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Single-molecule DNA digestion in various alkanethiol-functionalized gold nanopores

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ABSTRACT

This paper presents the alkanethiol-functionalized environmental effects of individual DNA molecules in nanopores on enzyme digestion at the single-molecule level. A template consisting of gold deposited within a solid-state nanoporous polycarbonate membrane was used to trap individual λ-DNA and enzyme molecules. The gold surfaces were modified with various functional groups (–OH, –COOH, $–NH_3$). The enzyme digestion rates of single DNA molecules increased with decreasing nanopore diameters. Surprisingly, the digestion rates in the L-cysteine chemisorbed nanopores were 2.1–2.6 times faster than in the mercaptoethanol chemisorbed gold nanopores, even though these nanopores had equivalent interspacial areas. In addition, the membrane of chemisorbed cysteamine with ionized functional groups of H_3N^+ at pH 8.2 had a greater positive influence on the enzyme digestion rate than the membrane of chemisorbed mercaptoproponic acid with ionized carboxyl groups (COO^-). These results suggest that the three-dimensional environment effect is strongly correlated with the functional group in confined nanopores and can significantly change the enzyme digestion rates for nanopores with different internal areas.

1. Introduction

Most studies of single-molecule reactions have been conducted in free solutions. Single-molecule measurements have revealed information about the kinetic processes and the distribution of molecular properties within a large ensemble [1–5]. Of these single-molecule studies, single-molecule enzymatic assays have attracted the attention of many researchers [6–9]. In previous single-molecule DNA digestion studies, the enzyme hydrolysis of individual nucleotides was analyzed and identified using optical microscopy [10,11]. Depending on the single-molecule technique used, average DNA digestion rates of 12 bp/s [12], 15–20 bp/s [13], and 32 bp/s [14] have been reported in different confined environments with differing electrostatic, hydrophobic, and steric surface properties. However, the effects of a three-dimensional (3-D) environment on these rates at the single-molecule level are still not well understood.

The manipulation and control of molecules or ions through the use of nanoporous structures has been utilized in DNA translocation studies and in ultra-fast genome sequencing analysis [15–17]. In particular, the reaction mechanism of enzymatic DNA degradation in a confined environment is a key issue in the field of nanopore technology [18–20].

The goal of this work is to investigate the three-dimensional environmental effects of chemicals with various functional groups chemisorbed by nanopores on single-molecule DNA digestions. Specifically, alkanethiols ($HS–(CH_2)_n–R$), which have a thiol group on one end, spontaneously form bonds within gold (Au) nanopores. This spontaneous reaction organizes chains on the surface in a well-defined pattern with the variable head group (R) forming the surface of the monolayer. Therefore, alkanethiols with different terminal R-groups are important in determining the functionality and properties of the monolayer. The chemical environment within the Au nanopores can be altered by the chemisorption of mercaptoethanol (ME), cysteine, mercaptopropioinc acid (MPA), and cysteamine to the inner walls of the pores. The enzymatic digestions of DNA molecules trapped in the pores through interaction with the hydrophilic tail group (–OH, –COOH, $–NH_3$) were detected using the first optical imaging system to integrate total internal reflection fluorescence microscopy (TIRFM) and real-time confocal microscopy (RT-CM). This system was developed in our laboratory [21]. The activity of enzymatic digestion of DNA was analyzed at the single-molecule level by measuring the decreasing relative fluorescence intensity of fluorescent dye (YOYO-1)–DNA. In addition, the relationship between the digestion rate of DNA and the pore functional group was studied by varying the buffer solution pH using L-cysteine

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chemisorbed polycarbonate (PC)/Au nanopores. The results demonstrated for the first time that individual DNA molecules had different enzyme digestion rates depending on the reaction conditions and the 3-D environment.

2. Materials and methods

2.1. Reagents and materials

Various track-etched PC membrane filters (Sterlitech Co., Kent, WA, USA), with various pore sizes (ϕ=200 nm–5.0 μm) and thicknesses (9–11 μm), were used as the synthetic pores. The λ-DNA (M_w=48,502 bp) was obtained from Promega (Madison, WI, USA). The λ-exonuclease enzyme (M_w=28 kDa, pI=5.5) was obtained from New England BioLabs (Ipswich, MA, USA). The H_2SO_4 and $SnCl_2$ were obtained from Fisher Scientific (Rockford, IL, USA). The trifluoroacetic acid, $AgNO_3$, Na_2SO_3, $NaHCO_3$, 37% formaldehyde, 99% mercaptoethanol (ME), 99% 3-mercaptopropinoic acid (MPA), and 98% cysteamine were obtained from Sigma-Aldrich. The commercial gold-plating solution ($Na_3Au(SO_3)_2$: Oromerse SO Part B) and 99% L-cysteine hydrochloride were obtained from Technic Inc. (Cranston, RI, USA) and TCI (Tokyo, Japan), respectively.

2.2. Preparation of samples

The λ-exonuclease (0.2 μg/mL) enzyme was diluted in a 1 × reaction buffer (pH 9.4, 2.5 mM of $MgCl_2$, 67 mM of glycine–KOH, 50 μg/mL of BSA). The λ-DNA was labeled with the YOYO-1 (Molecular Probes, Eugene, OR) intercalator at a molar ratio of 1:50 to minimize the photo-cleavage effect for the dye to nucleotide pairs (base pairs) in a 10 mM Gly–Gly buffer solution (pH 8.2) prior to enzyme digestion in a porous chamber. The DNA sample was further diluted to 1 pM using a suitable buffer solution (a 10 mM Gly–Gly solution buffered at pH 8.2 or a 20 mM sodium phosphate solution buffered at pH 2, 6, and 10) before the trapping process. All buffer solutions were filtered through a 0.2 μm membrane filter prior to use.

2.3. Electroless gold deposition and chemisorption

Gold was deposited in the pores and on the face of the PC template membrane using an electroless deposition method similar to that described previously [22]. The PC membrane was immersed in methanol for 5 min and then immersed in a solution of 0.026 M $SnCl_2$ and 0.07 M trifluoroacetic acid in 50% methanol for 45 min. The membrane was washed in methanol two consecutive times for 2.5 min and was then immersed in an aqueous ammoniacal $AgNO_3$ solution (0.035 M) for 5 min. After washing by immersion in water for 5 min, the membrane was placed in a Au-plating bath containing 7.7 mM $Na_3Au(SO_3)_2$, 0.127 M Na_2SO_3, 0.625 M formaldehyde, and 0.025 M $NaHCO_3$ (pH was adjusted to 10 by the dropwise addition of 0.5 M H_2SO_4) with constant stirring. The temperature of this bath was maintained at 2 °C for 12 h. The Au-deposited membranes were washed with water for 5 min and then dried at room temperature (Fig. 1A).

Chemical solutions were chemisorbed to the pore walls in order to change the chemical environment of the pore surface. L-cysteine chemisorption was accomplished by immersing the Au-deposited PC membranes in 2 mM L-cysteine hydrochloride in 80% ethanol for 24 h (Fig. 1A). The membrane was then rinsed with ethanol and dried in air. Thiol chemisorption was accomplished by immersing the Au-deposited PC membranes in a solution of 1 mM ME, 1 mM MPA, and cysteamine in absolute ethanol for 24 h (Fig. 1A). The membrane was then rinsed with ethanol and dried in air.

2.4. Characterization of Au nanoporous membrane filters

Field emission scanning electron microscope (FE-SEM) measurements were performed using a SUPRA 55 (Carl Zeiss) microscope in the secondary electron image mode in order to measure the membrane filter surface and pore diameter. The various functional groups of the Au deposited within the pores were confirmed by Fourier transform infrared (FT-IR) spectroscopy with an advanced grazing angle (PIKE Technologies Inc., Madison, WI, USA). All measurements were performed at room temperature.

2.5. Trapping and digestion of individual DNA molecules

Note that thiols with hydrophobic properties, propanethiol, decanethiol, and hexadecanethiol, chemisorbed to the inside of nanopores do not permit trapping of hydrophilic species (i.e., buffer solution, DNA, and enzyme molecules). However, the ME and L-cysteine chemisorbed PC/Au membranes were hydrophilic with wetting characteristics that helped the filters stick to the surface of the cover glass (22 × 60 mm) (Fig. S1, Electronic Supplementary Information). A suitable buffer solution (pH 2, 6, 8.2, and 10) was added to the center of the membrane filter on the cover glass such that it perfectly adhered to the surface. The cover glass was matched with immersion oil (n=1.78, Cargille Labs, NJ, USA) on the objective lens made for total internal reflection fluorescence (TIRF). Individual DNA molecules were easily trapped and digested by the following procedure. First, 2 μL of DNA diluted to 1 pM using the appropriate buffer solution was added to the nanopore chambers. Subsequently, a 0.2 μg/mL enzyme in a 1 × reaction buffer solution was added to the region between the chemisorbed PC/Au membrane and the cover glass (ϕ=12 mm) using capillary force. The λ-exonuclease enzyme digestion rate was measured from the integrated TIRF and confocal fluorescence intensity of a single coiled DNA in the nanopore chamber at 37 °C (Fig. 2B). The homogeneity and purity of the λ-exonuclease was confirmed by slab gel electrophoresis (Fig. S2, Electronic Supplementary Information).

2.6. Optical imaging system integrated by TIRFM and RT-CM

A modified integrated optical imaging system [21] was used for real-time detection of single molecules. Construction of the hybrid TIRFM and RT-CM with a conventional epifluorescence microscope is shown in Fig. 2A. An Olympus IX71 inverted microscope (Olympus Optical, Tokyo, Japan), coupled with a Plan Apo 100 × /1.65 HR objective lens (W.D.=0.10 mm, oil type, Olympus), used transmitted light to visualize the morphology of the nanoporous chamber. A 473 nm laser (SL-473 nm-50T, Shanghai Laser Century Technology Co., Ltd., China) was used as the light source for TIRFM studies. The filter cube was composed of 520/15 nm bandpass filters (Olympus). The optical system was integrated with the confocal system by mounting confocal scanners (CSU 22, Yokogawa Electric Co., Tokyo, Japan) on the inverted microscope platform of an Olympus IX71. A 488 nm argon ion laser (35-LAB-431-220, Melles Griot) was used as the excitation source for the real-time confocal studies. A cooled charge-coupled device (CCD) camera (Cascade 512B, Photometrics, Tucson, AZ, USA) was mounted on the microscope confocal scanner. The CCD exposure time was 100 ms. The temperature of the membrane pores was maintained at 37 °C using a temperature controller (CU-105, Live Cell Instruments, Seoul, Korea) that was placed on the microscope stage for the enzyme reactions. To reduce photobleaching, a UNIBLITZ mechanical shutter (model LS3Z2, Vincent Associates, Rochester, NY, USA) with a VCM-D1 shutter driver (Vincent Associates) was used to block the laser beam when the