Mechanical unfolding of α_2-macroglobulin molecules with atomic force microscope

Keita Mitsui[a], Masahiko Hara[b], Atsushi Ikai[a,*]

[a]*Laboratory of Biodynamics, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 226, Japan*
[b]*Riken Institute, Wako, Saitama 351-01, Japan*

Received 24 January 1996; revised version received 13 March 1996

Abstract **α_2-Macroglobulin was derivatized with a sulfhydryl cross-linker and sandwiched between a mica substrate and a solicon nitride tip, both coated with gold, of an atomic force microscope and force curve measurement was carried out. An extensive downward deflection of the cantilever was observed in the retracting realm of the curve, when and only when the substrate was covered with the derivatized protein. The result was interpreted in terms of the mechanical stretching and unfolding of a single or a few protein molecules.**

Key words: Atomic force microscopy; Force curve measurement; Protein stretching; Protein unfolding

1. Introduction

Since its invention by Binnig et al. [1], the versatility of the atomic force microscope (AFM) has been amply demonstrated in various disciplines in the biomedical science. An AFM is equipped with a sharp tip at the free end of a soft cantilever which scans over a sample keeping the tip at almost touching distance from the sample surface. The tip and cantilever movement thus digitally reproduces the topography of the sample surface and displays it on a computer screen. Since the AFM tip can make contact with the sample surface, it is also capable of measuring physical properties of the specimens.

Its imaging capacity of soft and non-conducting specimens under physiologically relevant conditions has contributed to its enormous popularity among biologically oriented researchers. To mention only a few examples from many exciting reports, we think (1) the imaging of live cells and living processes as summarized in [2], (2) the growth and division of yeast cells [3], and (3) the polymerization processes of biopolymers [4] have much to offer in terms of the usefulness of AFM in life science. Another very important and exciting application of AFM was demonstrated by Florin et al. [5]. Exploiting the measuring capacity of AFM, they reported a force measurement between single molecules of avidin and biotin, a system of wide biochemical use due to their unusually strong interaction. They coated agarose beads with cross-linked biotin and approached one of them in an aqueous buffer with an AFM tip covered with avidin molecules. They obtained force curves that showed stepwise changes in the deflection of the cantilever, indicating that a quantized amount of force was measured in the process of dislodging avidin from biotin or vice versa. They kept their analysis to the magnitude of the force required to free the tip from the substrate, and concluded that a force of approximately 160 pN was required to separate a single pair of avidin and biotin. It was the first of such measurements using AFM. Recently a similar measurement of the interaction between peptide glycan molecules has been reported [6], where both the glass substrate and the silicon nitride tip were coated with the same kind of peptide glycans and the force curve between them was measured in sea water with and without calcium ions. The average force required to separate two peptide glycans was 1–2 nN. Another interesting approach to the single molecule measurement has been reported by Lee et al. on a strand separation process of hydrogen bonded polynucleotide pairs [7].

In this paper, we tried to sandwich protein molecules between the substrate and the tip by covalent bonds. For this purpose a serum glycoprotein, α_2-macroglobulin (α_2-M) was derivatized with *N*-succinimidyl pyridyldithio propionate (SPDP) and free sulfhydryl groups were exposed immediately before use. Both the substrate and the tip were coated with gold. The protein was first cross-linked to a gold substrate in a buffer solution and gold-coated tips were used for the subsequent force curve measurement. The resulting force curve demonstrated that strong bonds, presumably Au-S covalent bonds, were formed during the brief contact period between the tip and the sample and, when the sample stage was lowered, the cantilever was pulled downward until the exerted force on the cantilever reached a certain limit. The result indicated that the sample sandwiched between the tip and the substrate was extended up to 50–400 nm before the tip was freed. Since such force curves were obtained only when the derivatized protein with SPDP was used, we concluded that our experiments demonstrated a case of mechanical stretching of protein molecules using AFM. We occasionally observed processes that could be interpreted as involving a single molecule of protein being stretched from 50 to 150 nm. We think such cases can be regarded as the first examples of the mechanical unfolding of a single protein molecule. We show possible ways to extract useful information on single molecule mechanics from the force curves obtained in our experiments.

2. Materials and methods

2.1. Proteins

α_2-M was prepared from human blood serum according to the established method [8]. It was derivatized with SPDP as previously described [9] and reduced with dithiothreitol to expose sulfhydryl groups immediately before use for AFM measurement and passed through a short column of Sephadex G-25 to remove the reducing agent. The average number of sulfhydryl groups on a subunit of the proteins was between 4 and 5 as determined by spectrophotometric methods [9]. The reduced sample was made 10 μg/ml in protein concentration and about 20 μl of the solution was applied to a clean surface of gold-coated substrate. The solution was blotted out after 5 min and the gold surface was rinsed, first with buffer solution and

*Corresponding author.

SSDI S0014-5793(96)00319-5

with distilled water for imaging in air. For force curve measurements, the protein sample was applied on a gold surface in a similar manner but the rinsing step with distilled water was eliminated. The protein sample was used within 1 h of elution from the G-25 Sephadex column to avoid possible cross-linking between protein molecules. *N*-(7-Dimethylamino-4-methyl coumarinyl) maleimide (DACM) was purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

2.2. AFM

A Nanoscope III multiprobe microscope (Digital Instruments, Santa Barbara, CA) was used in tapping mode when images were taken in air, and in the contact mode when the force curve was measured. Freshly cleaved mica was coated with thin layers of gold, approximately 100 nm in thickness under epitaxial growth condition at approximately 580°C [10], and silicon nitride tips and cantilevers were also coated with gold (less than 100 nm thick) at a lower temperature. Absolute values of the spring constant of the cantilevers used in this study were measured by pressing a macroscopic cantilever made from a piece of gold wire with known dimensions and Young's modulus. The deflection of the macroscopic cantilever was compared with that of the AFM cantilevers to calculate the force constants of the latter from the theoretical value of the former. The measured force constants ranged from 20 to 100% of the supplied value of 0.12 N/m by the manufacturer and the accuracy of the force constant for individual cantilevers was within ±15%. The force values reported in this study have therefore an ambiguity of ±15% [11]. The substrate coated with a protein layer was immediately mounted on the AFM sample stage and the liquid cell was constructed over it with the buffered saline inside. The time for one cycle of force curve measurement was between 10 s and 0.05 s. Experiments were done at 25°C.

3. Results

Fig. 1 shows a tapping mode AFM image in air of gold surface covered with α_2-M. The surface was covered with small particles with diameters of 30–50 nm. The particles were stably imaged when they were cross-linked to the gold surface, but when non-derivatized protein was used, similar particles were observed but at a much reduced frequency and those imaged were easily removed after 2 or 3 scans. The average size of the particles corresponded to that of α_2-M imaged under a transmission electron microscopy [12]. From the above observations we concluded that the particles of 30–50 nm in diameter imaged under AFM were single molecules of α_2-M. The size was slightly larger than the value reported for electron microscopic images of the protein but it is within a reasonable range when the tip sample convolution effect is considered. It is apparent from its size that the protein was not extensively denatured and probably retained most of its native structure but we did not check whether the protein was still active as a proteinase inhibitor or not.

Fig. 2a shows a reference force curve obtained on a protein free substrate. The ordinate of the diagram represents the deflection of the cantilever from its flat equilibrium position and the abscissa is the distance covered by the piezo movement which drives the sample stage up or down relative to the cantilever. In the case of Fig. 2a, the approaching realm of the force curve, as represented by a dotted line, is initially flat until it reaches the substrate surface, where it starts to be deflected linearly. We will call the flat part of the line the 'base line', representing the free position of the cantilever. The retreating realm of the force curve, as represented by a solid line in the figure, closely follows the approaching realm as expected for an elastic cantilever that is free of hysteresis.

Fig. 2b is another reference force curve taken on a substrate covered with non-derivatized protein. The force curve is noisier than the one in Fig. 2a, but it is generally very similar to the latter.

Fig. 2c represents a force curve obtained on a substrate uniformly covered with derivatized α_2-M with SPDP. The approaching realm of the curve is not much different from the previous one in Fig. 2a, except that the curve shows a gradual increase in the curvature in the contact zone between the tip and the substrate. The phenomenon is a typical one when the tip is making contact with a soft, deformable surface. A similar behavior can be noted in Fig. 2b. The retracting realm of the force curve closely follows, up to the contact zone, the approaching curve but deviates from it thereafter, extending below the base line. A negative cantilever deflection such as this one can be noted in previous publications [5,6], but not much effort has been paid to interpret it in physical terms. Since the sample stage is moving down steadily following the dashed line and the position of the tip is given by the solid line, the distance between the tip and the substrate is given by 'D'. The distance D keeps on increasing to approximately 200 nm below the base line where the tip is abruptly freed of the tension and snaps back to the base line. We call the maximum length of D at the moment of release D_{max}. Before the final release, the force curve shows several sharp edges preceded by smooth increases in D for some distances. It suggests that the tip is steadily pulled down following the sample stage movement until some unknown bond is stretched to its limit. The bond is then torn and the tip tries to snap back to the base line but it is resuspended with a reduced negative deflection because other bonds are now keeping it

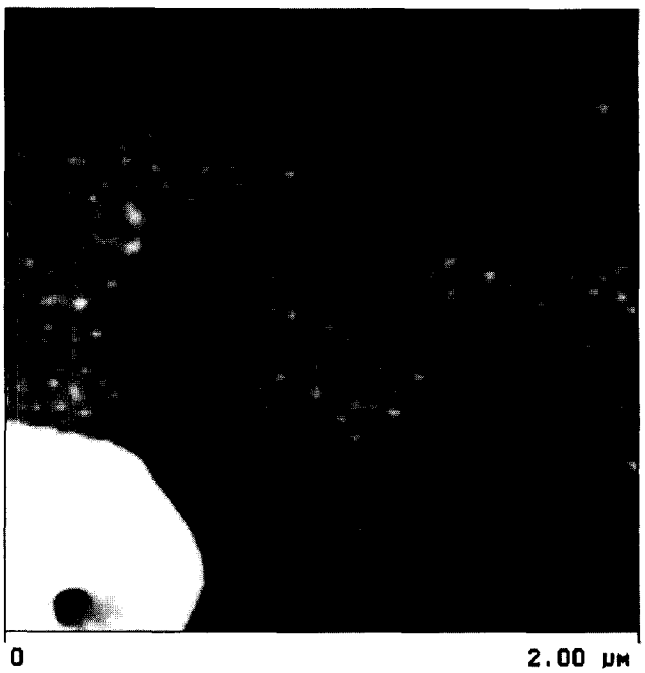
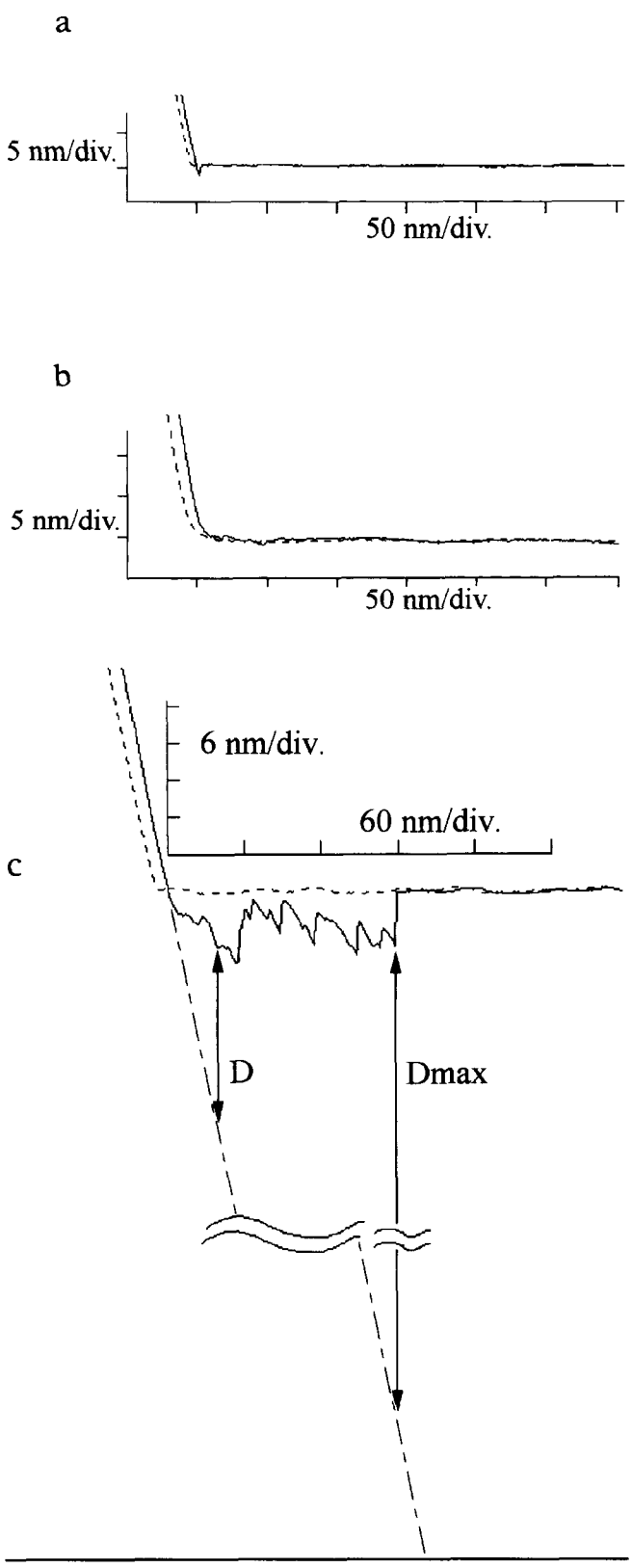


Fig. 1. Tapping mode AFM image of gold-coated mica substrate covered with derivatized α_2-M with SPDP. The substrate was treated exactly the same way as those for the force curve measurement but rinsed with deionized water and dried in the air. The AFM image was taken with an untreated silicon nitride tip. Small dots on the flat gold surface are proteins. This figure gives the approximate density of the protein layer over the gold surface when the force curve measurement was done. The height difference between the brightest and the darkest parts in the figure is 50 nm.



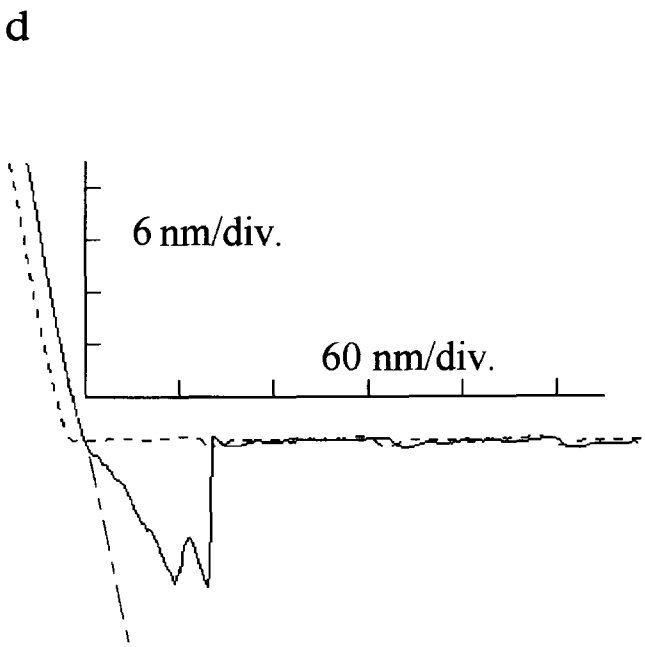


Fig. 2. Force curves obtained in buffer solutions under various tip and substrate conditions. In all cases the tip and the substrate were coated with gold. (a) A representative force curve taken on a bare gold substrate, (b) one obtained on a substrate covered with non-derivatized protein with a similar density as Fig. 1, (c) one obtained on derivatized protein with SPDP with the same protein density as in Fig. 1. The length of the suspended sample between the tip and the substrate is defined as D at any moment and the maximum length attained immediately before the final release of the tip is defined as D_{max}. (d) An example of the force curves that were occasionally obtained on a substrate with reduced density of protein and interpreted in the text as representing the force curve of single molecule unfolding event.

from the base line position. After repeated rupture of several bonds in the above manner, the tip is finally freed and the force curve recovers its base position. The force required to rupture the individual bond is proportional to the deflection of the cantilever and calculated with the knowledge of its spring constant. When the majority of the sulfhydryl groups of the derivatized protein on the gold surface were inactivated with DACM, we obtained force curves such as the one in Fig. 2b, and only rarely force curves with downward deflection were obtained.

Fig. 2d represents a simpler force curve where the protein was stretched to the length of 40 nm before the first bond was broken at the force of 1.1 nN, then came the second release at D_{max} = 67 nm and the release force of 1.1 nN again. We regard force curves such as this one represent stretching events of a single protein molecule (see section 4).

In the above experiments, the position of the tip was not changed for a single series of force curve measurement. Due to the large size of the tip (expected to be at least 100 nm in the radius at the apex after gold coating of thickness 100 nm) and to the random drift in the tip position relative to the substrate, the tip was capable of forming new bond(s) almost as often as it hit the derivatized protein layer. After repeated measurements of more than 20 force curves, however, the response became rather flat indicating that the reactive sulfhydryls were exhausted. It is therefore likely that the tip often hits protein molecules that were once stretched and most likely denatured. The response from denatured protein is another project to be investigated in the future.

Fig. 3a and b show, respectively, the distribution of D_{max}, the value of D measured immediate before the final release of the cantilever, and that of the force required at the final release of the tip. The distribution curve in Fig. 3a has two

peaks, at 150 nm and 300 nm, within a permissible range of estimated stretch length of one and two subunits of α_2-M (see section 4). Since we do not know the exact location of the cross-linking sites on α_2-M, it was not possible to get the precise length to be expected from single molecule stretching. The result in Fig. 3b will be discussed in section 4.

The force curve was measured at different frequencies ranging from 0.1 to 20 Hz. When the frequency was too low, the force curve was not stable due to the mechanical drift of the AFM sample stage and, at higher frequencies, the shape of the force curve became severely frequency dependent. We chose a frequency range between 0.5 to 5 Hz where force curves most often showed downward deflections of similar magnitude. It was difficult to confirm the reproducibility of the force curve at this stage because the sandwiching bonds were broken for every measurement and the stretching of the protein was most likely irreversible. Still, stable measurements of force curves of similar shape within the given frequency range implies that the protein sample was acting as a quasi-elastic rather than a plastic material.

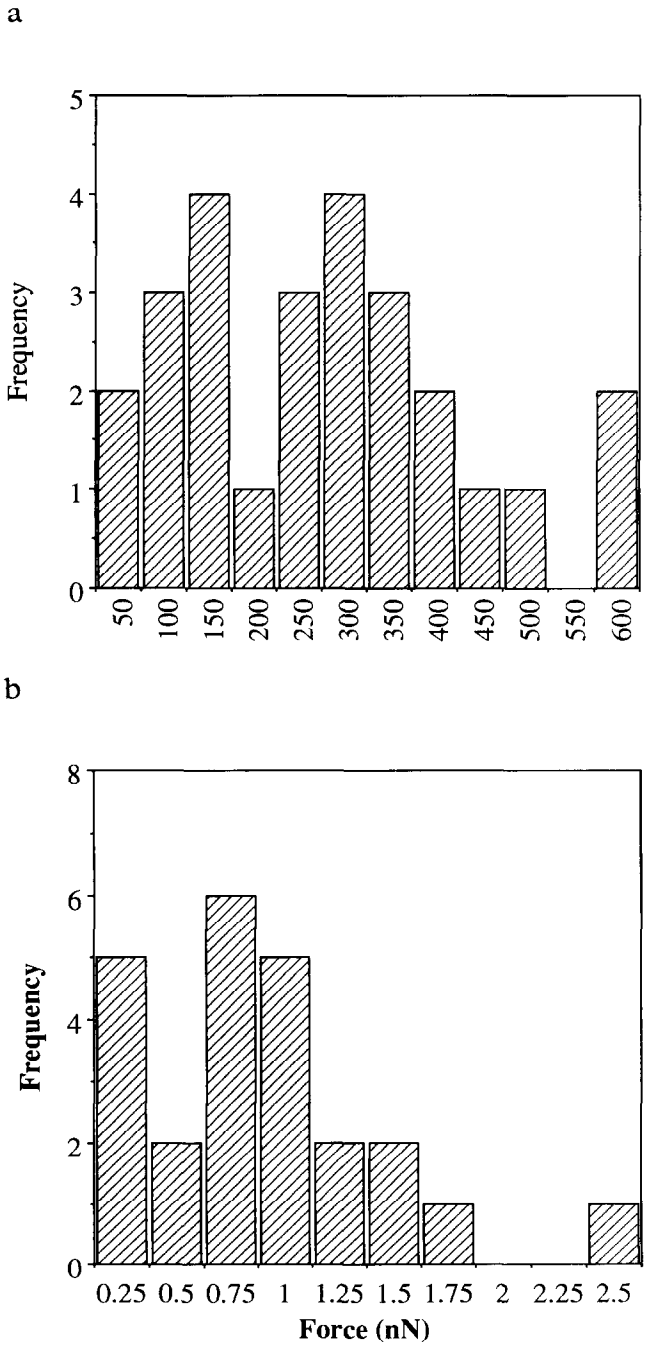


Fig. 3. Distribution of the force and the stretched length of the sandwiched sample at the moment of final detachment of the tip. (a) Distribution of the force required to free the tip finally to the base position, (b) distribution of D_{max} as defined in Fig. 2c for 30 measurements.

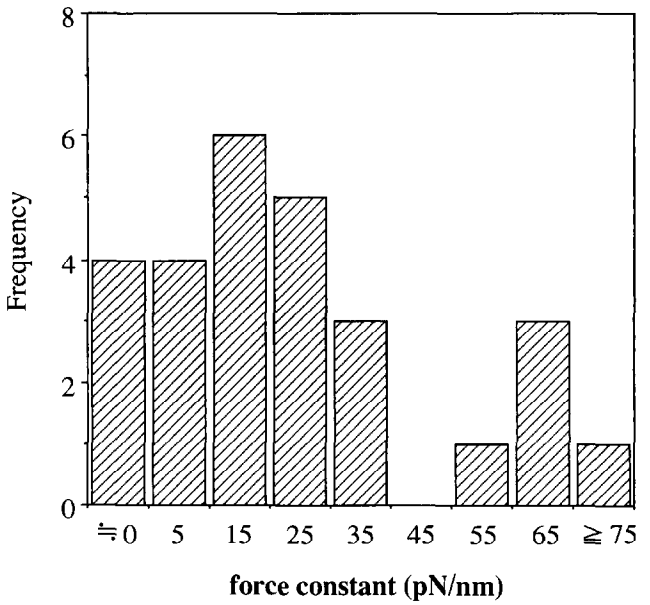


Fig. 4. Distribution of the spring constant of protein molecules suspended between the tip and the substrate. The spring constant was calculated from the average slope of the force curve in the initial stretching of up to 25 nm ($n = 27$).

Another series of experiments were performed where the tip, not the substrate, was covered with derivatized protein and the force curve was measured. Here, we do not go into details of the results obtained under these conditions, except to mention that we obtained force curves very similar to the one in Fig. 2d and reference experiments using uncoated tip and substrate gave similar force curves as in Fig. 2a.

4. Discussion

α_2-M is a large glycoprotein with a molecular weight of 725 000 and consists of four identical subunits, each having 1451 amino acid residues and 11 intra- and 2 inter-subunit disulfide bonds. Its amino acid sequence is known [13] and the low resolution crystallographic analysis of a chemically treated form has recently reported [14]. The native protein has a diameter of 20–30 nm under an electron microscope [12]. In our experiment, the protein was adsorbed to the gold substrate thus it is possible that the protein was somewhat denatured. We admit that it is not an ideally simple system for the study of structural mechanics of the protein, but the very large size of this protein allowed us to obtain the first case of single molecule stretching data by atomic force microscopy.

Several criteria were met in the reference experiments described in the previous section to establish our observation as representing the mechanical strain in response to the stress

exerted to protein molecules suspended between the substrate and the tip with fairly strong S-Au covalent bonds. It is not possible at this moment to determine the number of protein molecules trapped between the tip and the substrate. It is, however, reasonable to attribute some of the force curves such as the one in Fig. 2d to the deformation of a single protein molecule, though not necessarily that of a single subunit. The only problem to be avoided when we try to regard the force curves obtained in the present work as representing the stretching event of a single protein molecule is the possibility of having more than two molecules sandwiched between the tip and the substrate in parallel. In such cases the average spring constant as defined below, and not D_{max}, would be twice or more than what should be expected for the single molecule stretching. We think the majority of the data obtained in the present work represent the properties associated with the single molecule stretching of α_2-M because the distribution of the force constants obtained below was not significantly different from the ones obtained under different protein densities.

The force required to free the cantilever from its maximum stretch length of D_{max} was in the range of 0.25 to 1.8 nN (Fig. 3b) except for an isolated case of 2.5 nN. We tentatively assign higher values to rupturing of an S-Au bond and the lower values to that of non-covalent subunit interactions but the distinction between the two events was not possible. It is interesting to notice that the distribution curve of D_{max} has two peaks corresponding to the maximum stretch length of one and two subunits. Cases of two subunits stretching most likely involved that of disulfide linked dimers. Although each subunit has a highly constrained structure due to the presence of 11 intrasubunit disulfide bonds, it is still possible to extend it to about 180–200 nm without breaking disulfide bonds. Thus it is not unreasonable that the AFM often measured the extension in the range of 100–200 nm for one and up to 400 nm for two subunits in series. More complex situation should be considered when D_{max} exceeded 400 nm.

The slope of the force curve, σ, is related to the spring constant κ of the material being pulled between the tip and the substrate in the following manner: $\kappa = \sigma\varepsilon/(1-\sigma)$, where ε is the spring constant of the cantilever [16]. The exact slope of the force curve is difficult to define because of its change during a single force curve measurement and the change from one measurement to another. We obtained an average slope of each force curve and converted it to the apparent force constant of the sample in the following manner and plotted its distribution in Fig. 4.

The force constant ranged between 0 and 100 pN/nm with the most frequent values of κ at 15 pN/nm and 65 pN/nm. The bimodal distribution observed for Fig. 4 may represent the inhomogeneous distribution of non-covalent interactions within a protein molecule, but another possibility of differential adhesion force to the substrate cannot be excluded. Compared to the ordinary covalent bonds such as C-C or C-N with force constants in the range of 50–100 nN/nm [17], the force constant of α_2-M is roughly 1000 times smaller. It is not unreasonable for non-covalent force constants to be of such small values if one considers that the vibrational frequencies of global elastic motions of a protein are often considered to be in the range of 100 cm^{-1} compared with the vibrational frequencies of covalent bonds such as C-C or C-N which are usually in the range of 1000–3000 cm^{-1}. Within the harmonic approximation, the force constants responsible for such vibrations are proportional to the square of their vibrational frequencies [15]. The lower end of the force constant distribution is likely to include cases of those molecules that were already stretched and denatured. Denaturation of α_2-M in solution has been reported to be irreversible [18], and the molecule most likely lay denatured after 5-fold mechanical stretching in the length. Smaller irregularities in the force curve may reflect the distribution of spring constants within a protein molecule depending on which part of the protein is responding to a given stress at a given time. More refined experiments and the analysis of the force curves obtained therein will give the information on the internal mechanics of the sample and its differential response to the external force. We believe that the atomic force microscope is capable of determining more accurate force curves and the development of biochemical methods to securely sandwich a single molecule of a protein at N- and C-termini between the tip and the substrate will open the exciting field of the mechanics and dynamics of single molecule deformation of biopolymers, among others single molecule unfolding and refolding of proteins and DNA, not only in aqueous media but also in organic solvents and even in vacuum.

Acknowledgements: The authors express their sincere gratitude to Dr. Hideo Arakawa for his helpful discussions during the course of this experiment.

References

[1] Binnig, G., Quate, C.F. and Gerber, C. (1986) Phys. Rev. Lett. 56, 930–933.
[2] Hoh, J.H. and Hansma, P.K. (1992) Trends Cell Biol. 2, 208–213.
[3] Gad, M. and Ikai, A. (1995) Biophys. J. 69, 2226–2233.
[4] Drake, B., Prater, C.B., Weisenhorn, A.L., Gould, S.A.C., Albrecht, T.R., Quate, C.F., Cannell, D.S., Hansma, H.G. and Hansma, P.K. (1989) Science 243, 1586–1589.
[5] Florin, E.L., Moy, V.T. and Gaub, H.E. (1994) Science 264, 415–417.
[6] Dammer, U., Popescu, O., Wagner, P., Anselmetti, D., Güntherodt, H.J. and Misevic, G.N. (1995) Science 267, 1173–1175.
[7] Lee, G.U., Chrisey, L.A. and Colton, R.J. (1994) Science 266, 771–775.
[8] Arakawa, H., Osada, T. and Ikai, A. (1986) Arch. Biochem. Biophys. 244, 447–453.
[9] Mitsuda, S., Nakagawa, T., Nakazato, H. and Ikai, A. (1995) Biochem. Biophys. Res. Commun. 216, 399–405.
[10] Goss, C.A., Brumfield, J.C., Irene, E.A. and Murray, R.W. (1993) Langmuir 9, 2986–2994.
[11] Sarid, D. (1994) Scanning Force Microscope: With Applications to Electric, Magnetic and Atomic Forces. Oxford University Press, New York.
[12] Ikai, A., Nishigai, M., Osada, T., Arakawa, H. and Kukuchi, M. (1987) J. Prot. Chem. 6, 81–93.
[13] Sottrup-Jensen, L., Stepanik, T.M., Kristensen, T., Wierzbicki, D.M., Jones, C.M., Lonblad, P.B., Magnusson, S. and Petersen, T.E. (1984) J. Biol. Chem. 259, 8318–8327.
[14] Andersen, G.R., Koch, T.J., Dolmer, K., Sottrup-Jensen, L. and Nyborg, J. (1995) J. Biol. Chem. 270, 25133–25141.
[15] McCammon, J.A. and Harvey, S.C. (1987) Dynamics of Proteins and Nucleic Acids. Cambridge University Press, Cambridge.
[16] Ikai, A. and Mitsui, K. (1995) manuscript in preparation.
[17] Hollenstein, H. and Güthard, H.H. (1980) J. Mol. Spectrosc. 83, 457–465.
[18] Garel, J.R., Martel, A., Muller, K., Ikai, A., Morishima, N. and Sutoh, A. (1984) Adv. Biophys. 18, 91–113.