Atomic Force Microscopy Detects Changes in the Interaction Forces between GroEL and Substrate Proteins

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ABSTRACT The structure of the *Escherichia coli* chaperonin GroEL has been investigated by tapping-mode atomic force microscopy (AFM) under liquid. High-resolution images can be obtained, which show the up-right position of GroEL adsorbed on mica with the substrate-binding site on top. Because of this orientation, the interaction between GroEL and two substrate proteins, citrate synthase from *Saccharomyces cerevisiae* with a destabilizing Gly→Ala mutation and RTEM β-lactamase from *Escherichia coli* with two Cys→Ala mutations, could be studied by force spectroscopy under different conditions. The results show that the interaction force decreases in the presence of ATP (but not of ATPγS) and that the force is smaller for native-like proteins than for the fully denatured ones. It also demonstrates that the interaction energy with GroEL increases with increasing molecular weight. By measuring the interaction force changes between the chaperonin and the two different substrate proteins, we could specifically detect GroEL conformational changes upon nucleotide binding.

INTRODUCTION

In vivo the accumulation of misfolded species and aggregates is prevented by the action of molecular chaperones. In this context the chaperonin GroEL and its cochaperonin GroES (Hartl, 1996; Fenton and Horwich, 1997; Xu et al., 1997) play an important role by assisting protein folding in two different ways. First, folding of the substrate proteins can occur in the central cavity of GroEL capped by GroES (Mayhew et al., 1996; Rye et al., 1997). Second, the substrate proteins are released from GroEL and reach the final native state in solution. In this case GroEL prevents aggregation of misfolded protein molecules by releasing less aggregation-prone states and keeping the concentration of folding intermediates low in free solution by rebinding them (Todd et al., 1996).

GroEL is a tetradecameric protein, consisting of two stacked rings with seven identical 57-kDa subunits in each ring (Braig et al., 1994; Chen et al., 1994). Each subunit consists of three domains: apical, equatorial, and intermediate. The apical domain facing the channel shows a higher percentage of hydrophobic amino acid residues than the other domains and is presumed to bind directly to the substrate. Previous studies have demonstrated that the interaction of a polypeptide chain with GroEL is based on hydrophobicity (Fenton et al., 1994; Zahn and Plückthun, 1994; Zahn et al., 1994; Itzhaki et al., 1995; Lin et al., 1995), although recent reports show that electrostatic interactions also could be important for the (rapid) binding of the substrate protein with GroEL (Itzhaki et al., 1995; Perrett et al., 1997; Aoki et al., 1997). When ATP cooperatively binds to seven equatorial domains of the same GroEL ring, the apical domains rotate and move upward and reach the so-called R-state (Roseman et al., 1996; White et al., 1997). This structural change is the reason for the reduced affinity of unfolded (or partly folded) proteins for GroEL in the presence of ATP, because some of the hydrophobic residues of GroEL will no longer contact the substrate. This structural change is enhanced by GroES, which contacts the hydrophobic residues after the equatorial domains have moved upward.

Different techniques, such as the surface force apparatus (Israelachvili, 1989; Leckband, 1995), pipette suction (Evans et al., 1991), or flow chamber technology (Pierres et al., 1996a,b), have been used to measure biological interactions. Recently the use of the atomic force microscope (AFM) to detect specific interaction forces has been described by several groups and shown to be very sensitive (Hoh et al., 1992; Stuart and Hladly, 1995; Dammer et al., 1995, 1996; Hinterdorfer et al., 1996; Roberts et al., 1996; Allen et al., 1996, 1997; Fritz et al., 1997; Nakajima et al., 1997). The measurements of specific interaction forces with AFM were reported for, e.g., the avidin-biotin system (Lee et al., 1994a; Florin et al., 1994; Ludwig et al., 1994; Moy et al., 1994) or for complementary DNA strands (Lee et al., 1994b; Boland and Ratner, 1995; Noy et al., 1997). The measured forces are due to noncovalent interactions leading

Received for publication 25 November 1997 and in final form 17 February 1998.

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0006-3495/98/06/3256/08 $2.00

to measured interaction force values far weaker than that of a covalent bond, which is ~1000 pN (Evans et al., 1995).

In this paper, the interaction between GroEL and two substrate proteins has been studied by measuring the interaction forces. The two substrate proteins are citrate synthase from yeast, carrying the destabilizing Gly276Ala mutation (Zahn et al., 1996; Lindner et al., unpublished observations) and RTEM β-lactamase, in which both cysteines forming a disulfide bond have been changed to alanines (Cys-Ala β-lactamase) (Gervasoni and Plückthun, 1997; Gervasoni et al., 1997). Force distributions have been obtained by measuring the interaction force from recorded force-distance curves under different conditions of pretreating the substrate protein, and in the presence or absence of ATP or ATPγS, which enabled us to obtain information on the conformational features of the chaperonin GroEL. We have also shown that with tapping-mode AFM under water, highly resolved images can be obtained.

MATERIALS AND METHODS

Instrumentation

Atomic force microscopy

Tapping-mode images under liquid have been obtained with a NanoScope III (Digital Intruments, Santa Barbara, CA), modified as described in Vinckier et al. (1996b). A frequency of 8.5 kHz and an amplitude of 2.9 nm were applied to standard commercially available silicon tips with a cone angle of 20°. For the functionalization in the force spectroscopic experiments, the same silicon tips were used as for AFM imaging. The spring constant of each cantilever was determined by the resonant frequency method (Cleveland et al., 1993), and the exact length of the cantilevers was measured by transmission electron microscopy (TEM) and found to be 450 ± 2 μm (± SD). The spring constants were found to be within the range of 0.02–0.2 N/m, with an error of ~5%. In each experiment we used the appropriate force constant for that particular silicon cantilever.

The AFM force measurements have been obtained by using a BioScope (Digital Intruments). A simple homemade liquid cell was constructed by placing a small amount of a silicone glue (Forbo-CTU AG, Schönenwerd, Switzerland) around the mica sample of interest before the start of any experiment. Many force-distance curves under buffer—as described below—have been measured at several places in the sample to obtain the force distribution. The force experiments were always performed with a scan rate of 1 Hz. This scan rate must be kept constant, because it determines the interaction time between the substrate protein and the GroEL. We found a small decrease in the interaction force at higher scan speed (32 Hz), because the molecules may have less time to interact. Slower scan rates showed a slightly higher interaction force. For example, for the GroEL- (Gly-Ala) citrate synthase interaction, with a scan speed of 0.1 Hz, a mean force of 620 ± 130 pN was obtained, whereas at 1 Hz the force was 440 ± 100 pN. The mean force at 0.1 Hz is different, after statistical calculations, from that at 1 Hz, with $p < 0.0001$. At higher scan rates the force decreases further. This is reproducible with all of the tips. Therefore, we paid particular attention to maintaining a constant scan rate in all of the experiments, to compare different events.

Transmission electron microscopy

Transmission electron microscopy (TEM) experiments were performed with a Philips CM 100 operated at 80 kV. The sample preparation was based on the method described by Detrich et al. (1985), and the sample was negatively stained with uranyl acetate.

Protein expression and purification

The double mutant (Cys-Ala) β-lactamase (28.8 kDa) was produced and purified by methods described elsewhere (Laminet and Plückthun, 1989; Gervasoni and Plückthun, 1997). The (Gly-Ala) citrate synthase, a homodimer of 100 kDa, which carries a N-terminal and a C-terminal his_5-tail, was produced and purified as described by Lindner et al. (1992). The chaperonin GroEL was overexpressed in *Escherichia coli* strain W3110 and purified as described by Gervasoni et al. (1997). Protein concentrations were measured with the bicinchoninic acid assay (Pierce, Rockford, IL) and are always given for the oligomeric states. All measurements in this work, unless stated otherwise, were carried out in 3-[N-morpholino]propane-sulfonic acid (MOPS) buffer (50 mM MOPS, 50 mM KCl, 5 mM $MgCl_2$, pH 7.2).

Sample preparation

GroEL (1.4 μM) in MOPS buffer was allowed to adsorb on freshly cleaved mica (Goodfellow, Cambridge, England). To obtain a complete coverage of the GroEL on the mica substrate (for the force measurements), 0.2% (v/v) polyethyleneglycol 6000 and 1% (w/v) ammonium molybdate were added to the MOPS buffer (Zahn et al., 1993). After 30 min the GroEL-covered mica was intensively rinsed with the MOPS buffer. When the sample was prepared for AFM images, this step was followed by fixation in 2.5% glutaraldehyde in phosphate buffer (pH 7, 0.1 M) for 5 min. In contrast, when the sample was prepared for the force measurements, GroEL was not fixed with glutaraldehyde. In both cases the samples were investigated under liquid and were never air-dried.

For the investigation of the interaction between GroEL and the substrate protein in the presence of nucleotides, either 2.5 mM ATP or 3 mM ATPγS was added to the solution and incubated for 1 h. During the course of the whole experiment (1–2 h), the nucleotides were always present and were kept constant in the MOPS buffer with the concentrations indicated above.

Tip preparation and functionalization

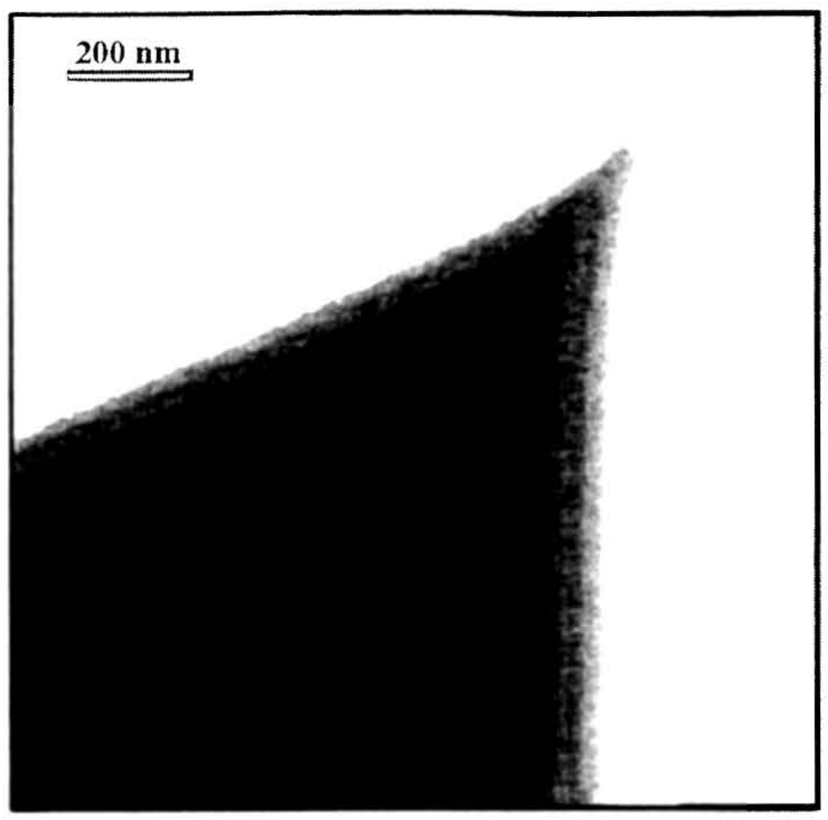
Before functionalization, the AFM tip was first flattened by fast scanning on a silicon oxide surface under a high load for 3 min. The tip was then cleaned with UV light (λ = 254 nm) for several hours. The tip shape at the apex was determined by TEM, following the method of DeRose and Revel (1997), before and after the experiment. A typical TEM image of such an AFM tip is shown in Fig. 1 *A*.

The functionalization of the tip is based upon the method proposed by Weetall et al. (Weetall and Filbert, 1974; Weetall, 1976) and is schematically presented in Fig. 1 *B*. In a first step the tip was silanized with a 5% solution of 3-aminopropyltriethoxysilane (3-APTES) (Fluka Chemie, Buchs, Switzerland) in 5% ethanol/95% water at room temperature for 15 min. The tip was then rinsed with the 5% ethanol/95% water solution, followed by air drying for 15–30 min. In a second step, the tip was immersed in a 2.5% glutaraldehyde solution in 100 mM phosphate buffer (pH 7.0) for 45 min and then extensively rinsed with water. Surface analytical investigations, such as AFM roughness measurements and ellipsometry, can be found in Vinckier (1996). In the last step the proteins ((Gly-Ala) citrate synthase (10 μM) and (Cys-Ala) β-lactamase (14 μM) in 50 mM MOPS (pH 7.2) bind covalently to the activated tip via their amino groups after a 30–60-min incubation. It is most likely that the proteins are immobilized randomly in different orientations.

The denaturation of these substrate proteins, covalently bound to the tip, was performed by an overnight incubation at 4°C in 8 M urea or 6 M guanidinium hydrochloride in MOPS buffer.

Hydrophobic tips were obtained by first cleaning the tip in UV light as described above and then reacting them with octadecyltrichlorosilane (OTS) (Fluka). The tip was immersed in an *n*-hexane solution of 10% OTS for 30 min. Afterward, the tip was rinsed with *n*-hexane and briefly air-dried, followed by a 2-h curing in an oven at 160°C. Because the tip modification cannot be monitored, we analyzed this reaction on pieces of

A



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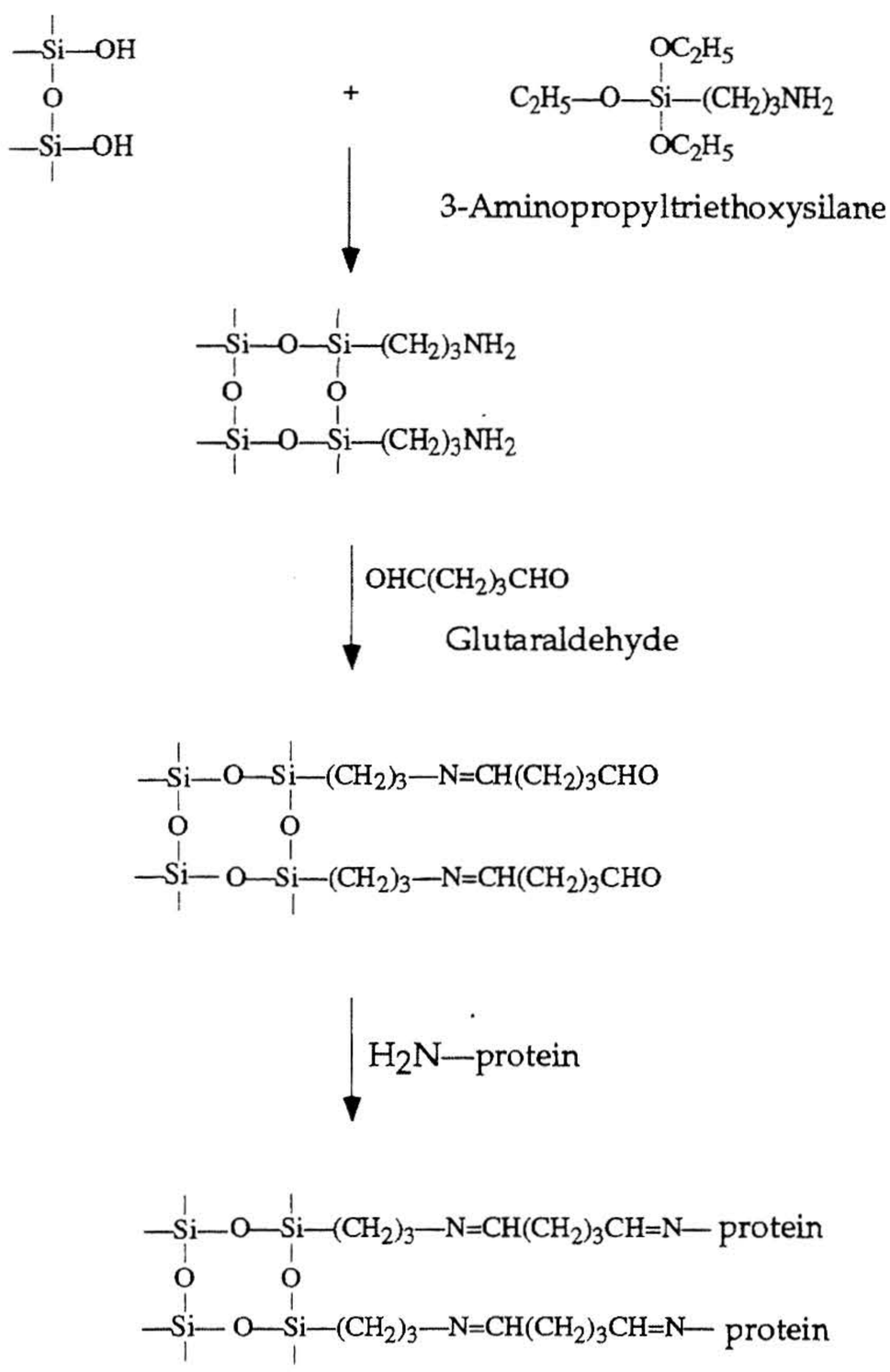

FIGURE 1 (*A*) TEM image of a flattened silicon tip for binding several biomolecules onto the top. The diameter of the tip at the flat area is 50 ± 1 nm and remained unchanged during the experiment. (*B*) Reaction scheme for the functionalization of the tip, following the method of Weetall and Filbert (1974).

silicon wafer. Contact mode AFM showed a relatively homogeneous layer with a root mean square roughness of 6.0 ± 0.4 Å; however, a minor polymerization resulting in particle contamination cannot be excluded. The thickness by ellipsometry was 4.3 ± 0.6 nm, which is a hydrophobic layer with a contact angle of 102 ± 3°, whereas the hydrophilic SiO_2 showed a contact angle of 28 ± 2°.

RESULTS AND DISCUSSION

Structural studies of GroEL by AFM

To study the interaction of GroEL with a substrate protein, it was first necessary to determine the orientation of the chaperonin, which was allowed to adsorb onto mica. The orientational direction was investigated by AFM. Fig. 2 *A* shows a tapping-mode (acoustically driven; Vinckier et al., 1996b) AFM image of GroEL in water. The "doughnut-like" structure of GroEL can be observed. The outer diameter of the rings is 48 ± 4 nm, and the height is 2.5 ± 0.3 nm. The inner diameter (the apparent "hole") observed here was 5 ± 1 nm. The corresponding values from the x-ray structure of GroEL are 13.7 nm for the diameter, 14.6 nm for the height, and 4.5 nm for the inner diameter (Braig et al., 1994).

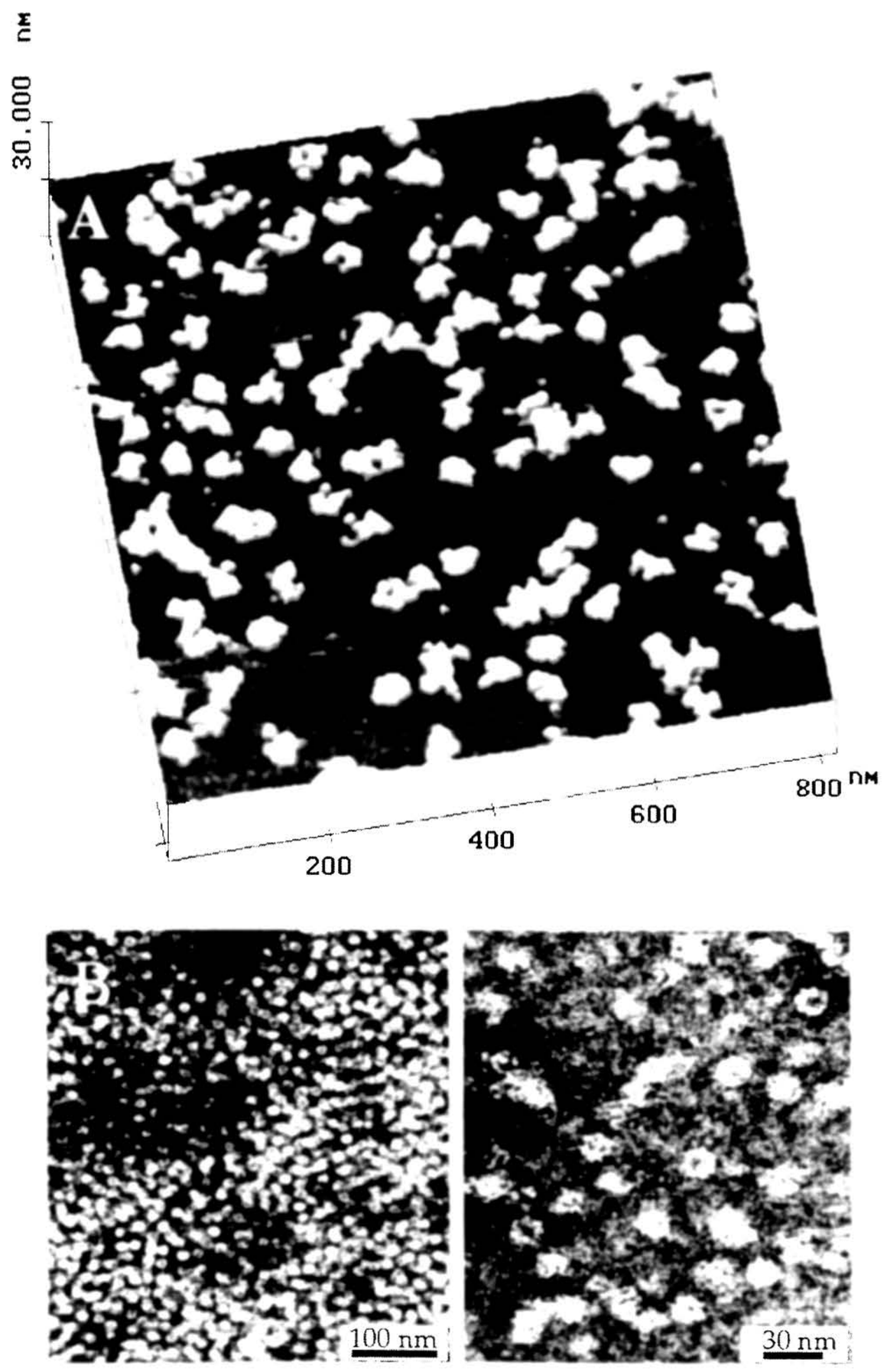

FIGURE 2 (*A*) Acoustically driven tapping-mode AFM image under water of mica-adsorbed GroEL, which was fixed with 2.5% glutaraldehyde. The MOPS buffer did not contain polyethyleneglycol 6000 or ammonium molybdate. The characteristic ring structure is discernible, and some fine structure can be made out. The GroEL binds to mica in an upright position. (*B*) Typical TEM images of GroEL (negative staining with uranyl acetate).

The discrepancy between the height measured by AFM and that measured by x-rays is most probably due to squeezing the GroEL by the tip under high vertical force. To explore this, we measured the indentation and the average height in contact mode AFM images at the same exerted force, because it has been shown that the sum of the indentation and the average height gives a height close to the true value (Vinckier et al., 1996a). The indentation obtained was 10 ± 1 nm, and the average height was 4 ± 1 nm, which results in a 14-nm-high molecule, which is in excellent agreement with the value obtained from x-ray crystallography (14.6 nm). This result confirms that GroEL is bound in a native conformation, with the two rings stacked back to back.

The apparent larger width is due to convolution, because of the finite size at the apex of the tip (Vinckier et al., 1995, and references therein). For the single molecules in Fig. 2 *A*, a lateral resolution of 4 nm and a vertical resolution of 0.3 nm have been found. Although the highest resolution has been obtained with contact-mode AFM under liquid, using 2-D crystals of GroEL (Mou et al., 1996a,b), we used tapping-mode AFM for imaging in the present work, despite its lower resolution, to prevent "moving" of the molecules, because we did not deal with a closely packed monolayer. In the presence of polyethyleneglycol 6000 and ammonium molybdate, we obtained a relatively closely packed layer, which was used for the force measurements (data not shown).

When the structure of GroEL was investigated by TEM, the chaperonin was bound to formvar-coated grids, and negatively stained with uranyl acetate. The TEM images in Fig. 2 *B* show the ring structure of GroEL, with the subunits clearly visible. Moreover, under these conditions there also was a tendency toward an upright orientation, as in the case of the AFM images of GroEL.

AFM images show that GroEL tends to orientate itself in the upright position, i.e., with the channel (almost) normal to the supporting substrate. Our results are therefore in good agreement with the work of Mou et al. (1996a,b) as well as that of Scheuring (1996).

Interaction of (Gly-Ala) citrate synthase and (Cys-Ala) β-lactamase with GroEL

We used flattened tips to permit several proteins to bind, and therefore to improve the chances that a molecule on the tip will interact with GroEL. The flatness also reduces the danger of damage to the tip. Before each experiment, the shape of the flattened tip was checked by TEM, as described in Materials and Methods. Fig. 1 A shows a TEM picture of the tip apex with a flat area and a diameter of 50 ± 1 nm, which was unchanged at the end of the experiment.

Because of the upright orientation of GroEL, we investigated the interaction between GroEL and (Gly-Ala) citrate synthase, and between GroEL and (Cys-Ala) β-lactamase by recording force-distance curves under different physiological conditions with a functionalized tip. A typical force-distance curve is shown in Fig. 3. The resulting forces for the interaction between GroEL and (Gly-Ala) citrate synthase, and between GroEL and (Cys-Ala) β-lactamase, are shown in Figs. 4 and 5, respectively. The force distributions shown in Figs. 4 and 5, however, are due to several simultaneous molecular interactions.

In Figs. 4 *A* and 5 *A*, the interaction of the native enzyme with GroEL was recorded in the absence of any nucleotide, and it gives a distribution whose maxima are 420 ± 100 pN for (Gly-Ala) citrate synthase and 240 ± 70 pN for (Cys-Ala) β-lactamase. This interaction may be due to hydrophobic patches on the surface of the native protein, and/or may involve those protein molecules that are partially denatured by the immobilization procedure or by being compressed in the approach phase of the force measurement. In a following step (Figs. 4 *B* and 5 *B*) ATP was added to the solution in the cell at room temperature and incubated for 1 h. The results presented in Figs. 4 *B* and 5 *B* show a marked decrease in the interaction force, i.e., both proteins covalently bound to the tip interact more weakly with GroEL.

In Figs. 4, *C* and *D*, and 5, *C* and *D*, the same tips with (Gly-Ala) citrate synthase or (Cys-Ala) β-lactamase molecules, respectively, had been incubated overnight in urea or guanidinium hydrochloride (GdmCl). The force distribution between the denatured (Gly-Ala) citrate synthase or (Cys-Ala) β-lactamase and GroEL in the absence of nucleotides is shown in Figs. 4 *C* and 5 *C*. A higher maximum and a wider distribution curve were observed. For the interaction with (Gly-Ala) citrate synthase, we found a mean force of 770 ± 190 pN, and for that with (Cys-Ala) β-lactamase, the force was 350 ± 100 pN. In Figs. 4 *D* and 5 *D*, the same experiment was performed in the presence of ATP, which again resulted in a marked decrease in the interaction force. Repeating this sequence of steps in Figs. 4 and 5, *A–D*, with different tips always showed a similar, reproducible behavior (data not shown). The tip shape was controlled after each experiment by TEM to ensure that the tip apex had not undergone alterations. Furthermore, the packing of the GroEL molecules on the sample before, during, and after the experiments was checked by tapping-mode AFM. We found that the GroEL molecules were closely packed, with almost no space between them. This densely packed layer was obtained by using polyethyleneglycol 6000 and ammonium molybdate, but no glutaraldehyde fixation was used.

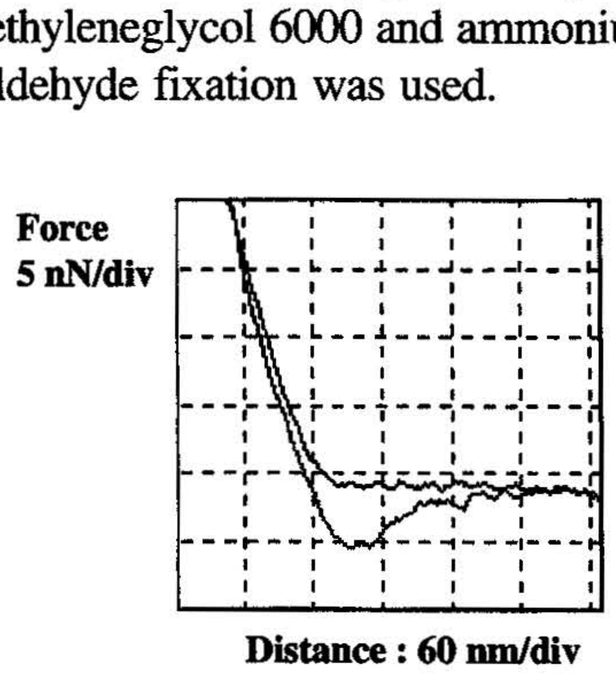

FIGURE 3 An example of a typical force-distance curve between a modified tip and mica-adsorbed GroEL in the absence of nucleotides. The tip was modified with "native-like" (Gly-Ala) citrate synthase.

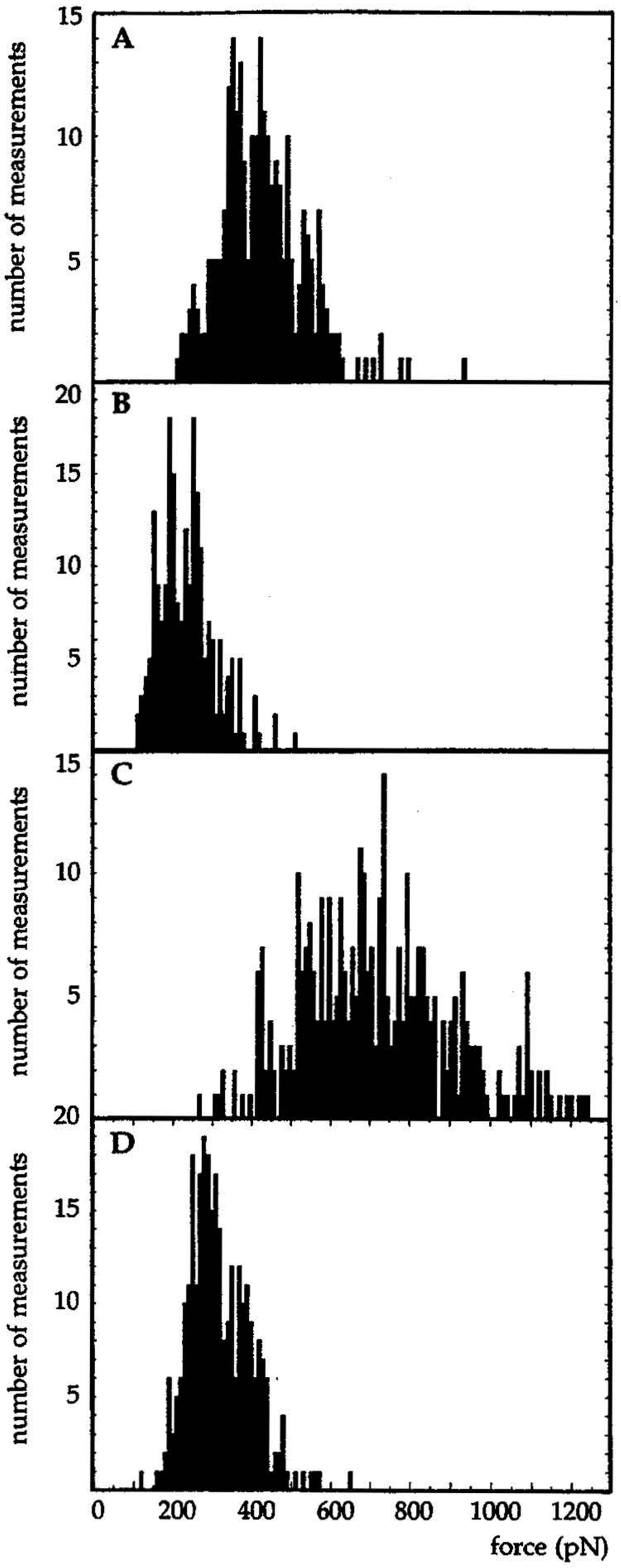

FIGURE 4 Force distributions of GroEL with (Gly-Ala) citrate synthase. The tip diameter was 70 ± 1 nm. In *A* the native protein was bound onto the tip, and in *B* the interaction was measured in the presence of 2.5 mM ATP. (*C* and *D*) Result obtained by using denatured proteins in the absence (*C*) and presence (*D*) of ATP.

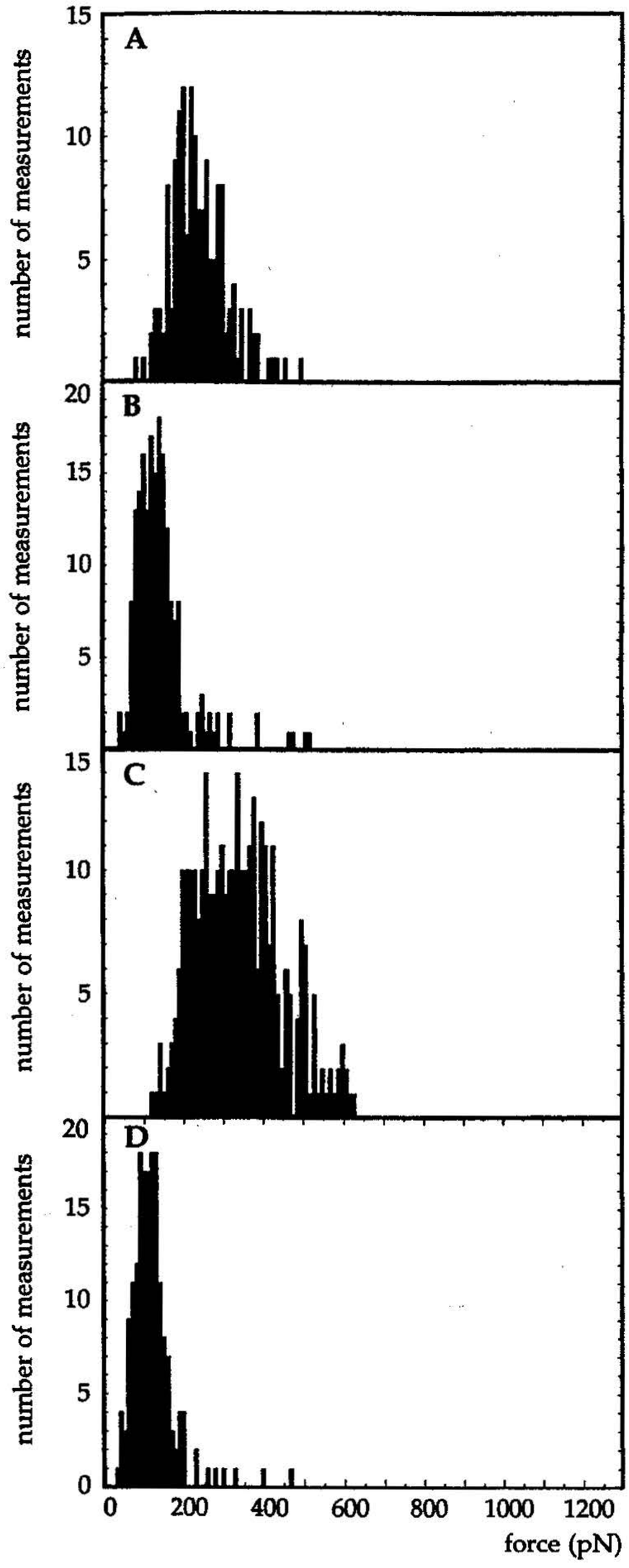

FIGURE 5 Force distributions of GroEL with (Cys-Ala) β-lactamase. The tip diameter was 80 ± 1 nm. In *A* the native protein was bound to the tip, and in *B* the interaction was measured in the presence of 2.5 mM ATP. (*C* and *D*) Result obtained by using denatured proteins in the absence (*C*) and presence (*D*) of ATP.

Control experiments were performed by measuring the interaction between the tip and mica during all steps of the functionalization of the tip, and by measuring the interaction of the substrate protein, immobilized on the tip, with freshly cleaved mica, as well as the interaction between a glutaraldehyde-activated tip with GroEL adsorbed on mica. Almost no interaction was measured (Table 1). Thus, although we cannot totally exclude very small interactions of the mica background with the modified silicon tip, these interactions must be negligible; the force distribution plots in Figs. 4 and 5 describe the specific interactions between GroEL and the substrate proteins.

By using bovine serum albumin (BSA) or horseradish peroxidase, a behavior similar to that of (Gly-Ala) citrate synthase or (Cys-Ala) β-lactamase was observed. For the tip functionalized with peroxidase, we found in the absence of any nucleotide that the native-like protein feels a force of 130 ± 30 pN (tip diameter 25 nm), whereas the force between BSA and GroEL was 570 ± 60 pN (tip diameter 30 nm). It is known that GroEL interacts with exposed hydrophobic patches on many proteins and, therefore, as our results demonstrate, potentially with any partially unfolded proteins.

TABLE 1 Mean interaction forces in the control experiments

Tip surface	Bottom surface	Forces ± SD (pN)
(Gly-Ala) citrate synthase	Mica	20 ± 20
(Cys-Ala) β-lactamase	Mica	130 ± 10
Hydrophilic silicon tip	GroEL	80 ± 20
Hydrophilic silicon tip	GroEL in the presence of ATP	970 ± 370
Hydrophobic silicon tip	GroEL	430 ± 100
Hydrophobic silicon tip	GroEL in the presence of ATP	140 ± 40
Glutaraldehyde-activated tip	GroEL	80 ± 30

Comparison between native-like and unfolded substrate proteins on the interaction of GroEL

There is a clear difference in the force distribution between the native-like and the unfolded form of both substrate proteins (Gly-Ala) citrate synthase and (Cys-Ala) β-lactamase, which is consistent with biochemical experiments (Zahn et al., 1996; Gervasoni and Plückthun, 1997; Gervasoni et al., 1997). We observed a shift in the maximum of the distribution curve (770 ± 190 pN for (Gly-Ala) citrate synthase and 350 ± 100 pN for (Cys-Ala) β-lactamase), as well as a broadening of the distribution curve for the interaction forces. This can be explained by the fact that the hydrophobic amino acids of soluble, globular proteins become more exposed and therefore accessible for binding to the chaperone only in nonnative states. However, it was not possible to distinguish whether there are different steps in the distribution curve, which might be due to several molecules bound to the tip or to multiple interaction steps of a single molecule. The already rather strong interaction between the native-like proteins and GroEL (420 ± 100 pN for (Gly-Ala) citrate synthase and 240 ± 70 pN for (Cys-Ala) β-lactamase) suggest that hydrophobic patches on the surface of the native substrate protein interact with GroEL and/or that some partial denaturation of the proteins bound to the tip has occurred because of the immobilization step or the applied force during the measurements.

Effect of ATP on the interaction forces between GroEL and substrate proteins

In the presence of ATP, the apical domains of GroEL move upward and rotate, and the substrate protein is released (Rye et al., 1997). Therefore, the interaction force between GroEL and the substrate protein is expected to decrease. In the experiments shown in Figs. 4, *B* and *D*, and 5, *B* and *D*, the interaction force indeed decreases in the presence of 2.5 mM ATP. Under the conditions used in this work, the ATP hydrolysis by GroEL has a half-life of 10 s, and this is the rate-limiting step of the whole ATP cycle: ATP binding, hydrolysis, and ADP·P release. Therefore, the GroEL structure observed here in a steady-state hydrolysis represents largely an ATP-bound state, i.e., the R-state (Burston et al., 1995; Roseman et al., 1996). For the interaction of GroEL with native (Gly-Ala) citrate synthase, we found an interaction force of 230 ± 70 pN, and for the denatured protein an interaction force of 320 ± 80 pN, both in the presence of ATP. In the case of native-like (Cys-Ala) β-lactamase, the measured force was 140 ± 60 pN, and with GdmCl-denatured (Cys-Ala) β-lactamase it was 120 ± 50 pN, also both in the presence of ATP. The minor changes can be related to the exact orientation of the molecules on the tip and to the GroEL occupation on the mica. Consequently, there is no large difference between the native protein and the denatured protein when ATP is present.

To test the effect of ATP hydrolysis on the interaction forces, a nonhydrolyzable ATP analog, ATPγS, has been used. We found that both the native and denatured substrate proteins show the same interaction force in the presence of ATPγS as in the absence of any nucleotide. The data are summarized in Table 2. Interestingly, the x-ray structure of GroEL in the presence of ATPγS shows that the binding of the nonhydrolyzable nucleotide analog results in only small conformational changes, compared to the free GroEL (Boisvert et al., 1996). This unexpected result was rationalized by Aharoni and Horovitz (1996), who showed that the negative cooperativity between the two rings of GroEL was reduced in the GroEL mutant (R13G/A126V) used in the x-ray crystallography studies. In addition, cryo-EM observations of GroEL in the presence of the nonhydrolyzable ATP analog AMP-PNP also showed a conformation intermediate between the ADP- and ATP-GroEL bound state (Roseman et al., 1996). This suggests that the interaction forces between GroEL and the substrate protein are not necessarily effected by the presence or absence of the nonhydrolyzable ATP analogue ATPγS. Taken together, these results with different nucleotides strongly support the conclusion that we are observing specific substrate-GroEL interactions.

Comparison of the two substrate proteins

When we compared the interaction forces obtained for the two substrate proteins, a smaller force was always found in Figs. 4 and 5 for (Cys-Ala) β-lactamase than for (Gly-Ala) citrate synthase. Usually the interaction force between GroEL and (Cys-Ala) β-lactamase was roughly half of that obtained between GroEL and (Gly-Ala) citrate synthase,

TABLE 2 Mean forces (± SD) of the interaction between GroEL and (Cys-Ala) β-lactamase in the absence of any nucleotide or in the presence of ATP or ATPγS

(Cys-Ala) β-lactamase	Without ATP (pN)	With ATP (pN)	With ATPγS (pN)
With tip 1 from Fig. 5			
Native-like	240 ± 70	140 ± 60	
GdmCl denatured	350 ± 100	120 ± 50	
With tip 2			
Native-like	260 ± 60		280 ± 70
GdmCl denatured	390 ± 80		390 ± 70

TABLE 3 Mean forces in pN between GroEL and (Cys-Ala) β-lactamase, depending on the diameter of the functionalized tip

Diameter of the tip (nm)	Mean force ± SD (pN)
35	110 ± 30
54	220 ± 60
80	240 ± 70
110	280 ± 70
160	380 ± 100

with the same tip diameter. The forces measured may be related to the molecular size and the interaction surface of the substrate protein.

Results from several interaction experiments between GroEL and the native-like form of (Cys-Ala) β-lactamase and the varying tip diameter are listed in Table 3. A tip with a larger diameter can accommodate more substrate proteins, which results in a higher interaction force.

Hydrophobic and hydrophilic tips

To check the importance of hydrophobic effects on the GroEL-substrate protein interaction, the tips were modified into hydrophilic and hydrophobic tips. We compared the interaction with GroEL on mica with a cleaned (hydrophilic) silicon tip as well as with a hydrophobic tip, i.e., a silicon tip modified with OTS, as described in Materials and Methods. These experiments were performed to understand the hydrophilic and hydrophobic interactions between GroEL and chemically well-characterized samples (Table 1). We found that the hydrophilic tip itself interacts poorly with GroEL and gives an interaction force of only 80 ± 20 pN, whereas the hydrophobic tip shows an interaction force similar to that of the substrate proteins immobilized on the tip. In fact, the interaction force with hydrophobic tips is 430 ± 100 pN, which lies in the range for the native-like proteins (Figs. 4 and 5). The tip diameter was also 50 nm, and thus was in the same range as the functionalized tips. In the presence of ATP, the hydrophilic tip shows an increased interaction with GroEL, which gives an interaction force of 970 ± 370 pN. The hydrophobic tip, however, showed a decreased interaction force of 140 ± 40 pN in the presence of ATP. Both results indicate that the interaction between GroEL and the substrate proteins is mostly hydrophobic, and that the forces measure the conformational state of GroEL.

CONCLUSIONS

In this work we show that tapping-mode AFM under liquid leads to resolved images of GroEL. We were able to obtain images of the characteristic ring structure of the chaperonin, in which some fine structure can be made out. Because of the upright orientation of GroEL on mica, AFM allowed quantitative, reproducible measurements of the interaction force between GroEL and the substrate proteins, (Gly-Ala) citrate synthase and (Cys-Ala) β-lactamase, by covalently immobilizing them on the surface of the tip. We could measure by AFM the changes in the interaction forces upon the addition of ATP, which results in conformational changes in the GroEL apical domains: in the presence of ATP, the interaction force between the two substrate proteins and GroEL decreased. Similarly, we found that denatured proteins give rise to a higher interaction force than the native-like proteins. Finally, the experiments also prove that hydrophobicity is important for the interaction of the substrate proteins with GroEL.

The authors thank P. Kernen, J. A. DeRose, and P. Wagner for helpful discussions; H.-P. Lipp for statistical advice; and B. Klinger for help with the purification of the proteins. This work was supported by SNSF (NFP 36), SNSF 31-037720.93/1, and O. Mayenfisch-Stiftung.

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