Measurement of the Length of the α Helical Section of a Peptide Directly Using Atomic Force Microscopy

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Using atomic force microscopy (AFM), the length of the α-helix structure of poly-L-lysine was investigated by stretching the peptide directly, one molecule at a time. In the absence of urea, many rupturing points that seemed to be due to the breaking of some hydrogen bonds were observed in force–extension curves, while these points were never observed in the presence of 8 M urea. In the presence of 0.4 or 1.6 M urea, both force–extension curve types were observed. Total peptide elongation for each condition was calculated from force–extension curves reflecting the α-helix rupturing process. The experimental value of total elongation divided by the theoretical value of total α-helix elongation yields the α-helix content. This value was compatible with circular dichroism (CD) measurement results. This suggests that peptide conformation and content of the α-helix on a single molecule scale can be investigated by direct mechanical measurement using atomic force microscopy.

Key words   atomic force microscopy; single molecule; poly-L-lysine; peptide; force–extension curve

The conformation of a peptide or a protein strongly dominates its properties *in vivo*.[1,2] Averaged conformations of large numbers of molecules are obtained using NMR, X-ray crystallography, circular dichroism (CD), and so on. It is difficult to investigate the conformation of a single molecule by these methods. Up to now, atomic force microscopy (AFM) has been developed to measure not only surface topography,[3] but also to measure the molecular interaction between the tip and a substrate by measuring force extension curves.[4—6] Now, AFM can be used as a tool to handle individual peptides. Analysis of the protein rupturing process has been done using AFM on the single molecule scale. Also, qualitative analysis of the secondary structure of proteins or peptides has already been attempted.[7—11] However, detailed quantitative analysis of the secondary structure has still proven to be problematic. One reason for difficulty in quantitative analysis seems to be molecule size. Proteins and DNA are not good samples for investigating the rupturing process in detail because their structure is usually very complex. A much simpler peptide such as poly-L-lysine (PLL) is suitable for detailed analysis of the rupturing process. One peptide with well understood properties is PLL. It is known that PLL can produce an α-helix, β sheet, and random coil forms under limited conditions. Above pH 10.5, its form is an α helical structure at 4 °C.[12,13] Further PLL properties seem to depend on peptide length.[14]

Previously, Lantz *et al.*[15] succeeded in measuring the energy of a hydrogen bond by stretching a single helical peptide molecule, Lysine₃₀–Cysteine, in a self-assembled peptide monolayer (SAM). In their work, it was difficult to change conditions around the peptide because it was completely surrounded by other peptides. In this paper, during force measurements, one single peptide surrounded by a buffer or water molecules was stretched between a mica substrate and gold-coated AFM tips. We investigated the rupturing process of a peptide molecule and single peptide molecule conformation using AFM in the presence of various concentrations of urea as a first step towards detailed analysis of the protein un-

folding process. Evaluation of the α-helix contents of the single peptide molecule indicated that peptide conformation was not uniform and that hydrogen bonds did not disperse homogeneously during peptide molecule stretching.

Experimental

Synthesis of Peptide Analogue   The peptide used in this work, Lysine₃₀–Cysteine, was synthesized by a solid-phase method using fluorenylmethoxycarbonyl chemistry carried out with a PepPlus 9050 synthesizer (PerSeptive). The peptide N-terminal was connected to thioctic acid by an amide bond. The resin (100 mg, 0.16 mmol/g) to which synthesized peptides were connected was soaked in tetrahydrofuran (THF). Thioctic acid (8 mg) was dissolved in a small amount of THF in the presence of dicyclohexylcarbodiimide (8 mg) and then added to the suspension of resins. The mixture was incubated for 12 h at room temperature after drying the resin under reduced pressure. The peptide analogue was cleaved from resin by using 95% trifluoroacetic acid/2.5% ethanedithiol/2.5% pure water. The peptide analogue was initially purified by diethyl ether precipitation and then purified using a reversed-phase HPLC system with an RPC18 column (Shimadzu). Peptide analogue purity was checked using an analytical reversed-phase HPLC system.

Preparation of SAM of Thioctic Acid and Peptide Analogue   A monolayer of thioctic acid was made on mica coated with gold by immersion in 0.1 mM of thioctic acid solution (5 mM Hepes–Caps, pH 11) for 2 h at room temperature. After rinsing with 2—3 ml pure water, it was soaked in 0.5 mg/ml of the peptide and 0.1 mM of thioctic acid solution (5 mM Hepes–Caps, pH 11) for 1 min. After rinsing with 2 ml pure water, it was dried using nitrogen gas and used for force measurement within 5 h.

CD spectroscopy was used to investigate peptide structure under AFM measurement conditions. The peptide was found to adopt the α-helix structure as indicated by peaks at 206 and 222 nm in the absence of urea in spectra, as shown in Fig. 1. The α-helix structure was partially broken in the presence of 0.4 M urea, and the peptide was no longer helical in the presence of 8 M urea, as clearly indicated by the CD spectrum. The content of the α-helix was estimated using the method reported by Greenfield *et al.*[12]

Fourier transformed infrared (FTIR) spectroscopy was used to investigate the secondary structure of the peptide binding onto gold substrates. The FTIR spectra were measured in D_2O solution at pH 11. A monolayer of thioctic acid on gold substrate was also prepared, and its FTIR absorbance was measured as a reference spectrum before measuring the FTIR absorbance of the AFM measurement sample. The FTIR spectra exhibited an amide I absorption peak at 1639 cm⁻¹, indicating that the absorbed peptide adopted the α-helix conformation[16] (data not shown).

AFM Measurement   A Nanoscope IIIa (Digital Instruments, Santa Barbara, CA, U.S.A.) with a fluid cell containing buffer (5 mM Caps at pH 11)
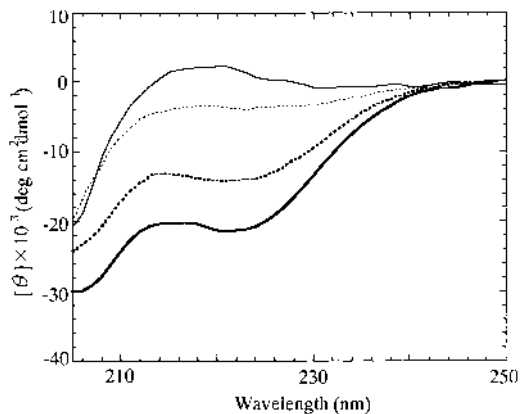
Fig. 1.   CD Spectra of the Peptide Measured in 10 mM Caps Buffer at pH 11 in the Absence of Urea (Thick Solid Line), in the Presence of 0.4 M of Urea (Thick Dotted Line), in the Presence of 1.6 M of Urea (Dotted Line), and in the Presence of 8 M of Urea (Thin Solid Line)

was employed to measure force–extension curves. Cantilever force sensors had spring constants of 0.06 and 0.12 N/m. All measurements were carried out at room temperature. All tips were coated with gold before use. The peptide sample was set on the piezo and the tip was far from the sample before measuring. First, the sample was advanced toward the tip and contacted at the surface of the tip. Then the sample was retracted from the tip. The maximum tip–sample separation during the force curve measurement was 86 nm. Advance/retraction cycles were performed as gently as possible (10 Hz). A force–extension curve was obtained by plotting an applied force as a function of sample displacement for the retraction process.

Results and Discussion

Peptide analogue length is about 120 Å in the elongated state and 55 Å in the α helical state. The helical peptide length is 50 Å, and the length of the 1,2-dithiolane-3-pentanoyl parts is 5 Å. The peptide analogue consisted of Lysine$_{30}$–Cysteine and 1,2-dithiolane-3-pentanoyl residues. The 1,2-dithiolane-3-pentanoyl residue was linked to the N-terminal of the peptide. The peptide property is similar to PLL; also the peptide forms an α helical structure at pH 11. The
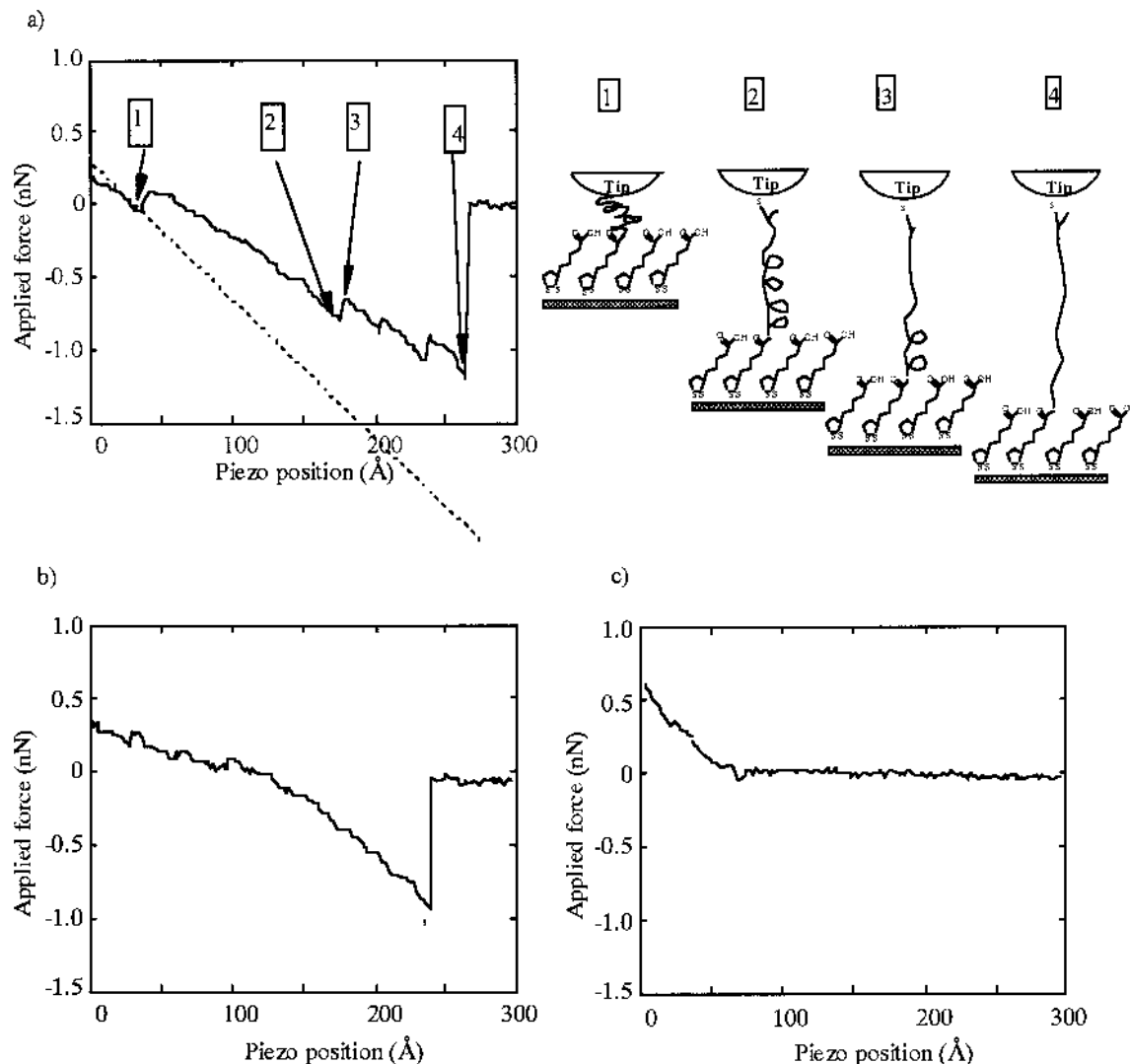

Fig. 2

a) One typical force–extension curve of a retraction process, including peptide stretching at pH 11, in the absence of a detergent (Type I).
The dotted line shows the position of the cantilever traveling with the piezo. Schematic models at the position indicated by thin arrows are shown at the right of Fig. 2a.
b) Another type of force–extension curve of a retraction process while stretching a peptide (Type II).
c) A typical force–extension curve without peptide stretching.

peptide analogue includes a sulfur atom at both peptide terminals. The peptide can bind to the Au/mica surface by an Au–S bond at one peptide terminal. During force measurement, the other end of the peptide analogue bound to the gold coated tip to enable peptide molecule stretching. This measurement process allowed stretching of a single peptide molecule as implied by the following experimental observations. First, the chance of detecting large forces reflecting the stretching of a peptide or peptides was one in 30 during a series of measurements. This probability of detection decreased systematically with decreased incubation time of the SAM preparation, though the Au–S bond rupturing force remained almost constant. Second, the observed rupturing force of the Au–S bond was comparable to the value reported by Grandbois *et al.*[17]

A typical force–extension curve for the retraction process at pH 11 in the absence of urea is shown in Figs. 2a and b. Figure 2c shows a typical force–extension curve without peptide stretching. In Fig. 2c, only adhesion force between the tip surface and the monolayer of thioctic acids was observed. This adhesion force seemed to depend strongly on the tip contact area. This nonspecific adhesion force is also shown in Figs. 2a and b.

Rupture points during the stretching process, which occurred at 180, 205, and 217 Å of the sample displacement, are indicated in Fig. 2a. Similar rupture points were also observed in the previous paper.[15] These rupture points seemed to be due to the rupturing of hydrogen bonds during the stretching process, as shown in Fig. 2a. This kind of force curve will be referred to as Type I in this paper. The final large rupture point at 264 Å of the sample displacement is believed to correspond to Au–S bond rupture. The rupture force of the Au–S bond was 1.2 ± 0.4 nN. This value compares well with that reported by Grandbois *et al.*[17] of

1.4 ± 0.3 nN.

Rupture points did not always occur at the same value of tensile force, except for the final large rupture point. One explanation for this result could be a fluctuation of the peptide molecule by thermodynamic vibration. Alternatively, the conformation of each peptide molecule may differ. In some cases, as in Fig. 2b, no rupture points were observed prior to the Au–S bond rupture. These occurred under the same experimental conditions and will subsequently be referred to as Type II force curves. The probability of observing this type of curve was very low at pH 11 in the absence of urea. This case suggests that the random coil of the peptide might be stretching.

Force–elongation curves were calculated from these force–extension curves. Total peptide elongation was estimated by subtracting cantilever displacement from sample displacement. Force–extension curves in the presence of 0.4, 1.6, and 8 M urea were measured (data not shown) and force–elongation curves were calculated for each. Figures 3a—c show typical Type I force–elongation curves in the presence of 0, 0.4, 1.6 M urea, respectively. Type II force–elongation curves were also observed for each condition. In presence of 8 M urea, Type I was never observed. In Figs. 3a—c, no rupturing point was observed below *ca.* 60 Å peptide elongation. This 60 Å is compatible with the value for the theoretical length of the α helical structure before stretching. In this region, it is plausible that the peptide compressed by the tip stretched and reverted to an α helical structure. A few rupture points, due to hydrogen bond breaking, were observed above *ca.* 60 Å of peptide elongation in Figs. 3a—c.

The α-helix content was estimated from Figs. 3a—c for each condition. Arrows in each figure show the elongation distance of the peptide that has formed an α-helix before hydrogen bond rupture. We attributed the jump to a rupture
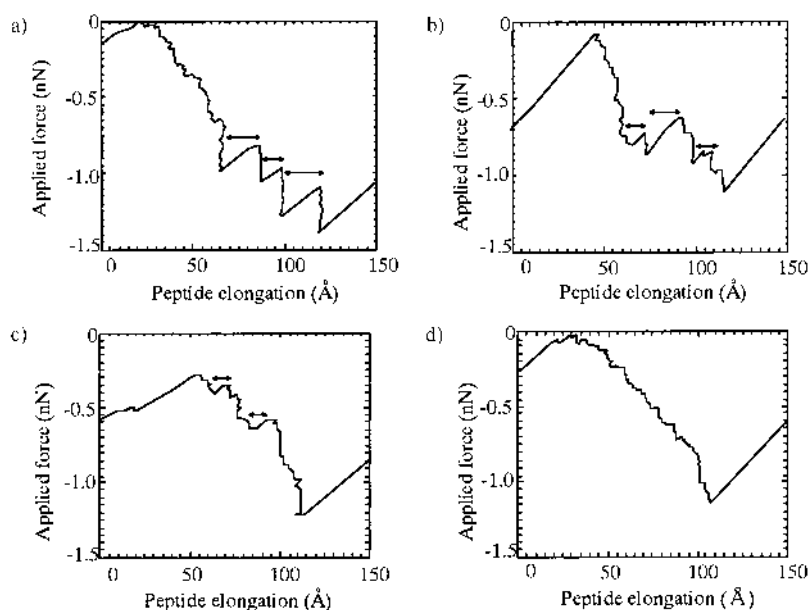

Fig. 3

a) A typical force–elongation curve of Type I in the absence of urea
   Arrows show the elongation distance of a peptide that has formed an a-helix before hydrogen bond rupturing.
b) A typical force–elongation curve of type I in the presence of 0.4 m of urea
c) A typical force–elongation curve of type I in the presence of 1.6 m of urea
d) Typical force–elongation curve in the presence of 8 m of urea

Table 1. α-Helix Content Observing a Type I Force–Extension Curve at Each Condition

Condition	Total elongation of α-helix (Å)	α-Helix content by AFM (%)	α-Helix content by CD (%)
No urea	45±4	68±6	61±6
0.4 M urea	35±3	51±5	30±4
1.6 M urea	13±3	19±4	4±2
8.0 M urea	0	0	0

point when the jump in the deflection signal of the cantilever was twice that of the noise level. The total length of the peptide in its fully stretched form in Figs. 3a—c was *ca.* 120 Å. This value was compatible with the theoretical value of the stretched peptide length, and it suggested that peptides were stretched approximately vertically in each case. Force–elongation curves were sometimes obtained in which the total peptide length was less than 100 Å. In this case, the peptide might not be stretched vertically. Such data was omitted from the calculation of total peptide elongation.

Total elongation of the peptide under each condition was calculated and is shown in Table 1. Total elongation of the peptide that has formed an α-helix decreased with increasing urea concentration. As the length of the elongated peptide analogue is about 125 Å and the helical peptide length is 60 Å, the theoretical value of the total elongated α helical form of this peptide is 65 Å. Total elongation of the peptide at each condition divided by 65 Å yields the α-helix content. These values are also shown in Table 1. The content of the α-helix decreased with increased urea concentration. In the absence of urea, the content of the α-helix was 66%. In the presence of 1.6 M urea, the content of the α-helix was reduced to 19±4%. Apparently, the content of the α-helix at pH 11 without urea is much lower than the value of PLL in references.[12,13] One reason may be the effect of higher temperature. In this work, we measured force curves at room temperature (*ca.* 24 °C) though the value in reference[12,13] was obtained at 4 °C. Another reason may be the effect of peptide length. The length of the peptide is more than 10 times shorter than PLL. It is reported that a short helix shows a broad differential scanning calorimetry (DSC) curve, suggesting that the structure of the helical peptide is not stable above 20 °C.[14] So, the α-helix structure may be partially broken at pH 11 at room temperature.

The content of the α-helix estimated from CD spectra shown in Table 1 is similar to AFM measurement results, but the AFM results were slightly overestimated. In the presence of 0.4 or 1.6 M urea, the probability of observing Type II was high. One reason for overestimation may be that Type II force curves were neglected. To evaluate the α-helix content using CD results, a large number of force–extension curves are needed, along with the necessary consideration of Type II force curves.

Peptide conformation can be also discussed from force–elongation curves in the presence of urea. In Figs. 3a—c, the jumps in the deflection of the cantilever at rupture points became smaller with increased urea concentration. The jump corresponding to the breaking of hydrogen bonds shows elongation of the peptide that formed an α-helix. From this result, it was found that the length of the α helical part was

reduced with increased urea concentration. The rupture process of a single hydrogen bond cannot be detected using this system because the jump is too small compared to the noise level. If hydrogen bonds are dispersed homogeneously, then detection of the rupture processes becomes impossible when the α-helix content is below 50%. However, in the presence of 1.6 M urea, the hydrogen bond rupture process was observed even though the α-helical content was estimated at only 19% from CD analysis and AFM measurement. This suggests that hydrogen bonds do not disperse homogeneously in the α-helix during peptide stretching and that peptide conformation is a mixture of two states—the α-helical state and random coil—in the presence of urea.

Clarke *et al.* reported that the α-helix folded on an millisecond time scale,[18] although it is known that the folding of a peptide from a random coil to an α helical state occurs on the sub-microsecond scale in a solution. In our case, the peptide molecule was fixed at both terminals and was stretched during force measurement. This will strongly restrict peptide movement. It will take a longer time than in solution to change the peptide molecule conformation because of this restriction. Unfolding pathways of individual bacteriorhodopsin or poly-L-glutamic acid peptide were investigated[8,19] by AFM. It is difficult to find the hydrogen bond rupture process in these results. The difference might be due to the velocity of the piezo (the rate of extension) during force–extension curve measurement. The rate of extension was 0.1—40 nm/s in that study, while it was 1.72 μm/s and the time taken to stretch a peptide was less than 0.01± 0.004 s in our study. The effect of extension rate on the rupture process will be studied in a future work.

This study suggests that the α helical content of the peptide on the substrate could be measured using AFM on the single molecule scale. We also infer that hydrogen bonds do not disperse homogeneously in the α helical peptide, and that peptide conformation is a mixture of two states—the α helical state and a random coil. Quantitative analysis of biomolecular form based on AFM measurement will be a powerful tool.

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