Specific Interaction between GroEL and Denatured Protein Measured by Compression-Free Force Spectroscopy

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ABSTRACT We investigated the interaction between GroEL and a denatured protein from a mechanical point of view using an atomic force microscope. Pepsin was bound to an atomic force microscope probe and used at a neutral pH as an example of denatured proteins. To measure a specific and delicate interaction force, we obtained force curves without pressing the probe onto GroEL molecules spread on a mica surface. Approximately 40 pN of tensile force was observed for ~10 nm while pepsin was pulled away from the chaperonin after a brief contact. This length of force duration corresponding to the circumference of GroEL's interior cavity was shortened by the addition of ATP. The relation between the observed mechanical parameters and the chaperonin's refolding function is discussed.

INTRODUCTION

It is generally accepted that the three-dimensional structure of a protein is dictated by its amino acid sequence, and the folding of a nascent polypeptide is a spontaneous event at least for small, simple proteins under controlled conditions (Anfinsen, 1973). Inside of a living cell, however, there are many and diverse kinds of proteins for which misfolding and abortive aggregate formation are not rare events (Schubert et al., 2000; Ellis and Hartl, 1996). Molecular chaperones have been postulated to help such proteins fold correctly by preventing their aggregation and to mediate the degradation of misfolded proteins (Wickner et al., 1999). The chaperonin GroEL from *Escherichia coli* is one of the best-studied molecular chaperones. The GroE system consisting of GroEL and GroES assists misfolded proteins to refold correctly in an ATP-dependent manner (Rye et al., 1999). Elementary reaction steps of the GroE system, namely, catching and releasing of misfolded proteins, binding and dissociation of ATP and GroES, and conformational changes of GroEL involved in the foregoing sequences have been well studied (Sigler et al., 1998). How GroEL assists the protein folding is, however, still under discussion (Ellis and Hartl, 1996; Coyle et al., 1999; Brinker et al., 2001). One of the key points to clarify the mechanism of GroEL is to investigate how GroEL interacts with a nonnative protein. In this aspect, Farr et al. have shown, using a mutant GroEL ring genetically linked as a single peptide, that nonnative protein binds to multiple subunits of the apical domains of a GroEL (Farr et al., 2000). As a next step, we tried to obtain quantitative information on the interaction between a nonnative protein and a GroEL by force measurement of atomic force microscopy (AFM).

AFM is increasingly being used in biological sciences not only for imaging but also for measuring the force of interaction between biomolecular pairs. Such forces can be measured by immobilizing specific receptor molecules to a substrate surface and corresponding ligands to an AFM probe. Interaction has been measured in this way for ligand-receptor pairs of biotin and avidin (Florin et al., 1994; Moy et al., 1994), complementary DNA pairs (Lee et al., 1994), and antigen-antibody pairs (Hinterdorfer et al., 1996). In our application of this method to measure the force of interaction between GroEL and a denatured protein, we tried to solve the following two problems. The first problem is how to reduce nonspecific adhesive interactions between the probe and the substrate surface, both of which were covered with various organic molecules. The second was how to keep sample molecules biologically active against a large loading force inflicted on them through the cantilever. Our solution to these problems is the ''compression-free'' force spectroscopy measurement (Sekiguchi et al., 2002) where the piezo movement was reversed from approaching the tip to retraction immediately before the start of the upward deflection of the cantilever. Since the immobilized protein on the tip was denatured and flexible, such an operation still gave rise to force curves signifying positive interactions.

In this article, we show the results obtained from ''compression-free'' experiments between a denatured protein and GroEL on the probe and the substrate surface, respectively. The result is expected to provide new quantitative information on the mechanism of the GroEL reaction.

Submitted May 14, 2002, and accepted for publication February 12, 2003.

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0006-3495/03/07/484/07 $2.00

MATERIALS AND METHODS

Proteins

The GroEL mutant protein, E315C, was produced by site-directed mutagenesis using the Kunkel method (Kunkel, 1985). The mutant was

expressed in the *E. coli* strain BL21(DE3) Gold (Stratagene, La Jolla, CA), bearing the expression plasmid pET21-GroEL(E315C). GroEL (E315C) was purified using a protocol similar to that described for the purification of wild-type GroEL (Motojima et al., 2000). The purified GroEL was stored in a 65% saturated ammonium sulfate suspension until used. GroEL (E315C) behaved like the wild-type GroEL in every property examined, including the rate of steady-state ATP hydrolysis (Morii et al., 1996) and the efficiency of assisting the refolding of green fluorescent protein (Makino et al., 1997).

We used porcine pepsin (Sigma, St. Louis, MO) as a model of the denatured protein because it is known to lose its native conformation at a neutral pH and interact with GroEL (McPhine, 1989; Aoki et al., 1997).

Preparation of functionalized substrate and tip

An aliquot of a GroEL solution (0.5 mg/ml, 100 μl) in buffer (50 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT, pH = 7.2) was deposited on a freshly cleaved mica surface, and GroEL molecules were left to adsorb on the surface for 1 h at room temperature. The mica surface was then rinsed with HEPES buffer and kept in the same buffer until used. When the sample was prepared for AFM imaging, GroEL solution (0.05 mg/ml) was first deposited on a mica surface and rinsed with HEPES buffer as above, then fixed with 1.0% glutaraldehyde for 3 min and rinsed again with HEPES buffer.

Gold-coated AFM tips (OMCL-TR400PB 200 μm long cantilevers, Olympus, Tokyo, Japan) were functionalized with pepsin through the crosslinker, Sulfo-LC-SPDP (sulfosuccinimidyl 6-[3′-(2-pyridyldithio)-propionamido] hexanoate) (Pierce, Rockford, IL), which forms an Au-S bond with the tip. For this purpose, pepsin was reacted with Sulfo-LC-SPDP in a 1:1 molar ratio and reduced with DTT (1,4-dithiothreitol) according to a standard method (Cumber et al., 1985). The modified pepsin was desalted by subjecting it to gel chromatography on a Sephadex G-25 column immediately before use. Gold-coated AFM probes were cleaned in a UV ozone cleaner (NL-UV253, Nippon Laser & Electronics Lab., Nagoya, Japan) and in a series of solvents (chloroform, ethanol, and water) to remove contaminants completely, and immersed in a solution of modified pepsin for 1 h at room temperature to react sulfhydryl groups on pepsin with the gold-coated surface of the probe. As pepsin contains only one Lys residue per molecule, the protein should be bound to a probe through the amino groups either of the N-terminus or Lys-320 near the C-terminus, or both. The cantilever spring constant was calibrated by the thermal vibration method (Hutter and Bechhoefer, 1993) to be 0.025–0.035 N/m.

Atomic force microscopy

Tapping mode images of GroEL in HEPES buffer (50 mM HEPES, 100 mM KCl, and 5 mM $MgCl_2$, pH = 7.2) were taken with a NanoScope IIIa (Digital Instruments, Santa Barbara, CA). V-shaped cantilevers with a stated spring constant of 0.15 N/m (OMCL-TR800PSA 200 μm long cantilevers, Olympus) were operated at 9.8 kHz in the drive frequency.

Force curves were recorded on an SPM-9500-J2 (Shimadzu, Tokyo, Japan) equipped with a liquid cell containing HEPES buffer. We used a special program for the force spectroscopy measurement, Force Curve Software version 2.54 (Shimadzu). By using this program, we were able to control the holding time of the piezo tube at its approach end, which was considered as the reaction time between pepsin on the tip and GroEL on the substrate surface during their encounter. To obtain high resolution force curves, we used the ''sensitivity × 5 mode,'' which amplified the output signal five times. To calibrate the response of cantilever deflection signal as a function of piezo movement, standard force curve measurements were repeated after the compression-free measurements (Sekiguchi et al., 2002). In all force curve measurements, we set the scanwidth to 5 V (~60 nm), the scan speed to 1 Hz (~120 nm/s), and the holding time to 0.5 s. All force curves whose data had attractive interactions with no pressing region of the probe onto a sample surface in approaching process were analyzed.

Competitive inhibition experiments

Force curves in the absence and the presence of free pepsin in solution were recorded in series as follows for an accurate comparison of the frequency of binding between the denatured protein and GroEL under the two different conditions. First, force measurements were performed in an experimental buffer (50 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, and 10 μM cysteine, pH = 7.2) for 30 min in the absence of free pepsin; then free pepsin (2.0 mg/ml) was added to the sample solution to a final concentration of 1.0 mg/ml, and the solution was incubated for 20 min at room temperature. Force measurements were then restarted in the presence of free pepsin (to be called the inhibition experiment) and continued for 30 min. This series of experiments was repeated four times, replacing functionalized tips and modified mica substrates for each series.

ATP-dependent experiments

Force measurements with and without ATP in solution were done in a series of experiments similar to the inhibition experiment described above. Force curves were first obtained without ATP for 30 min, and then ATP was added into the experimental buffer to a final concentration of 5 mM. Force curve recordings were restarted after 20 min and repeated for 30 min. This series of experiments was repeated nine times, replacing the functionalized tips and modified substrates for each series.

RESULTS

An AFM image of GroEL molecules adsorbed on a mica surface in HEPES buffer is given in Fig. 1. Uniformly distributed circular dots were identified as GroEL from their diameter and from the presence of a central cavity in most of them. The average diameter defined as the half height width in this image was 13.1 ± 1.7 nm (n = 555)

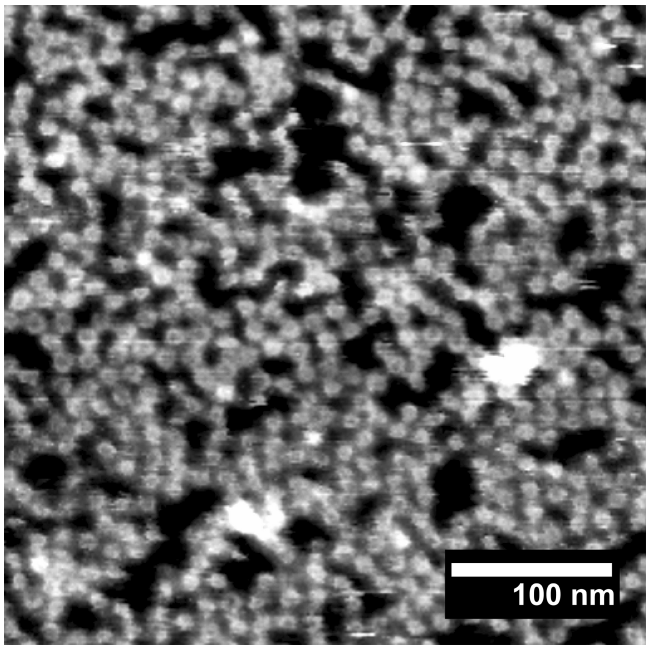

FIGURE 1 AFM image of GroEL on a mica surface. GroEL was adsorbed on a cleaved mica substrate, which was imaged by tapping-mode AFM under HEPES buffer. The characteristic ring structure of GroEL is observed.

which compared favorably with the reported value of 13.7 nm, based on x-ray analysis (Braig et al., 1994). In usual AFM imaging, the width of isolated molecules measures larger than the expected width of the molecules because of the tip-sample convolution effect. However, when the tip scans over a closely packed array of molecules, the tip-sample convolution effect is minimized and the measured width of molecules is comparable to the expected width of molecules (Hansma, 1995). In this case, GroEL molecules were almost closely packed and the tip was expected to be scanning the top surface of GroEL, therefore the derived diameter was less or comparable to the expected diameter of GroEL. The resulting images indicated that the conformation of GroEL on the mica surface was similar to that of native GroEL.

In Fig. 2 *A*, a schematic diagram of a usual force curve recording is given where the relation between the deflection of the cantilever (ordinate) and the sample position along the *z*-axis (abscissa) is illustrated. The rupture force that was required to break the adhesion between the probe and the sample was calculated by multiplying the cantilever deflection at point 5 in the figure with the force constant of the cantilever and was identified as the force of interaction between the tip and the sample. The distance between the substrate surface and the tip under an applied tension was calculated by subtracting the cantilever deflection from *z*-piezo movement and transformed to a force-distance curve which is a more direct presentation of the mechanical properties of the sample under tensile deformation (Heinz and Hoh, 1999).

In this article, we obtained force curves by the compression-free method which was done without pressing a probe against the sample and, as a result, we obtained force curves lacking region 2 and 3 in Fig. 2 *A* as shown in the lower force curve of Fig. 2 *B*. Details of the compression-free method are described previously (Sekiguchi et al., 2002). In practice, standard force curve measurements (upper inset of Fig. 2 *B*) were needed after the compression-free measurements to calibrate the response of the cantilever deflection signal as a function of piezo movement and to confirm whether or not recorded data in the compression-free method were indeed obtained on a GroEL surface. All the force curves obtained in this way showed elastic curves in the pressing region (point 3 in Fig. 2 *A*) which had ~15 nm in indentation (data not shown) corresponding to the height of GroEL layer.

When GroEL was pressed under the loading force of a modified tip, the final rupture forces observed were much larger than those obtained by the new compression-free method, and neither the value of the rupture force nor the profile of the force curve were reproducible. This result indicated that direct contact of the tip with GroEL resulted in strong physical adsorption which was precisely what was to be avoided in our experiments.

Fig. 2 *C* shows examples of force-distance curves between

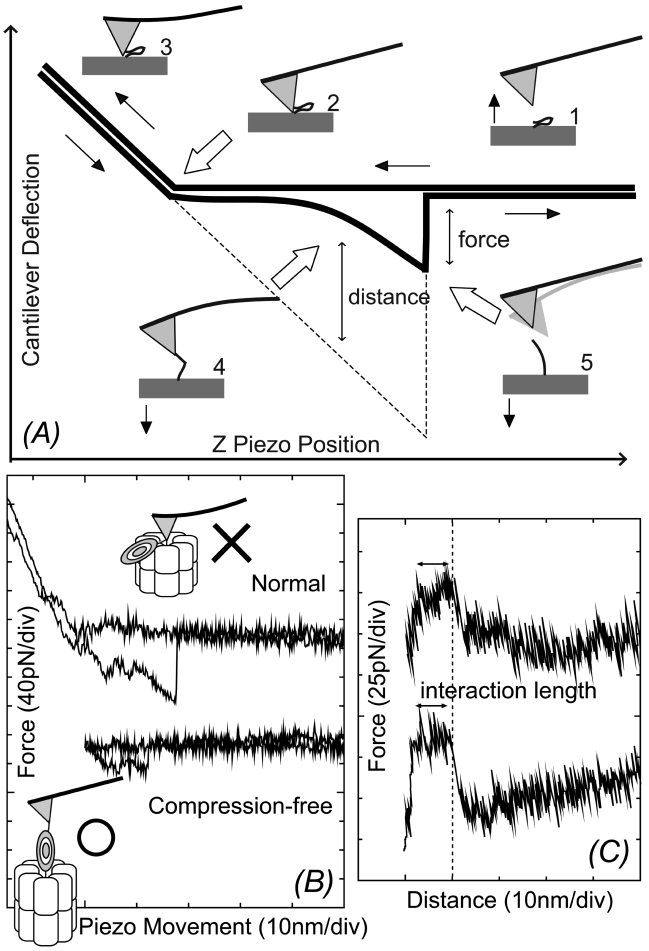

FIGURE 2 Methods for measuring force using AFM. (*A*) Schematic drawing of an AFM force curve. When the sample stage approaches the AFM tip, the force curve is initially flat (point *1*) until it reaches the tip surface (point *2*), where it starts to be deflected linearly upward (point *3*). After the substrate stage starts to retract at the left end of the diagram, the curve closely follows the previous approach curve until the deflection returns to the initial level. When the substrate stage releases, the adhesion force between the tip and the surface causes the cantilever to deflect toward the sample (point *4*). The rupture force that was required to break the adhesion between the probe and the sample was calculated by multiplying the cantilever deflection at point *5* with the force constant of the cantilever. The distance between substrate surface and the tip, that is, the extension of the sample, is estimated by subtracting the cantilever deflection from *z*-piezo movement. (*B*) Comparison of force curve measurements obtained by normal (*upper*) and compression-free (*bottom*) methods. (*C*) Force-distance curves for denatured pepsin and GroEL obtained by the compression-free method. The *x*-axis is the distance between the tip and the substrate, and the *y*-axis is the tensile force.

denatured pepsin and GroEL obtained in compression-free measurements. The force spectra had a common profile with a plateau of ~10 nm in width and 40 pN in force.

Throughout this article, we set the final separation point of each force curve as a reference point on the abscissa (*dotted line*) because we could not determine the absolute distance between the tip and the substrate surface by the compression-free method, and we defined the interaction length as the

plateau distance where the cantilever deflects downward, as indicated with arrows in Fig. 2 *C*.

The distribution of the average force and the width in the plateau region showed a clear peak around 41 ± 14 pN and 8.6 ± 4.0 nm, respectively, as shown in Fig. 3. To confirm that the observed force curves and the mechanical parameters were specific for the interaction between pepsin and GroEL, we added free pepsin to the sample solution as a competitive inhibitor and counted the frequency of positive responses. As shown in Fig. 3, the frequency of such responses under the same experimental conditions decreased dramatically from 78 times in the absence of free pepsin to 9 times in its presence during four repeats of the 30-min experimental series.

Fig. 4 *A* shows force-distance curves obtained in experiments with and without ATP in solution. In the absence of ATP, force spectra with a distinctive plateau as described above were obtained, whereas in the presence of ATP, the plateau of interaction changed into a sharp peak after an extension profile similar to that for the stretching of a random coil-like chain. As shown in Fig. 4 *B*, the mean maximal force was 44 pN in the absence of and 48 pN in the presence of ATP, whereas the interaction length shown in Fig. 4 *C* had different distributions with mean values of 9.2 and 2.5 nm in the absence and presence of ATP, respectively.

DISCUSSION

The interaction between GroEL and a denatured protein was studied by AFM in the force curve mode. Before starting force spectroscopy measurements, we checked the orientation of GroEL on a mica substrate by imaging the sample surface (Fig. 1).

The well-known central cavity of GroEL was observed in most cases, confirming an upright orientation for the majority of GroEL on the surface consistent with previous reports (Vinckier et al., 1998; Mou et al., 1996; Viani et al., 2000). Since these reports showed that GroEL proteins adsorbed on a mica surface retained their activity, we proceeded to perform force measurement experiments. When the conventional force curve mode was employed in our experimental set-up, reproducible force curves were not obtained (data not shown). Possible reasons for the difficulty in obtaining reproducible data are: 1), deformation of GroEL under the loading force of the cantilever; 2), a direct and nonspecific adsorption by the tip surface to the sample or substrate surface; and 3), the possibility of multiple pair formation between GroEL and pepsin involving the tip and the substrate surface. All of these problems would be avoided if a modified AFM tip with pepsin could be brought close enough to GroEL for the pepsin to interact with GroEL but not close enough for the tip to touch or compress GroEL molecules. To obtain such ''compression-free'' force curves,

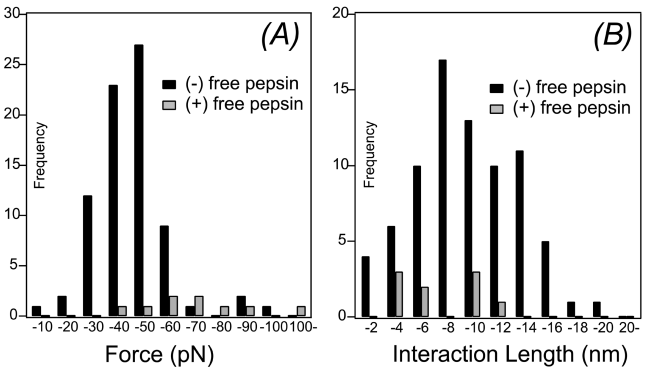

FIGURE 3 Histogram of the interaction force and length; Histograms of the interaction force (*A*) and length (*B*) in the presence and absence of free pepsin as a reagent competing for the binding. The force and the interaction length were 41 ± 14 pN and 8.7 ± 4.0 nm ($n = 78$), respectively.

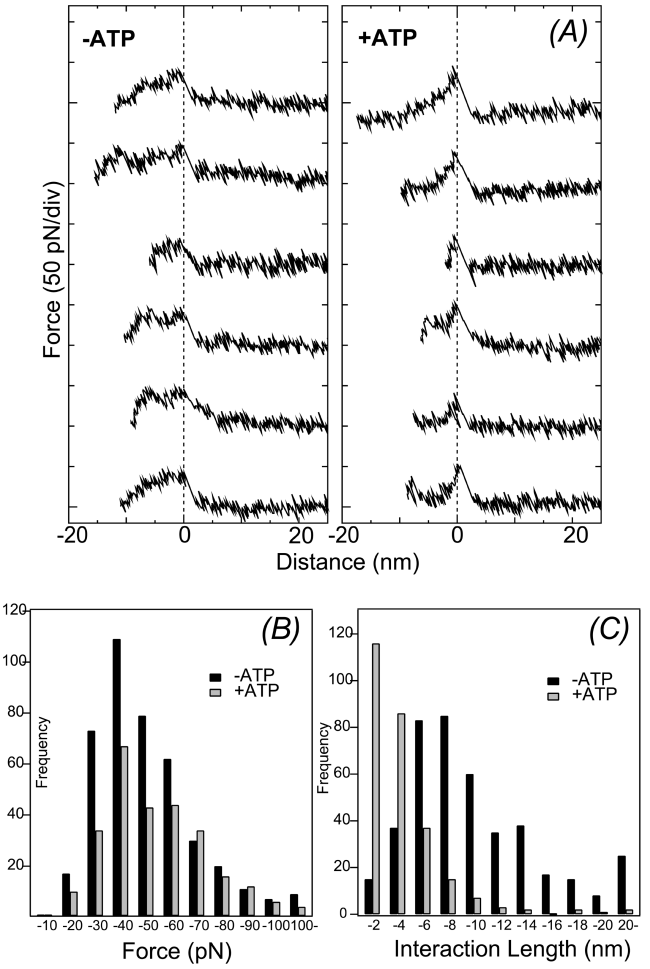

FIGURE 4 Comparison of the interaction with and without ATP. (*A*) ATP-dependency of force-distance curves. Six examples of force-distance curves obtained in the absence (*left*) and presence (*right*) of ATP are shown. We set the rupture point in each force-distance curve as the reference point. Histograms of the interaction force (*B*) and length (*C*). In the absence of ATP, the force and the interaction length were 44 ± 20 pN and 9.2 ± 5.9 nm ($n = 418$), respectively. In the presence of ATP, the force and the interaction length were 48 ± 20 pN and 2.5 ± 3.4 nm ($n = 271$), respectively.

we made sure that the sample stage was brought close to but not in contact with the tip and then retracted it back after a specified reaction time. This approach and retraction cycle was repeated several times, each time moving the point of return for retraction closer to the tip by a small amount, until the AFM started recording force curves representing adhesive interactions between the tip and the sample, represented by downward deflections of the cantilever. Force curves were continuously recorded while there was no sign of direct contact of the tip with the sample surface represented by an upward deflection of the cantilever. Since the exact distance between the tip and the sample cannot be estimated from the force curve in the compression-free method, and, since the piezoelectric that realizes the precise up-and-down movement of the substrate stage has a creep and hysteresis in its characteristics, a careful operation was needed in this method. In compression-free experiments, the contact area between pepsin and GroEL was very small because the tip was not in contact with the sample surface. Therefore, the interaction was expected to involve at most a few molecules and, in many cases, a single molecule of GroEL and/or pepsin.

In actual measurements, the distribution of force (Figs. 3 *A* and 4 *B*) was unimodal with the most probable value around 40 pN, which supported our expectation that the measured force originated from single pair interactions. Compared with the results of Vinckier et al. in 1998 (Vinckier et al., 1998) who reported forces larger than 200 pN for the interaction between GroEL and denatured citrate synthase or β-lactamase, we observed much smaller rupture forces. The disagreement may be due to the difference in the choice of denatured protein used for experiments, but more likely to the use of the compression-free type force measurement in our experiment which avoided interference from nonspecific and/or multiple pair interactions more effectively than the conventional method.

As shown in Fig. 3, the frequency of appearance of force curves showing positive interactions clearly decreased when free pepsin was added, proving that the observed forces were specific to the pepsin-GroEL interaction. In addition, as the structure and binding properties of GroEL are known to change after ATP binding, the change observed in force-distance curves after the addition of ATP (Fig. 4 *A*) was also convincing evidence of the specific nature of the observation. Details of these phenomena will be discussed later in this section.

Pepsin is a gastric aspartic proteinase (34,550 Da) with an optimum pH < 2 and contains two conformationally homologous domains. At a neutral pH, it loses its enzymatic activity and its conformation is denatured irreversibly (Lin et al., 1993). The total length of an extended form of a denatured pepsin is $\sim$100 nm considering the existence of 3 disulfide bridges (45-50, 206-210, and 249-282), assuming the contour length of one amino acid residue to be 0.37 nm (total number of amino acid residues is 326). Actually, pepsin does not have a string-like conformation at neutral pH because some secondary structure persists (Aoki et al., 1997) and denaturation occurs in the N-terminal domain at a neutral pH (Lin et al., 1993). All one can say is that pepsin can be extended for 100 nm at most.

The structures of GroEL and its complexes with various other molecules have been determined by x-ray crystallography (Braig et al., 1994; Xu et al., 1997). Fig. 5 *A* shows the structure of a GroEL subunit in the absence (*left*), and presence of ADP and GroES (*right*), respectively. The filled parts are H and I helices of GroEL which, together, compose the binding site for a nonnative protein. Seven such subunits form a ring, and two such rings form a homo 14-mer of

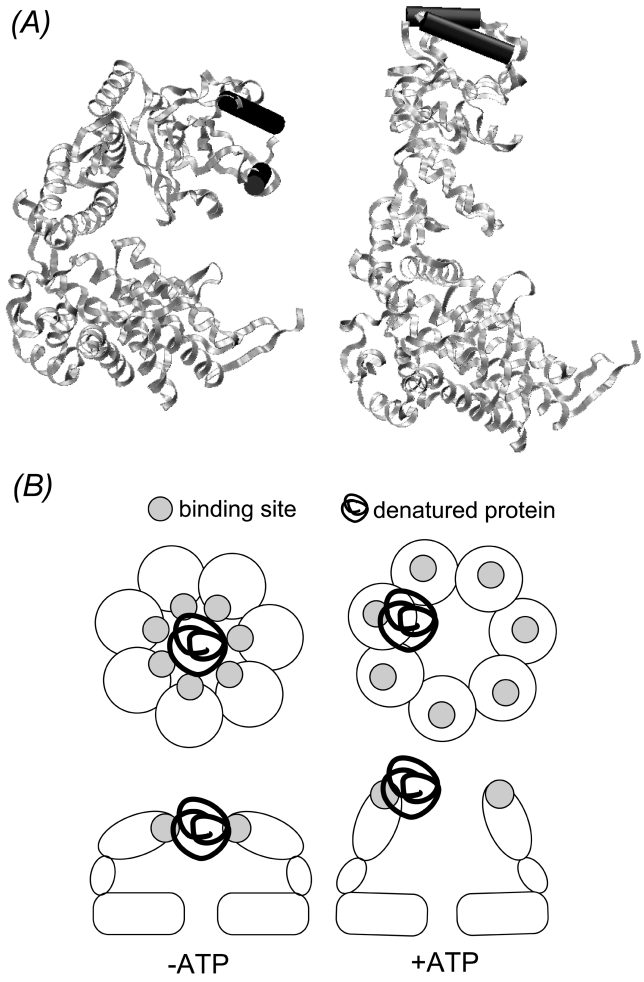

FIGURE 5 Crystal structure of a GroEL and models for the interaction between GroEL and a substrate protein. (*A*) The crystal structure of a GroEL subunit: without ATP (*left*; PDB data 1OEL) and with GroES and ADP (*right*; PDB data 1AON). The binding site for substrate protein (helices H and I) is in black. Both figures were drawn using VMD (Humphrey et al., 1996). (*B*) Proposed models for the interaction between GroEL and a substrate protein showing how GroEL interacts with pepsin in the absence (*left*) and in the presence (*right*) of ATP in this measurement. The binding sites and substrate proteins are indicated by gray circles and black curves, respectively.

GroEL. The inner diameter of the ring is 4.5 nm, and the binding sites for substrate protein face the interior cavity as shown in Fig. 5 *B* (*left*). The seven binding sites of a ring structure thus form a circle with a circumference of ~14 nm. Most of the interaction as summarized in Fig. 3 *B* occurred within 14 nm. Based on the structure of GroEL, we interpret this observation to mean that most of the seven binding sites of GroEL together catch a denatured pepsin. When pepsin is pulled away from the ring by force, the multiple bonds between pepsin and GroEL are broken one after another, giving rise to a force-distance curve that would have a sawtooth pattern like the one observed for the existence of the titin molecule (Rief et al., 1997), but we observed force curves with a plateau region in most of them, as shown in Figs. 2 *C* and 4 *A*. Some curves seemed to have multiple peaks (for example, the second, fourth, and fifth curves from the top in Fig. 4 *A*, *left column*), but it is difficult to resolve each peak. The sawtooth pattern was thought to be hidden in noise because the signal-to-noise ratio is not enough to detect it. The signal of cantilever deflection had noise of ~30 pN in standard deviation as seen in Figs. 2 *C* and 4 *A*, whereas the detected force in this study was only 40 pN in average. If the interaction force between each binding site and pepsin is assumed to be ~40 pN, this model explains the results of our experiments performed in the absence of ATP (Fig. 4 *A*).

Binding of ATP to GroEL is known to move the binding sites for substrate protein outward from the central cavity and spread them far apart, as shown in Fig. 5 *B* (*right*). When a denatured pepsin approaches GroEL in such a conformation, pepsin can bind only to a smaller number of the binding sites, possibly one or two, than in the absence of ATP, because the binding sites are now separated far from each other. This explains the observed shortening of the interaction length in the force-distance curve while the interaction force remained almost unchanged.

In conclusion, compression-free force measurement which can reduce sample damage and tip contamination during measurement is useful for measuring interaction between proteins. This method was applied for the GroEL system, and we were successful in measuring the specific interaction forces between GroEL and denatured pepsin, and in detecting the ATP-dependency of force duration in the force-distance curve. These results suggested that denatured pepsin was bound to GroEL subunits through multiple bonding, and the number of binding sites for denatured pepsin to GroEL was decreased in the presence of ATP.

Further investigation, especially into the mechanical properties of misfolded proteins, is essential to clarify the functional mechanics of the chaperonin.

We thank Ms. J. Suzuki for technical assistance in expressing and purifying mutant GroEL.

This work was supported in part by grants-in-aid to A.I. from the Japan Society for the Promotion of Science (Research for the Future Program 99R167019) and from the Japanese Ministry of Education, Culture, Sports, Science and Technology (Scientific Research on Priority Areas (B) 11226202).

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