Nanomechanics of Protein-Based Biostructures

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Nanomechanics of Protein-Based Biostructures

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In this article, we review recent studies on nanomechanics of biostructures performed in the Laboratory of Biodynamics at Tokyo Institute of Technology. We employed the force spectroscopy mode of the atomic force microscope, to determine the hidden mechanical properties of protein-based biostructures that have made life on the earth so successful. We investigated the mechanical heterogeneity of the internal structure of globular proteins and cell membranes. Single molecules of globular proteins were stretched from their two ends after being sandwiched between the probe of the atomic force microscope and the substrate through a covalent crosslinking system. The resulting force-extension curve revealed mechanical heterogeneities in the conformation of globular proteins. The covalent crosslinking system withstood a tensile force of up to 1.8 ± 0.33 nN (loading rate $= 11.7$ nN/s) while most of the noncovalently folded protein sub-structures were completely stretched out with less force. The result of force spectroscopy supported a long-standing conjecture that an enzyme cannot simply be a *soft* material because it must catalyze chemical reactions involving the formation and breakdown of mechanically rigid covalent molecules. Next, the AFM force spectroscopy was applied to determine the force needed to disrupt noncovalently assembled biostructures such as composite biomembranes composed of lipids and proteins. We were able to show that intrinsic membrane proteins that are securely anchored to a lipid bilayer could be pulled out of the membrane with a significantly less force than that required for covalent bond breakdown, but with a force in the comparable range required for the disruption of the internal structures of globular proteins. From the available results from our group and other groups, a new concept of force-based biostructure assembly is emerging. [DOI: 10.1143/JJAP.43.7365]

KEYWORDS: atomic force microscopy (AFM), force spectroscopy, protein stretching, receptor mapping, GroEL and protein interaction, membrane protein extraction

1. Introduction

In this article, we review recent studies on nanomechanics of biostructures performed in the Laboratory of Biodynamics at Tokyo Institute of Technology. We like to summarize the importance of studying the nanomechanics of biostructures to understand the basic architecture of life and its processes. At the macroscopic level, gravitational and hydrodynamical forces are the two major external stresses acting on biological structures. Because of the small size of such structures, however, the gravitational effect becomes of minor importance as one goes down to the cellular and molecular level, and the effect of hydrodynamic stress tends to be restricted to special circumstances where cells and molecules are subjected to a strong shear flow as in the blood vessels of animals. That is, elemental biological structures on the micrometer and nanometer scales are more or less in equilibrium states most of the time, indicating that mechanical stresses and their corresponding strains do not persist for long periods in these systems. This is because biological systems are soft and can relax rather rapidly to their equilibrium states compared with systems made of rigid materials such as metals or concrete. We aim at determining how soft *soft* biological structures are, and at developing appropriate methods of measuring the overall and local stiffness of protein-based biostructures using recent nanomechanical measurement technologies. This is where the theories and experimental techniques developed and used in applied physics and engineering fields for the mechanical characterization of macroscopic materials find their applications in biomedical innovations.[1)]

As stated above, the behavior of nanometer-scale biological systems can be described by mechanisms based on equilibrium thermodynamics, but this does not mean that biomolecules and related systems are not under mechanical stresses in any circumstances. In fact, they are likely subjected to a strong mechanical stress, be it for a very short time, during activation from one equilibrium state to another.[2,3)] An enzyme reaction is effective in illuminating this situation. Before and after the reaction of converting the substrate S to the product P as catalyzed by the enzyme E, both (E+S) and (E+P) states are in chemical equilibrium and no mechanical stress accumulates in any of the chemical species involved. However, as the reaction progresses, E binds S to form an activated complex where both E and S are in different conformations from their equilibrium ones suggesting that some chemical bonds in the complex are displaced from their equilibrium lengths and/or angles, thereby generating mechanical stresses in the complex. The time scale of this mechanical stress cannot be readily estimated, but is likely to be much longer, *e.g.*, from micro- to milli-second range, than the time scale of molecular relaxation processes which take place in the picosecond range.[4)] It has, indeed, been argued that, since an enzyme acts as a catalyst, its internal structure must have sufficient rigidity to sustain the mechanical stress imposed on it during activation,[5)] but there is no appropriate method to detect the presence or absence of rigid substructures within a single molecule of protein until the recent advent of atomic force microscope (AFM). To address this problem, we employed the force measurement mode of the AFM. As illustrated in Fig. 1, the AFM is composed of a small cantilever with an integrated probe at its free end, an optical lever sensing system for cantilever deflection, and a sample stage on top of a piezomotor that drives the sample stage horizontally (x and y) and vertically (z).[6)] When the AFM is operated in the force curve mode, the piezotube under the sample stage makes only a vertical (z) movement at a fixed lateral position

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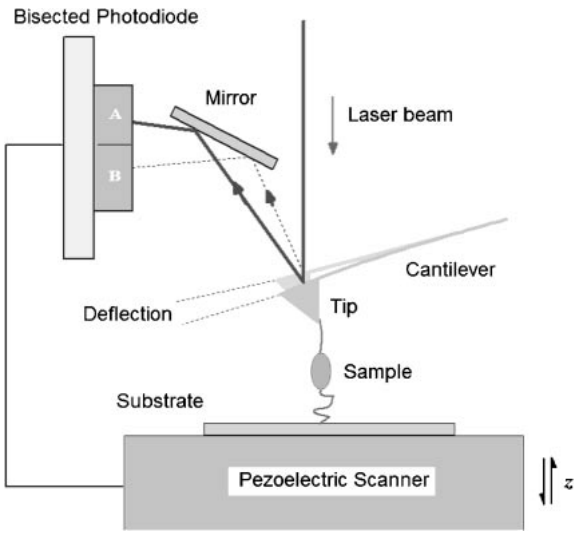

Fig. 1. Schematic view of operating principle of typical commercial AFM. The laser beam reflected from the back of the gold-coated cantilever is received by a bisected (or quadrasepted) photodiode detector. The difference between the outputs of the top and bottom photodiodes is kept at near zero level by changing the height of the sample stage through the feedback control system. The input voltage from the feedback system to the piezo scanner below the sample stage is recorded and interpreted to represent the topography of the sample surface.

so that it is possible to stretch a polymer chain from its two ends while constantly monitoring tensile force as a function of chain extension.[7–9)]

To ensure the correct analysis of the force versus extension relationship of a protein molecule, it must be established, at least to a reasonable level, that the observation is made on a single chain of the sample protein and not on multiple chains. We and most other researchers in this field use the following two criteria. First, the percentage of successful recording of chain elongation must be less than 10–20% of the total number of trials, the rest showing no positive indication of the presence of tensile materials between the AFM tip and the subsrate. If the success rate of forming connections between the sample protein and the tip is small, the probability of double or even triple capture events will be negligibly small, i.e., less than 1–4%.

The mechanical unfolding of globular proteins using the AFM was initiated by Mitsui *et al.*[7)] and later substantially extended by several groups using the giant molecule titin (connectin).[8,10)] Mitsui *et al.* stretched an immobilized protein molecule on a gold surface through –S–Au– bonds by forming another set of similar bonds with a gold-coated cantilever that approached the protein from the top. They reported the average slope of the *F–E* curves that show for the first time that a globular protein could be stretched out with a force in the range of a few hundreds of pNs. Titin extension was first reported by Gaub and his colleagues as cited above showing a train of force peaks corresponding to the successive breakdown of the modular subunits of the giant protein which has hundreds of such subunits connected by very flexible linker peptides. Titin turned out to be a convenient sample in many respects for use in new types of force spectroscopy methods because of its modular structure that allows researchers to stretch any part of the protein giving the same results. The globular modules of titin was shown to break down with a force of a few tens and a few hundreds of pNs depending on loading rate.[11)]

The force curve mode of the AFM was also applied for harvesting protein molecules embedded in the cell membrane. These proteins have hydrophobic segments that span the hydrophobic interior of the lipid bilayer and function as mediators of chemical information from the outside to the inside of the cell and vice versa.[12)] In addition to these membrane spanning segments, such proteins usually have hydrophilic extracellular and cytoplasmic domains and appear to be securely anchored to the membrane. If the protein-protein interaction is stronger than the protein-lipid interaction, protein should form aggregates in the membrane, otherwise, proteins would be strongly solvated by lipid molecules. Many, if not all, of the membrane proteins are in association with sub-membranous protein layers called the cytoskeleton through noncovalent interactions and mediate the transfer of extracellular information to the intracellular enzyme systems. In order to harvest membrane proteins by extraction from the cell membrane, both lipid-protein and protein-cytoskeleton interactions must be disrupted.

In his pioneering work on the adhesive interactions between cells in 1978, Bell extended his theoretical study on cell adhesion to the estimation of the force required to disrupt the interaction between an intrinsic membrane protein and a lipid bilayer, and predicted that a force of about 100 pN is required to uproot a single molecule of glycophorin, the protein on the red blood cell membrane.[13)] If boundary lipids are extracted together with glycophorin, the force would be in the range of 250 pN. We wanted to test Bell's estimate of the anchoring force of a membrane protein to the cell surface using an AFM.

Another promising application of nanomechanical measurement at the molecular level is the detection of specific receptor molecules on viable cell surface using an AFM probe modified with covalently immobilized specific ligand molecules.[14)] The probe makes a 2D survey over the cell surface and records the interaction force between the probe and the surface as the indication of the presence of receptor molecules with biochemical specificity to the ligand on the probe.[15–17)] Such an application of the AFM for receptor mapping is based on the assumption that the unbinding force between ligands and receptors is substantially less than the force required to extract or uproot proteins from the cell surface. If not, the force measured every time the probe makes contact with the cell surface and is retracted, is not the unbinding force of the ligand from the receptor but the uprooting force of the latter from the membrane, and mapping will not be possible. All the ligand molecules on the AFM tip will be eventually covered with receptor molecules and no response is expected from the ligand-receptor interactions. It is therefore important to establish the range of force required to extract membrane proteins from the viable cell surface in comparison to the unbinding force of ligand-receptor pairs of interest. We allowed bifunctional covalent crosslinkers immobilized on the AFM tip to form covalent bonds with cell surface proteins and then pulled out the membrane proteins taking advantage of on the strength of a covalent bond.[18–20)]

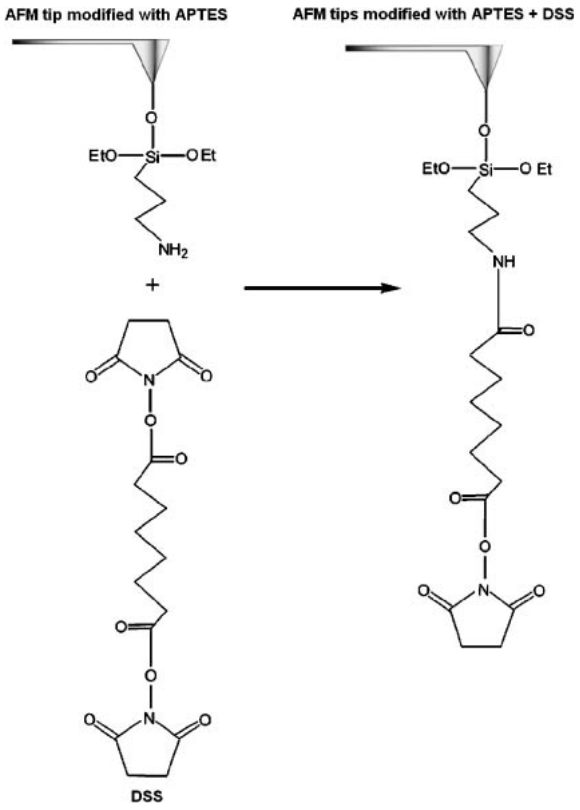

Fig. 2. Covalent crosslinking system used for the measurement of noncovalent interaction forces (cited from ref. 18 with permission).

2. Methods and Materials

AFM probes and silicon substrates were chemically functionalized so that the macromolecules caught between them are covalently crosslinked to the tip on one end and to the substrate on the other. The chemical modification method of cantilevers is given in Fig. 2. The substrate used was modified in a similar manner. The sample molecule thus sandwiched between the tip and the substrate will then be put under a continuous tensile stress by increasing the tip-stage distance. The sandwiched sample will then be stretched until one or more of the weaker bonds in the system are broken. During the stretching of the protein molecule, the mechanical response of the sample against the tensile stress produces a downward deflection of the cantilever which holds the probe at its free end.[7] The extent of cantilever deflection, d, is detected by a bisected photodiode detector and, by knowing the cantilever force constant k, the tensile force applied to the sample is calculated using $F = -k \times d$. The extension of the sample E during the process of increasing the probe-stage distance D is calculated using $E = D - d$. The experimental parameters obtained from a force spectroscopy experiment are schematically shown in Fig. 3 and explained in the legend.

2.1 Chemicals and proteins

The silanization reagent used 3-aminopropyltriethoxy silane (APTES) was obtained from Shin-Etsu Chemical Co. (Tokyo, Japan). Disuccinimidyl suberate (DSS) and *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) were purchased from Pierce (Rockford, IL). They were stored under a highly dehydrated and low-temperature conditions and used without further purification. Phosphate-buffered saline as a liquid medium for cells during atomic force microscopy experiments was obtained from Gibco (Langley, OK). Other chemicals were bought from Sigma (St. Louis, MO). The proteins used in the stretching experiments were produced by the recombinant method with the insertion of cysteine residues outside the N- and C-termini together with N-terminal histidine tags to facilitate purification by affinity chromatography.[21]

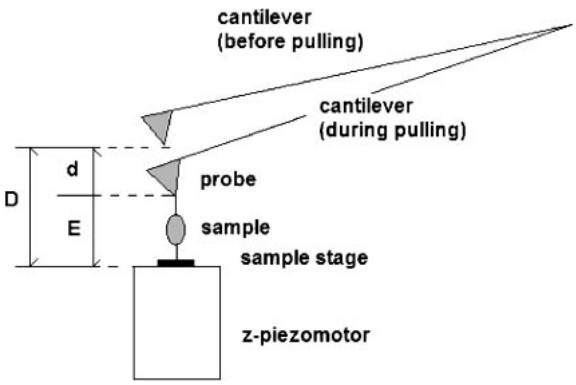

Fig. 3. Force-curve-mode operation of AFM. The relationship given in the text, i.e., $E = D - d$ is explained.

2.2 Cells

Mouse 3T3 fibroblast cells were grown in Dulbecco's modified Eagles' minimum medium (DMEM) for several days and in the final stage of AFM experiments, the medium was changed to phosphate-buffered saline.

2.3 Atomic force microscope

Several commercially available AFM instruments were used including a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA), an NVA 100 AFM (Olympus, Tokyo, Japan), and a BioScope (Veeco, Woodbury, NY). Cantilevers were purchased from Olympus and Veeco. The cantilevers and crystalline silicon substrates were silanized with APTES and further modified with SPDP to make their surface reactive with -SH groups on the engineered proteins with cysteine residues. For the extraction of membrane proteins, AFM tips were modified with DSS so that the proteins could be immobilized through their amino groups to the tips. Gold-coated cantilevers (Olympus) were also used after direct modification with SPDP.

2.4 Loading rate dependence

It has been pointed out that the measured force should be dependent on how fast one loads the tensile force on the sample, and the exponential dependence of the mean force f on the loading rate has been established both theoretically and experimentally [22] as

$$f = \frac{k_B T}{x_\beta} \ln[\text{loading rate}],$$

where k_B, T and x_β are the Boltzmann constant, the temperature (K), and the activation distance of the bond to be broken.

Cantilever force constant was calibrated based on the

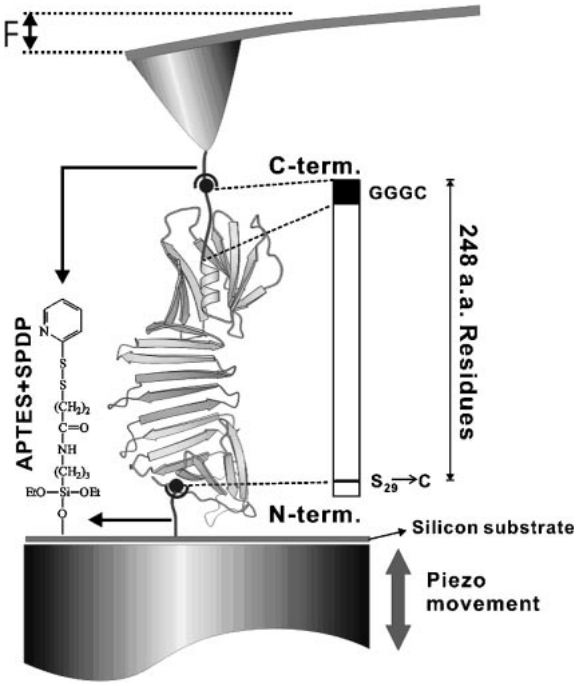

Fig. 4. Schematic view of OspA stretching on AFM sample stage (cited from ref. 24 with permission).

equi-partition theorem of the thermal energy and the kinetic energy of the cantilever.[23)]

3. Results

3.1 Protein stretching experiments

First, we describe the stretching experiment of several globular proteins where we found the presence of highly resistant substructures against tensile force within a single protein molecule. We expect that this finding will provide experimental evidence of what has been anticipated from the theoretical point of view as described in Introduction.

When the globular protein OspA was sandwiched between the AFM tip and the substrate using bifunctional covalent crosslinkers, as illustrated in Fig. 4, and subsequently stretched from the two ends as the distance between the tip and the substrate was increased, we observed *F–E* curves with two force peaks such as those given in Fig. 5.[24)] Since this protein, out of its total 248 amino acid residues, has two terminal globular domains connected by the central β-sheet as shown in Fig. 4, it is most natural to presume that the two force peaks correspond to the breakdown of the two domains and the low stress stretching in the initial 30–40 nm corresponds to the unzipping of the central β-sheet. To establish the correspondence between the substructures of the protein and the two force peaks, two types of amino acid substitution were constructed. The introduction of an amino acid replacement in the middle of the primary structure (F126A, that is, phenylalanine at the 126 position was replaced by an alanine residue) abolished one of the two force peaks (see Fig. 5). OspA + 1BH, a mutant OspA protein having an extra β-hairpin inserted between strands 8 and 9 also abolished one of the two force peaks. It is thus apparent that the central flat region is mechanically strongly associated with the appearance of one of the peaks. An interpretation based on a simple visual inspection needs to

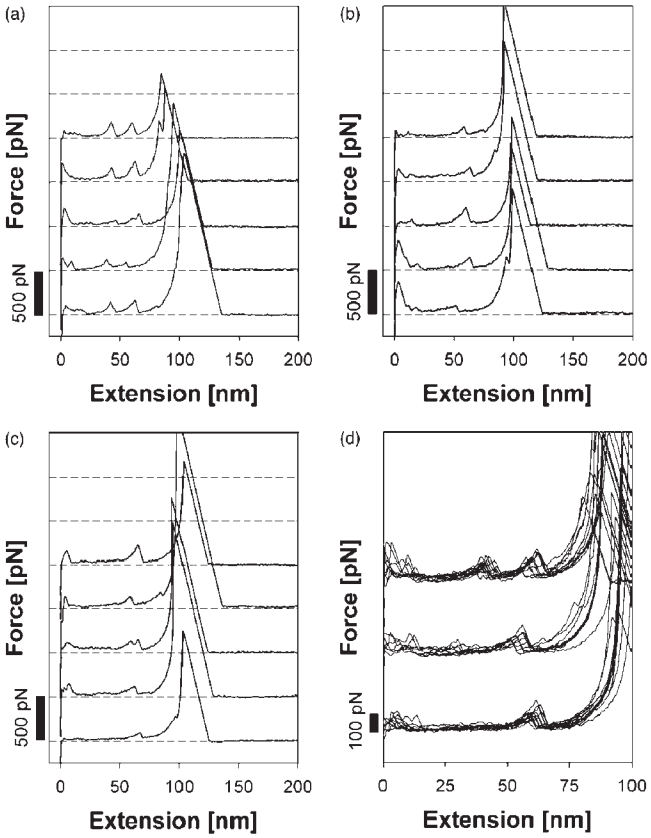

Fig. 5. *F–E* curve of OspA. Wildtype OspA (a), OspA-F126A (b) and OspA 1BH (c). In (d), superimposition of curves of wild-type OspA (top panel), OspA-F126A (middle panel) and OspA + 1BH (bottom) are given. Abbreviations OspA-F126A and OspA + 1BH are given in the text (cited from ref. 24 with permission).

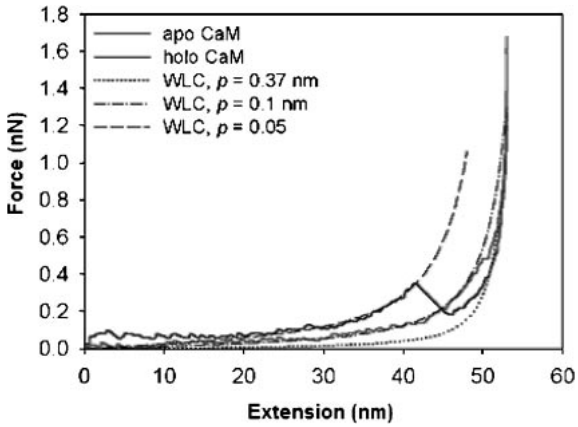

Fig. 6. *F–E* curves of apo- and holo-calmodulin together with fitting curves based on the wormlike chain with different values of parameters (cited from ref. 25 with permission).

be augmented by obtaining more results on amino acid replacement in other areas.

Figure 6 shows the results of the experiment on calmodulin in two forms, one with Ca^{2+} and biological activity, the other with neither Ca^{2+} nor activity. The *F–E* curve in the presence of Ca^{2+} showed a single force peak and the one without Ca^{2+} did not show any force peaks. The 3D conformations of the two forms of calmodulin are α-helical counterpart structures of OspA in which two globular

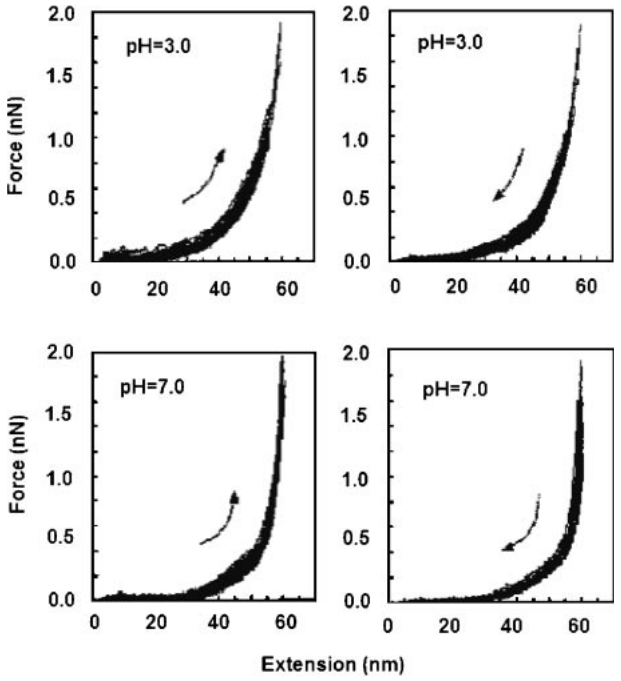

Fig. 7. *F–E* curves of poly-L-glutamic acid at pHs 3.0 (top) and 7.0 (bottom). The left panels represent stretching curves while the right panels retracting curves (cited from ref. 26 with permission).

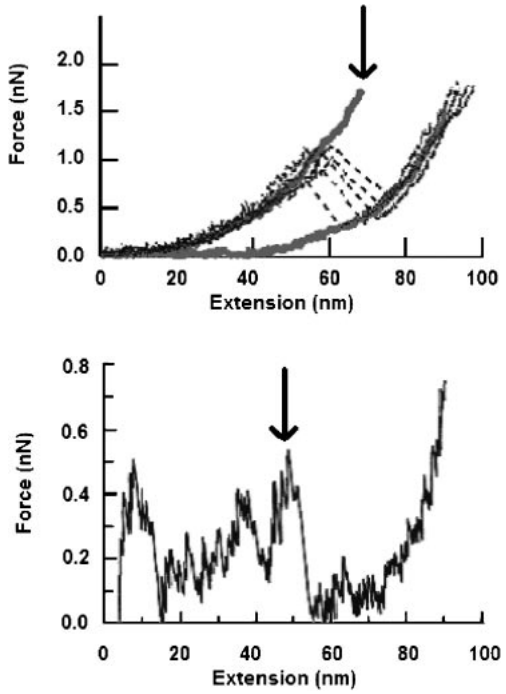

Fig. 8. Experimental (top) and SMD simulation (bottom) results of carbonic anhydrase stretching showing the appearance of a very large force peak (marked with an arrow) signifying a major structural transition (cited from ref. 21 and 29 with permission).

subdomains are connected by a single rod of α-helix but the *F–E* curve in this case showed only one peak.[25)]

To understand the *F–E* curves of protein stretching, we obtained similar curves for the stretching of substantially helical poly-L-glutamic acid at acidic pH and randomly coiled ones at neutral and alkaline pHs.[26)] As given in Fig. 7, the stretching and retraction curves of both helical (at pH 3.0) and randomly coiled (at pH 7.0) poly-L-glutamic acid were completely reversible and repeatable for more than 100 traces. In the helical conformations expected, the polymer was stiffer than that in the randomly coiled state. Our result on a poly-L-alanine-based helical polymer showed similar force curves when it is stretched. Such results help us understand the very smooth *F–E* curves of apo-calmodulin (without Ca^{2+}) as given above (R. Afrin to be published).

3.2 Molecular dynamics simulation

In the application of computer simulation to protein stretching, the steered molecular dynamics (SMD) has been used in several cases with some success.[27,28)] In SMD simulation, a virtual spring was placed between protein and the pulling point. As the tip is moved at a constant velocity, the protein unfolding may not take place at the same velocity. The difference between the two velocities will stretch the hypothetical spring whose extension will be interpreted as the tensile force on the protein. We applied this method to the unfolding of carbonic anhydrase[29)] and obtained the result shown in Fig. 8. In the top panel of the figure, the experimental *F–E* curves cited from Alam *et al.*[21)] are given and the simulation result is given in the bottom panel. The large force peak observed after 50 nm stretching is reproduced in the simulation. Simulation results with many small and medium force peaks seem to disclose more details of the stretching mechanics and sometimes, under special experimental circumstances, fine structures of force spectroscopy have been observed.[30)]

Another interesting aspect of the mechanical stretching of carbonic anhydrase was that the protein in its native, has a knotted conformation as revealed by X-ray crystallography.[31,32)] As a mechanical consequence of this knot structure, it was very difficult to stretch the protein due to knot tightening at the molecular level.[9,33)] The above result was obtained for a mutant protein in which knot tightening was avoided. We found that this protein has a mechanically very tight internal core in addition to the knot structure. This finding was in agreement with the previous work on this protein in solution.[34)] Using the same protein, Mitsui *et al.*[35)] and Okajima *et al.*[36)] studied its dynamic response against a slowly oscillating tensile force, and reported that in a certain range of extension, phase inversion is observed between the sinusoidal input signal and the output signal from the protein molecule. It was interpreted that the protein becomes particularly viscous in a certain extension range implying that the driving force for refolding or misfolding inverts the phase.

3.3 Brittle fracture

When force peaks appear in the *F–E* curves of globular protein extension, we interpreted them as reflecting brittle fractures of submolecular structures. In macroscopic mechanics, a sudden breakdown of glass or ceramics has been treated in the theoretical framework first laid out by Griffith in 1921.[37,38)] According to Griffith's theory, the size of a small cavity is elongated under stress and reaches a critical length over which a crack starts propagating. In the following equation, a_c, E, Γ_S, and σ_0 are: the critical length of the cavity for crack propagation, Young's modulus of the protein, the surface energy of the newly created surface

after fracture, and the threshold stress for the fracture to propagate. Since force peaks have been observed for primarily β-sheet proteins, we apply the simple theory on a piece of sheet material.

$$a_c \approx \frac{2E\Gamma_S}{\pi\sigma_0^2}$$

Γ_S may be taken as the hydrophobic Gibbs energy of the surface and σ_0 may be approximated by the peak stress observed in the *F–E* curve of protein stretching. If we insert as a trial, $E = 100\,\mathrm{MPa}$, $\Gamma_S = 3000\,\mathrm{J/mol/nm^2}$ and $\sigma_0 = 100\,\mathrm{MPa}$, we obtain $a_c = 0.2\,\mathrm{nm}$ which is not an unreasonable value. Another interpretation has also been put forward. It has been proposed on the basis of experimental observation that the appearance of a force peak in the case of stretching the all β-sheet protein E2lip3 depends on the pulling direction of the polypeptides in a given β-sheet.[39] When two stands were separated diagonally to the chain axis as in a fashion of unzipping, no force peaks were observed but when they were pulled along the chain axis in a shearing mode, a force peak appeared.

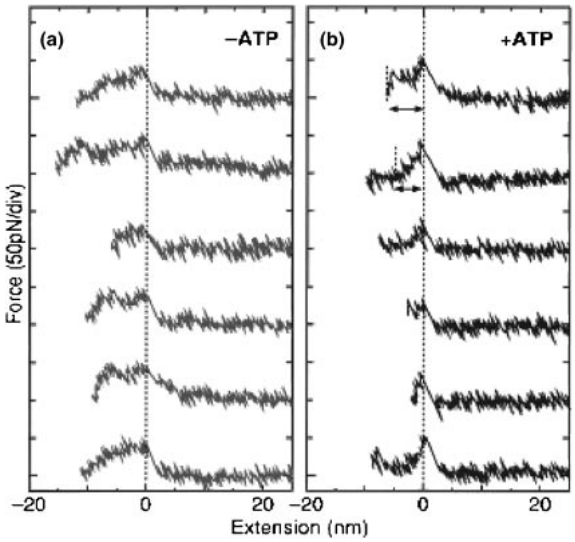

Fig. 9. Noncompressive force measurement in GroEL-pepsin system in the absence (left) and presence (right) of ATP (cited from ref. 43 with permission).

3.4 Protein-protein interactions

The measurement of protein-protein interaction is another popular application of force spectroscopy. As the first example of such an experiment, the well-characterized ligand-receptor system of biotin-avidin has been investigated.[40] This ligand-receptor pair forms a very stable complex and is widely used in biological experiments as a tool for immobilizing biomolecules on solid surfaces or on other molecules. When the experimental result of the force measurement was analyzed in terms of the loading force dependence, an activation distance of 0.3–0.4 nm was obtained. Under a similar loading rate range, the unbinding force of the pair is not as large as expected from its exceptionally high thermodynamical stability and aroused interest among concerned researchers.

This aspect of force spectroscopy was studied, for example, in antibody-antigen systems. The force required to separate a single antigen-antibody complex has been of great interest and determined for various different combinations. It is now accepted that the force required to mechanically disrupt a molecular system does not directly correlate with the free energy for the stabilization of the system, but rather, it more closely correlates to the enthalpy of the system.[41] This conclusion was obtained by comparing the observed force required to disrupt ligand-protein interaction with the result of a thermodynamic study on various mutant proteins with different affinities to specific ligands. Details are found in the literature cited above.

3.4.1 Chaperonine system studied by noncompressive force spectroscopy

Chaperonines are known to help newly synthesized proteins correctly form biologically active, specific 3D structures from their disordered conformation in the initial stage.[42] GroEL, a chaperonine, has been shown to entrap in its cavity an unfolded form of a protein and to keep it isolated until the protein spontaneously forms the correct 3D structure without being sidetracked to accumulate incorrectly folded structures by, for example, associating with other protein molecules. It has been shown that GroEL has a seven-fold rotational symmetry arising from a circular arrangement of seven subunits in one tire and each subunit has a single binding site for an unfolded polypeptide. After binding to such sites, the denatured protein is transferred into the molecular cavity of the GroEL with the help of ATP where the protein folding reaction takes place. Because the unbinding of the denatured protein from the binding site on the subunit plays a crucial step in the internalization of unfolded proteins into the GroEL cavity, it is of interest to determine the magnitude of unbinding force at this site.[43]

A modified method of force spectroscopy has been applied to this system. The AFM tip modified with denatured protein molecules was brought in close proximity so that the denatured protein starts making contact with immobilized GroEL on the surface of the substrate. The tip and substrate were kept at a noncompressive distance during the force curve measurement. The resulting *F–E* curves are reproduced in Fig. 9(a). They do not have the repulsive force signifying the noncompressive operation but show attractive force with a more or less flat plateau of about 11 nm which is terminated to the horizontal position by a jump of the cantilever. The appearance of the plateau was interpreted as a result of multiple and successive unbinding events and the force of unbinding was calculated from the deflection of the cantilever in the plateau region. Histograms of the height and the length of the plateau gave mean $\pm$ SE of $42 \pm 17\,\mathrm{pN}$ and $11 \pm 3\,\mathrm{nm}$, respectively. In the presence of ATP, the conformation of GroEL changes and the seven binding sites for unfolded peptides are more spread out. The force curve obtained under such conditions were changed into those shown in Fig. 9(b). The *F–E* curve now shows a sharp force peak without a plateau. The result implies that because the distance between binding sites on a GroEL is now large, the denatured protein can interact only with one or at most two binding sites, reducing each unbinding event to more or less an isolated event. The numerical value of the unbinding force was similar to that obtained in the absence of ATP, confirming that the effect of ATP is restricted to the change

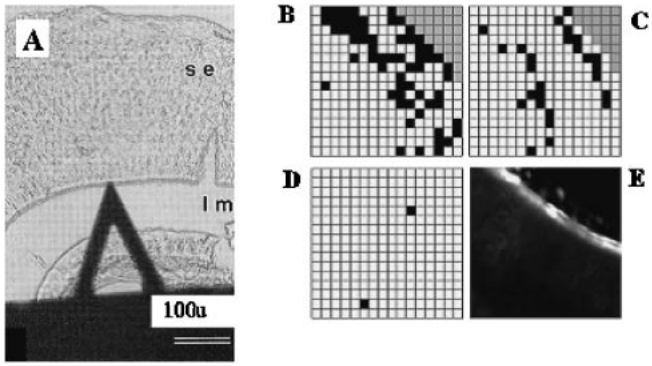

Fig. 10. Result of force mapping of specific polysaccharides on fixed tissue section probed by galactoside specific lectins. Optical microscopic image of AFM cantilever closing in on a small area of sectioned sample (A), and on the right, two diagonal rows of dark squares representing positive response to lectin molecules on the tip (B) and (C), and area of almost no response as empty squares (D). Fluorescence microscopic image of stained sample with the same lectin as that used for mapping (E) (cited from ref. 16 with permission).

in the geometry of GroEL and has no significant influence on binding force at individual sites.

3.5 *Membrane protein mapping*

The method of measuring the force required to unbind ligand-receptor pairs was applied to the cell surface receptors as a new method of mapping the presence of a particular type of receptor on a surface.[15–17] In one of such applications, the AFM tip was covered with the protein α_2-macroglobulin and scanned over the surface of a living cell in the culture medium point by point to determine the presence of its specific receptor. Ideally the force required to separate the cantilever from the cell surface should be zero when there is no receptor, and a non-zero, finite value when ligand-receptor interaction is established.[15]

This mapping method was later extended to several different membrane systems, and the advantages and disadvantages of the AFM-based mapping method in contrast to conventional methods based on receptor labeling with fluorescent dyes were discussed. Some of the results of the mapping of receptors are given in Fig. 10.[16]

If the force was due to some other events such as the uprooting of receptor molecules from the cell surface, the force mapping of receptors loses its validity. If the unbinding force is comparable or even larger than the force required to extract receptors from the cell membrane, receptors would have been extracted with a finite probability, which obscures the result of force mapping.

It is therefore necessary for us to determine the range of force required to unbind a ligand from the receptor and that for extraction of membrane proteins from the cell surface. This problem is addressed in the next section.

3.6 *Membrane protein extraction*

The cell membrane is a composite structure of phospholipids and proteins. Among cell-surface-associated proteins, intrinsic proteins are those that span the lipid membrane from one side to the other having a central hydrophobic segment connecting its extracellular domain to the cytoplasmic one and are thus securely anchored to the membrane. In general, they are not displaced from the membrane unless the latter is destroyed by, for example, treatment with detergents. Another type is called the peripheral membrane proteins, which are bound to the lipid surface or to the extracellular domain of intrinsic membrane proteins and thus easily displaced by the addition of, for example, chelating agents.

It is of fundamental and practical interest to measure the force required to dislodge the intrinsic membrane proteins from the cell surface by forcibly pulling them out using the AFM. For this purpose, we have to make it sure that the weakest bond in the force spectroscopy system between the tip and the substrate is the targeted bond or interaction. We therefore used the modified AFM tip with the amino-reactive bifunctional covalent crosslinkers, such as SPDP, Sulfo-SPDP and DSS. Once covalent bonds are formed between the crosslinkers on the tip and the amino-bearing molecules on the cell surface, the subsequent separation of the tip from the substrate should disrupt the noncovalent interactions between the membrane and the intrinsic protein provided that such interactions could be ruptured with a force significantly less than the force required to sever a covalent bond, i.e., in the range of 1.8 ± 0.33 nN.[30,44]

Some of the experimentally observed force curves under such an experimental setup are given in Fig. 11. When an unmodified tip was used, the force curve often showed a downward deflection in the retraction regime with a gradual decrease in the degree of deflection without any jumps to the horizontal position of the cantilever. This is probably due to the almost continuous unbinding events of multiple weak adhesions between the tip and the cell surface. For a modified tip with covalent crosslinkers, a similar downward deflection was prolonged and terminated abruptly with a jump to the horizontal position. Sometimes, two or three such jumps were observed before the final jump. Some of the force curves even ended with a large downward deflection signifying that the tip was never released from the cell surface.

When the force change associated with the jumps was collected as a histogram, the majority of the force values clustered at approximately the mean with an SE of 450 ± 220 pN. It was shown that the AFM tip used in the above experiment actually carried extracted proteins as tensile materials on its surface, which was removed by the treatment with proteolytic enzymes. The identification of the extracted proteins was attempted by the immunofluorescence method and integrins and fibronectin were identified.[18]

The histogram of the final rupture force is given in Fig. 12 together with the loading rate dependence of the mean force value of the histogram in Fig. 13. An almost flat dependence of the extraction force on the loading rate is apparent and it confirms the theoretical framework of Bell's proposition[13] that the activation distance of protein extraction from the lipid bilayer is as long as a few nanometers thus reducing the slope of the plot to as low as a few piconewtons. Since, under a similar loading rate condition, the force of uprooting intrinsic membrane protein must be at least five times larger than the unbinding force of ligand-receptor interaction involving integrins and fibronectin. Thus, it is safe to conduct receptor mapping experiments based on the force spectroscopic method.

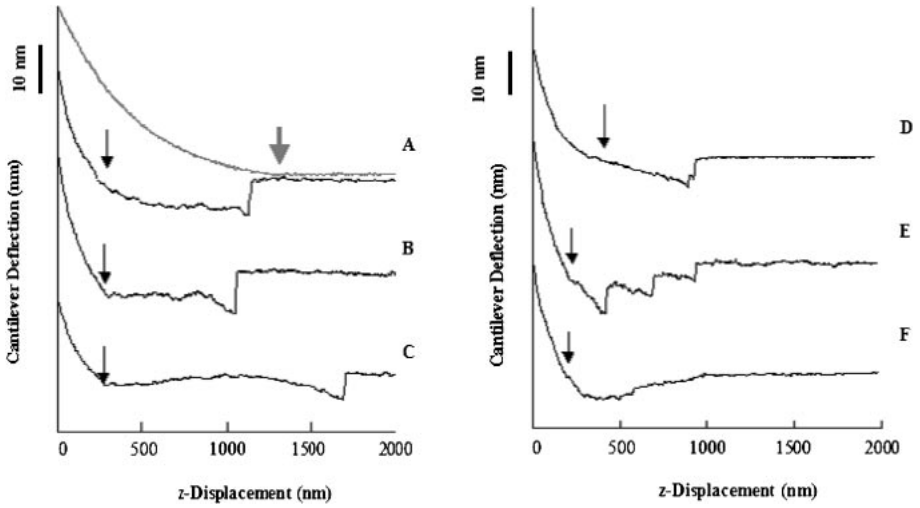

Fig. 11. Force curves obtained on the viable cell surface using AFM tips modified with bifunctional covalent crosslinkers. Left: a force curve depicting contact with the cell surface (top) and retracting curves after contact (bottom three curves). Right: retracting curves with double (top), triple (middle) and zero jumps. The gray arrow in curve A shows the approximate position of the tip in contact with the cell surface, and solid arrows in other figures represent the position where compression turned to stretching of the sample (cited from ref. 19 with permission).

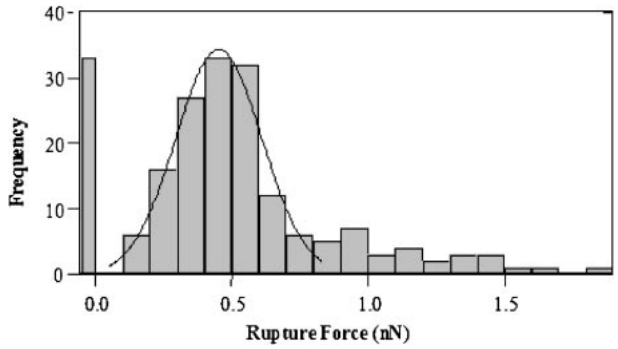

Fig. 12. Histogram of the force obtained for protein extraction from the living cell surface. Result between 100 to 700 pN bins was approximated by a Gaussian curve in solid (cited from ref. 19 with permission).

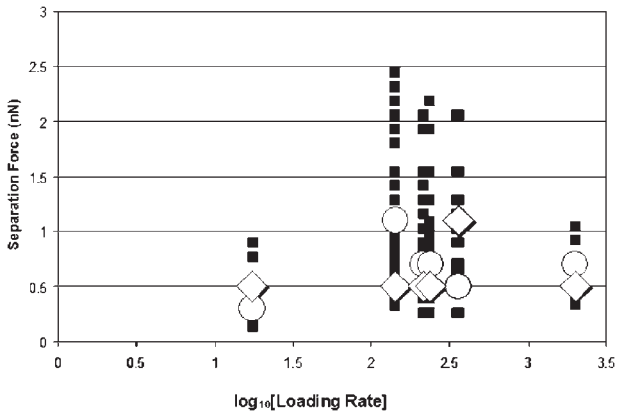

Fig. 13. Loading rate dependence of protein extraction force from viable cell surface. Diamonds represent the most probable value in each set of data, and circles the second most probable (cited from ref. 18 with permission).

The extraction of a well studied membrane protein, bacteriorhodopsin, has been reported to show several force peaks corresponding to the separation of helical loops from each other.[45] Since membrane proteins are crucially important in the origin of cellular life, detailed studies on their anchoring force to the lipid bilayer will shed light on principle of compartmentalization of biochemical molecules.

How the membrane proteins are extracted from the cell membrane is not a simple matter because they interact with membrane lipids rather strongly. It has been suggested and broadly accepted that membrane proteins are generally surrounded by phospholipid molecules with a higher affinity than that involved protein-protein interaction so that proteins would not form large aggregates in the membrane. In fact, membrane proteins may form specific dimers or oligomers but not large aggregates except for a very special case of bacteriorhodopsin where protein molecules form patches of 2D crystals. Such observation suggests that protein-lipid interaction is generally sufficiently strong so that when protein molecules are extracted, some lipid molecules are likely to be extracted with the protein molecules. In addition, it is also possible that the associated lipids are detached from the cell surface as a small vesicle rather than as a flat patch. Although the estimate of the magnitude of the force required in each possible process is not yet established, Bell gave a simple method of estimation for the first two cases by providing hydrophobic free energy for the transfer of constituent amino acid side chains on the membrane spanning segment of glycophorin A, a typical membrane protein. His estimates of the force required for the extraction of a bare protein segment stripped of all the boundary phospholipids was about 100 pN, and was 250 pN if extracted with a monolayer of such lipids.[13]

To estimate the force in the case of vesicle detachment, we need to determine the size of the lipid vesicle that is cleaved off from the cell surface with the target protein molecule. It is also necessary to determine the shape of the cell membrane immediately before vesicle formation. The newly created vesicle has completely different principal and Gaussian curvatures from the original rather flat cell surface. Since both of the curvatures are larger than those of original

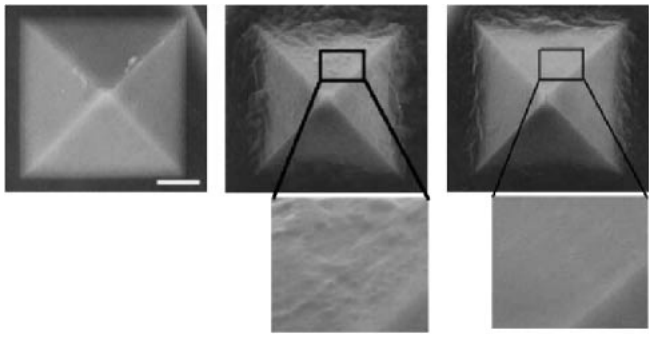

Fig. 14. SEM images of AFM tips used for extraction of cytoplasmic components. (Left) unused tip with a scale bar of 1 μm. (Middle) used tip with cytoplasmic components adsorbed on the surface. (Right) used tip that was brought close to the cell but not into the cell. The bottom figures are magnified imaged of the corresponding top figures (cited from ref. 47 with permission).

surface, the free energy increase due to vesicle formation is positive. During the deformation of the cell membrane before the detachment of the vesicle, the bending free energy increases but that due to the Gaussian curvature remains constant. When the positive bending energy is divided by the activation distance in the uprooting process, one gets the estimate of the force required for the detachment of membrane protein from the cell surface by creating a small new vesicle. Much of the detailed information necessary for obtaining the numerical estimate is still lacking in this area.

3.7 Extraction of mRNA from live cells

Harvesting mRNA from the cytoplasm of live cells and genomic DNA from isolated chromosomes has been successful, but are still being developed in our group.[46–48] In the case of mRNA extraction, an unmodified AFM tip was forcibly inserted into the cell interior and, after a specified time, pulled out and recovered in a test tube. The tip surface was undoubtedly covered with various components of the cytoplasm, especially proteins and mRNAs among others as the SEM image of one of such tips given in Fig. 14 suggested. The tip recovered in the test tube was then subjected to PCR (for polymerase chain reaction) for the amplification of specific mRNAs by adding primer DNAs with known targeted sequences, for example, a partial base sequence matching that of β-actin mRNA. After PCR, the amplified mRNA was identified by electrophoresis followed by hybridization with fluorescence-labeled DNA fragments with a partial β-actin sequence. If due precaution is taken to eliminate the contamination of RNAs from the culture medium, it is possible to identify the presence of several types of mRNA in various parts of a single viable cell. Figure 15 shows the result of one of such experiments that showed the localization of β-actin mRNA in the regions close to the nucleus in a resting cell and its spreading to perinuclear zones upon stimulation with fetal calf serum.

Xu and Ikai succeeded in extracting DNA fragments from a single piece of mouse chromosome using a modified AFM tip covered with amino groups so that at pH 11, the tip was uncharged and used to image a targeted chromosome. After imaging, the pH of the solution was changed to 7.4 so that the tip became positively charged thereby generating strong interactions with negatively charged DNA. The tips that recorded force curves indicating prolonged pullout of DNA from the chromosome were individually recovered in test tubes and adsorbed DNA was PCR amplified. The amplified DNA was fluorescence-labeled and used for fluorescence in situ hybridization (FISH). The result shown in Fig. 16 shows the image of the chromosome with a mark on the spot where the AFM tip was pushed in together with the result of FISH which confirmed that the PCR-amplified DNA had indeed come from the corresponding spot marked on the targeted chromosome. This line of research has potential for application to the identification of individual genes and their genetic aberrations on specific loci of the chromosome.

The extended use of probe microscopy for the actual harvest of a small number of macromolecules from a single viable cell will open a new field in biochemistry and will contribute to the understanding of cells' individual behaviors and basic medical research including drug tests.

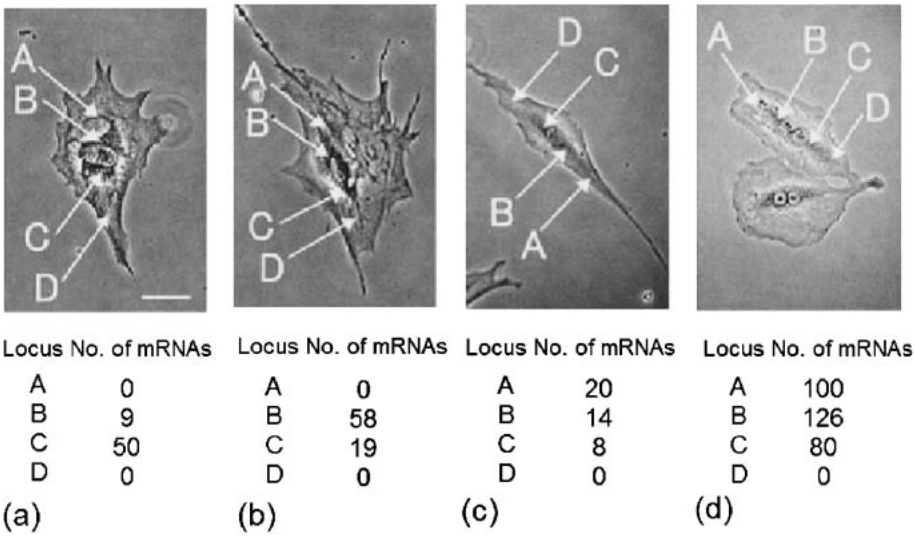

Fig. 15. Detection of β-actin mRNA in several different locals of viable cell by new method using AFM tip. (a) and (b): Extracted from non-stimulated cells at indicated positions of A, B, C, and D with a scale bar of 10 μm. (c) and (d): Extracted from activated cell with fetal calf serum at positions A, B, C, and D (cited from ref. 47 with permission).

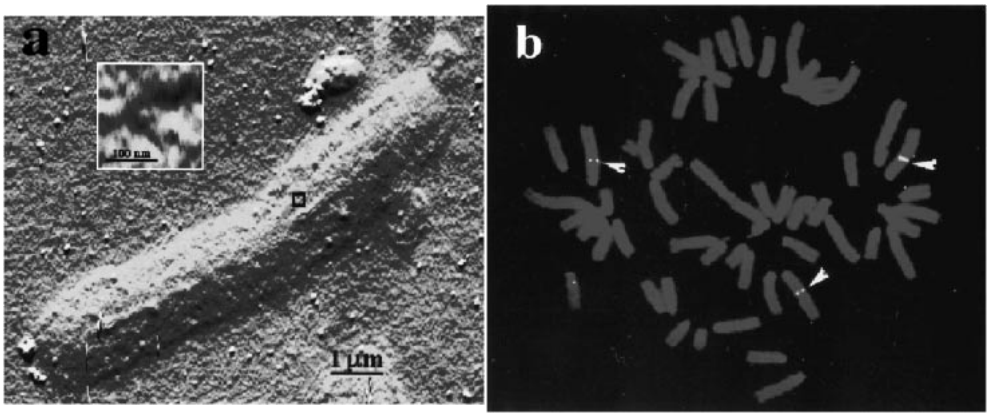

Fig. 16. Extraction of DNA from a specified locus of mouse chromosome. (Left) An AFM image of chromosome at pH 11 where tip-sample interaction is minimal. After changing the solution pH to 7.4, the tip was pushed into the chromosome at the position indicated by a small square. (Right) Result of in-situ hybridization showing that PCR-amplified DNA hybridized with chromosomal DNA at estimated position of original extraction (cited from ref. 48 with permission).

4. Discussion

The measurement of the force required to disrupt biostructures based on protein-protein and protein-lipid interactions is the focus of this article. The result of recent measurement is expected to reveal the mechanical principles of the internal architecture of these structures, which cannot be explicitly measured using conventional technology and therefore has not been investigated in the past.

The *F–E* curves obtained during the forced unfolding of several globular proteins gave a rare insight into the presence of rigidly folded local substructures inside the native conformation of such proteins. Whether this observation represents a general feature of protein molecules is a question to be answered after studying many more cases. It is still noteworthy that the classical question of how proteins can be soft and yet sustain the activated state of covalently bonded molecules has been partially answered by new evidence of the presence of locally rigid substructures. Due to the presence of such locally rigid substructures, the internal mechanics of protein is rather more complex than hitherto imagined. It is also true, as has been sufficiently demonstrated, that proteins cannot only be rigid. The rigid parts of a protein molecule must also coordinate with more flexible and soft parts of the same molecule for the whole molecule to perform its biologically significant roles. We are now encountered with several new questions as follows.

(1) How can rigid and soft parts be created on the basis of a small number of noncovalent interactions?
(2) Are the composite structure of soft and hard parts of protein molecules really necessary for these molecules to fulfill their biologically significant roles such as an enzyme?
(3) How do the two mechanically different parts of a protein molecule work in enzymatic catalysis?
(4) How does the force of anchoring of membrane proteins to the lipid bilayers compete with the force exerted by cellular movement like locomotion?
(5) How effectively can we harvest membrane proteins on an AFM tip to perform precise in situ analysis of their identity, distribution, and relative abundance?

Answers to these questions will be obtained when technology for mechanical measurements on nano-biostructures has made considerable improvements in terms of accuracy and 3D resolution.

5. Conclusions

We have established a method of investigating hitherto unexplored physical properties of globular proteins using force spectroscopy based on the force curve mode of the AFM. Globular proteins have been shown to possess mechanically rigid sub-structures which, when under tensile stress, showed fracture mechanics similar to that of brittle materials. Membrane proteins can be extracted from the cell surface by a force less than that required to break a covalent bond but significantly larger than that required to separate ligand-receptor pairs on the cell surface. Receptor mapping and cell membrane harvest can, therefore, be performed in distinct force ranges.

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