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AFM structural study of the molecular chaperone GroEL and its two-dimensional crystals: an ideal "living" calibration sample

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Abstract

Supramolecular complexes, such as chaperonins, are suitable samples for atomic force microscope structural studies because they have a very well defined shape. High-resolution images can be made using tapping mode in liquid under native conditions. Details about the two-dimensional structures formed onto the surface upon adsorption and of the single protein can be observed. Dissection of the upper ring of the supramolecular complex as a result of the applied lateral force through scanning tip is observed. Finally, the combination of lateral convolution and tip penetration into the cavity of chaperonins offers a direct evaluation of the tip convolution effect on images of macromolecular samples.

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1. Introduction

Atomic force microscopy (AFM) [1] is widely used for studying biological samples, in their native environment, because it achieves nanometer scale resolution [2]. In fact, the microscope is able to scan the sample in a situation as close as possible to an in vitro biological assay, while working in solution with proteins simply adsorbed onto a surface.

However, it is very difficult to extract sub-molecular informations from the AFM topography for globular proteins. One is only able to identify the single globular protein as a more or less defined spherical/ellipsoidal object at the best [4]. On soft biological samples it is not possible to achieve atomic resolution as with solid state flat materials or two-dimensional crystals [3]. An improvement of the resolution can be obtained by fixing the protein with a chemical reagent, such as glutaraldehyde [5,6], but at the expense of rendering the protein non-functional and risking a modification in structure.

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In recent years, there has been a growing interest around molecular chaperones. These very important proteins, involved in the folding of unfolded or newly synthesized polypeptides [7], have been widely studied with biochemical and biophysical techniques [8,9]. In particular, the subclass of chaperones called hsp60, or chaperonin, has been investigated with many imaging techniques [10,11] and it was shown that they are assembled into complexes with a characteristic toroidal shape. The number of monomers in the complex depends upon the organism to which they belong. In the case of GroEL, the chaperonin of *E.coli,* the toroid is constituted of 14 identical subunits [12] arranged in two sevenfold symmetric rings stacked in the form of a barrel-shaped object with a central cavity. The height of the barrel is 14.6 nm, the diameter 14 nm, while the diameter of the internal cavity is about 4.5 nm [9].

This particular shape is necessary for the GroEL activity. GroEL binds and folds, with the help of a cochaperone GroES, unfolded or newly synthesized proteins in order to hasten their folding into the correct native state through an ATP driven cycle. Unfolded proteins are bound within the cavity, which is originally hydrophobic, and which, upon the hydrolysis of ATP, becomes mainly hydrophilic providing a favorable environment for protein folding [13]. Electron microscopy techniques [11], as well as, X-ray crystallography [12,14] have provided very high resolution structures of this chaperonin, but the samples were always in a non-native environment. The atomic force microscope has also provided high resolution images of GroEL. For AFM imaging, the GroEL complex was fixed with gluteraldehyde and bound to the substrate [6,15], because it becomes more rigid and "not" deformable by the AFM tip. With the AFM operating in tapping mode, proteins can be imaged without binding to the substrates or chemical fixation [16,17]. In this mode of operation the cantilever with the tip is oscillated above the sample at frequencies of some KHz, touching the sample just at the very end of its oscillation. The lateral stimulation of the sample is greatly reduced and the proteins are not pushed around by the tip, as in the case of the AFM operating in contact mode. Imaging can be performed with active proteins. However, the resolution is obviously reduced if compared to measurements on hard and flat samples. It has been shown that GroEL can be imaged after simple adsorption onto a surface [16] giving good resolution results and maintaining its activity [18]. As such, GroEL is also a very good sample for both imaging and functional analysis by AFM [6].

A detailed structural study of GroEL is thus possible by the AFM and can lead to a better understanding of its physical properties.

In our study reported here, the mechanical stability and the two-dimensional structures formed on a surface are examined.

2. Methods

2.1. Purification

The GroEL was purified from French press lysates of cells harboring the plasmid pOF39 [19] as described previously [20,21]. The main steps in this procedure were DEAE-Sephacel chromatography followed by gel filtration on a Sephacryl S-300 column and Q-Sepharose chromatography. All column material was purchased from Pharmacia Biotech.

2.2. Sample preparation

GroEL sample was initially diluted from the stock solution to a final concentration of 0.4 μM in the deposition buffer (20 mM Tris pH 7.3, 10 mM $MgCl_2$, 30 mM KCl) then 10 μl of it were deposed onto freshly cleaved mica for about 30 min at room temperature. After the sample was gently rinsed with buffer in order to remove any unbound excess of proteins, it was immediately put into the fluid cell for imaging.

Mica was fixed, before deposition, on the disk support by melted wax which, once solid, forms a hydrophobic seal and does not allow water to penetrate between the sample and the support.

2.3. Imaging

Images were collected using a nanoscope III (Digital Instrument, Santa Barbara, CA) operated in tapping mode with a liquid cell. The probes used were commercially available DI nanoprobes and carbon supertips (Nanotools Gmbh, Lenting, Germany) grown onto the former. The cantilevers were driven into oscillation at the frequency of 9.00 kHz and the set point was regulated in order to have the minimum possible force.

The cantilever used was 100 μm long, with a nominal spring constant of 0.32 N/m. The scan rate was kept between 2 and 3 lines/s.

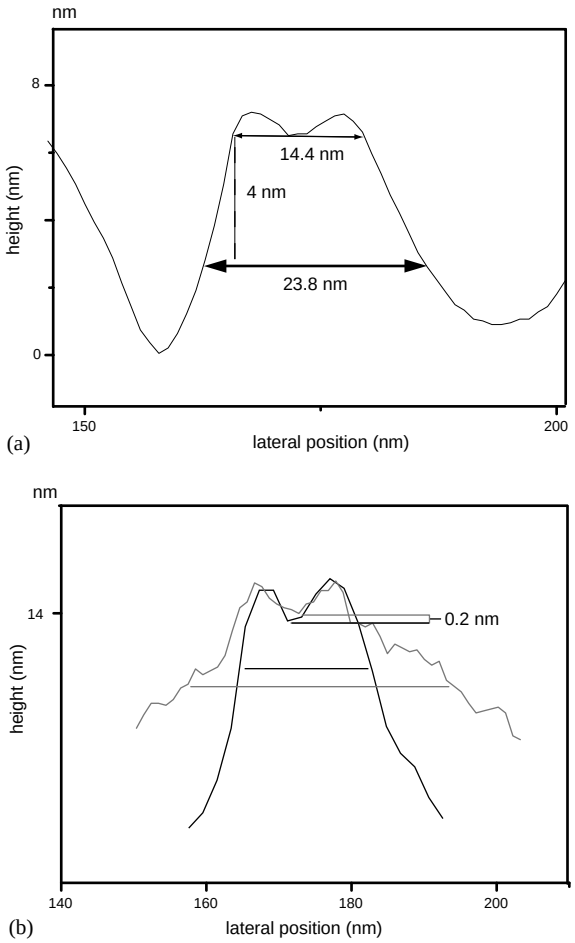

Fig. 1. Section analysis of a single molecule of GroEL. In Fig. 1a, it is possible to see the lateral dimension of the complex and how it changes depending upon where it is measured. At the top of the double ring it is 14.4 nm (where the top has been defined as the points 1 nm below the upper level; the 93% of the total GroEL height) while at 4 nm from the top, it is already increased to 23.8 nm. In Fig. 1b, a comparison between two profiles of GroEL measured with different tips is depicted: it is evident that when the tip radius is larger, a bigger lateral convolution correlates with a shallower penetration into the cavity. The difference of 10 nm in lateral convolution and of 0.2 nm in cavity depth is coherent with the model of a spherical tip probing a hole large 4.5 nm.

2.4. Image analysis

Raw images were just flattened and then section analysis was performed with the Nanoscope IIIa software.

3. Results

Depending on the conditions of deposition, it is possible to find regions on the surface where GroEL is present as a single molecule as well as regions where it is embedded in densely packed two-dimensional layers [22].

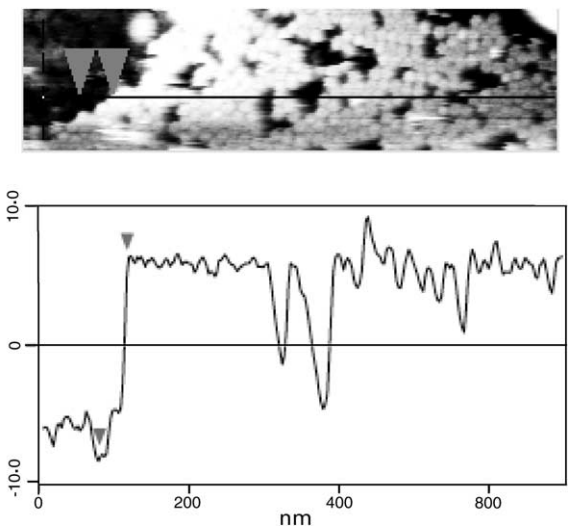

Fig. 2. Section analysis of a GroEL monolayer. From the topographic images it is easy to see that the proteins are packed two dimensionally. From the profile a height of the layer of 14 nm is determined, which agrees well with data reported in the literature.

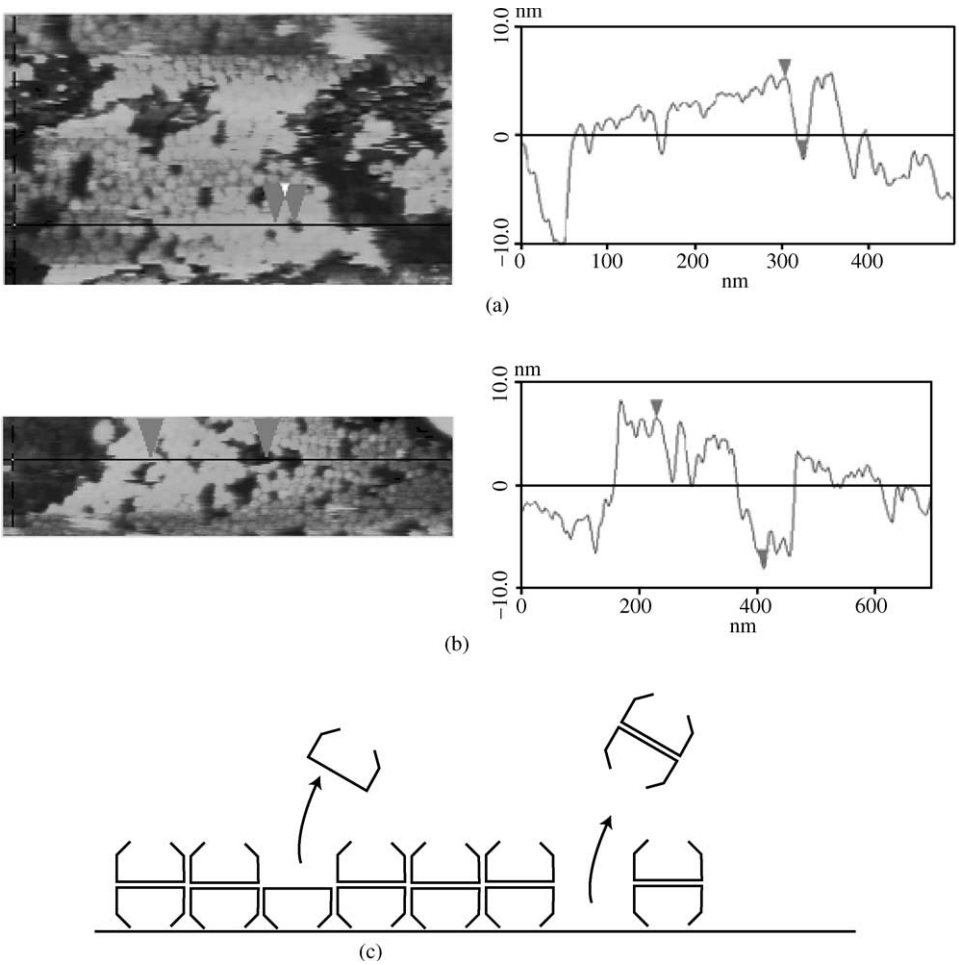

Fig. 3. In the two images, depth measurements of defects within the two-dimensional GroEL array are shown. In Fig. 3a the defect is constituted by a single ring, whose height is exactly half of a complete molecule (the vertical distance between the two cursors is 7.4 nm). In Fig. 3b there are more molecules missing and it is evident that a full double ring is missing within the array (the vertical distance between the two cursors is 14.6 nm). In Fig. 3c a model describing the two previous defects is proposed.

The single molecules are always oriented upward exposing the central cavity. In the images, it is possible to observe the characteristic toroidal shape, which can be sometimes a little bit deformed because of the interaction of the external walls of the molecules with the scanning tip. The external diameter measured at the very top of the molecules is 14.4 ± 0.5 nm (here the top has been chosen to be the points lying 1 nm below the measured highest one), in perfect agreement with crystallographic data [12], but if measured at different heights of the cylinder, the tip convolution effect becomes relevant (Fig. 1).

The internal diameter (cavity) is also in good agreement with other structural data (4.5 nm) if measured at the very top of the cavity (in this case, the cavity depth is affected by the convolution of the tip). The penetration of the probe into the cavity in fact can be larger or smaller if the tip is more or less sharp (Fig. 1). In Fig. 1b, to a 10 nm bigger lateral convolution, there corresponds a 0.2 nm smaller penetration into the cavity, in good

agreement with the model of a spherical tip probing a hole 4.5 nm large. The latter result can also be affected by the interaction of chemically different tips with the inner surface (results in preparation). The height of GroEL is close to the value reported in literature (14 nm) as well: this measurement is not affected by tip convolution.

Within the monolayer, the oligomer appears incorporated into an ordered structure. In this case, the single protein is probed just with the very end of the tip, allowing to image details of the upper side of a single complex but preventing to image between neighboring proteins. It is possible to measure the height of the layer only along its perimeter (Fig. 2). On the best images the disposition of a single GroEL molecule within the array can be determined especially in the presence of some defect. As it can be seen in Fig. 3 one protein within the monolayer is missing creating a hole in the structure. A local hexagonal packing can be deduced looking at the marked arrays in Fig. 4. This elementary two-dimensional structure can be seen also as an isolated complex indicating it as the unit cell of two-dimensional layer formation (Fig. 4b).

A more accurate analysis of the defects created by the tip shows that both a complete double ring (complete complex) and just one single ring (half complex) can be pulled out of the layer [23] by the scanning tip or are already present at the moment of the layer formation. In Fig 3, both situations can be observed: the full molecule is missing in Fig. 3b, while just the upper ring has been pulled out in Fig. 3a. Isolated single rings can be found, as well, on the surface and they are easy to identify via height difference measurements.

The effect of separating the two rings by the scanning probe, was already observed by Mou et al. [15], but in their experiments the sample was strongly fixed with gluteraldehyde and scanned in contact mode, so the lateral force exerted on the proteins was quite high. In our case, the lateral force is very small, the molecules are not fixed and the interaction forces keeping the two rings together are those existing in vivo.

In many experiments, after the very first scan, it was evident that proteins were missing from the previously scanned region. This effect was, at the beginning, attributed to the displacement of whole GroEL molecules onto the surface, but after a more detailed analysis, it was evident that the AFM probe had displaced just the upper part of the molecules. The measurement of the height difference between the areas scanned multiple times and those scanned once leads to this conclusion (Fig. 5).

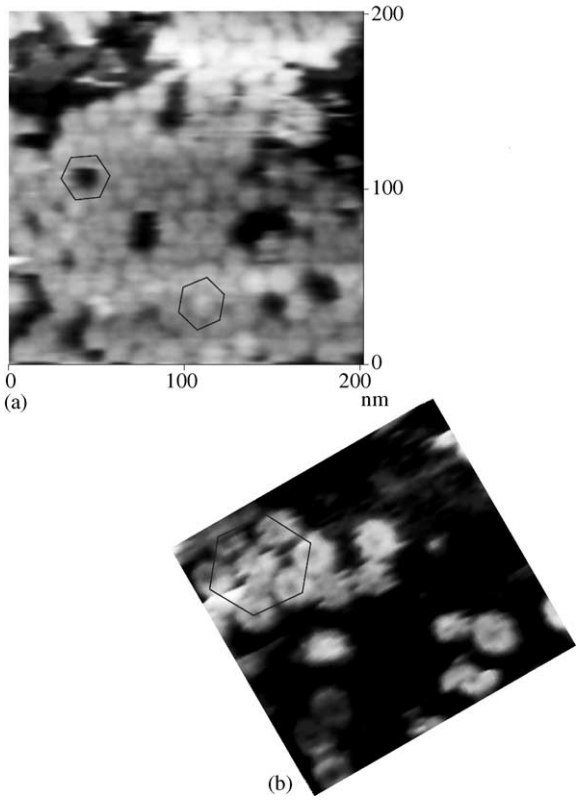

Fig. 4. Packing of the GroEL molecules within a monolayer. In Fig. 4a, a two-dimensional layer is shown where it is possible to identify the hexagonal unit cell typical of dense packing. Two examples of hexagonal packing are indicated: on the top of the image, one where the central protein is missing, on the lower part of the image a complete unit cell. In Fig 4b, one single hexagonal unit cell has been found on the surface isolated from other proteins.

This example shows how important the comparison between crystallographic data and AFM images can be, the latter provides information about the structure of the biomacromolecules in solution which can then be compared with the details of X-ray image leading to a better insight.

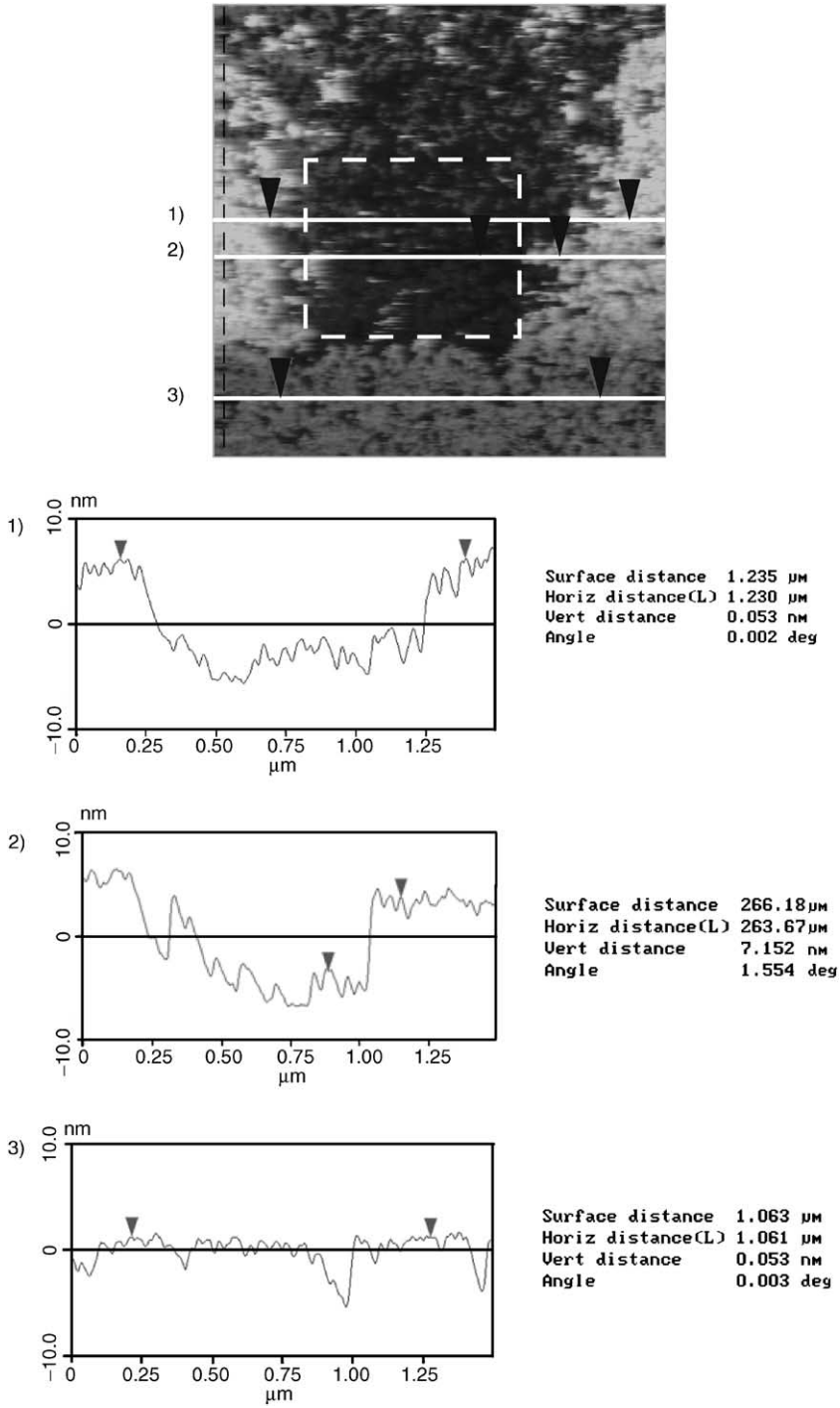

Fig. 5. Effect of scanning with too much force on the GroEL monolayer. Fig. 5a shows a large scale (2 µm) image of the protein monolayer collected after a previous scan of a smaller region within it. The upper line section shows that, on the sides of the scan area, the height of the sample is the same, the middle line section shows a difference of exactly one ring in height between the region scanned once and the one scanned multiple times, and the lower line section shows the homogeneity of the sample scanned only one time.

In this case knowing from crystallography the height of the two rings composing the complex we were able to understand what was displaced by the tip.

4. Conclusion

In this work we have presented a detailed AFM study of the *E. coli* molecular chaperone GroEL. It has been shown that upon simple deposition onto a surface, GroEL forms (locally) quite regular two-dimensional structures with a characteristic hexagonally packed arrangement. Single molecules, as well as, single rings (half molecules) are missing within this two-dimensional locally ordered structure, indicating that they probably have been displaced by the tip.

On a larger scale, the effect of scanning with high force has been demonstrated and indicates that the upper ring of the adsorbed molecules is dissected in the entire scanned surface. An understanding of the best scanning conditions, for a biomacromolecule, thus can be obtained by the evaluation of this effect.

It can be concluded that GroEL is not only a very suitable sample whose structure and activity can be studied by atomic force microscopy, but it offers an ideal "living" calibration sample.

Besides the evaluation of the scanning force used, the analysis of the topography of the single molecule allows one to determine the tip shape through the lateral convolution and the tip penetration into the cavity at a level of few nanometers, a level and a complexity difficult to achieve with the commercial calibration samples.

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