mM Tris-HCl (pH8.0), 100 mM NaCl, and 10 mM $MgCl_2$ for 30 min at room temperature. The ratio of avidin monomers to DNA molecules in the reaction tube was ~10.

Uncoiling and Immobilizing DNA Samples. The glycosylated, end-biotinylated DNA templates labeled with bio-ARP and avidin monomers were gently stretched and immobilized on a freshly cleaved mica surface (25 mm × 25 mm, Ted Pella Inc.) by the method previously described.[18] We also used control DNA molecules without glycosylation or end-biotinylation. Briefly, the mica sheet was mounted on the custom-made spin-stretcher, and the mica plate was spun at ~5000 rpm. A series of solutions was gently dispensed on the spinning center in the order of 50 μL of H_2O, 50 μL of 500 mM $MgCl_2$, 50 μL of H_2O, 10 μL of the DNA sample, and 50 μL of H_2O with a 15-s interval.

Imaging by Atomic Force Microscopy. A Nanoscope III atomic force microscope (Digital Instruments, Inc.) was used to capture topographical images of monomeric avidin, DNA templates, and avidin−DNA complexes immobilized on the mica surface. AFM was operated in the ambient air at 15−20% humidity. The tapping mode was used to reduce any damage to biological samples caused by physical contact with the tip, and the tapping frequency was set to ~290 kHz. A scanning field of view was 2 μm × 2 μm (coarse scanning) or 500 nm × 500 nm (fine scanning) with the scanning rate of 0.5−1 Hz and 512 scanning lines. The silicon tips we used had an estimated curvature of 10−20 nm. Height images in the range of 0−5 nm were flattened to remove the background curvature of the mica surface, and the images were analyzed using NIH Image 1.60 image analysis software. The normalized position corresponding to AP sites was defined as $d_1/(d_1 + d_2)$, where d_1 and d_2 are the lengths of DNA segments bisected by a bound avidin monomer.

RESULTS

Labeling of AP Sites with Monomeric Avidin. Prior to locating biotin-labeled AP sites on individual DNA molecules, we first determined a geometric size of 250-bp DNA templates as well as monomeric avidin and evaluated proper sample preparations for the AFM-based detection of the AP sites. In a scanning field of 2 μm × 2 μm, ~30 DNA molecules were detected, and the mean and the standard deviation of end-to-end DNA length was measured as 77 ± 5 nm (N = 45, sample number) (Figure 2). The predicted length in B-form was 82 nm. Monomeric avidin was elliptically shaped with the mean and the standard deviation of 16 ± 2 (major axis, N = 30) and 9 ± 1 nm (minor axis, N = 30) (Figure 2). The dimensions of a natural form of avidin consisting of four monomer units were measured as 23 ± 3 (major axis, N = 30) and 16 ± 2 nm (minor axis, N = 30). We used a monomer form of avidins in this study in order to enhance resolution in determining avidin's binding sites. When the DNA template was not biotinylated at the 5′-end, ~20% of the molecules were detected as a DNA−protein complex (Figure 2). Avidin's binding positions were found in the vicinity of one of the two AP sites at 163 and 183 bp from the end. Approximately 1% of the DNA molecules exhibited two avidin monomers closely spaced next to each other. For the DNA templates end-labeled with biotin, over 90% of the molecules was end-marked with avidin when the ratio of DNA molecules to avidin monomers was set to 10.

Determination of AP Sites. We next determined the position of 58 avidins bound on the 250-bp DNA templates and examined the specificity of the binding of avidin monomers to the two AP sites. Using the DNA template without having the biotinylated 5′-terminus, we chose the straightened DNA molecules whose end-to-end length was in the range of 77−84 nm. Since the DNA templates were not end-marked in this experiment, the DNA-bound avidins could be on the segment close to the 5′-terminus or the 3′-terminus. The binding site was therefore assigned between 0 and 0.5 in a normalized scale where the end-to-end length of an individual DNA template was set to 1. The histogram exhibited two peaks located at 0.28 ± 0.02 (mean ± standard deviation) (N = 29) and 0.35 ± 0.02 (N = 29) (Figure 3). These observed peaks were in good agreement with the predicted values at 0.27 and 0.35.

Simultaneous Labeling of AP Sites and DNA End. To remove directional ambiguity and to distinguish one AP site located on a DNA segment close to the 5′-terminus from the other on the 3′-terminus, we conjugated biotin to the 5′-end of one DNA single strand. Since biotin was incorporated at the 5′-end as well as the two AP sites, all three sites can be simultaneously labeled

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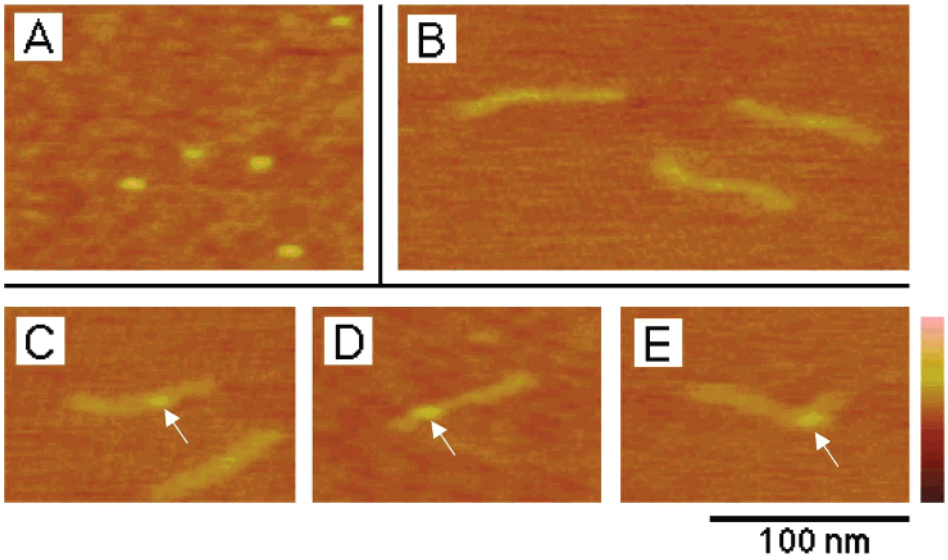

Figure 2. AFM height image of avidin, DNA, and avidin-bound DNA. Height is indicated by a color code with dark (0 nm) and light (3 nm). (A) Monomeric avidin, 16 kDa in mass. (B) Spin-stretched 250-bp DNA templates without any labeling. The mean and the standard deviation of the end-to-end length was measured as 77 ± 5 nm ($N = 30$). (C−E) The avidin−DNA complexes formed after incubation with bio-ARP and monomeric avidin, respectively.

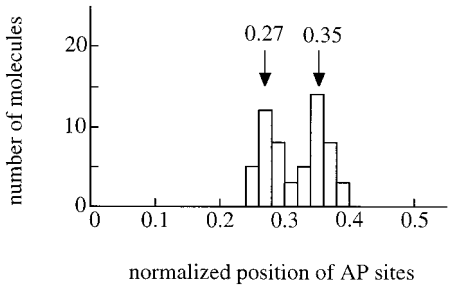

Figure 3. Histogram showing the distribution of bound avidins on the stretched DNA templates. The mean and the standard deviation for the two AP sites were measured as 0.28 ± 0.02 ($N = 29$) and 0.35 ± 0.02 ($N = 29$) in good agreement with the predicted value of 0.27 and 0.35, respectively.

with avidin monomers. AFM was able to detect avidin monomor bound to the DNA end (Figure 4). The efficiency of DNA end-labeling was ~90%, and about one out of four end-labeled DNA molecules exhibited bound avidin at sites close to the two AP sites. The AFM images clearly showed that the AP sites were located away from the 5′-end marked by avidin (Figures 1 and 4). The observed distance between the two AP sites illustrated in Figure 4C was 8 nm, with good agreement to the predicted distance of 7 nm.

DISCUSSION

We describe a rapid and sensitive ARP-mediated method of identifying the position of DNA abasic sites using AFM. For the 250-bp DNA templates, the two AP sites were determined from the site of bound avidins on the uncoiled DNA. The binding of avidin to the two AP sites as well as the biotinylated DNA end was specific in our AFM-based assay. The average difference between the observed AP site and the predicted AP site was within a few nanometers. The standard deviation of the distribution was ~6 bp (~2 nm) in the observed population of 58 bound avidins. A pair of avidins, apparently bound tandemly at the two AP sites separated by 20 bp, was clearly identifiable from a shape resembling a cluster of two globular structures.

AFM is becoming a powerful tool in analyzing individual DNA molecules and human genomics. Useful applications include mapping a recognition site of restriction enzymes, constructing a physical DNA map of protein-binding sites, and identifying base-pair mismatches.[19−22] Unlike conventional molecular tools such as electrophoretic mobility shift assays or DNase footprinting, the AFM-based method allows us to use large DNA templates from a minute amount of sample. DNA molecules over 100 kbp in length can be straightened easily by the stretching apparatus we have developed, and determining sites of bound proteins such as avidin along uncoiled DNA molecules is straightforward. By end-labeling the 5′-terminus or the 3′-terminus of a single DNA strand, we have shown that binding sites of interest can be identified without directional ambiguity. The described AFM-based method allows us to detect abasic sites at a higher resolution than other methods using fluorescent dyes. To make the described method a practical assay tool, avidin at an abasic site should be discriminate from any kink or twist of DNA molecules. Although a resolution is lowered, using a large molecule such as an avidin−gold complex may contribute to reduce false negative signals.

The described AFM-based method allows us to analyze the biochemical reactivity at specific AP sites and to determine efficiency of AP-site labeling. An AP site exists as three tautomeric forms such as an open-chain aldehyde, an open-chain hydrate, and hemiacetals, and it can be opposed to any of the four different bases on its complementary strand. In this study, the open-chain

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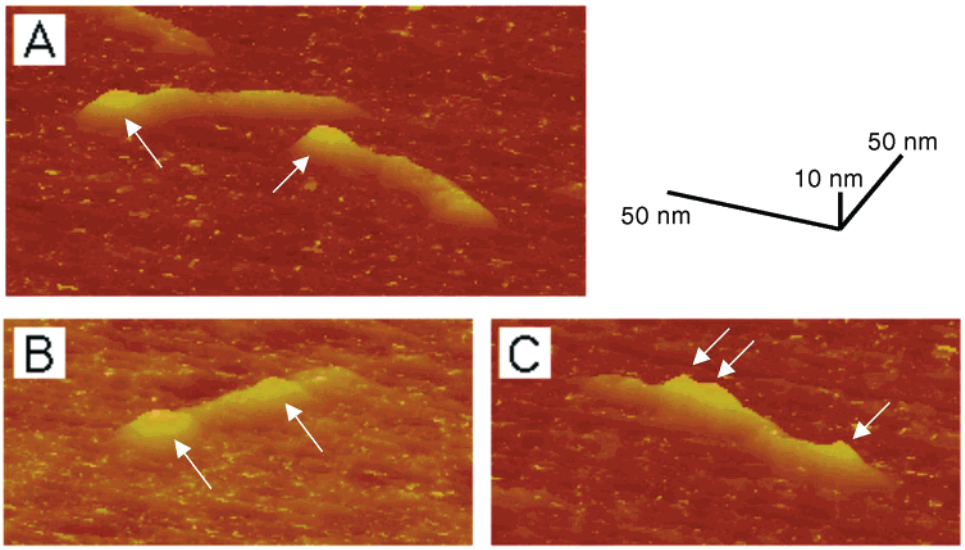

Figure 4. AFM height image of the end-labeled avidin−DNA complexes. To illustrate three-dimensional features, the topographical images are displayed with tilting the mica surface by 20°. The white arrow indicates the bound avidin monomer. (A) DNA templates end-labeled with avidin. (B) End-labeled DNA template. One of the two AP sites was labeled by avidin. (C) End-labeled DNA template. Both of the two AP sites were labeled by avidin.

aldehyde group was targeted and labeled with bio-ARP and the two AP sites were both opposed to an A residue. Because of tautomerization, any AP site takes the aldehyde form ~1% of time and therefore it can be labeled by aldehyde-reactive probes.[15] Alternatively, there is an enzyme that specifically cleaves the AP site and generates a 5′-end base-free deoxyribose phosphate.[23,24] Using the AFM-based detection scheme, it would be interesting to develop a unique AP-site labeling method via an AP-site specific endonuclease.

In conclusion, we have described the AFM-based method of detecting DNA abasic sites mediated by bio-ARP and avidin monomers. Formation of AP sites is a relatively frequent event under physiological conditions. The number and the specific locations of AP sites are reported to vary widely among tissues and age groups, serving as an important indicator of cellular stress, aging, and the effects of radiation therapy.[25−27] The described method provides a rapid and sensitive tool for identifying individual AP sites by placing a protein landmark at a nanometer resolution. This method is expected to complement currently available colorimetric or fluorescence-based assays, most of which are designed to quantify a mean number of AP sites from a population of DNA molecules.

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