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ORIGINAL PAPER

# The Effect of Physical Form of DNA on ExonucleaseIII Activity Revealed by Single-molecule Observations

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**Abstract** Single-molecule studies have revealed molecular behaviors usually hidden in the ensemble and time averaging of bulk experiments. Single-molecule measurement that can control physical form of individual DNA molecules is a powerful method to obtain new knowledge about correlation between DNA-tension and enzyme activity. Here we study the effect of physical form of DNA on exonucleaseIII (ExoIII) reaction. ExoIII has a double-stranded DNA specific 3′→5′ exonuclease activity and the digestion is distributive. We observed the ExoIII digestion of individual stretched DNA molecules from the free ends. The sequentially captured photographs demonstrated that the digested DNA molecule linearly shortened with the reaction time. We also carried out the single-molecule observation under random coiled form by pausing the buffer flow. The digestion rates obtained from both single-molecule experiments showed that the digestion rate under the stretched condition was two times higher than the random coiled condition. The correlation between physical form of DNA and digestion rate of ExoIII was clearly demonstrated by single-molecule observations.

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## Introduction

Single-molecule fluorescent observations of DNA–protein interactions have been investigated by many researchers. These studies show that single-molecule techniques are effective in analyzing DNA–protein interactions. Especially, real-time fluorescent observation assists direct understanding of phenomena, as DNA–protein interactions can be directly detected by fluorescent labeling for proteins and DNA. For example, sliding motion of fluorescent-labeled RNA polymerases on double-stranded DNA (dsDNA) was observed [1, 2], and dsDNA hydrolysis by RecBCD helicase [3–5] was analyzed using a single-molecule real-time fluorescent imaging system.

Stretching DNA molecules is a useful method in a single-molecule DNA observation system. Because DNA molecules takes a random coiled conformation in solution by Brownian motion, single-molecule manipulation of DNA is essential to measure the length of individual fluorescent-stained DNA and position of fluorescent-labeled proteins. DNA stretching methods using a flow of solution [3–8] or electrostatic force [9, 10] are widely used. Optical trapping is also a powerful technique [3–6, 11, 12] in single-molecule measurements. These techniques have been also used on studies of elasticity of DNA [13, 14]. In addition, single-molecule DNA synthesis [15, 16] and polymerization of RecA proteins on an individual dsDNA molecule [17] are analyzed by monitoring elasticity of an individual DNA.

In recent years, the correlations of tension on DNA with enzymatic reaction have been studied using these techniques. For example, the effect of template tension on DNA polymerase activity [15, 18] and the DNA-tension dependence on restriction enzyme activity [19, 20] were reported. In addition, Kleckner and his coworkers showed that chromatin function is governed by internal mechanical forces obtained from *in vivo* study [21]. Thus these studies suggested the possibility that DNA form is involved in regulation of biochemical reaction in cell.

In this report, we describe single-molecule DNA digestion by *E. coli* exonucleaseIII (ExoIII) using fluorescent microscopy. ExoIII is a well-known DNA digestive enzyme, and the property has been well studied [22–25]. ExoIII is a 3′→5′ dsDNA-specific exonuclease. Therefore, it is possible to digest from the blunt end, 3′-recessed end and nicking sites, but it is impossible to digest from the 3′-protruding end. The final product is single-stranded DNA and 5′-P mononucleotides. Salt concentration and temperature both significantly affect ExoIII activity [23]. At 23–28 °C, the exonucleolytic digestion is processive, and under saturating enzyme concentrations at this temperature, 5′ mononucleotides are removed at a rate of ~100 nucleotides/min/3′end. At 37 °C, the digestion is distributive, and digestion rate is ~400 nucleotides/min/3′end at saturating enzyme concentrations [25].

In our previous report, we observed single-molecule DNA digestion by ExoIII using two-layer micro-channel and optical trapping [26]. The observed digestion rate from the single-molecule experiment was much faster than bulk experiments. It is interesting what caused the difference in the digestion rates between single-molecule experiments and bulk experiments. We supposed that the physical form of DNA molecules affected digestion by ExoIII. In this paper, "physical form of DNA" means a higher-order conformation of DNA (stretched form or random coiled form). In bulk experiments, all DNA molecules are in random coil structure and always change their shape randomly. On the other hand, individual DNA molecules were stretched by buffer flow in our previous single-molecule experiment. We deduce that the physical form of DNA may affect three elementary steps in DNA digestive reaction by ExoIII; (1) ExoIII association to DNA, (2) digestion of DNA by ExoIII (digestion rate) and (3) ExoIII dissociation from DNA (processivity). Although we cannot control the physical form of individual DNA in test tubes, the effect of DNA tension on ExoIII activity can be demonstrated only by single-molecule experiments that can control tension of DNA.

In this report, we demonstrated that the physical form of DNA affects DNA digestion of ExoIII by single-molecule experiments. We immobilized one-ends of fluorescently stained DNA on a glass surface, and then they were digested by ExoIII under both conditions: stretched state by flow and relaxed state. The sequentially captured photographs demonstrated that the digested DNA molecules were linearly shortened with the reaction time. The digestion rate obtained from the stretched condition was two times higher than the relaxed condition.

## Materials and methods

### Preparation of DNA substrate for bulk assay

DNA substrates for bulk assay were prepared as follows: 100 ml cultures of *E. coli* DH5α carrying pUC19 DNA (2686 base pairs (bp)) were incubated in CircleGrow media (Qbiogene) with 50 μg/ml of ampicillin. The culture was harvested and the plasmid was extracted and purified by QIAGEN Plasmid Maxi Kit (QIAGEN). Purified pUC19 DNA was resuspended in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) and the concentration was determined using $A_{260}$. The purified pUC19 DNA was linearized by restriction with BamHI (TaKaRa) and then purified by phenol/chloroform extraction and ethanol precipitation. The DNA was resuspended in TE buffer. The concentration of DNA was also determined using $A_{260}$.

### Electrophoretic analysis for bulk assay

The effects of the fluorescent dye and anti-fade reagents on DNA digestion by ExoIII should be evaluated prior to single-molecule observation. In this assay, we used the linearized pUC19 plasmid DNA. Both ends of the linearized pUC19 were 3′-recessed end. DNA molecules were stained with the bis-intercalating fluorescent dye YOYO-1 iodide (Y-3601; Invitrogen), which is used for fluorescent observation of DNA in the single-molecule experiment. The ratio of dye:nucleotide base pair was 1:20. The stained DNA molecules (920 ng) were incubated in 50 mM Tris–HCl pH 8.0, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 90 U ExoIII (0.9 U/μl) (TaKaRa) and anti-fade reagents (2.3 mg/ml D-(+)-glucose, 0.1 mg/ml glucose oxidase, 18 μg/ml catalase) at 25 °C in 100 μl. Portions of 12 μl were recovered from the sample in intervals of 3 min and immediately mixed with 3 μl of 0.5 M EDTA and 3 μl of a gel loading buffer consisting of 0.3 M NaOH, 6 mM EDTA and 18% Ficoll Type 400. The digestion products were analyzed by 1.2% denatured agarose gel electrophoresis in 50 mM NaOH and 10 mM EDTA. The final products consisted of both double-stranded region and single-stranded region. Denatured agarose gel electrophoresis was carried out to estimate the length of both regions. After the gel electrophoresis, the gel was neutralized with the buffer consisting of 1 M Tris–HCl

pH 7.6 and 1.5 M NaCl. The neutralized gel was stained with SYBR green II (Invitrogen) according to the manufacturer's manual and detected by variable image analyzer (Typhoon9400; GE Healthcare Bioscience).

## Fabrication of flow cell for single-molecule observation

Single-molecule experiments were performed with a flow cell, which was made of a poly (dimethylsiloxane) (PDMS)-sealed coverslip. Design of the flow cell is shown in Fig. 1. The coverslip was treated with dichlorodimethylsilane for one-end immobilization of individual thiol-modified DNA molecules [10]. The hydrophobic coverslip was also prepared as described previously [10]. PDMS was prepared as our previous report [26] with some modifications. The ratio of base polymer:curing reagent was 10:1. Small holes (3 mm in diameter) were drilled into the PDMS using a borer to produce an inlet and an outlet. The inlet port (NanoPort N-124S, Upchurch) was bonded on the hole using silicone adhesive (Shin-etsu chemical). A PEEK tube (I.D. 100 μm, O.D. 360 μm, 1571–12X, Upchurch) was used for connecting the inlet port and 1 ml syringe (Hamilton). The syringe was controlled by a syringe pump (Kd-scientific).

## Thiol-modification of λ DNA for single-molecule observation

Thiol-modification of DNA molecules was carried out as our previous report [10]. 0.4 pmol (12.6 μg) of λ phage DNA (48,502 bp; Nippon gene) and 0.4 nmol of 27 mer oligonucleotides modified with a phosphate group and disulfide chain at 5′-end and 3′-end (5′ P-GGGCGGC GACCTAGATAGGACACTACG 3′-$(CH_2)_3$-S-S-$(CH_2)_3$-OH), respectively, were mixed in ligation buffer (66 mM Tris–HCl pH 7.6, 6.6 mM $MgCl_2$, 10 mM Dithiothreitol (DTT), 0.1 mM ATP). The solution was incubated for 5 min at 65 °C and rapidly cooled on ice to prevent linking among λ DNA molecules. One recessed end of λ DNA was linked specifically to the two oligo DNA by overnight incubation at 16 °C in the ligation buffer containing 15.6 U/μl (final concentration) of T4 DNA ligase (TaKaRa). The DNA solution was then incubated for 10 min at 65 °C to inactivate T4 DNA ligase. The sample was then purified by gel filtration column (CHROMASPIN-S1000; clontech) to remove the free oligo DNA.

The disulfided λ DNA was reduced with 40 mM DTT in 0.17 M sodium phosphate buffer (pH 8.0) for 16 h at 25 °C to convert the disulfide into sulfhydryl group. Finally, the DNA sample was purified by removing DTT using gel filtration column (NAP-5 Column; GE Healthcare) equilibrated with TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). The concentration of the thiol-modified λ DNA solution was determined by absorbance of 260 nm. The sample was stored at −20 °C.

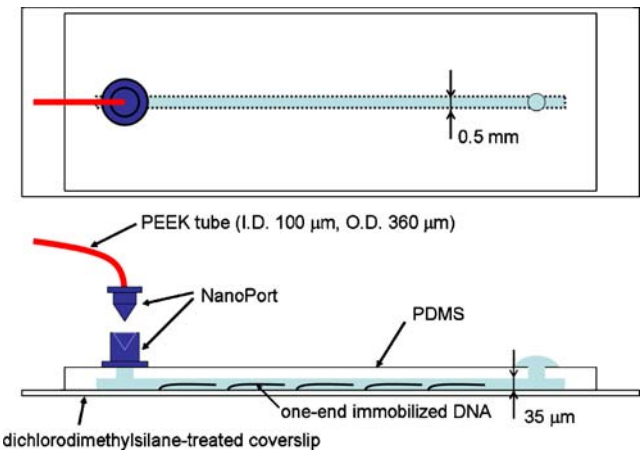


**Fig. 1** Top-view and side-view of the flow cell for single-molecule observation of DNA digestion by ExoIII. The flow cell was made of poly (dimethylsiloxane) (PDMS)-sealed coverslip. The coverslip was treated with dichlorodimethylsilane for one-end immobilization of thiol-modified DNA molecules. The inlet port was bonded on the hole using silicone adhesive. A PEEK tube (I.D. 100 μm, O.D. 360 μm) was used for connecting the inlet port and 1 ml syringe. The syringe was controlled by a syringe pump

## Optical setup

DNA molecules were observed using a fluorescence microscope (ECLIPUSE TE-2000U; Nikon) equipped with a 100×, 1.4 numerical aperture (NA) oil immersion objective lens (PlanApo; Nikon). To produce sharp fluorescent images, we used a white-light TIRF (Total Internal Reflection Fluorescence) system (Nikon). The white-light TIRF system enables TIRF microscopy using a mercury lamp. The TIRF illumination system was mounted on the TE-2000U microscope. The excitation light and emission light were selected with a filter set GFP-B (suitable for Green Fluorescent Protein (GFP), EX480/40, DM505, and EM535/50) purchased from Nikon. Fluorescent images of DNA molecules stained with YOYO-1 (excitation 491 nm, emission 509 nm) were visualized by a high sensitivity EB-CCD camera (C9100-43; Hamamatsu Photonics) and recorded with an AQUACOSMOS imaging system (Hamamatsu Photonics). The length of individual DNA molecules was also measured using the length measurement tool of the AQUACOSMOS.

## Single-molecule DNA digestion by exonucleaseIII

Our single-molecule experiment for measuring DNA digestion is shown schematically in Fig. 2. First, the thiol-modified λ DNA molecules (0.28 μg) were stained with YOYO-1 in 50 mM Tris–HCl pH 8.0, 1 mM $MgCl_2$,

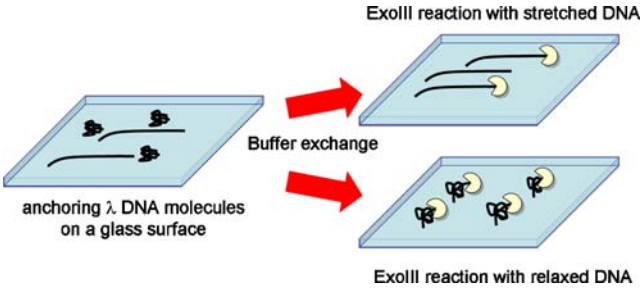


**Fig. 2** Schematic of the single DNA digestion observation. One-ends of DNA are anchored on a glass surface. For the stretched state, the single-molecule observation is performed with the flow of reaction buffer. For the relaxed state, in contrast, the observation is performed without buffer flow. When we capture the fluorescent image of DNA, the buffer flow is applied for a few seconds. After the recording, the flow is stopped, and then the immobilized DNA becomes random coiled state

10 mM 2-mercaptoethanol, 0.21 μM YOYO-1 and the anti-fade reagents for at least 1 h at a dye/bp ratio of 1/20. The solution containing the DNA stained with YOYO-1 was introduced to the flow channel at the flow rate of 5 μl/h (linear flow rate of 80–100 μm/s) for 1 h. The end of the thiol-modified λ DNA molecules were immobilized on the glass surface during the slow speed injection of the solution. After immobilization of λ DNA, ExoIII reaction buffer (50 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, the anti-fade reagents and 0.9 U/μl ExoIII) was injected into the flow cell at the flow rate of 105 μl/h (linear flow rate of 1.67 mm/s). At the same time, unimmobilized DNA molecules were removed and the entire chamber was uniformly filled with enzyme solution. The buffer exchange was directly monitored by observing high fluorescent intensity of unimmobilized DNA. The reaction buffer extremely reduces background fluorescence from unimmobilized DNA, in contrast to the DNA immobilization buffer.

After the injection of ExoIII, we started single-molecule observation of DNA digestion. The single-molecule experiments were carried out with the immobilized DNA either held stretched by buffer flow or relaxed (Fig. 2). For the stretched state, the single-molecule observation was performed with the flow of reaction buffer at the flow rate of 105 μl/h. The fluorescent images were captured every 1 min. For the relaxed state, in contrast, the observation was performed without buffer flow. When we captured the fluorescent image of DNA, the buffer flow was applied for a few seconds at the flow rate of 105 μl/h. After the recording, the flow was stopped, and then the immobilized DNA became random coiled state. This procedure was continued every 10 min until 50 min had elapsed. We used a lens heater (MATS-LH, Tokai Hit) to control temperature of the reaction. All single-molecule experiments were carried out at 25 °C.

## Results and discussion

Figure 3 shows the result of denatured agarose gel electrophoresis of the bulk experiment. In the "Control" reaction, the reaction buffer did not contain the fluorescent dye YOYO-1 and the anti-fade reagents. In addition, this reaction was performed in the presence of 5 mM $MgCl_2$ as recommended by the supplier's instruction. ExoIII reactions with YOYO-1 and the anti-fade reagents were carried out under 1 mM and 5 mM of $MgCl_2$ because higher concentration of $Mg^{2+}$ prevents fluorescent observation of single-molecule DNA. All the bands migrated faster with increasing the reaction time. The band patterns of the gel electrophoresis were almost the same between the control and that containing the fluorescent dye and the anti-fade reagents. In addition, fluorescent intensities of each band decreased with the reaction time. The estimated digestion rates of these reactions were 60–100 nt./min. This result shows that DNA digestion by ExoIII is not interfered with by the addition of the fluorescent dye and anti-fade reagents under this condition. For the real-time observation of ExoIII reactions with individual DNA, this condition was used.

The results of single-molecule observations are shown in Fig. 4. Sequential photographs from typical reactions in the absence and presence of ExoIII are shown in Fig. 4A and B, respectively. Individual DNA molecules were stretched by the buffer flow during these reactions. These photographs were captured every 1 min by opening a shutter of

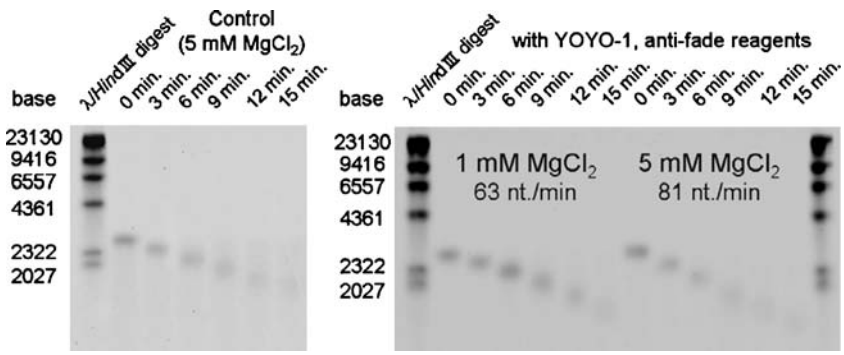


**Fig. 3** Denatured agarose gel electrophoresis. The lanes indicated "*control*" contain no fluorescent dye and anti-fade reagents. The lanes "*with YOYO-1, anti-fade reagents*" are reaction products containing the dye at a ratio of dye/bp=1/20. 2.3 mg/ml D-(+)-glucose, 0.1 mg/ml glucose oxidase and 18 μg/ml catalase were contained as anti-fade reagents. Both concentration of 1 mM and 5 mM $MgCl_2$ were carried out

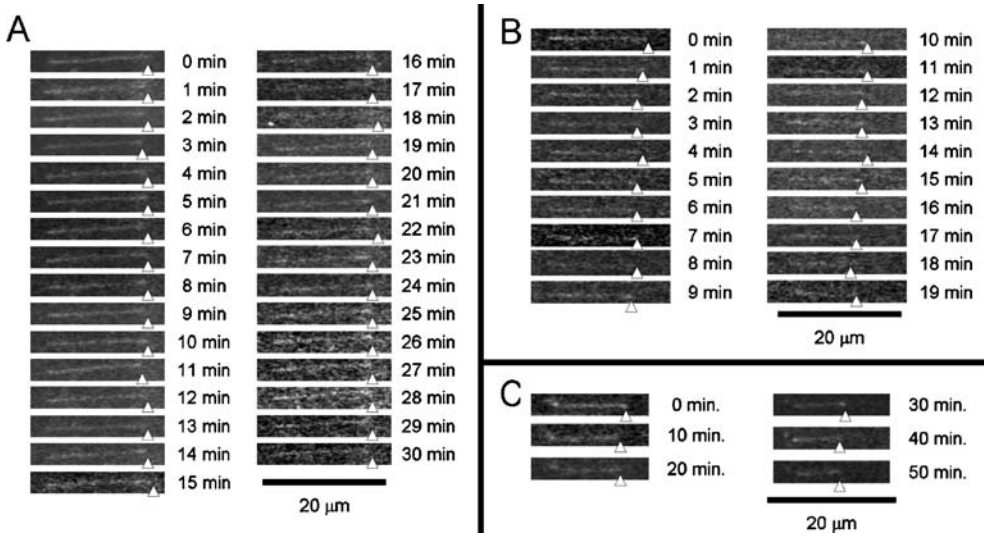


**Fig. 4** Sequential photographs of the real-time observation of DNA digestion by exonuclease III. **a** In absence of ExoIII with buffer flow, **b** in presence of ExoIII with buffer flow, **c** in presence of ExoIII without buffer flow. "*White triangle*" shows the position of free-end of stretched DNA

excitation light. Figure 4C shows the result of ExoIII reaction without buffer flow. These photographs were captured every 10 min, and DNA molecules were stretched by buffer flow only during the capturing. One-end immobilized DNA molecules on a coverglass were stretched near the surface. Although fluorescent dye YOYO-1 is not a perfect dye for the long time observation, the TIRF illumination system produced sharp fluorescence images with a high signal-to-noise ratio.

In the absence of ExoIII (Fig. 4A), the length of DNA during the reaction was almost constant with a fluctuation less than 2.0 μm. In contrast, in the presence of ExoIII under both conditions of stretched or relaxed DNA, DNA shortened with elapsed time (Fig. 4B and C). Figure 5 shows the time course of length of the digested DNA molecules shown in Fig. 4. In the reaction for stretched DNA, the observed rate of DNA shortening was 0.23 μm/min. This rate was different from that for the relaxed DNA, which was 0.11 μm/min.

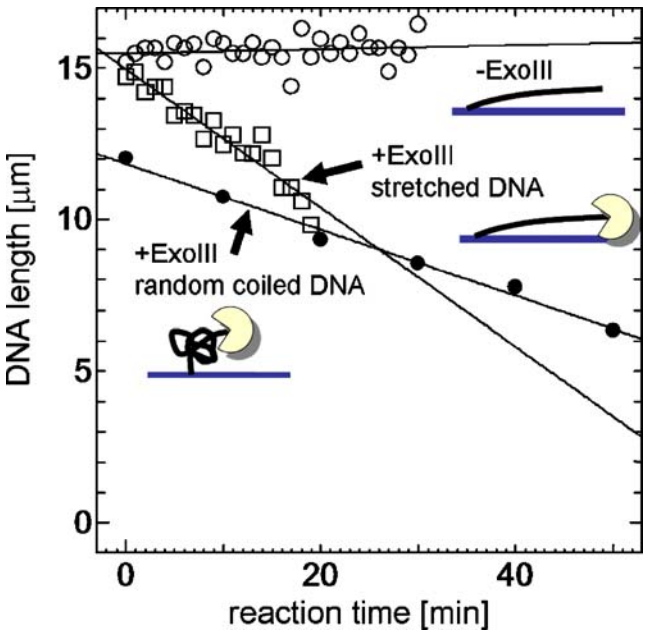


**Fig. 5** Time course of length of the digested DNA molecule. The experimental conditions of each sample are illustrated. The digestion rates of each reaction were calculated from gradients obtained from a least-square method

We analyzed DNA digestion by ExoIII based on gel electrophoresis and single-molecule observation. First, we determined the "bulk" digestion rate of ExoIII by electrophoretic assay. Digestion rates of ExoIII have been determined by previous reports [23, 25]. However, we also determined the digestion rates in the presence of chemicals for fluorescence observation, because DNA should be stained with a fluorescent dye for real-time observations using a fluorescent microscopy. Some fluorescent dyes and anti-fade reagents could interfere with certain interactions between the stained DNA and proteins involved in transcription, replication, repair and digestive enzyme reactions. Figure 3 demonstrates that YOYO-1 and anti-fade reagents slightly reduced digestion rate, but inhibition in ExoIII reaction by these reagents is negligible. In addition, we also modified $Mg^{2+}$ concentration for single-molecule observation. According to the supplier's instructions, 5 mM $MgCl_2$ is recommended, however, high concentration of $Mg^{2+}$ prevents clear fluorescent observation. Therefore, we performed electrophoretic analysis for products of ExoIII digestive reaction under 1 mM $MgCl_2$. This analysis demonstrated that these digestion rates in both concentrations were almost the same (Fig. 3). For this reason, $Mg^{2+}$ concentration in single-molecule observations is sufficiently high for ExoIII reaction, and there is little influence on single-molecule ExoIII digestion rate. All digestion rates obtained from gel electrophoresis (60–100 nt./min) agreed with both the previous reports [23, 25] and the catalogue data from the supplier.

In single-molecule experiments, digestion by ExoIII was observed. In Fig. 4A, the length of DNA in the absence of ExoIII was almost constant and fluctuated less than 2.0 μm. In addition, the observed average length of a λ DNA molecule was 14.6±1.1 μm (mean±SD, $n$=29). Thus, the average number of base pairs per μm can be calculated as 48,502/14.6=3,328 bp/μm. In contrast, in the presence of ExoIII, shown in Fig. 4B, the decrease rate in the length of stretched DNA was 0.23 μm/min. Based on the average number of base pairs per μm, ExoIII digestion rate for stretched DNA was estimated as 765 nt./min. In addition, we also carried out single-molecule observation for random coiled DNA digestion by ExoIII. The result of the experiment is shown in Fig. 4C. The decreased rate of DNA length was observed as 0.11 μm/min. This result suggests that the digestion rate of ExoIII for random coiled DNA was 366 nt./min.

Figure 6 shows distributions of digestion rate of ExoIII under three conditions. In the absence of ExoIII (Fig. 6A), the mean digestion rate was 49.4±81.8 nt./min (mean±SD, $n$=29). In the presence of ExoIII (Fig. 6B), the mean digestion rate for stretched DNA was 741±538 nt./min ($n$=39). Such variation in digestion rate is typically observed in single-molecule observation. In Fig. 6B, we can see the samples of which digestion rate were almost zero. ExoIII can digest from the blunt end, the 3′-recessed end and the nicking sites, but, it is impossible to digest from the 3′-protruding end. Therefore, the free ends of samples of which digestion rate were almost zero might be the 3′-protruding end. In contrast, the mean digestion rate for random coiled DNA was 349±212 nt./min ($n$=34) (Fig. 6C). To test the statistically significant difference between the distributions shown in Fig. 6B and C, this data was compared by means of Welch's $t$ test. Before Welch's $t$ test, the hypothesis of homoscedasticity was tested using $F$ test. The $F$ test rejected this hypothesis with a $p$-value of less than 0.0001. In this case, Welch's $t$ test is suitable to test hypotheses about two groups with different variances. Therefore, we tested the two distributions about Fig. 6B and C with a null hypothesis having a same average by using Welch's $t$ test. As a result of the test, $p$ value was calculated as 0.0000107 and this value is small enough to conclude to reject this null hypothesis. These results show that the digestion rate under the stretched condition was two times higher than the relaxed condition.

In this report, we carried out single-molecule observation for random coiled DNA digestion by ExoIII. The result of the experiment showed the digestion rate for random coiled DNA was 349 nt./min. This result disagrees with the result of electrophoresis (60~100 nt./min), although the physical form of DNA is random coiled state in both experiments. The reason for this disagreement might be caused by the difference of DNA/enzyme ratio in both experiments. We discussed this difference in our previous report [26]. In the electrophoretic assay, the number of terminus of substrate DNA is much higher than the number of ExoIII. In contrast, the number of ExoIII is excess for immobilized DNA in our single-molecule experiments. Because ExoIII is a distributive enzyme [23, 24], frequency of association for terminus of DNA may affect the digestion rate of ExoIII. For that reason, we suppose that the higher digestion rate is attributable to higher association frequency, which is caused by higher DNA/enzyme ratio in single-molecule experiments. In addition, DNA molecules in single-molecule observations were stretched during the measurements of those lengths. The buffer flow was applied for a few seconds at the flow rate of 105 μl/h for the measurements, and then the flow was stopped after capturing the images. It took about 30 s to convert from stretched state to random coiled state (data not shown). The digestion rate for stretched DNA was two times higher than for random coiled DNA, as described above. Therefore, we were apprehensive that the measurement operation of DNA length with the buffer flow interfere digestion rate of random coiled state in single-molecule

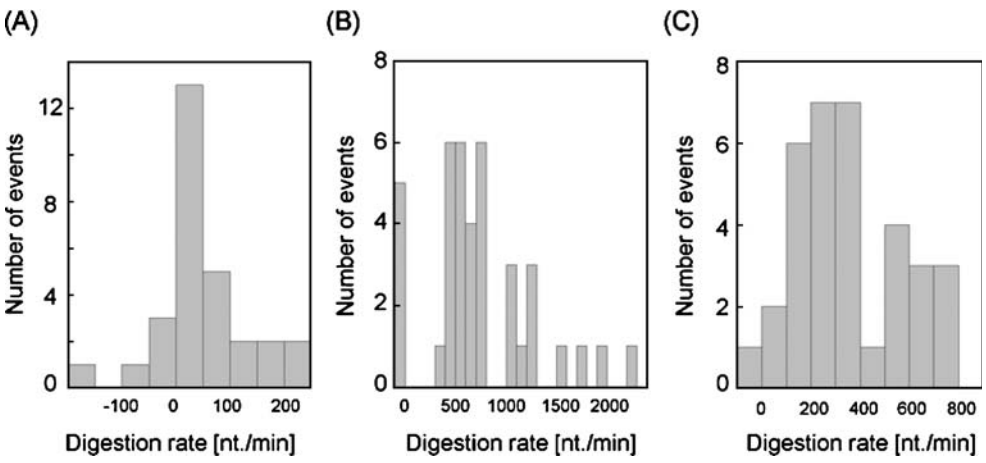


**Fig. 6** Distributions of digestion rate under three conditions **a** in the absence of ExoIII, **b** in the presence of ExoIII with buffer flow and **c** in the presence of ExoIII without buffer flow

observations. However, the ratio of time duration of stretched state to random coiled state was 1/20. To estimate the 'true' digestion rate of random coil state, we assumed that DNA was digested with the 'true' digestion rate of random coil state during 19/20 duration (random coil state) and that for stretched state during 1/20 duration. Under this assumption, we calculated the 'true' digestion rate, which could be faster by only 6%. For these reasons, the disagreement between the result of electrophoresis and single-molecule experiments might be the cause of the difference of DNA/enzyme ratio in both experiments.

Our single-molecule observation demonstrates that the digestion rate of ExoIII for stretched DNA is two times higher than for random coiled state. This observation suggests that the physical form of DNA affects DNA digestion by ExoIII. We have to consider which step is affected by the physical form of DNA in digestive reaction by ExoIII; (1) ExoIII association to DNA, (2) digestion of DNA by ExoIII ("actual" digestion rate) and (3) ExoIII dissociation from DNA (processivity). First of all, we would like to discuss the first step (association to DNA) described above. Even though association rates for DNA are primarily affected by DNA/enzyme ratio, the ratio is the same between the experiments for stretched state and random coiled state. So we consider an interference of random coil DNA to the free end of DNA, which is a binding site for ExoIII as another mechanism of the physical form dependency. Because a free end and DNA string in random coiled state rapidly move by Brownian motion, the ExoIII binding to a free end might be interfered by Brownian motion of DNA string closed to free end. On the other hand, stretching of DNA suppresses free Brownian motion of DNA string; therefore the interference of the DNA string to the free end must be weakened. Furthermore, in the digestions of relaxed DNA, the reactions products (ssDNA) might be present during most of the reaction except when flow was applied. These products may inhibit the enzyme, thereby slowing the digestion of DNA not under tension. We consider this is a plausible mechanism for the physical form dependency. Of course, we consider the second step to also be important. Therefore, we would like to discuss effects of the physical form of DNA on DNA digestion reaction or processivity also (corresponding to the second step). Many researchers already demonstrated that tension of DNA affected several reactions as follows. Maier and his colleagues reported that the rate of replication depends strongly on the stretching force applied to the template [15]. The replication rate increases at low forces, decreases at forces greater than 4 pN, and ceases when the single-stranded DNA substrate is under a load greater than ~20 pN. Wuite et al. demonstrated the effect of template tension on T7 DNA polymerase activity [18]. The replication rate also increases at low force and the digestion rate of exonuclease activity at high force (>40 pN) is ~100 times faster than observed at zero tension on dsDNA. We demonstrate that the digestion rate of ExoIII for stretched DNA is higher than for relaxed state. Our results agree with those previous reports. For these reasons, we suppose that exonucleolytic digestion is generally affected by the physical form of DNA. However, to make further reference to this problem, movement of fluorescently labeled ExoIII must be observed at the single-molecule level.

Using single-molecule observation and controlling the form of DNA by flow, we clearly demonstrate that the physical form of DNA affects ExoIII activity. It is very important for understanding the mechanism of DNA–protein interactions to compare ExoIII digestions both by bulk assay and the single-molecule approach. Single-molecule measurement that can control the physical form of individual DNA molecules is a powerful method for obtaining new knowledge about the correlation between DNA-tension and enzyme activity. Our experimental setup is very simple and can be easily applied for other enzymes. A relationship between a physical form and biochemical reaction is very interesting. Further single-molecule experiments should elucidate the mechanism of force-related DNA–protein interactions and provide additional insight into these interactions in living cells.

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