Chaperonins GroEL and GroES: Views From Atomic Force Microscopy

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ABSTRACT The *Escherichia coli* chaperonins, GroEL and GroES, as well as their complexes in the presence of a nonhydrolyzable nucleotide AMP-PNP, have been imaged with the atomic force microscope (AFM). We demonstrate that both GroEL and GroES that have been adsorbed to a mica surface can be resolved directly by the AFM in aqueous solution at room temperature. However, with glutaraldehyde fixation of already adsorbed molecules, the resolution of both GroEL and GroES was further improved, as all seven subunits were well resolved without any image processing. We also found that chemical fixation was necessary for the contact mode AFM to image GroEL/ES complexes, and in the AFM images, GroEL with GroES bound can be clearly distinguished from those without. The GroEL/ES complex was about 5 nm higher than GroEL alone, indicating a 2 nm upward movement of the apical domains of GroEL. Using a slightly larger probe force, unfixed GroEL could be dissected: the upper heptamer was removed to expose the contact surface of the two heptamers. These results clearly demonstrate the usefulness of cross-linking agents for the determination of molecular structures with the AFM. They also pave the way for using the AFM to study the structural basis for the function of GroE system and other molecular chaperones.

INTRODUCTION

The *Escherichia coli* chaperonin GroEL is a member of the heat shock protein cpn 60 family (Hendrix, 1979; Hemmingsen et al., 1988; McMullin and Hallberg, 1988; Trent et al., 1991; Gao et al., 1992), and forms a double ringed tetradecamer with a total molecular weight of 840 kDa (Hutchinson et al., 1989; Hohn et al., 1979; Pushkin et al., 1982; Harris et al., 1994; Schmidt et al., 1994a; Llorca et al., 1994; Braig et al., 1994). The co-chaperonin GroES (Tilly et al., 1981; Chandrasekhar et al., 1986), a member of the cpn 10 family, forms a single heptameric ring of only 70 kDa (Chandrasekhar et al., 1986; Zondlo et al., 1995; Hunt et al., 1996; Mou et al., 1996), and binds to GroEL in the presence of ATP or one of its non-hydrolyzable analogs (Azem et al., 1994; Langer et al., 1992; Harris et al., 1994; Schmidt et al., 1994a; Llorca et al., 1994). Together, GroEL and GroES constitute a fascinating molecular machine that facilitates the folding of denatured proteins under various stress conditions (for reviews, see Hendrick and Hartl, 1993; Landry and Gierasch, 1994; Lorimer, 1994) with the GroES playing a modulatory/regulatory role (Jackson et al., 1993; Todd et al., 1994; Gray and Fersht, 1991; Schmidt et al., 1994b; Todd et al., 1993; Todd et al., 1995; Kawata et al., 1995). Chaperonins are essential components of a cell because of their protective functions (Fayet et al., 1989). Although the structure of both GroEL and GroES has been solved to atomic resolution by x-ray diffraction (Braig et al., 1994; Boisvert et al., 1996; Hunt et al., 1996; Mande et al., 1996) and their functions have been studied extensively with biochemical and biophysical methods (for recent reviews, see Hendrick and Hartl, 1993; Landry and Gierasch, 1994; Lorimer, 1994), there are still essential questions that remain unanswered (Lorimer and Todd, 1996; Saibil, 1996; Mayhew and Hartl, 1996). A molecular model of protein folding with the GroEL/ES complex has not been firmly established (Lorimer and Todd, 1996). Therefore, it is important to develop other, complementary structural approaches, in addition to x-ray diffraction and electron microscopy (EM), to address these complicated, yet fascinating issues. From this point of view, atomic force microscopy (AFM) appears to be a particularly attractive approach, for its ability to acquire images under physiological conditions (Hansma and Hoh, 1994; Shao and Yang, 1995; Shao et al., 1995). In addition to comparing structures with that of x-ray diffraction (Mou et al., 1996), the chaperonin system can be imaged during its reconstitution. Low resolution structures acquired by AFM can also be compared with predicted structural features by other less direct methods, such as neutron scattering (Thiyagarajan et al., 1996).

However, high resolution AFM imaging was only achieved with a limited number of biological specimens (Yang et al., 1993, 1994a; Mou et al., 1995a, b; Hoh et al., 1991; Schabert et al., 1995; Muller et al., 1995), despite the much increased use of the AFM in biology (for recent reviews, see Hansma and Hoh, 1994; Lal and John, 1994; Shao and Yang, 1995; Shao et al., 1995). For soluble proteins, AFM imaging in solution has only achieved moderate or low resolution in most cases (Yang et al., 1994b; Ill et al., 1993; Ohnishi et al., 1992; Radmacher et al., 1994), suggesting that the high molecular weight tetradecamer GroEL and its complex with GroES could be a formidable task for the AFM, although the smaller co-chaperonin

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GroES was recently successfully imaged at high resolution ($\lesssim$ 1 nm) with glutaraldehyde fixation (Mou et al., 1996).

In this paper, we report high resolution images of GroEL, GroES, and their complexes obtained with the AFM in solution at room temperature. Our results show that GroEL can be directly imaged in solution without additional treatment, such as fixation. However, for GroES, glutaraldehyde fixation was effective in the resolution of the sevenfold symmetry near the roof region (Mou et al., 1996). The resolution with GroEL was also improved to some extent by glutaraldehyde fixation. With nonfixed GroEL specimens, we also found that the upper heptameric ring could be dissected away with the AFM tip to expose the surface of the equatorial domains, an ability rather unique with the AFM (Hoh et al., 1991). Furthermore, we show that with GroEL and GroES complexes in the presence of nucleotide, glutaraldehyde fixation became essential for AFM imaging, otherwise the bound GroES "cap" could be easily removed. An important conclusion from these studies is that these AFM structures are in excellent agreement with those from x-ray diffraction and EM, providing a necessary basis for the study of less well-characterized structures of the GroE system. The high resolution and excellent specimen stability also suggest that the AFM will be capable of resolving some of the outstanding issues, such as the substrate binding site and the connectivity of the two chambers in GroEL.

MATERIALS AND METHODS

Materials

All chemicals used in these experiments were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO). The cross-linker, glutaraldehyde, was obtained from SPI Supplies (West Chester, PA) (microscopy grade). Deionized water (18 MΩ) was used for the preparation of various solutions. *E. coli* GroEL and GroES were obtained from Sigma Chemicals, and the purity was better than 95% (SDS-PAGE). After the lyophilized powder was reconstituted (0.25 mg/ml for GroES in 25 mM Tris, 75 mM KCl, 0.5 mM DTT and 1.25% trehalose, pH 7.5; 1 mg/ml for GroEL in 50 mM Tris, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 2.5% trehalose, pH 7.5), both GroEL and GroES were first examined with EM after the procedure of Engel et al. (1995), with a Philips CM200FEG (data not shown). For GroEL, both end-up and horizontal orientations were present, which was essentially the same as other EM observations of GroEL (Engel et al., 1995; Harris et al., 1994; Langer et al., 1992; Schmidt et al., 1994a). For the end-up orientation, a nominal diameter of 14–15 nm was resolved as expected. The diameter of GroES was 7–8 nm in the EM image, but the sevenfold symmetry was not resolved. In addition to checking the quality of the materials, these EM images also serve as a useful comparison with the AFM images.

Specimen preparation

For the preparation of AFM specimens, a small droplet of the protein solution was directly applied to a freshly cleaved mica surface. After incubation at room temperature (for 30 min or more), the specimen was washed with deionized water or a low salt buffer for several times to remove proteins that were not adsorbed. Normally, a full coverage can be achieved with this method. The prepared specimen should not be dehydrated during handling. These specimens were either imaged directly by the AFM in solution, or fixed with 2% glutaraldehyde before AFM imaging in a low salt solution. For the preparation of the GroEL/GroES complexes, the GroEL was first adsorbed to a mica surface in 50 mM Tris, 50 mM KCl, and 50 mM $MgCl_2$ at pH 8. After the specimen was washed with the same buffer, the final solution was adjusted to include 3 mM AMP-PNP (Azem et al., 1994; Langer et al., 1992; Harris et al., 1994; Schmidt et al., 1994a; Llorca et al., 1994) before 20 μl of GroES at a concentration of 0.25 mg/ml were added to the solution covering the specimen, and incubated for 10–30 min at room temperature. The binding efficiency of GroES to GroEL was also examined with negatively stained EM. In Fig. 1, a typical image of GroEL/ES complexes is shown (with 15-min incubation). It is seen that GroES binds to GroEL in both the symmetric and asymmetric fashion (Harris et al., 1994; Schmidt et al., 1994a; Llorca et al., 1994), with a nearly 100% occupancy. For the GroEL/GroES complexes, the specimen was also fixed with 2% glutaraldehyde in a buffer containing both AMP-PNP and Mg^{2+}. If fixation was performed in a buffer without sufficient AMP-PNP, most GroES was found dissociated from GroEL (EM and AFM, unpublished observations).

AFM imaging

All AFM images were obtained with a NanoScope II AFM (Digital Instruments, Santa Barbara, CA) in a homemade fluid cell (Yang et al., 1993) with the contact mode. The AFM was suspended from the ceiling without additional vibration isolation. Commercial cantilevers with oxide-sharpened tips and a nominal spring constant of 0.06 N/m (Digital Instruments, Santa Barbara, CA or Park Scientific, Sunnyvale, CA) were used. Typical scanning speed was 5–10 Hz. The probe force was maintained at $<$0.5 nN. All images contain 400$\times$400 pixels, and are presented without image processing, except flattening. The scales of the piezo scanner were calibrated against a grid of known dimensions.

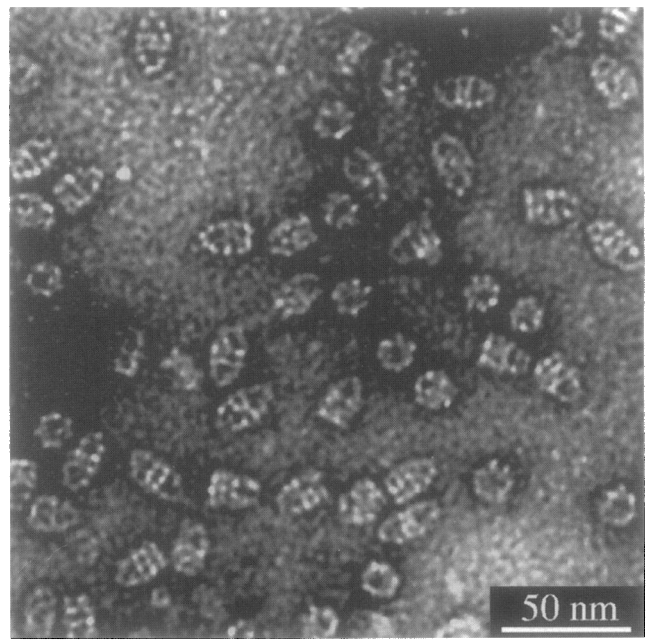

FIGURE 1 An image of GroEL/ES complexes in the presence of AMP-PNP obtained by transmission electron microscopy (TEM) via negative staining. It is clearly seen that nearly all GroEL had one or two GroES bound, indicating that a high occupancy of GroEL sites is achieved. This result is consistent with previous observations (Harris et al., 1994; Engel et al., 1995). For the end-up orientation, the binding of GroES could not be differentiated.

RESULTS AND DISCUSSION

Structure of GroEL in solution

It is well established that GroEL forms a double-ringed tetradecamer in vivo and in vitro, based on EM observations and biochemical studies (Hendrix, 1979; Hemmingsen et al., 1988; McMullin and Hallberg, 1988; Trent et al., 1991; Gao et al., 1992; Hutchinson et al., 1989; Hohn et al., 1979; Pushkin et al., 1982; Harris et al., 1994; Schmidt et al., 1994a). The structure of the tetradecamer, both in the absence of nucleotide (Braig et al., 1994) or with ATPγS (Boisvert et al., 1996), has been solved to atomic resolution by x-ray diffraction, confirming the double-ringed architecture. According to the x-ray model, the intact GroEL has an outer diameter of 14 nm, a height of 15 nm, and a central channel with a diameter of 4.5 nm (Braig et al., 1994; Boisvert et al., 1996). The binding of ATPγS caused only very moderate structural changes in GroEL (Boisvert et al., 1996). Such tall, multi-subunit structures have been considered difficult to image with the AFM in the past (Yang et al., 1994b; Ill et al., 1993).

With freshly cleaved mica, GroEL was found to adsorb readily to the surface in most buffers, and most of them appeared to have an end-up orientation. However, AFM imaging in high ionic strength buffers turned out to be difficult, and the central channel opening was only occasionally resolved. In low salt buffer or deionized water, the stability of the specimen and the quality of the images were markedly improved. An example is shown in Fig. 2 *a* where the image was taken in deionized water at room temperature. With these specimens, the sample was extremely delicate in that, very often, they could sustain the scanning tip only once, and were thereafter either damaged or dissected (see below). The diameter measured from these images was 13.4 ± 0.9 nm, in good agreement with the x-ray model (Braig et al., 1994; Sigler and Horwich, 1995), indicating that the effect of tip broadening was rather insignificant. The quality of these images is quite close to that of EM with negatively stained specimens (Harris et al., 1994; Engel et al., 1995). The height of GroEL, as measured from the edge of closely packed patches (13–14 nm), is close to the known value (15 nm), indicating that at least for large scale scans, the vertical compression was not severe, consistent with a recently proposed model (Yang et al., 1996; Shao et al., 1996). To improve the stability of these large complexes, various additives frequently used in other methods to precipitate or stabilize protein structures, such as polyethylene glycol (PEG) and trehalose, were introduced into the imaging buffer. We found that among the various sizes of PEG, the one with the molecular weight of 1450 Da (PEG1450) was somewhat helpful for improving the quality of the AFM images, and no advantage with trehalose (up to 10 mM) was found in these experiments, although the latter was thought to have a stabilizing effect on the molecular structure (Harris et al., 1994).

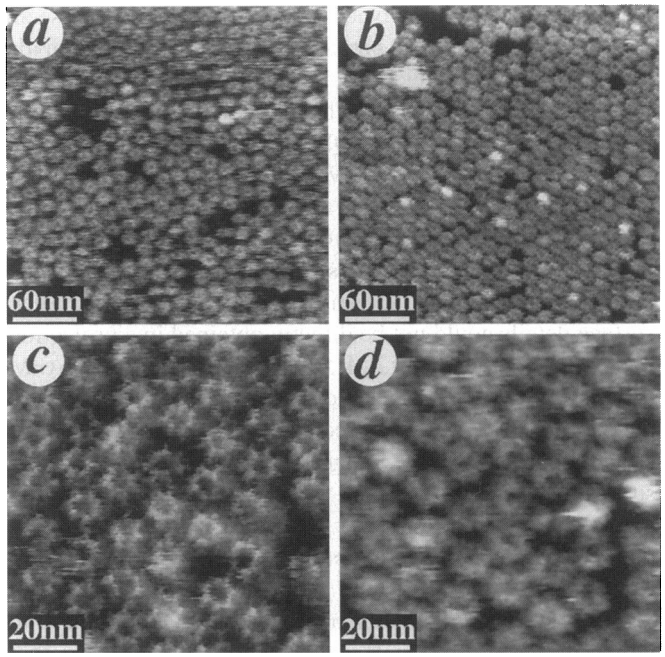

FIGURE 2 AFM images of GroEL obtained in aqueous solution. (*a*) An image obtained in deionized water, after GroEL was allowed to adsorb to a mica surface. Individual GroEL tetradecamers, each with a central channel, could be seen, but the specimen was very unstable. To obtain these images, the probe force must be kept to the minimum, and in most cases, only a strip of the image could show individual GroEL with a reasonable resolution. With glutaraldehyde fixation, the molecular stability was improved, and repeated scans, even at a slightly larger probe force, did not appear to damage these molecules. (*b*) An image of GroEL with fixation. It is seen that the specimen was in a quasi-closely packed form, and nearly all molecules were in the end-up orientation. The central channel was resolved in almost every GroEL. (*c*) A high resolution image of GroEL in the absence of AMP-PNP. In this image, the seven subunits in the upper heptamer are clearly resolved. It should be noted that such a resolution was achieved without any image averaging. The contrast achieved by the AFM is much higher than that of negatively stained EM. (*d*) A high resolution image of GroEL in the presence of AMP-PNP. No profound structural differences can be discerned, when compared with that in the absence of AMP-PNP, which is consistent with the model based on reconstructed images of EM (Chen et al., 1994) and x-ray diffraction (Braig et al., 1994). For some reason, the image quality was not as high as that without AMP-PNP.

Because GroEL appeared seriously damaged by the AFM tip after only one scan under the smallest probe force we could manage, the molecular structure must be further strengthened to achieve a resolution sufficient to resolve the individual subunits. For this purpose, we have experimented with the most commonly used fixation reagent, glutaraldehyde (Glauert, 1975), to chemically fix the molecules that were already adsorbed to a mica surface. As reported earlier with the gap junction plaques (Hoh et al., 1991), we also found that prefixed GroEL did not adhere well to the substrate, but that the adhesion was not affected significantly if the molecules were already on the substrate surface. With this type of in situ fixation, the quality of the AFM images was significantly improved. In Fig. 2 *b*, the specimen is seen closely packed with a flat clean surface. At a smaller scale

(Fig. 2 *c*), the well-resolved subunit structure can be seen. These specimens were prepared with 2% glutaraldehyde fixation without nucleotides, and AFM imaging was performed in deionized water. The signal to noise ratio shown in Fig. 2 *c* is quite high and the image quality of AFM has now exceeded that of negatively stained EM. It is particularly interesting to notice that each subunit has an elongated envelope, which compares well with that of x-ray diffraction if the surface profile is computed from the atomic coordinates (data not shown) (Braig et al., 1994). Such surface details could not be directly obtained in a projection image, such as that from EM. From this point of view, AFM images can also be used as a complementary comparison to the surface envelope of the reconstructed three-dimensional structure based on EM images (Chen et al., 1994). Measurements with these AFM images show that the outer diameter of GroEL is 13.3 ± 0.6 nm which is the same as that without fixation. Therefore, glutaraldehyde did not cause significant structural alteration at the resolution achieved. However, the channel opening has a somewhat larger diameter (4.3 ± 0.5 nm) when compared to that without fixation. This might be because the structure near the channel opening of unfixed GroEL was deformed more severely, resulting in a smaller pore. It may be noted that the channel diameter from x-ray diffraction is also 4.5 nm (Braig et al., 1994). Because the fixed specimens could withstand repeated scans of the AFM tip even with a slightly increased probe force, we suggest that the improved image quality was primarily due to the increased specimen rigidity and stability. When the GroEL was imaged in the AFM in the presence of AMP-PNP, it was found extremely difficult to obtain clean images, perhaps due to the conformational changes at the channel opening that resulted in an increased flexibility (Chen et al., 1994; Langer et al., 1992; Saibil et al., 1993). When 2% glutaraldehyde was used to fix the adsorbed GroEL in the buffer containing AMP-PNP, the image quality was much improved (see Fig. 2 *d*). The overall structure is very similar to that without AMP-PNP (see Fig. 2 *c*). However, the channel opening was found somewhat smaller than that without bound AMP-PNP. This observation is consistent with the suggested model based on EM studies (Chen et al., 1994) and x-ray diffraction (Boisvert et al., 1996), although we must emphasize that the possibility of a tip-related effect cannot be ruled out at present. It is important to realize that with the available technology, the size of the tip is basically uncontrollable, not only because of the fabrication limitations, but also because in situ contamination (Shao and Yang, 1995).

Molecular dissection of GroEL in solution

In the crystal structure, there are about 30 unresolved amino acid residues within the equatorial domain of each GroEL subunit (Braig et al., 1994). It is of interest, even at a lower resolution, to determine the location of the missing mass, because of its potential implications in the function of GroEL (Triyagarajan et al., 1996). It was recently suggested that these missing residues formed a "plug," so that the two chambers in GroEL are disconnected (Triyagarajan et al., 1996), contrary to the current view (Braig et al., 1994). Direct imaging with single ring mutants should be a useful approach to address this question (Viitanen et al., 1992; Weissman et al., 1996). Alternatively, intact GroEL could be dissected in a manner similar to that of the gap junctions (Hoh et al., 1991). With unfixed GroEL specimens, such an approach proved to be successful. In Fig. 3a, a closely packed specimen was imaged; the height of the GroEL was 14.8 ± 1.3 nm (measured from the edge of occasional

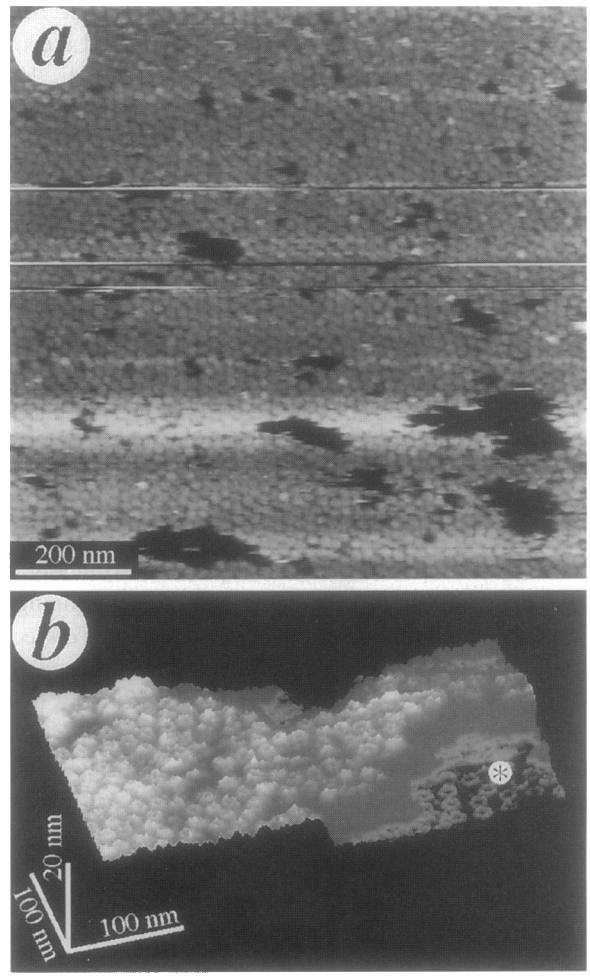

FIGURE 3 Removing of the upper heptamer of GroEL by the AFM tip. (*a*) A nearly perfect closely packed specimen shows a flat surface. With these specimens, individual GroEL molecules can be resolved at smaller scan sizes, without fixation. (*b*) With the specimen shown in (*a*), repeated scanning with an increased probe force can remove the upper heptamer, although the lower heptamer remained bound to the mica surface. These regions appeared as a surface defect. A surface plot is shown here and the height difference is 7.5 nm, precisely the same as a single heptamer ring based on x-ray diffraction (Braig et al., 1994). The height of the intact GroEL measured from these specimens is 14.8 nm. The structural difference between the apical domain (high surface) and the equatorial domain (low surface, denoted by *) can be seen (see Fig. 4 for more details). When the specimen was fixed with glutaraldehyde, such dissection was difficult to perform, and the resulting specimen was normally "dirty."

packing defects) and the specimen surface was uniformly flat. After scanning with an increased probe force (≥2 nN), the upper ring was removed in the scanned area, appearing as a surface "defect" (see Fig. 3 *b*). The height profile indicates that the removed layer was about 7.5 ± 0.5 nm thick, which is the height of a single GroEL heptameric ring (Braig et al., 1994; Harris et al., 1994; Schmidt et al., 1994a; Langer et al., 1992; Chen et al., 1994; Saibil et al., 1993). The exposed surface, therefore, is the contact interface between the equatorial domains in the two heptamers. With glutaraldehyde-fixed samples, such an operation became more difficult, and the exposed surface was found with a great deal of adsorbed debris.

At a higher resolution, the exposed equatorial domain surface displayed a very different structure than that of the apical domain surface, as shown in Fig. 4 *a*, but the sevenfold symmetry is also clearly resolved at a resolution of 1–2 nm. A comparison with that of the apical domains is quite interesting (Fig. 4 *b*). With the apical domains, a circular opening is seen, with seven elongated domains radiated outward. With the equatorial domains, the outer profile is somewhat circular, but the sevenfold symmetry is more profound within the opening. It is particularly interesting to see the details of each subunit at the rim of the opening. These observations are in agreement with those from the x-ray model (Braig et al., 1994), including all dimensions. Preliminary examination of the data indicates that an additional depression at the center of the opening of the equatorial domains could not be consistently resolved (see *inset* of Fig. 4 *a* for a sectional plot), suggesting that there may not be a channel at the center, consistent with the results of neutron scattering (Triyagarajan et al., 1996). However, additional experiments and detailed data analysis are required to confirm this conclusion. These structural details of the equatorial domain could not be resolved by EM with negative staining.

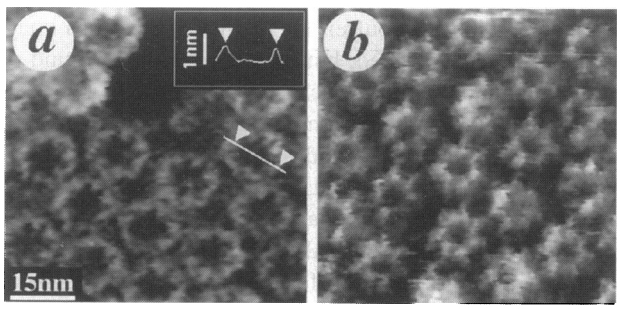

FIGURE 4 High resolution structure of the contact interface between the GroEL heptamers. (*a*) High resolution can be obtained of the exposed equatorial domain surface without additional treatment of the specimen after dissection. In this image, the sevenfold symmetry is well resolved, and the dimensions and the surface morphology are consistent with that from x-ray diffraction (Braig et al., 1994). The resolution achieved in these images is about 1–2 nm. The same tip used for dissection was also used to acquire these images. A central depression was not consistently resolved, suggesting that the two chambers of the GroEL was not connected. *Inset*: Cross-sectional profile along the line indicated; arrowheads are the corresponding points. Notice the flat bottom inside the rim of the equatorial domains. (*b*) As a comparison, the surface structure of the apical domains is shown at the same scale. The structural differences are apparent.

Structure of GroES in solution

Although GroES is a much smaller heptamer with only 10 kDa for each subunit (Chandrasekhar et al., 1986), AFM imaging in solution was also successful. In Fig. 5 *a*, an AFM image is shown with a sample prepared with direct adsorption on mica. It is seen that with such nearly closely packed specimens, individual GroES heptamers were well resolved with a diameter of ~8 nm, although the sevenfold symmetry could not be identified. There is a hint of a protrusion at the center of each GroES heptamer. The structure of GroES revealed by AFM appeared rather different from that of negatively stained EM of two-dimensional crystals after image averaging, where a ring-like structure was resolved (Harris et al., 1994). However, the failure to detect each individual subunit in EM with two-dimensional crystals

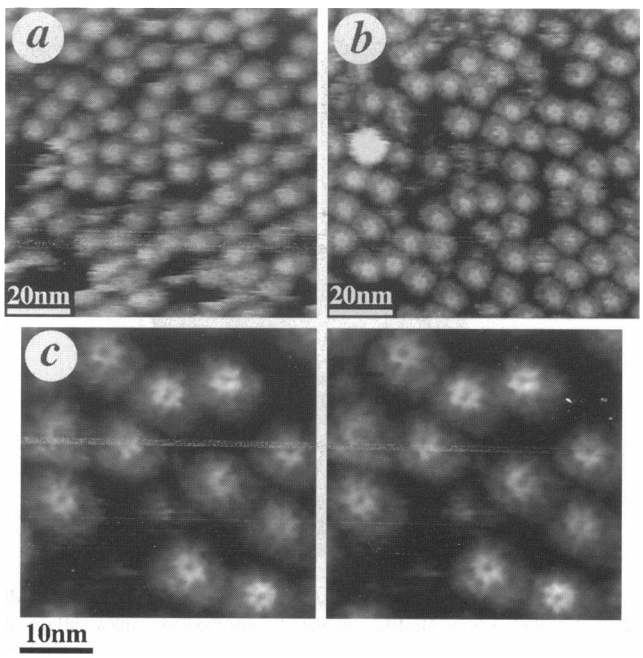

FIGURE 5 AFM images of GroES in aqueous solutions. (*a*) An image of GroES in deionized water without fixation. The diameter of GroES is 8.4 nm, in good agreement with the result from electron microscopy. The central channel is not resolved, but a protrusion at the center can be seen. This morphology is quite different from that of EM of negatively stained two-dimensional crystals (Harris et al., 1994). (*b*) An image of GroES in deionized water after 2% glutaraldehyde fixation. It is seen that the subunits of the heptamer are now clearly resolved without image processing, representing a higher resolution than that achieved with EM after image averaging. The outer diameter of the GroES is the same as that without fixation, and the central channel has a diameter of 1.1 nm. It is also interesting to note that the seven fold symmetry is much more profound near the channel opening. (*c*) A stereogram of GroES, prepared with the method of Shao and Somlyo (1995). In this stereo image, the overall shape and the central protrusion are clearly presented. The structure obtained by the AFM, including both vertical and lateral dimensions, is nearly identical to that of x-ray diffraction (Braig et al., 1994).

may indicate that only hexagonal packing was achieved in these so-called crystals, but the angular orientation was rather disordered. When glutaraldehyde fixation was applied to preadsorbed GroES, a significant improvement in AFM resolution was observed. A typical AFM image is shown in Fig. 5 *b*. In this image, the sevenfold symmetry was well resolved with an excellent contrast (Mou et al., 1996). The structure around the central channel demonstrated a lateral resolution of 1 nm or more. This is so far the highest resolution achieved by AFM of a soluble protein. The channel appeared to have a diameter of 1.1 nm, which is much smaller than that of GroEL. This is one of the clearest examples where the AFM achieved a spatial resolution that is higher than that of EM without crystallization. One may also notice that there is a 4- to 5-nm wide protrusion (~0.8 nm high) near the central pore in the GroES heptamer (see Fig. 6c for a stereogram). The height of fixed GroES is about 3 nm, as measured from the edge of closely packed patches. Both the dimensions and the surface features are found nearly identical to that of x-ray diffraction (Hunt et al., 1996; Mande et al., 1996), suggesting that the AFM is fully capable of high resolution structural studies. Because these adsorbed GroES failed to bind GroEL in the presence of AMP-PNP and Mg^{2+}, the GroEL binding sites were proposed to be on the other side (Landry et al., 1993; Mou et al., 1996). The fact that the central protrusion in GroES could be detected with AFM even without fixation indicates that the roof region is rather stable.

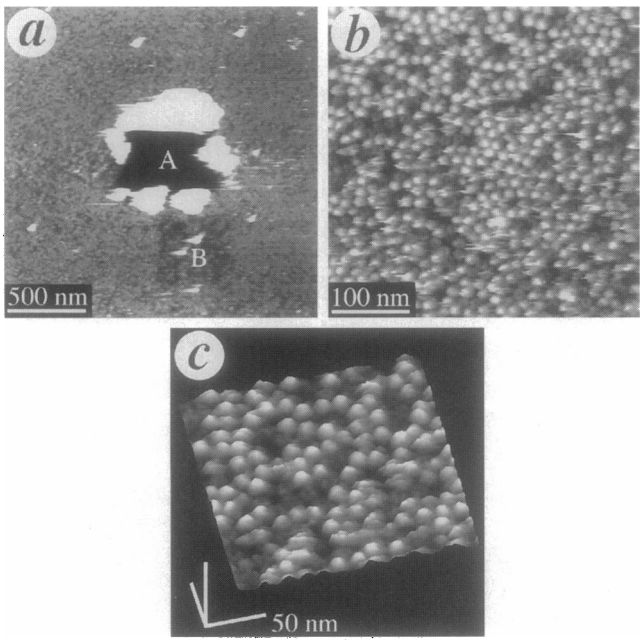

FIGURE 6 AFM images of GroEL/ES complexes in the presence of AMP-PNP and Mg^{2+}. After GroEL was already adsorbed to a mica surface, the specimen was fixed after incubation with GroES. (*a*) With excessive GroES in solution, the binding to GroEL is nearly 100%. The intermolecular distance is the same as with GroEL alone, but the height of the complex, as measured from region A where the adsorbed molecules were removed by the AFM tip with large force scanning, is about 20 nm, about 5 nm higher than that of GroEL. Without fixation, GroES could be easily displaced. But even with fixation, repeated scanning can still remove the bound GroES, as seen in region B. (*b*) At a higher resolution, individual GroEL/ES complexes were resolved, and the central protrusion of the bound GroES could be discerned directly, although further details of the GroES were not observed, perhaps due to the reduced stability of the complex. The few GroEL without bound GroES could also be seen with a central pore in these images. One should notice that the center to center distance of the complexes was essentially the same as GroEL alone. (*c*) The height difference, with or without the bound GroES, is clearly shown in the surface plot. The measured height difference is ~5 nm, consistent with the results from (*a*). Because GroES alone is only 3 nm high, the apical domains of GroEL must move upward by about 2 nm. These results demonstrate that large conformational changes can be detected with the AFM directly.

Complexes of GroEL and GroES: conformational changes

The AFM was also successful in imaging GroEL and GroES complexes in solution. We first allowed the GroEL to be adsorbed to a freshly cleaved mica surface, so that the majority of the molecules could adopt an end-up orientation (see Fig. 2). After the excess GroEL was removed, the sample was examined by the AFM to make sure that the GroEL coverage was adequate and uniform, before an excess amount of GroES in 3 mM AMP-PNP and 50 mM Mg^{2+} was added to the solution covering the sample surface. After a short incubation, most GroEL should be GroES bound based on our tests with EM and other published results (Harris et al., 1994; Engel et al., 1995; Schmidt et al., 1994a; Langer et al., 1992; Chen et al., 1994; Saibil et al., 1993). However, when the excess GroES was removed, it was difficult to obtain clean and high resolution images in the buffer containing AMP-PNP with the AFM. Although individual molecules were discernible from time to time, the poor reproducibility and low resolution prevented any conclusion from being drawn. When the imaging buffer was changed to a low-salt buffer, the quality of the AFM images was somewhat improved, but most GroES were found dissociated from the GroEL, because a clear pore in a molecule with a diameter of 13–14 nm could be seen on most of the molecules and the height and morphology were essentially the same as that of GroEL. This observation was also consistent with our preliminary EM studies, where dilution of the GroEL/ES in the AMP-PNP/Mg^{2+} buffer into a low-salt, nucleotide-free buffer resulted in GroES dissociation from GroEL. Additives, such as PEG and trehalose, did not appear to be sufficient to stabilize the complex structure.

However, when 2% glutaraldehyde was used to fix the GroEL/ES complexes formed on a mica surface in 3 mM AMP-PNP and 50 mM Mg^{2+}, the stability of these complexes was significantly improved. An example is shown in Fig. 6 *a*, where the image was obtained in deionized water after fixation. In this image, the adsorbed GroEL/ES complex was scraped away by the AFM tip via large force scanning (Fig. 6 center, denoted by *A*). The height of the complex was determined from such "defects" to be 19–20 nm. Even with such fixation, prolonged scanning at sub-nN

forces can still remove the bound GroES (see the region, denoted by *B*). At a higher resolution (see Fig. 6 *b*), the coverage was nearly 100%. The central protrusion of the bound GroES could be discerned in this image, and the few GroEL without the bound GroES could also be recognized with a central pore. The height difference, with and without GroES, is clearly demonstrated in the surface plot (Fig. 6 *c*), and was measured to be ~5 nm. Because GroES is only 3 nm high, the additional 2 nm height measured from these images indicates that the apical domains must move upward, as suggested by EM observations (Chen et al., 1994; Saibil et al., 1993; Langer et al., 1992). In fact, if the dimensions from EM micrographs are calibrated with that from x-ray diffraction, the amount of domain movement measured with the AFM is the same as that determined from EM. The resolution of the pore in GroEL also indicates that the tip was in reasonably good condition. It is not clear why details of the bound GroES were not resolved, but one could argue that the rigidity of the complex must be less than that of individual oligomers. The fact that GroES can indeed bind to adsorbed GroEL with similar efficiency also demonstrated that the interaction between the substrate (mica) and the lower GroEL heptameric ring did not interfere with the interaction between GroEL and GroES significantly. It is also interesting to compare Figs. 6 *b* and *c* with that obtained by EM (see Fig. 1) (Engel et al., 1995; Chen et al., 1994). With negatively stained EM, it cannot be distinguished whether GroES was present if GroEL had an end-up orientation because of the difficulty to detect a small mass difference in a projection. Although the mass difference could be detected with scanning transmission electron microscopy of unstained specimens (Thomas et al., 1994), the resolution was normally much lower (Engel et al., 1995). Because AFM is strictly a surface imaging technique, this type of specimen is particularly suited for the AFM. These studies also strongly suggest that by taking advantage of the excellent vertical resolution in the AFM, conformational changes can be detected even if the lateral resolution is not sufficient. The requirement for glutaraldehyde fixation also suggests that the probe force in the contact mode AFM is still larger than desired, so that noncovalent association of macromolecules is not sufficiently strong to sustain the disturbance of the probe. With chemical cross-linking, the interaction between the subunits is certainly strengthened, even if the elastic modulus was only slightly improved. Whether the tapping mode AFM (Hansma et al., 1994) can significantly improve the resolution in this case remains to be demonstrated. The resolution achieved with these delicate molecular complexes indicates that the question of the protein substrate binding, whether it is *cis* or *trans* with regard to GroES (Saibil, 1996), may be resolved with sequential reconstitution of the GroE system.

CONCLUSION

We have shown that AFM can be used to image the chaperonins GroEL and GroES in solution at a high resolution. With both GroEL and GroES, chemical fixation was found effective for improving the molecular stability, resulting in the resolution of the individual subunits without image processing or enhancement. The resolution achieved is already higher than that from negatively stained EM with two-dimensional crystals after image averaging. Chemical fixation was also found necessary for the successful imaging of GroEL/ES complexes with the AFM, because without such fixation, the bound GroES was readily removed by the AFM tip during imaging. These results clearly demonstrate the usefulness of fixatives for structural determination with the AFM at molecular resolution. Although chemical fixation is not suitable for the study of conformational changes in real time, one can overcome this difficulty by fixing the specimen under different conditions (conformation) to understand the changes that have happened. Therefore, it is still feasible to image the different steps in the GroEL/ES reaction cycle with multiple specimens. The successful dissection of GroEL also demonstrates that the interior of a large molecular complex can be accessible to the AFM to obtain useful structural information. The excellent agreement between AFM and x-ray diffraction and EM clearly establishes the validity of the methodology, providing a necessary basis for the study of the mechanism of molecular chaperones. The high resolution achieved also suggests that a detailed comparison of the AFM structures with that from x-ray diffraction can be used to evaluate surface deformation caused by the AFM tip and the extent of structural preservation by chemical fixation. If the results presented in this report are any indication, the AFM will be a competent structural method in biology.

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REFERENCES

Azem, A., M. Kessel, and P. Goloubinoff. 1994. Characterization of a functional GroEL14(GroES7)2 chaperonin hetero-oligomer. *Science.* 265:653–656.

Boisvert, D. C., J. Wang, Z. Otwinowski, A. L. Horwich, and P. B. Sigler. 1996. The 2.4A crystal structure of the bacterial chaperonin GroEL complexed with ATPγS. *Nat. Struct. Biol.* 3:170–177.

Braig, K., Z. Otwinowski, R. Hegde, D. C. Boisvert, A. Joachimiak, A. L. Horwich, and P. B. Sigler. 1994. The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature* 371:578–586.

Chandrasekhar, G. N., K. Tilley, C. Woolford, R. Hendrix, and C. Georgopoulos. 1986. Purification and properties of the GroES morphogenetic protein of Escherichia coli. *J. Biol. Chem.* 261:12414–12419.

Chen, S., A. M. Roseman, A. S. Hunter, S. P. Wood, S. G. Burson, N. A. Ranson, A. R. Clarke, and H. R. Saibil. 1994. Location of a folding protein and shape changes in GroEL-GroES complexes imaged by cryo-electron microscopy. *Nature.* 371:261–264.

Engel, A., M. K. Hayer-Hartl, K. N. Goldie, G. Pfeifer, R. Hegerl, S. Muller, A. C. R. da Silva, W. Baumeister, and F. U. Hartl. 1995. Functional significance of symmetrical versus asymmetrical GroEL-GroES chaperonin complexes. *Science.* 269:832–836.

Fayet, O., T. Ziegelhoffer, and C. Georgopoulos. 1989. The GroES and GroEL heat shock gene products of E. coli are essential for bacterial growth at all temperatures. *J. Bacteriol.* 171:1379–1385.

Gao, Y., J. O. Thomas, R. L. Chow, G. H. Lee, and N. J. Cowan. 1992. A cytoplasmic chaperonin that catalyzes beta actin folding. *Cell.* 69: 1043–1050.

Glauert, A. M. 1975. Fixation, Dehydration and Embedding of Biological Specimens. Elsevier North-Holland, New York.

Gray, T. E., and A. R. Fersht. 1991. Refolding of barnase in the presence of GroE. *FEBS Lett.* 292:254–258.

Hansma, H. G., and J. Hoh. 1994. Biomolecular imaging with the atomic force microscope. *Annu. Rev. Biophys. Biomol. Struct.* 23:115–128.

Hansma, P. K., J. P. Cleveland, M. Radmacher, D. A. Walters, P. E. Hillner, M. Bezanilla, M. Fritz, D. Vie, H. G. Hansma, C. B. Prater, J. Massie, L. Fukunaga, J. Gurley, and V. Elings. 1994. Tapping mode atomic force microscopy in liquids. *Appl. Phys. Lett.* 64:1738–1740.

Harris, J. R., A. Pluckthun, and R. Zahn. 1994. Transmission electron microscopy of GroEL, GroES, and the symmetrical GroEL/ES complex. *J. Struct. Biol.* 112:216–230.

Hendrix, R. 1979. Purification and properties of GroE, a host protein involved in bacteriophage assembly. *J. Mol. Biol.* 129:375–392.

Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix, and R. J. Ellis. 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature.* 333:330–334.

Hendrick, J. P., and F. U. Hartl. 1993. Molecular chaperone function of heat shock proteins. *Annu. Rev. Biochem.* 62:349–384.

Hoh, J., R. Lal, S. A. John, J.-P. Revel, and M. F. Arnsdorf. 1991. Atomic force microscopy and dissection of gap junctions. *Science.* 253: 1405–1408.

Hohn, T., B. Hohn, A. Engel, M. Wurtz, and P. R. Smith. 1979. Isolation and characterization of the host protein GroE involved in bacteriophage lambda assembly. *J. Mol. Biol.* 129:359–373.

Hutchinson, E. G., W. Tichelaar, G. Hofhaus, H. Weiss, and K. Leonard. 1989. Identification and electron microscopic analysis of a chaperonin oligomer from Neurospora crassa mitochodria. *EMBO J.* 8:1485–1490.

Hunt, J. F., A. J. Weaver, S. Landry, L. Gierasch, and J. Deisenhofer. 1996. The crystal structure of the GroES co-chaperonin at 2.8A resolution. *Nature.* 379:37–45.

Ill, C. R., V. M. Keivens, J. E. Hale, K. K. Nakamura, R. A. Jue, S. Cheng, E. D. Melcher, B. Drake, and M. C. Smith. 1993. A COOH- terminated peptide confers regiospecific orientation and facilitates atomic force microscopy of an IgG1. *Biophys. J.* 64:919–924.

Jackson, G., R. A. Stainforth, D. J. Halsall, T. Atkinson, J. J. Holbrook, A. R. Clarke, and S. G. Burson. 1993. Binding and hydrolysis of nucleotides in the chaperonin catalytic cycle: implications for teh mechanism of assisted folding. *Biochemistry.* 32:2554–2563.

Kawata, Y., K. Hongo, K. Nosaka, Y. Furutsu, T. Mizobata, and J. Nagai. 1995. The role of ATP hydrolysis in the function of chaperonin GroEL: dynamic complex formation with GroES. *FEBS Lett.* 369:283–286.

Lal, R. and S. A. John. 1994. Biological applications of atomic force microscopy. *Am. J. Physiol.* 266:C1–23.

Landry, S. J. and L. M. Gierasch. 1994. Polypeptide interactions with molecular chaperones and their relationship to in vivo protein folding. *Annu. Rev. Biophys. Biomol. Struct.* 23:645–669.

Landry, S. J., J. Zeilstra-Ryalls, O. Fayet, C. Georgopolous, and L. M. Gierasch. 1993. Characterization of a functionally important mobile domain of GroES. *Nature.* 364:255–258.

Langer, T., G. Pfeifer, J. Martin, W. Baumeister, and F. U. Hartl. 1992. Chaperonin mediated protein folding: GroES binds to one end of the GroEL cylinder, which accommodates the protein substrate within its central cavity. *EMBO J.* 11:4757–4765.

Llorca, O., S. Marco, J. L. Carrascosa, and J. M. Valpuesta. 1994. The formation of symmetrical GroEL-GroES complexes in the presence of ATP. *FEBS Lett.* 345:181–186.

Lorimer, G. H.. 1994. GroEL structure: a new chapter on assisted folding. *Structure.* 2:1125–1128.

Lorimer, G. H. and M. J. Todd. 1996. GroE structures galore. *Nat. Struct. Biol.* 3:116–121.

Mande, S. C., V. Mehra, B. R. Bloom, and W. G. J. Hol. 1996. Structure of the heat shock protein chaperonin 10 of Mycobacterium leprae. *Science.* 271:203–207.

Mayhew, M., and F. U. Hartl. 1996. Lord of the rings: GroES structure. *Science.* 271:161–162.

McMullin, T. W., and R. L. Hallberg. 1988. A highly evolutionarily conserved mitochondrial protein is structurally related to the protein encoded by the E. coli GroEL gene. *Mol. Cell Biol.* 8:371–380.

Mou, J., J. Yang, and Z. Shao. 1995a. Atomic force microscopy of cholera toxin B oligomer bound to bilayers of biologically relevant lipids. *J. Mol. Biol.* 248:507–512.

Mou, J., D. M. Czajkowsky, Y. Zhang, and Z. Shao. 1995b. High resolution atomic force microscopy of DNA: the pitch of the double helix. *FEBS Lett.* 371:279–282.

Mou, J., D. M. Czajkowsky, S. Sheng, R. Ho, and Z. Shao. 1996. High resolution surface structure of E. coli GroES oligomer by atomic force microscopy. *FEBS Lett.* 381:161–164.

Muller, D. J., F. A. Schabert, G. Buldt, and A. Engel. 1995. Imaging purple membranes in aqueous-solution at subnanometer resolution by atomic force microscopy. *Biophys. J.* 68:1681–1686.

Ohnishi, S., M. Hara, T. Furuno, and H. Sasabe. 1992. Imaging the ordered array of water-soluble protein ferritin with the atomic force microscope. *Biophys. J.* 63:1425–1431.

Pushkin, A., V. Tsuprun, N. Solojeva, V. Shubin, Z. Evstigneeva, and W. Kretovich. 1982. High molecular weight pea leaf protein similar to the GroE protein of E. coli. *Biochim. Biophys. Acta.* 704:379–384.

Radmacher, M., M. Fritz, H. G. Hansma, and P. K. Hansma. 1994. Direct observation of enzyme activity with the atomic force microscope. *Science.* 265:1577–1579.

Saibil, H. R., D. Zheng, A. M. Roseman, A. S. Hunter, G. M. F. Watson, S. Chen, A. auf der Mauer, B. P. O'Hara, S. P. Wood, N. H. Mann, L. K. Barnett, and R. J. Ellis. 1993. ATP induces large quaternary rearrangements in a cage like chaperonin structure. *Curr. Biol.* 3:265–273.

Saibil, H. R.. 1996. The lid that shapes the pot: structure and function of the chaperonin GroES. *Structure.* 4:1–4.

Schabert, F. A., C. Henn, and A. Engel. 1995. Native E. coli Ompf porin surfaces probed by atomic force microscopy. *Science.* 268:92–94.

Schmidt, M., K. Rutkat, R. Rachel., G. Pfeifer, R. Jaenicke, P. Viitanen, G. Lorimer, and J. Buckner. 1994a. Symmetric complexes of GroE chaperonins as part of the functional cycle. *Science.* 265:656–659.

Schmidt, M., J. Buchner, M. J. Todd, G. H. Lorimer, and P. V. Viitanen. 1994b. On the role of groES in the chaperonin assisted folding reaction. *J. Biol. Chem.* 269:10304–10311.

Shao, Z., and A. P. Somlyo. 1995. Stereo representation of atomic force micrographs: optimizing the view. *J. Microsc. (Oxf.)* 180:186–188.

Shao, Z., and J. Yang. 1995. Progress in high resolution atomic force microscopy in biology. *Q. Rev. Biophys.* 28:195–251.

Shao, Z., J. Yang, and A. P. Somlyo. 1995. Biological atomic force microscopy: from microns to nanometers and beyond. *Annu. Rev. Cell Dev. Biol.* 11:241–265.

Shao, Z., J. Mou, D. M. Czajkowsky, J. Yang, and J. Y. Yuan. 1996. Biological atomic force microscopy: what is achieved and what is needed. *Adv. Phys.* 45:1–86.

Sigler, P. B., and A. L. Horwich. 1995. Unliganded GroEL at 2.8 A structure and functional implications. *Philos. Trans. R. Soc. Lond-Biol. Sci.* 348:113–119.

Thiyagarajan, P., S. J. Henderson, and A. Joachimiak. 1996. Solution structure of GroEL and its complex with rhodanese from small angle neutron scattering. *Structure.* 4:79–88.

Tilly, K., H. Murialdo, and C. Georgeopoulos. 1981. Identification of a second E. coli groE gene whose product is necessary for bacteriophage morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 78:1629–1633.

Thomas, D., P. Schultz, A. C. Steven, and J. S. Wall. 1994. Mass analysis of biological macromolecular complexes by STEM. *Biol. Cell.* 80: 181–192.

Todd, M. J., P. V. Viitanen, and G. H. Lorimer. 1993. Hydrolysis of adenosine 5′-triphosphate by E. coli GroEL: effects of GroES and potassium ion. *Biochemistry.* 32:8560–8567.

Todd, M., P. Viitanen, and G. H. Lorimer. 1994. Dynamics of the chaperonin ATPase cycle: implications for facilitated protein folding. *Science*. 265:659–666.

Todd, M. J., O. Boudkin, E. Freire, and G. H. Lorimer. 1995. GroES and the chaperonin assisted protein folding cycle: GroES has no affinity for nucleotides. *FEBS Lett.* 359:123–125.

Trent, J. D., E. Nimmesgern, J. S. Wall, F. U. Hartl, and A. L. Horwich. 1991. A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. *Nature*. 354: 490–493.

Viitanen, P. V., G. H. Lorimer, R. Seetharam, R. S. Gupta, J. Oppenheim, J. O. Thomas, and N. J. Gowan. 1992. Mammalian mitochodrial chaperonin 60 functions as a single toroidal ring. *J. Biol. Chem.* 267:695–698.

Weissman, J. S., H. S. Rye, W. A. Fenton, J. M. Beechem, and A. L. Horwich. 1996. Characterization of the active intermediate of a GroEL-GroES-mediated protein folding reaction. *Cell*. 84:481–490.

Yang, J., L. K. Tamm, T. W. Tillack, and Z. Shao. 1993. New approach for atomic force microscopy of membrane proteins: the imaging of cholera toxin. *J. Mol. Biol.* 229:286–290.

Yang, J., J. Mou, and Z. Shao. 1994a. Structure and stability of pertussis toxin studied by in situ atomic force microscopy. *FEBS Lett.* 338: 89–92.

Yang, J., J. Mou, and Z. Shao. 1994b. Molecular resolution atomic force microscopy of soluble proteins in solution. *Biochim. Biophys. Acta*. 1199:105–114.

Yang, J., J. Mou, J. Y. Yuan, and Z. Shao. 1996. The effect of deformation on the lateral resolution of atomic force microscopy. *J. Microsc. (Oxf.)*. 182:106–113.

Zondlo, J., K. E. Fisher, Z. Lin, K. R. Ducote, and E. Eisenstein. 1995. Monomer-heptamer equilibrium of the E. coli chaperonin GroES. *Biochemistry*. 34:10334–10339.