Nanotechnology and Protein Mechanics

A Review of the work from Biodynamics Laboratory of Tokyo Institute of Technology

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Abstract. The atomic force microscope is currently used in our and many other laboratories to measure the mechanical response of polypeptide and proteins against tensile forces applied to well defined positions in their chemical structures. The resulting force vs. extension (F-E) curves are analyzed in relation to their known conformations under various conditions. The method can be extended to study the mechanical responses of other, often much larger biological structures, and extract the component proteins and DNAs from cell membranes and chromosomes.

Key words: atomic force microscope, bionanomanipulator, nanomechanics of proteins, nanotechnology, protein extraction

1. Introduction

The atomic force microscope enabled us to measure the intra- and intermolecular forces that hold bio-molecular structures together. The nano-mechanics of proteins, DNAs and cellular structures has been intensively explored in the last decade and provided interesting insights into the mechanical properties of nanometer scale biological structures [1–4]. The atomic force microscope (AFM), when combined with an optical microscope and a nano-manipulator allows us not only to measure the nano-mechanical properties of bio-materials but also to operate on them at the same time. We are currently using an AFM alone or in combination with a total internal reflection fluorescence microscope and a manipulator with high precision movements when necessary:

1. to stretch single molecules of polypeptides and proteins and find their intra- and intermolecular mechanics in terms of the tensile force vs. chain elongation (F-E) relationship to be used in the future design of molecular machines. Polyglutamic acid (PGA) in its helical and randomly coiled conformations [5], the α -helical proteins calmodulin [6], and the globular enzyme carbonic dehydratase 11 [7–9] were mechanically stretched under various solution conditions. In these studies, sample proteins were first immobilized on an aminosilanized crystalline silicon surface through bifunctional covalent cross-linkers to en-

sure covalent holding of the sample molecules between the AFM tip and the substrate.

2. to measure the force responsible for the binding of receptor and ligand molecules on the cell surface [10]. A more general cell surface engineering is also scheduled using our bionanomanipulator [11].
3. to inject functional macromolecules into living cells at specific sites without increasing their volume. Functional molecules were covalently attached to a ZnO whisker using a photo-cleavable cross-linker and the latter was inserted into the cell. Irradiation of near UV light freed the molecules from the whisker that was subsequently pulled out of the cell [12].
4. to pull out DNA from a piece of chromosome for later identification of the base sequence of the extracted DNA after PCR amplification [13].

This is meant to be a review article of the recent work from our laboratory with some extensions of the original discussions. The readers are referred to the original articles cited in the text for experimental details.

2. Basic Experimental Strategies

In our stretching experiments of proteins and polymers, bifunctional covalent cross-linkers such as, disuccinimidyl suberate (DSS), *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), *N*-succinimidyl 6-[3'-(2-pyridyldithio)-propionamidol hexanoate (LC-SPDP) (all from Pierce, Rockford, IL, U.S.A.) and SPDP with a long spacer of polyethylene glycol PEG3400-MAL (Shearwater, Huntsville, AL, U.S.A.) were used. The succinimidyl and pyridyldithio moieties of the cross-linkers respectively react with amino and sulfhydryl groups on target molecules or with modified surfaces of AFM tips and substrates. The latter were usually treated with 3-aminopropyl-triethoxysilane (APTES) or 3-mercaptopropyl-triethoxysilane (MPTES) (Aldrich Chem., Milwaukee, WI, U.S.A.). Experimental procedures and preparation of materials are given in the original articles.

3. Major Results and Discussions

3.1. STRETCHING OF HELICAL PEPTIDE

The pH dependence of mechanical stretching of α -helical polypeptide, polyglutamic acid (PGA) in aqueous buffers of various pHs is summarized in Figure 1 in the form of force vs. extension relationships [5].

To avoid non-specific adsorption of PGA to a silicon substrate, a bifunctional covalent cross-linker having a polyethylene glycol (PEG) spacer was used in the actual experimental setup. After obtaining force vs. extension (F-E) curves of the combined system of PEG cross-linker and PGA, the contribution of the PEG cross-linker to the force-extension curve was graphically subtracted. The F-E curve of the PEG cross-linker was found to be independent of the solution pH. The resulting F-E curves were then normalized with respect to the total extension of 30 nm.

Figure 1. Mechanical stretching of polyglutamic acid at 6 different pHs between pH 8.0 and 3.0. The ordinate is the tensile force in nN and the abscissa is the extension of the chain in nm. The extension is normalized to 30 nm. The lowest line in each figure represents the F-E curve of entropic random coil with the persistence length of 0.37 nm.

Normalization of extension length has been used for other polymers and may be verified for homopolymers [14]. At an acidic pH of 3.0, PGA is presumably in a helical conformation and the stiffness of the chain is high, whereas at pH 8.0, the chain is in a non-helical randomly coiled state showing the lowest stiffness among the results obtained at 6 pHs between 3.0 and 8.0 [1]. One of the surprising

Figure 2. Schematic trajectories of helix extension on the Ramachandran plot. Circled numbers show two routes from alpha-helical conformation to all-trans conformation.

features of the F-E curves of PGA stretching was that the chain was stretched very smoothly without major breaks in the F-E curve except that only very occasionally there were one or two force peaks toward the end of stretching (data not shown). The result indicated that a helical chain was extended continuously to the all-trans form without discontinuous conformational transitions. On the Ramachandran plot of the dihedral angles, ϕ and ψ , the observed stretching corresponds to a change from around ($\phi = -57^\circ$, $\psi = -47^\circ$) to ($\phi = \pm 180^\circ$, $\psi = \pm 180^\circ$) [15]. Possible trajectories of this conformational change on the Ramachandran plot are schematically shown in Figure 2 with two arrows, numbered 1 and 2, respectively. We think that trajectory 1 is closer to the actual pathway because it goes through a lower energy basin compared to the alternative route, but more experimental as well as theoretical results are needed to confirm this point.

By limiting the extension length to less than the total contour length of the chain, the extension-retraction cycle of PGA can be repeated more than 200 times before the covalent cross-linking system was accidentally broken. From an engineering point of view, the above result suggests that PGA in an acidic media can be used as a reversibly responding, reliable nano-sized spring in the future construction of molecular machines.

Figure 3. Extension of BCAII under native conditions using PEG cross-linker. Curves 1, 2 and 3 correspond to F-E curve of PEG cross-linker, that of tethered BCAII with PEG cross-linker, and the resulting F-E curve after subtracting curve 1 from 3, respectively. Taken from [7] with permission.

3.2. CARBONIC ANHYDRASE II

We have also stretched the globular protein, bovine carbonic anhydrase II (BCAII), in three different ways. First, wild type BCAII with two cysteine residues at its N- and C-termini was cross-linked to an amino-silanized substrate and a similarly modified tip using the PEG cross-linker. After subtraction of the PEG extension, we obtained the result given in Figure 3 [7].

The extension of the protein under a native condition was as short as 14 nm (corresponding to 38 fully extended residues) after graphical subtraction of the average extension profile of the PEG cross-linker, whereas under a denaturing condition of 6 M guanidinium chloride it was between 90 and 100 nm [7], a close value to the theoretical length of the protein with $259+2$ (for cysteine residues) amino acid residues assuming that the contour length of a fully extended amino acid residue to be 0.37 nm. The force value at the end of stretching for both curves, being about 1.7 nN, is large enough to break a covalent bond in the cross-linking system.

Figure 4. Stretching of BCAII without using PEG cross-linkers. Group of curves in the left are for native protein and those on the right for denatured protein in 6 M guanidinium chloride. The solid line on the far right is a theoretical curve for the worm-like-chain. Taken from [8] with permission.

Second, we stretched the wild type protein without using PEG cross-linker but using an ordinary LC-SPDP and obtained the results in Figure 4 [8]. The extension of the native protein was about 14 nm and agreed with the result obtained above using a PEG cross-linker. Previous results using a PEG cross-linker assured that the steep rise of the F-E curve for the native protein in this case after 10 nm extension was not due to the adsorption of the protein to the substrate, but represented a true mechanical response of the protein.

We therefore concluded that the native conformation of BCAII was very difficult to unfold by applying a tensile force from its two ends, but it can be extended to its full length once denatured. The F-E curves of the protein under denaturing conditions sometimes gave a very close fit to the worm-like-chain (WLC) curve [16] but more often they were significantly different from the latter. It is probable that the protein in 6 M guanidinium chloride retains some residual structures, and the extent of such structure is different from chain to chain.

Addition of the specific inhibitor of the protein, *p*-aminomethylbenzene sulfonamide, changed the F-E curve as given in Figure 5 [7]. The initial low force region is now extended to about 28 nm before the tensile force steeply rose to 1.7 nN, indicating that an extra 14 nm extension from the inhibitor free form became available before the breakdown of the covalent system. We think the observed change in the F-E behavior in the presence of the inhibitor signified binding of

Figure 5. F-E curves of CABII in the presence of p-aminomethylbenzene sulfonamide, a specific inhibitor of the enzyme. Taken from [8] with permission.

the inhibitor to the protein with concomitant changes in mechanical properties of the latter and the force measuring mode of AFM is sensitive enough to record the change. It is interesting to observe that the extendable part of the protein is now 14 nm longer than in the absence of the inhibitor. Binding of the inhibitor made a part of the protein softer than in the absence of inhibitor binding.

The observed tightness of BCAII against the tensile force, both in the absence and presence of the inhibitor, may be attributed to the fact that the protein has a 'knot' structure in the C-terminal region as can be seen in Figure 6. Pulling the protein from its two ends might tighten the knot creating an unusually rigid protein structure.

We then constructed a mutant of BCAII by replacing Gln253 with a cysteine thus creating a Gln253Cys mutant having the other cysteine residue at its N-terminal. The mutant protein had 49% of the enzymatic activity of the wild type enzyme and very similar CD and fluorescence spectra to those of the native protein. When this protein was stretched from the two cysteine residues, the knot tightening explained above can be avoided. The result is exactly as we expected as shown in Figure 7 where the protein is extended smoothly almost to its full contour length of 96 nm.

Since the F-E curves of the mutant were more similar to those of denatured protein, we added one of the specific inhibitor of the protein to the solution and measured the F-E curves again. An interesting result was that about half of the F-E curves (Type I in Figure 7) became like the ones in Figure 5, indicating that

Figure 6. Knot structure in BCAII. The C-terminal segment goes under the N-terminal segment in such a way that a forced extension from C- and N-termini tightens the knot conformation. Taken from [9] with permission.

they corresponded to active proteins, whereas the rest of the force curves (Type II) were not changed to a significant degree suggesting they represent F-E curves of the protein that did not bind inhibitor molecules.

3.3. MEASUREMENT OF INTERACTION FORCE BETWEEN GROEL AND DENATURED PROTEIN

In addition to the measurement of intramolecular cohesive force as above, the interaction force in biomolecular systems can be measured with AFM as shown by Florin et al. on the biotin-avidin system [17]. We applied this method to two systems:

1. Force between a denatured protein (pepsin in a neutral buffer) and GroEL, a protein known as *E. coli* chaperonin. In this case, an AFM tip covered with covalently immobilized gastric pepsin was brought into a close proximity but not into actual contact with GroEL proteins sitting on the substrate. Pepsin is denatured at a neutral pH and when the tip was in proximity of the GroEL protein on the substrate, parts of a pepsin molecule are expected to come in

Figure 7. BCaII stretching without knot tightening. Many curves were obtained with a wide variety in the extension length before the final rupture and they were found to form two groups after histogram analysis. The two groups are designated as Type I and II.

touch with one of the GroELs and, when the sample stage is retracted, we could record the cantilever deflection accompanying the forced separation of pepsin from GroEL. In this way, it was assured that GroEL was not damaged by the applied force from the tip. The force-distance curves thus obtained were characterized by a plateau of about 40 pN in force and 13 nm in duration. Addition of ATP to the experimental solution reduced the plateau to a single peak but the force at the peak was still in the range of 35 to 40 pN. These observations were interpreted in terms of the known structure of GroEL and its structural changes in the presence of ATP [18].

2. Force between fibronectin, a protein that mediate adhesion of cells to matrix substrates, and the cell surface protein integrin [11]. In this case, the AFM tip was replaced with a latex sphere of an approximate diameter of 10 μm and covered with covalently anchored fibronectin molecules. Because of a wide area of contact between the latex bead and the cell surface, the force curves was analyzed after integrating them with respect to the extension length giving rise to a new quantity, which we called 'work of separation', having a dimension of energy. The magnitude of this quantity was dependent on the presence of free fibronectin in solution suggesting its specificity. A fairly large section of a living cell surface was scanned with a tip modified with fibronectin and

a map representing local strengths of interaction between the tip and the cell surface was constructed.

3.4. EXTRACTION OF MEMBRANE PROTEIN FROM THE LIVING CELL

An AFM was used to extract membrane proteins from the cell surface after covalent bond formation was completed between the amino groups on membrane proteins and those on the bifunctional cross-linkers on the tip [13]. For this purpose, an AFM tip was first amino- or mercapto-silanized with APTES or MPTES and functionalized with DSS or SPDP. The modified tip was brought to a brief contact with a living cell surface and the force required to rupture the contact between the tip and the cell surface was measured. The measured force of 300–1000 pN (the mean was 350 pN) was greater than the force known to break non-covalent interactions between ligand and receptors (50–200 pN) [19], but much less than the force to sever a covalent bond (1.7–2 nN) [5, 20]. We took the result as an indication that, during the separation process, membrane proteins were extracted from the cell surface. The presence of extracted proteins on the AFM tip was later proved by taking force curves between the tip and a modified glass surface with amino-reactive cross-linker, SPDP. Most of the force curves showed the presence of tensile material that could be extended up to 150–200 nm after brief contacts with the surface and the final rupture force was larger than 1.5 nN (details will be published elsewhere).

Extraction of proteins from bio-membranes will be an important technology when it becomes necessary to follow the time sequence of the expression pattern of membrane proteins on a living cell. The immuno-fluorescence method of protein identification is currently a powerful method for a large scale mapping of membrane proteins but it often requires fixing of cells as a necessary procedure. Since the method of protein extraction on an AFM tip described above can be applied repeatedly on selected areas of a living cell and, when combined with immuno-fluorescent identification of extracted molecules, will be a useful tool to monitor the time dependent change of membrane protein expression on a living cell.

Other types of application of the force measurement mode of the AFM including extraction of genomic DNA from chromosomes and insertion of functional molecules into a selected cell are also under way in our laboratory. Our previous work on the extraction of DNA was done from acid treated chromosomes using AFM tips [9]. In this work, mouse chromosomes were first imaged in the tapping mode in buffer at pH = 10 using an AFM tip modified with the amino-terminated silanization reagent, APTES, 3-aminopropyl-triethoxysilane (Aldrich Chem., Milwaukee, WI, U.S.A.), and after imaging, the buffer was replaced with a neutral one at pH 7. The AFM tip was then brought to a targeted small region on the chromosome and pushed into it by applying a large force. When the tip was retracted from the chromosome, a small amount of chromosomal DNA was expected to adsorbed to the tip due to electrostatic interactions between negatively charged DNA

and the positively charged amino-silylated tip in a neutral buffer. This operation was repeated several times, each time replacing a used tip with a new one and the used tips were collected. The PCR amplification protocol was performed on each used tip placed in separate test tubes and the presence of amplified DNA as well as its specificity was studied by electrophoresis and by the fluorescence *in situ* hybridization (FISH) method applied on a fixed set of chromosomes prepared from the same type of the cells. This is a promising method to extract genomic DNA from chromosomes for later amplification by PCR method and sequencing. The efficiency of extracting the targeted DNA was rather low and we are presently trying to improve the efficiency of DNA extraction. We are also improving the method so that genomic DNA may be extracted from native chromosomes which are not treated with an acid medium.

4. Conclusions

Mechanical handlings of proteins and DNA were done to obtain new information and to develop novel methodologies for the cellular and sub-cellular level operations. Proteins and polypeptides behave like non-linear springs with sensitive mechanical responses towards environmental changes such as solution pH, metal and inhibitor binding. Methods and knowledge developed and gained in such mechanical experiments were found useful in more applied fields of protein and DNA extraction from biological structures such as plasma membranes and chromosomes. Nano-mechanical manipulation of biological molecules and structures is expected to constitute an important field in biological physics as well as in medicine.

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