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Enzyme digestion of entrapped single-DNA molecules in nanopores

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ABSTRACT

The real-time digestion of entrapped single-DNA molecules by λ-exonuclease in nanoporous alumina membranes was observed using an epifluorescence microscope. The alumina membrane provides pL ($\sim 10^{-12}$ L) containers for confining single-DNA molecules without immobilization. When one end of the DNA molecule was inserted into a nanopore, it was possible to monitor the digestion process outside, near and inside the pore, where the individual DNA molecules exhibited different characteristic digestion modes. The digestion rates calculated from the decrease in fluorescence intensity showed different values according to the location of the individual molecules. Entrapment rather than immobilization allows the DNA strand to be fully exposed to the enzyme and the reaction buffer. These results confirm that the enzymatic digestion of DNA molecules is affected by their three-dimensional (3D) environment.

1. Introduction

Ultra-sensitive detection and real-time observation are among the most important techniques for examining the behaviors of individual molecules, such as their conformational changes, dynamics or reactivity. Such studies allow much more detailed information to be gained than by performing ensemble-averaged measurements. Many biological applications of single-molecule detection (SMD) have been demonstrated [1–12]. In particular, in studies of enzyme activity, single-molecule measurements have been invaluable because the various conformers do not interconvert very quickly [13–20]. It was also demonstrated at single-molecule level that highly purified enzyme molecules have been shown to have identical activity [21].

Enzyme molecules have been interrogated by using SMD in various environments, such as on the surface [22,23] and in liquids [24,25]. The entrapment of enzyme molecules using nanoporous membranes as a solid support holds great promise as a single-molecule analytical technique and as a biophysical model system [26,27]. Artificial membranes including porous silica [28], polymers [29,30] and alumina [31] are known to be thermally and mechanically stable, non-toxic, and highly resistant against microbes and organic solvents [32] compared to their biological counterparts. Such nano-size membranes in the form of a uniform array have the potential to be used for the separation of molecules according to their size [33,34] as well as for enzymatic catalysis [35,36]. Although many studies of enzymes have been carried out using solid-state nanopores, until now they have been limited to the immobilization of the enzyme or the deposition of the desired reagents within the pores.

Herein, we investigate the kinetic of the direct enzymatic digestion of entrapped single-DNA molecules by λ-exonuclease using a nanoporous alumina membrane, prepared *via* the electrochemical modification (anodization) of high purity alumina. One major difference to previous studies [37,38] is that the trapping of the single-DNA molecule inside the nanopore does not require an optical force or an electric field. Unlike surface combing, entrapment also means that a substantial part, if not all, of the DNA strand remain mobile and fully exposed to the reaction buffer [33]. Single-DNA molecules diffuse and randomly reside inside the pores at different depths. The location of the molecule, whether it is caught inside the pore or stays outside the pore, has a potent influence on the digestion rate due to the different local environments. Therefore, the exploration of enzyme digestion of single-DNA molecules using nanopores opens the way for probing the effect of the 3D environment on the digestion rates, including various steric, hydrophobic and electrostatic interactions.

2. Materials and methods

2.1. Reagents and materials

An alumina membrane filter (Whatman International Ltd., Maidstone, UK; 13 mm diameter, 60 μm thick, pore

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diameter = 200 nm, porosity = 75%) [39] was used to provide inorganic pores that tend to be neutral. λ-DNA (M_w = 48 502 bp) and circular-ΦX174 RF DNA (M_w = 5386 bp) were obtained from Promega (Madison, WI). The circular-ΦX174 RF DNA molecules were cleaved to produce linear-ΦX174 RF DNA molecules using a one-cut restriction enzyme (RE) according to the digestion conditions recommended by the Restriction Enzyme Usage Information of Promega. 0.1% poly-*l*-lysine (PLL, M_w = 150 000–300 000) solution and cetyltrimethylammonium bromide (CTAB) were supplied by Sigma–Aldrich (St. Louis, MO). λ-Exonuclease enzyme (M_w = 28 kDa; pK_a = 5.29) was obtained from New England BioLabs (Ipswich, MA).

2.2. Preparation of samples

The λ-exonuclease enzyme (0.2 μg/mL) was diluted with a 1× reaction buffer (pH 9.4, 2.5 mM $MgCl_2$, 67 mM glycine–KOH, 50 μg/mL BSA). The DNA sample was labeled with an intercalator, YOYO-1 (Molecular Probes, Eugene, OR) at a molar ratio of one dye molecule per 50 base pairs in 10 mM Gly–Gly buffer solution (pH 8.2) and then diluted to 1 pM in 25 mM CHES buffer (pH 10.5) for single-molecule imaging. Prior to their use, all buffer solutions were filtered through a 0.2-μm membrane filter.

2.3. Cationic-modification of substrate

To facilitate the entrapment of the biomolecules into the alumina pores, a glass slide was modified by immersing it in 2.0 mM cationic-CTAB surfactant at a concentration greater than the critical micellar concentration (CMC) of 0.9–1.1 mM [40] at 25 °C for 1 h. The poly-*l*-lysine (PLL)-coated glass was produced by immersing a glass slide into a 0.1% PLL solution at 25 °C for 1 h.

2.4. Entrapment and digestion of single-DNA molecule

Alumina membrane filters with a neutral charge and hydrophilic in nature (contact angle = 14.4°) were employed to facilitate their sticking to the surface of the bare- and cationic-modified glasses. The nanoporous wells were formed by attaching the membrane filters to the bare- or cationic-modified glass slide. An 8 μL volume of 1× exonuclease reaction buffer solution (pH 9.4) was dropped at the center of the membrane filter to achieve perfect wetting. Subsequently, 4 μL of 1 pM DNA was dropped onto the same area. The individual DNA molecules placed between the alumina sheet and the cover slip randomly approached (i.e., due to Brownian movement) or were sometimes entrapped on the surface of the alumina membrane (Fig. 1A). The cover slip was matched with an immersion oil (Immersol™ 518F, Zeiss, n = 1.518) for the observation of the epifluorescence image. After confirming that one end of the single-DNA molecule is still mobile while the other end is entrapped inside the pore (Electronic Supplementary Information, M1), 0.2 μg/mL enzyme in 1× reaction buffer solution was injected onto the membrane filter from the edge by using capillary force. The digestion rates of the individual molecules by λ-exonuclease were calculated from the decrease in the relative fluorescence intensity of the entrapped DNA recorded at 37 °C by using an epifluorescence microscope (Fig. 1A).

2.5. Single-molecule measurements by epifluorescence microscopy

The epifluorescence experiments were accomplished using a Zeiss Axioskop50 upright microscope (Zeiss, Germany) coupled with a Pentamax 512-EFT/1EIA ICCD camera (Princeton Instruments, Princeton, NJ). Light from a Hg lamp (power 40 W; AttoArc™ Instrumental, Rockville, MD) was used as the excitation source. A filter cube composed of an excitation filter (460–500 nm), a 50-nm wide bandpass filter centered at 535 nm and a dichroic beam splitter (DM505) was used for the detection of the DNA labeled with YOYO-1. Another filter cube composed of an excitation filter (540 nm), a 40-nm wide band-pass filter centered at 585 nm and a dichroic beam splitter (DM575) was used for the detection of the enzyme labeled with Alexa Fluor® 532. A dichroic beam splitter was used to reflect the light into the back aperture of the objective lens (EC Plan-NEOFLUAR, Zeiss 100×/1.3 N.A., W.D. = 0.2 mm, oil type). To reduce photobleaching, the beam was transmitted through an Uniblitz mechanical shutter (model LS2Z2, Vincent Associates, Rochester, NY) with a VMM-D1 shutter driver (model T132, Vincent Associates). The sampling frequency was 5 Hz with the shutter driver set to an exposure of 10 ms and a delay of 190 ms. The temperature was maintained at 37 °C by using a temperature controller (FRYER, A-50). The single-molecule fluorescence images were collected using WinView/32™ (Version 2.5.14.1, Princeton Instruments) and analyzed using Image J 1.41n software.

3. Results and discussion

3.1. Environment of nanoporous alumina membranes

Fig. 1B shows the scanning electron microscope (Amray 1845FE-SEM, USA) image of the regularly arrayed and uniform (diameter = 200 nm) cylindrical tubes of the alumina membranes used as the containers. The membrane charge is one of the most important factors affecting the interaction between the molecules and the membrane. Furthermore, it is well known that the charge of alumina depends on the pH, due to its being an amphoteric material with an isoelectric point (pI) [41]. Ma and Yeung showed that alumina membranes have a low affinity for DNA at pH 9.0–10 [33]. In this study, we monitored the digestion of DNA by the enzyme in 1× exonuclease reaction buffer (pH 9.4) to minimize any electrostatic effect between the nanoporous membrane and the DNA molecules.

3.2. Surfaces with neutral and cationic charges

To increase the interaction between the DNA molecules and the surface, the bare glass was modified by immersing it in CTAB solution as a cationic-surfactant or in PLL solution. The DNA molecules (pH 10.5) moved randomly on the bare glass slide (Fig. 2A, arrow), but was adsorbed in the linear form on the two cationic-modified glass substrates due to electrostatic force, as shown in Fig. 2B and C, respectively. However, the environment created by putting the nanoporous alumina membrane on the cationic-modified glass substrate did not allow the molecules to be trapped inside the nanopore (Fig. 2D and E, arrows). Therefore, as a substrate, we used a bare glass slide without any chemical modification. In this way the electrostatic force between the slide and the DNA inside the alumina pore (60 μm in depth) was relatively weak to allow entrapment.

3.3. Steric effect of alumina nanoporous membrane

Most of the DNA molecules moved randomly on the alumina membrane surface under bulk flow. By comparing λ-DNA molecules with a different size DNA (5.4 kb) molecules in both the neutral and cationic modified glass environments, we found that the electrostatic force on the DNA molecules was low, regardless of their size, under the same conditions as those indicated in Fig. 2. Although some 5.4 kb-DNA (1.84 μm) molecules were adsorbed on the cationic-modified glass slide (Fig. 3B and C), most of the 5.4 kb-DNA molecules moved randomly on the alumina surface