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Single-Molecule Imaging by Atomic Force Microscopy of the Native Chaperonin Complex of the Thermophilic Archaeon *Sulfolobus solfataricus*

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The chaperonin of the extremely thermophilic archaeon *Sulfolobus solfataricus* has been imaged for the first time under native conditions using the atomic force microscope. This technique allows to visualize the structure of biomolecules in solution under physiological conditions providing a nanometer resolution topographic image of the sample. Single molecule studies can reveal fine structural details, providing a powerful insight into the active conformation of a macromolecule, and also allowing to detect different conformational states corresponding to functional changes. © 2001 Academic Press

Key Words: **atomic force microscopy (AFM); tapping mode; chaperonin; TF55; *Sulfolobus solfataricus*; archaea.**

Chaperonins are ubiquitous oligomeric protein complexes essential for cell viability. They are barrel-shaped objects constituted by identical or different polypeptide subunits depending upon the organism to which they belong. This particular shape is achieved by the combination of two rings of subunits, each surrounding a central cavity, stacked back to back (1). The final toroidal structure is essential for chaperonin function, namely assisting the folding of unfolded and newly synthesized polypeptides (2). Two different families of chaperonins exist, with different amino acid sequences but similar in structure (2, 3). The first family includes the 60-KDa heat-shock proteins of the bacteria and mitochondria (hsp 60), and the chloroplast chaperonins which are not heat-shock proteins. The *Escherichia coli* GroEL is the best known example; it is constituted by 14 identical subunits arranged in two seven subunit rings stacked on one another (4).

The second family, called CCT-TriC family, includes the chaperonins of the archea and eukaryotes. They also form toroidal multimeric complexes, which however contain several different subunits. The chaperonin of the thermophilic archaeon *Sulfolobus solfataricus* (growing optimally at ~80°C) was formerly described as a homomultimeric complex containing 18 copies of a 55-kDa polypeptide, termed TF55, arranged as two stacked nine-subunit rings (5). It was later discovered that the complex contained two different TF55 subunits termed α-subunit and β-subunit (6) with similar molecular weight and related primary structure. Recent genomic sequencing data (7) suggest the existence of a third subunit, gamma, which could explain why *Sulfolobus* chaperonin contains a nine-subunit ring instead of the 8-subunit ring found in other archaea. *Sulfolobus* chaperonin is already very abundant under normal growth conditions and is over expressed upon heat shock, namely exposure to temperatures above 86–88°C (8). In addition to the protein-folding activity, *Sulfolbus* chaperonin has probably other functions; indeed, it was shown to be an RNA-binding protein (9).

Atomic force microscope (AFM) (10) allows the direct study of biomolecules in solution. By AFM, it is possible to reconstruct a nanometer topography of proteins and other complexes, as well as to study the interactions between macromolecules. When operating in tapping mode, the AFM can scan macromolecules without any chemical fixation onto the surface (11), namely under conditions in which they retain their full biological activity. In this operation mode, the cantilever with the tip is oscillated upon the sample at frequencies of some KHz, and the probe touches the latter just at the very end of its oscillation reducing at the mini-

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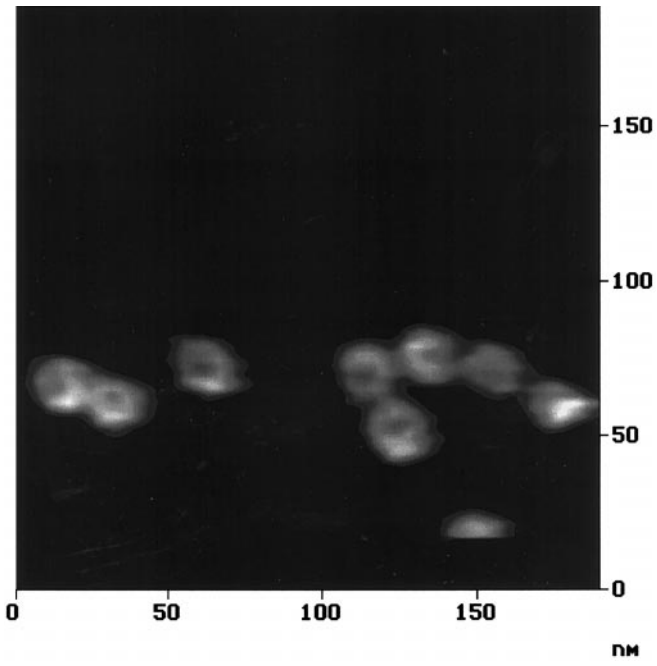

FIG. 1. Tapping mode AFM image in which are shown chaperonins of *Sulfolobus solfataricus* simply adsorbed onto the mica substrate. The toroidal shape is easy to recognize, they are present as isolated molecules not embedded within any two dimensional monolayer what allows a detailed single molecule study.

mum the lateral stimulation (11). When scanned in contact mode, instead, the unfixed molecules can be pushed around by the scanning tip, making it impossible to obtain good images. If compared to other imaging techniques, such as cryo-electron microscopy or X-ray crystallography (12, 13) tapping-mode AFM does not require any particular sample preparation protocol. So, although the resolution achieved is smaller if compared to those techniques or to contact-mode AFM with fixed samples, it is possible to visualize a macromolecule under conditions known to allow full activity in *in vitro* biological assays (14).

Other chaperonins have already been imaged both fixed with glutaraldehyde (15, 16) and in native conditions (14). Most efforts have been focused on *E. coli* GroEL, which has in fact been observed in all possible conditions. Very good images have been obtained with proteins simply adsorbed onto a surface, allowing to distinguish well the toroidal shape and to study its interactions with other proteins (16, 17).

In this work we have performed imaging experiments with Tapping Mode AFM on the chaperonin of *S. solfataricus* without any fixation. We report single-molecule images at good resolution. The images have allowed us to analyze in some detail the structural organization of this chaperonin, and to compare the present data with those obtained by means of other biophysical and biological techniques (12, 18).

MATERIALS AND METHODS

Purification. *S. solfataricus* chaperonin was purified essentially as described by Ruggero *et al.* (9). Briefly, the high-molecular weight material, including ribosomes and chaperonin, was collected by high-speed centrifugation from cell lysates (S-30 extracts). The intact 20S chaperonin complex was further purified by zonal centrifugation on sucrose gradients, selective fractionation with 50% ammonium sulphate and a final gel-filtration step on Sephacryl S-300.

The purified chaperonin was finally collected by ammonium sulphate precipitation, resuspended and stored in Tris/HCl 20mM pH 7 containing 5% glycerol.

Sample preparation. TF55 original solution (10 mg/ml) was initially diluted 100 times to a final concentration of 0.1 mg/ml in the deposition buffer (25mM Tris pH 7.5, 10 mM $MgCl_2$, 10 mM KCl) then 10 μl of it were deposed onto freshly cleaved mica for about 30 min at room temperature. Then, to remove any protein excess, the sample was gently rinsed with the same buffer and was immediately put into the fluid cell for imaging in order to avoid drying.

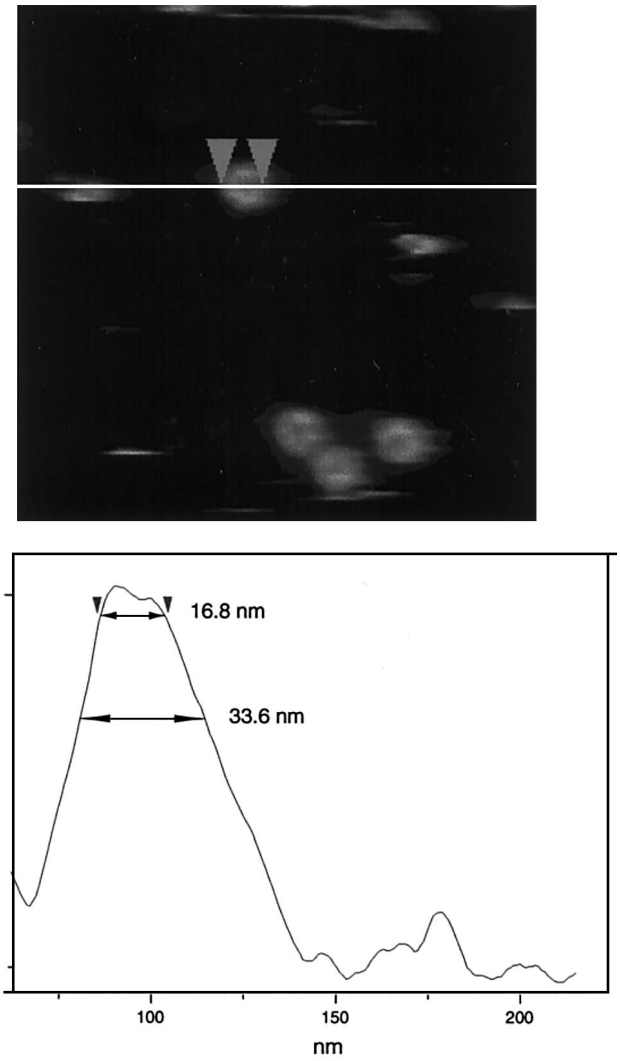

FIG. 2. Section analysis of a chaperonin showing that it is a single isolated protein on the surface. Lateral dimensions measured at the top of the complex are 16.8 nm in agreement with data reported in literature; the measured topography of the complex shows the typical enlargement due to the effect of the tip convolution.

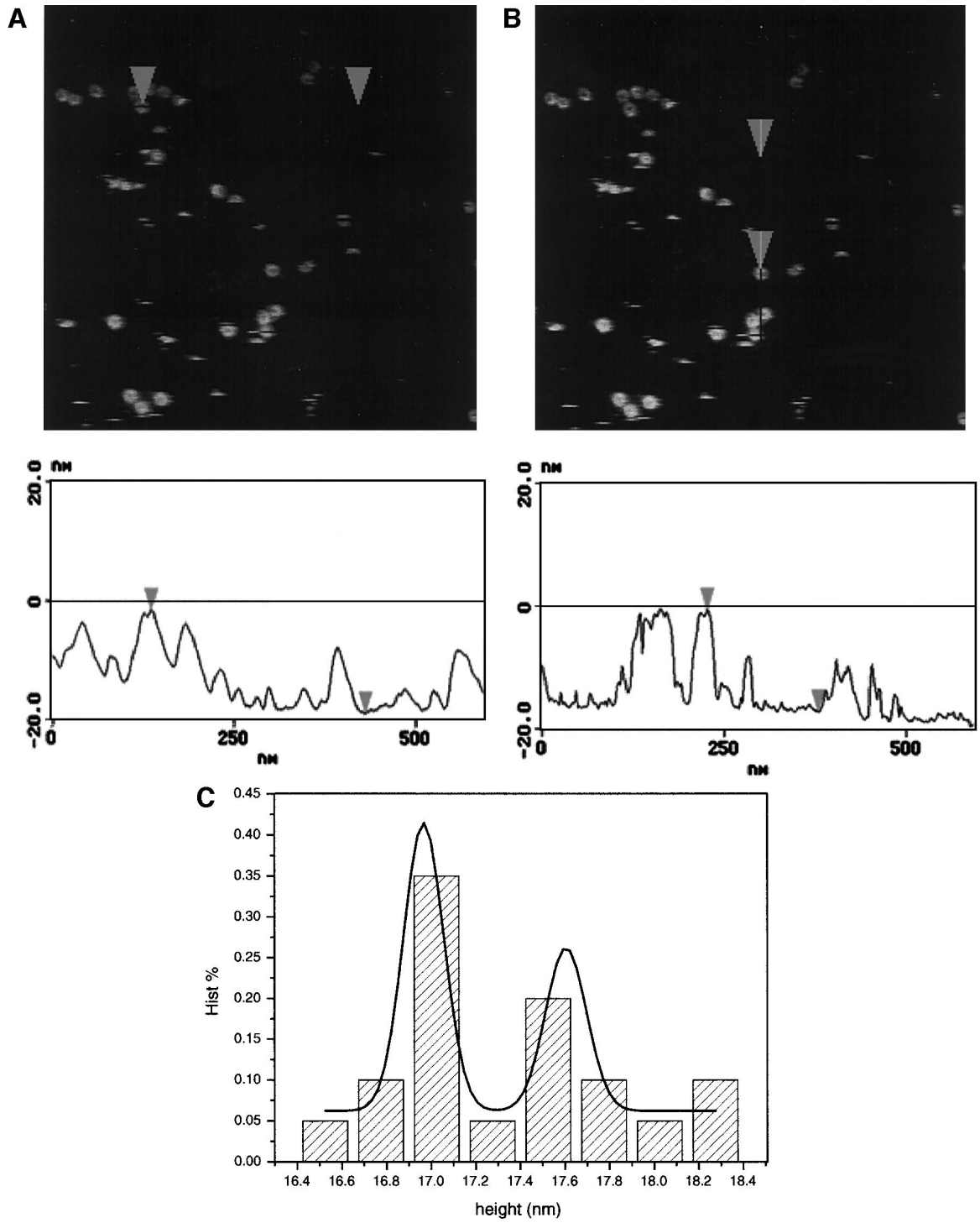

FIG. 3. Height of the chaperonins complexes meaasured by Tapping mode AFM. In a and b, the heigth of two single proteins are measured by the section analysis of the topographic image and they are, respectively, 17.0 ± 0.1 nm and 17.5 ± 0.1 nm. In c, is reported the distribution of the heights measured in one image. The vertical dimension was measured in comparison with exactly the same region of the mica substrate. Two close but separated peaks are present, which can represent the two different conformations of the archeal chaperonin.

Before deposition, mica was fixed on the support by melted wax; once solid, this is hydrophobic and does not allow any water to penetrate between sample and support.

AFM experiment. Images were collected using a nanoscope III (Digital Instrument, Santa Barbara, CA) operating in Tapping mode in liquid. The probes used where commercially available DI Nano-

probes (Digital Instrument, Santa Barbara, CA) and carbon supertips grown by (Nanotools Gmbh, Lenting, Germany) onto the former. The cantilevers were oscillated at the frequency of 9.00 KHz and the set-point was regulated in order to have the minimum possible force exerted on the sample.

The cantilever used was always the short and thin one with a nominal spring constant of 0.32 N/m. The scan rate was kept between 2 and 3 line per second.

Image analysis. Raw data where just flattened; section analysis and bearing analysis were performed with the software of Nanoscope III.

RESULTS

Sulfolobus chaperonin is often present as a single molecule onto the mica surface. Isolated complexes are quite easy to find when scanning the sample. Even if isolated molecules are usually difficult to image because of their high mobility on the substrate, we were able to achieve a good resolution when the proteins were not embedded into a two dimensional layer. The images of Fig. 1 show some individual chaperonin complexes: it is easy to distinguish the characteristic toroidal structure with the central cavity. As shown by section analysis (Fig. 2) the complexes were mainly surrounded by mica. Lateral dimension like internal and external diameter, measured at the top of each molecule, were in good agreement with data from other techniques: 16.8 ± 0.5 nm for the external, 4.5 ± 0.5 nm for the cavity (19). In Fig. 2 it is possible to see these lateral dimensions for one molecule, as well as the enlargement due to tip convolution which appears when diameter is measured below the top of the complex. In fact, it is well known in literature that AFM images are the convolution of the sample surface and the tip shape (20).

The cavity depth is also influenced by the shape of the tip, since a larger probe will penetrate less within the hole.

The height of the toroid has been measured for each molecule compared to the near surface. This is a very important point of the work, because, usually, molecules are present on the surface as more or less closely packed arrays (unpublished results); in these conditions the height is difficult to measure because the tip cannot reach the substrate surface. However, it is still possible to measure the height of the proteins located on the outside of the array or to estimate it by indirect comparisons.

Figure 3 shows the height measured for two isolated chaperonins together with the distribution of the heights measured in the same full image. The mean value of the distribution is 17.3 ± 0.1 nm. A notable feature, however, is the presence of two close but well resolved peaks, which suggest the existence of two distinct conformations of the chaperonin complex under the conditions employed. Indeed, *Sulfolobus* and other chaperonins are known to adopt two different

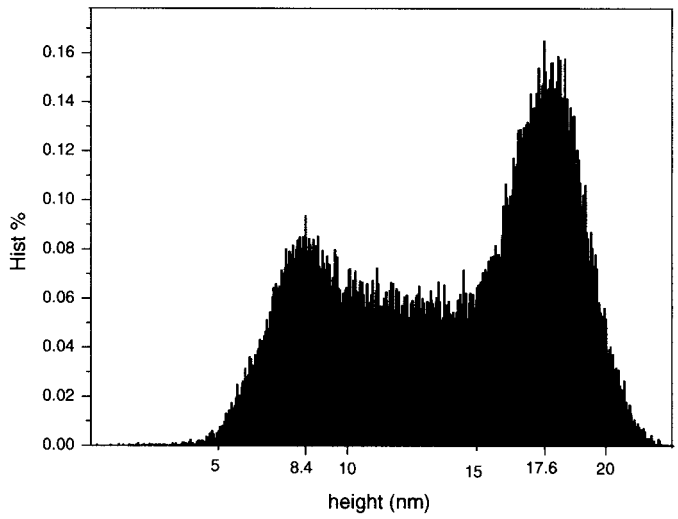

FIG. 4. Bearing analysis of an image where the chaperonins were present in higher concentration, the distribution of heights shows two peaks corresponding to the height of the single ring and of the complete double ring.

conformations, termed "open" and the "closed," revealed biochemically by electrophoresis on nondenaturing gels, whose relative amount depends on the environmental conditions (19). The two different heights we observe for the complex are suggestive of a certain flattening of the toroid associated with the conformational transition, although it is not possible to decide whether the "shorter" barrel corresponds to the open or the closed conformation. The Cryo-EM data of Schoen *et al.* (21) support our observation, reporting the contemporary presence of the conformations at room temperature with difficulties in shifting toward one of them because of the reduced ATPase activity of the protein below 50°C

In some cases a higher density of proteins has been found resulting in a tighter packing of the complexes; in this case a bearing analysis, which makes a statistic on the heights present within an image, shows without uncertainty the contemporary presence of both double-ring and single-ring chaperonin complexes. Therefore two well defined maximums are present in the plot of Fig. 4, corresponding to the heights of 17.5 nm and 8.5 nm of the two different association states. The splitting of the double ring chaperonin into two single-ring complexes has also been documented under certain physiological conditions (19).

DISCUSSION

In this work we have imaged for the first time the chaperonin of the thermophilic archaeon *Sulfolobus solfataricus* under native conditions by atomic force microscope. The structure of single chaperonin complexes has been observed at good resolution without

employing any preparation techniques that could alter the structure or the function of the protein. The dimensions of *Sulfolobus* chaperonin in solution thus measured were in perfect agreement with those obtained with other techniques. Importantly, by performing height measurements we could also obtain evidence for the presence of two distinct structures of the complex, which probably correspond to the "open" and "closed" conformations proposed to exist on the basis of biochemical experiments. Finally, our data lend support to the surmise, also advanced on the basis of biochemical studies, that *Sulfolobus* chaperonin in solution exists as both single-ring and double-ring complexes. Further studies with native AFM will allow us to explore the conditions promoting conformational changes and determining the prevalence of a given conformation, as well as to investigate the interaction of *Sulfolobus* chaperonin with other proteins and/or RNA molecules.

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