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Observation of the destruction of biomolecules under compression force

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Abstract

The atomic force microscope (AFM) has been eagerly employed in biology and various biomolecules have been measured by single molecule level. However, a biosample is generally flexible and weak against environmental change. Thus, the perturbation induced by an AFM probe should be observed and controlled to apply an AFM measurement to the biological study. In this study, we tried to observe the destruction of biosample under compression force. By using a force curve mode confocal laser scanning microscope/AFM (CLSM/AFM) system, the denaturation of green fluorescent protein (GFP) induced by the compression force was observed by the simultaneous measurement of the fluorescence change. Consequently, it is clarified that GFP molecule exhibited the reversible denaturation by the compression force of averaged 5 nN and that the fluorescence was quenched.

1. Introduction

The atomic force microscope (AFM) has been eagerly employed in biological study because it has no requirement of electric conductivity of the sample and it can operate under physiological condition. For example, the observation of a single membrane protein [1], protein–protein interaction [2], the mechanical unfolding of a single protein molecule [3–5] and the observation of the temporal change of mRNA concentration within the living cell [6] have already been reported. As there are no alternative experimental methods for single protein imaging or molecular manipulation under physiological condition, the AFM becomes an indispensable tool for nanobiotechnology.

It is ideal for a microscopic measurement to image the sample with no perturbation. Therefore, it is not desirable for a microscopic measurement that the sample is scratched from the substrate or it is denatured by the probe. However, a biosample is generally weaker against mechanical perturbation or environmental change than solid material. Thus, it is indispensable for the biological

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experiment to remove such an effect induced by an AFM probe, and various experimental techniques have been suggested. For example, the low-spring constant cantilever [7] was developed and a non-destructive experimental technique has been suggested [8]. Also, the solvent property on the sample surface is changed by the approach of a solid surface on an AFM probe and it may cause to be denatured. Thus, the experimental method has been reported to observe solvent molecules between an AFM probe and the sample surface using the fluorescent molecular probe [9]. Thus, it is important for the biological study to measure the effect of the perturbation induced by an AFM probe and to control them.

In this study, we demonstrate the experimental technique to observe the effect of the mechanical perturbation by the AFM measurement and the simultaneous spectroscopic measurement of biomolecules in the contact area. We measured the fluorescence change of green fluorescent protein (GFP) under compression force by using a force curve mode confocal laser scanning microscope (CLSM)/AFM system [9,10]. GFP is the most famous fluorescent protein and it has the chromophore in the center of the β-barrel protein structure. It is well known that the intense fluorescence is quenched by the denaturation of the protein structure [11,12]. Thus, GFP molecules are appropriate samples to measure the effect of the compression force.

2. Experimental

2.1. CLSM/AFM system and its experimental technique

We used the homemade CLSM (Nanofinder, Tokyo Instruments, Tokyo, Japan)/AFM (Smena liquid head, NT-MDT, Moscow, Russia) system developed in our previous study [9,10]. The status information during a force curve measurement can be obtained from the AFM and the optical spectrum can be synchronously measured from under a glass substrate using the CLSM/AFM controller. The position at which an optical spectrum is measured can be made to correspond accurately to the point in the force curve. The excitation light source is an Ar^+ laser (488 nm). The optical spectra of the samples are measured with a cooled charge coupled device (CCD, Andor Technology, Belfast, Northern Ireland) equipped with a spectrograph ($f = 56$ cm, 200 grooves/mm grating). In all experiments, the measurement point of the CLSM was adjusted to the contact area of an AFM probe beforehand and it was fixed during experiments. Only the AFM probe was vertically moved during the force curve measurement. The simultaneous spectroscopic measurements were performed with the force curve measurement and we mainly discussed the difference of the fluorescence intensity calculated from those spectra. The CLSM/AFM system and the experimental technique have been described in detail in our previous paper [10].

2.2. Sample preparation

GFP which was modified as follows was immobilized on the glass surface: S65T (Ser-65 → Thr), C48A (Cys-48 → Ala), introduce 12-histidine tag to C terminus and introduce cysteine to N terminus [13]. GFP was purified by the metal chelate affinity chromatography on Ni-NTA agarose. GFP molecules were attached to the substrate surface by the following method: (1) The glass substrate was modified with 3-(diethoxymethylsilyl)propylamine (Sigma, St. Louis, MO) by the silane coupling method [14]. (2). *N*-(6-Maleimidocaproxyloxy)-succinimide (EMCS, NH_2-SH cross-linker, Dojindo, Kumamoto, Japan) was reacted. (3) The Cys residue of the N terminus of GFP was attached to the substrate surface in pH 7.4 phosphate buffer saline. The sample substrate was sufficiently washed between each reaction process.

For observing the fluorescence change remarkably, we make the contact area enlarge by attaching a glass microbead (glass beads, $r = 5-15$ μm, Polysciences, Warrington, PA) to the end of a standard cantilever (NP-S, spring constant = 0.06 N/m, Digital Instruments, Santa Barbara, CA; NSG-11 B-type spring constant = 5 N/m, NSG-20 45 N/m, NT-MDT, Russia) with micromanipulators [9]. Also, the spring

constant was determined with the thermal vibration method [15] by ourselves when we used NP-S as a cantilever.

2.3. Experimental condition

In these experiments, the velocity of an AFM probe and the exposure time of the spectroscopic measurement should be set up as an appropriate value because these parameters determine the experimental accuracy. In this study, we set up the velocity of an AFM probe as 200 nm/s during the force curve measurement and the exposure time as 1 or 0.5 s. All experiments were performed in liquid (pH 7.5–8.0 with 100 mM phosphate buffered saline) and the input power of the excitation light was adjusted to 7 μW (Fig. 1).

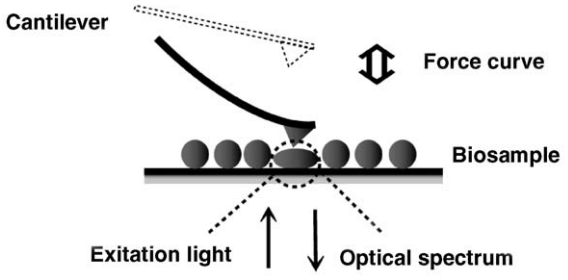


Fig. 1. Schematic of present measurement.

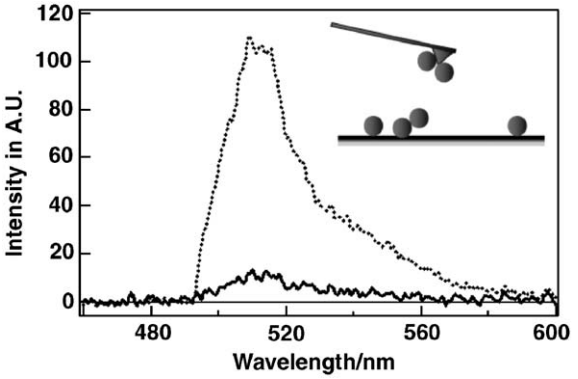


Fig. 2. Fluorescence spectra of GFP molecules measured when a large compression force of over 1 μN was applied. The solid line expresses the fluorescence spectrum in the contact area. It was acquired after applying the compression force to the measurement point of the CLSM and retracting the AFM probe completely from the substrate surface. The dotted line is the fluorescence spectrum of GFPs outside the contact area.

3. Results and discussion

First, the large compression force of over 1 μN was applied to GFPs. The fluorescence spectra of GFPs are shown in Fig. 2. The solid line in Fig. 2 expresses the fluorescence spectrum in the contact area. It is acquired after applying the compression force to the measurement point and retracting the AFM probe completely from the substrate surface. The dotted line shows the fluorescence spectra which was obtained outside the contact area. The great compression force was applied for the purpose of the remarkable observation of the sample destruction. These spectra show that the fluorescence intensity in the contact area is smaller than that outside the contact area. It is considered that the GFP molecules were scratched during the force curve measurement and that the number of GFP molecules was decreased. Moreover, the fluorescence intensity was enhanced when the AFM probe was moved again into the measurement region of CLSM. It is considered that GFP molecules scratched by the AFM probe were moved from the substrate to the surface of the AFM probe and that the number of GFP molecules in the measurement region of CLSM was increased. Thus, such a large compression force and vertical movement of an AFM probe cause the sample destruction.

Next, a compression force of below 30 nN was applied to GFPs and the change in the structure of GFPs were observed. The GFPs on the sample surface was not scratched from the substrate surface when such a small load was applied and the force curve measurements were repeatedly performed. Figs. 3a and b show the force curve and the fluorescence spectra which were acquired simultaneously in the contact area. All fluorescence spectra were measured sequentially from curve 1. The spectra indicated by curves 1 and 3 were obtained when the AFM probe was separated from the substrate surface and no compression force was applied to GFPs (noncontact spectrum). Also, curve 2 is a compression spectrum, which is

acquired when the compression force was applied by an AFM probe. The vertical position at which the compression spectrum was measured is indicated by the same number in the force scans. Curves 2 and 3 in Fig. 3b indicate that the fluorescence of GFP molecules was quenched when the compression force was applied to them and that it was recovered again when the AFM probe was retracted from the substrate. However, the fluorescence intensity of GFP is generally decreased because of the photobleaching. Therefore, the fluorescence spectra of the contact area were measured by repeating contact and noncontact alternately to remove the effect of photobleaching. The temporal change of the fluorescence intensity calculated from each spectrum was shown in Fig. 3c. The calibration curve of photobleaching was created on the basis of each fluorescence intensity of the noncontact spectrum. Although GFP molecules are gradually photobleaching, it is clarified from Fig. 3c that the fluorescence of GFPs is quenched under compression force and that those quenching are repeatedly observed. However, such fluorescence quenching was not always observed whenever the same experiments were performed because it is difficult to adjust the measurement point of the CLSM system to the contact area with sufficient reproducibility. However, when the adjustment of the measurement point of the CLSM to the contact area is successful, such a fluorescence change can be observed repeatedly if both the measurement point of the CLSM and the lateral position of the AFM probe are not shifted. Also, the perfect quenching of GFPs in the contact area could not be observed because the contact area of an AFM

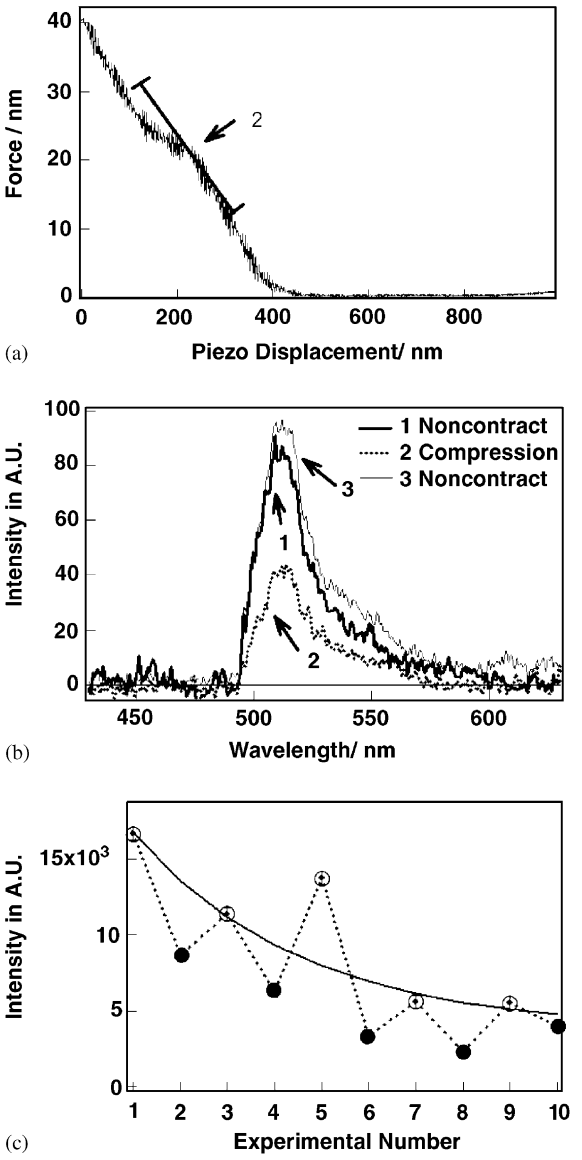


Fig. 3. Fluorescence change of GFPs under a compression force of below 30 nN. Panels (a) and (b) are the force curve and the fluorescence spectra measured simultaneously, respectively. The solid and the thin lines in (b) represent the noncontact spectra and the dotted line in (b) represents the compression spectrum. All fluorescence spectra were measured sequentially from curve 1. The vertical position at which the compression spectrum was measured is indicated by the same number in the force scans. The error bars of the position of an AFM probe are also shown because the AFM probe moved vertically during the spectroscopic measurement. (c) Temporal change in the integrated fluorescence intensity. Each value was calculated by integrating each spectrum from 500 to 600 nm. All spectroscopic measurements were carried out in the same exposure time (1 s) and excitation power (7 μW). The horizontal axis corresponds to the sequential number of the spectroscopic measurement from 1. The solid and the open circles represent the fluorescence intensity obtained when the AFM probe was in contact and in noncontact, respectively. The solid line is a photobleaching curve created on the basis of the open circles. The dotted line in (c) represents the change in the fluorescence intensity.

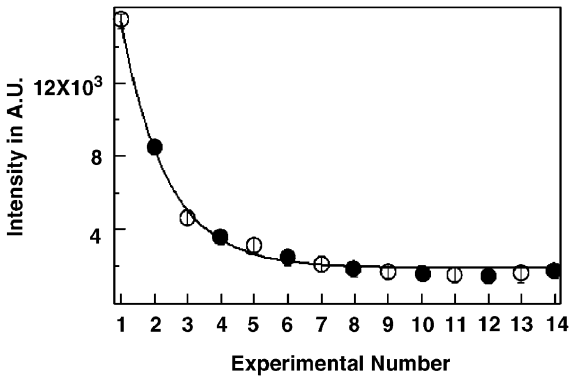
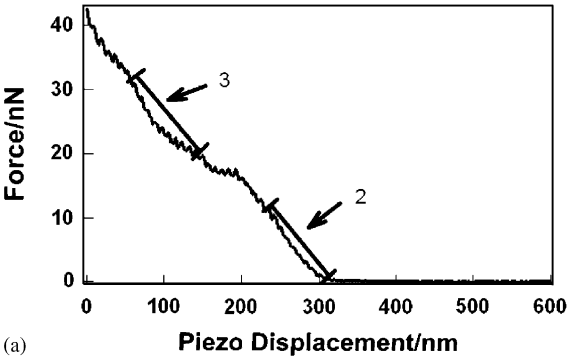
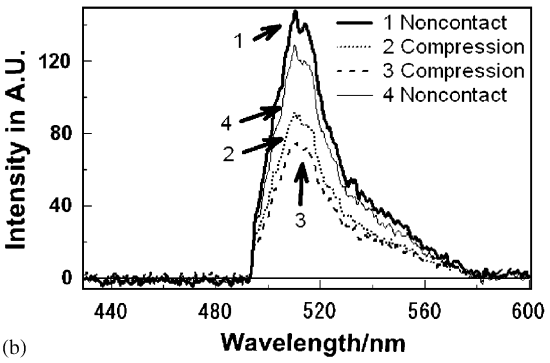


Fig. 4. Temporal change in the integrated fluorescence intensity observed when tetramethylrhodamine-5-maleimide was used as a sample instead of GFP. Each value was calculated by integrating each spectrum from 500 to 600 nm. All spectroscopic measurements were carried out in the same exposure time (1 s) and excitation power (7 μW). The horizontal axis corresponds to the sequential number of the spectroscopic measurement from 1. The solid and the open circles represent the fluorescence intensity obtained when the AFM probe was in contact and in noncontact, respectively. The solid line is a photobleaching curve created on the basis of the open circles.

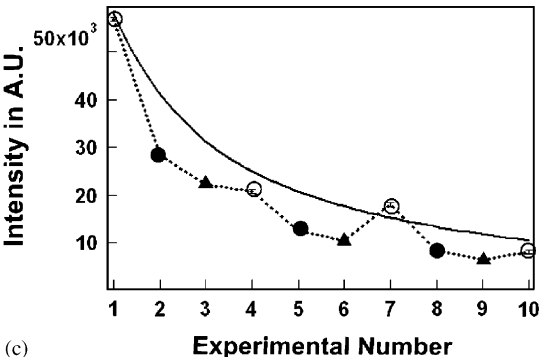
probe was smaller than the measurement region of the CLSM and the fluorescence of GFPs outside the contact area was also collected with that in the contact area. Also, such fluorescence quenching could not be observed in case the standard fluorescent dye (tetramethylrhodamin-5-maleimide, Molecular probes, Eugene, OR) was used as a sample instead of GFP (see Fig. 4). Thus, it is considered that such fluorescence quenching is due to the denaturation of GFPs induced by the compression force. It is well known that the β-barrel structure of GFP molecule is rigid and stable. It is clarified that the β-barrel structure of



(a)



(b)



(c)

Fig. 5. Force dependence of fluorescence change. Panels (a) and (b) show the force curve and the fluorescence spectra measured simultaneously. The solid and the thin lines in (b) represent the noncontact spectra. The dotted and the broken lines in (b) represent the compression spectrum 1 and 2, respectively. All fluorescence spectra were measured sequentially from curve 1. The vertical position at which the compression spectrum was measured is indicated by the same number in the force scans. The error bars of the position of an AFM probe are also shown. (c) Temporal change in the integrated fluorescence intensity. Each value was calculated by integrating each spectrum from 500 to 600 nm. All spectroscopic measurements were carried out in the same exposure time (0.5 s) and the excitation power (7 μW). The horizontal axis corresponds to the sequential number of the spectroscopic measurement from 1. The open circle represents the fluorescence intensity obtained when the AFM probe was in noncontact. Also, the solid circle and the solid triangle represent the fluorescence intensity calculated from the contact spectrum 1 and 2, respectively. The solid line is a photobleaching curve created on the basis of the open circles. The dotted line in (c) represents the change in the fluorescence intensity.

GFP is distorted by the compression force of 20 ± 10 nN and that the protein function is lost. To our knowledge, there was no previous study in which the fluorescence change of GFP was measured with the application of the direct mechanical perturbation to it. Also, it has been reported that the fluorescence intensity is not decreased with a high pressure of approximately 100 MPa in liquid [16]. It is considered that the structure of GFP was disrupted by the direct mechanical perturbation because the compression force is a unidirectional force. Therefore, when the AFM measurement is performed with such applied load, although the disruption of the sample from the substrate surface may not be occurred, it is difficult to perform the microscopic measurement for biomolecules under no perturbation condition.

Finally, the force dependence of the fluorescence quenching tried to be observed. The exposure time of the spectroscopic measurement was set up as 0.5 s and the fluorescence spectra were measured twice when an AFM probe was brought into contact during each force scan. Figs. 5a and b show the force curve and the fluorescence spectra measured simultaneously, respectively. The spectra indicated by curves 1 and 4 in Fig. 5b are noncontact spectra and curves 2 and 3 are compression spectra, respectively. The vertical position at which the compression spectra were measured is indicated by the same number in the force scans. Curve 2 was obtained under a compression force of 0–10 nN (hereafter called compression spectrum 1) compared with curve 3 (20–30 nN, hereafter called compression force 2). Fig. 5b shows that the fluorescence intensity had been quenching while the compression force was applied and it was recovered when the applied force was set at zero. The same experiments were performed repeatedly like previous experiments and the fluorescence intensity calculated from each spectrum was summarized in Fig. 5c. Fig. 5c shows that the fluorescence intensities of both compression spectra 1 and 2 are small compared with the values on the calibration curve. The ratio of the fluorescence change is almost the same between them. Consequently, it is considered that GFPs in the contact area were sufficiently denatured by applying the compression force of averaged 5 (± 5) nN and that there was no difference in the ratio of the fluorescence change between them. Thus, it is confirmed that compression force should be limited to below averaged 5 (± 5) nN to measure the GFP molecule with an AFM system.

4. Conclusion

In this study, we demonstrate the experimental technique to observe and control the denaturation of a biomolecule induced by an AFM probe. As a first trial, we observed the fluorescence change of GFP under a compression force applied by an AFM probe attached to the glass microbead. Consequently, it was clarified that GFP molecules in the contact area were disrupted under a compression force of averaged 5 (± 5) nN and the fluorescence intensity was decreased. Although the force dependence of the fluorescence change could not be observed, the threshold of the compression force would be obtained if the experimental accuracy could be improved, for example, the exposure time or the velocity of the force curve measurement was set up small. Such an experimental method can be applied to various biomolecules as well as GFP by introducing a fluorescent dye to them or combining tip-SERS technique [17–20]. The ideal AFM measurement will be performed by controlling the perturbation induced by an AFM probe.

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