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Imaging the native structure of the chaperone protein GroEL without fixation using atomic force microscopy

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Summary

Most sample preparation methods for scanning probe or electron microscopy require that biomolecules, such as proteins, be fixed. Fixation destroys the molecular functionality and can possibly affect the true molecular structure. Here we report sample preparation conditions that allow the imaging of an unfixed protein, GroEL, under *in-vivo* conditions, by atomic force microscopy. Under these conditions, the protein should maintain its native structure and biological activity. The typical toroidal shape with pore of the GroEL complex was easily visible in the images. Images of a single complex show dimensions that agree well with crystallographic data. Under *in-vivo* conditions, it should be possible to study the biological activity and function of proteins.

Introduction

Up to now, proteins have been imaged by scanning probe microscopy (SPM) and electron microscopy (EM) after a more or less destructive preparation. The aim of these sample preparations is to have a well fixed layer of molecules on the scanned surface. The standard preparation protocols involve chemical fixation of proteins. For SPM, the substrate (glass, mica, or Si) surface is usually chemically activated in order to bind the sample molecule. For these substrates, silanization is often done, followed by the use of other reagents, such as glutaraldehyde, to immobilize proteins on the surface (Janowski *et al.*, 1991).

For imaging of biological molecules by SPM, binding is often needed to enable one to scan them without them moving all around the substrate surface. In fact, the SPM tip can easily push aside unbound molecules, making it impossible to obtain a good image.

Sometimes the proteins are also treated with a fixative in addition to being bound to the surface. The fixative renders them rigid and normally better image results are obtained.

In the case of fixation, the protein is non-functional, but also proteins bound to a surface can have their biological activity strongly diminished. For example, the protein that we study, GroEL, needs to be unfixed to remain functional.

GroEL is a molecular chaperone of *Escherichia coli*, whose role is to participate in the folding of unfolded and newly synthesized polypeptides (Hartl, 1996; Xu *et al.*, 1997). It is a supramolecular complex composed of 14 identical subunits arranged in two sevenfold symmetric rings stacked in the form of a barrel (Braig *et al.*, 1994; Chen *et al.*, 1994; Fenton & Horwich, 1997). It is known (Sigler *et al.*, 1998) that it has two levels of cooperativity. In fact, kinetics studies have revealed positive cooperativity of ATP binding and release within the ring (Gray & Fersht, 1991) and negative cooperativity between the rings (Yifrach & Horovitz, 1994).

Binding the GroEL complex to the mica surface could lead to the inactivation of the protein and make it impossible to study its activity. In this study, we have tried to make images of GroEL without fixing them or binding them on the substrate. Best results are obtained using the atomic force microscope (AFM) in tapping mode (TM-AFM) for unbound molecules. In this mode, the cantilever oscillates, touching the sample only at the end of its downward movement, greatly reducing the contact time between the tip and sample surface compared with contact mode.

Proteins were simply adsorbed onto mica exploiting the van der Waals and electrostatic double layer forces

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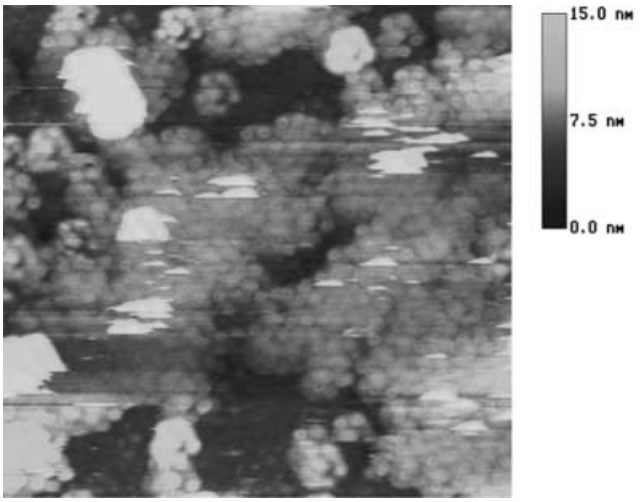

Fig. 1. TM-AFM image of GroEL aggregates adsorbed on mica. Their dimensions are about 10 times larger than a single protein molecule. The scanned area is 650 nm × 650 nm.

(Israelachvili, 1991). The latter depends upon the valence and concentration of solute ions, so it follows that adsorption is strongly influenced by electrolytes present in the solution. Thus, the first task was to find an optimal salt concentration that allowed strong adsorption onto the surface, but also did not affect the protein activity (Müller *et al.*, 1997). GroEL is active in the presence of K^+ and Mg^{2+} and within a pH range of 7.0–7.5. Higher ion concentrations help the adsorption process (Israelachvili, 1991), but can also strongly affect the regular activity of the protein.

Another important factor in the procedure for sample preparation is the concentration of the protein solution deposited onto mica. It can, in fact, have an effect on the quality of the protein layer formed on the surface.

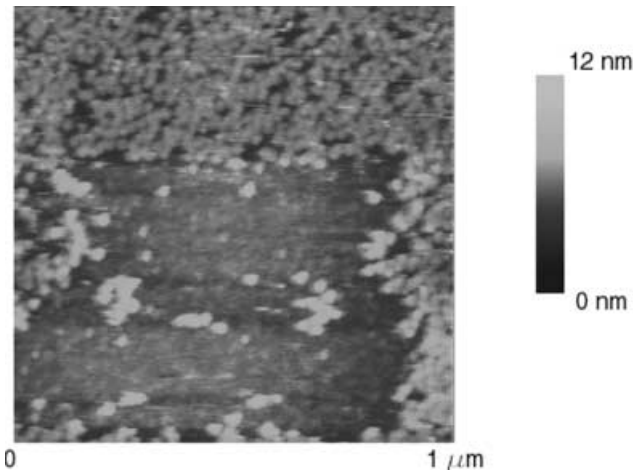

Fig. 2. TM-AFM image where, in the central part, a region has been cleared of protein due to the effect of the scanning tip. The scanned area is 1 μm × 1 μm.

Materials and methods

GroEL was purified from French press lysates of cells harbouring the plasmid pOF39 (Fayet *et al.*, 1989) as described previously (Viitanen *et al.*, 1990; Nieba-Axmann *et al.*, 1997). 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 Amersham Pharmacia Biotech, Piscataway, NJ.

Sample preparation

Stock solutions (20 μM) were diluted into the final buffer solution by 50–100 times in order to obtain a good protein layer strongly adsorbed onto the surface and without aggregates. We found the best imaging buffer to be an aqueous solution of 50 mM KCl, 10 mM $MgCl_2$ and 20 mM Tris/Trizma at pH 7.2. GroEL solutions at a final concentration of 0.04 μM were deposited onto freshly cleaved mica and left at room temperature for about 30 min. Afterwards, the sample was gently rinsed in the imaging buffer and immediately put in the microscope fluid cell (it is very important to avoid drying the sample).

The AFM used was a DI Nanoscope IIIa (Digital Instruments, Santa Barbara, CA, U.S.A.) used in tapping mode. The fluid cell was used without the o-ring. The resonance drive frequency chosen was a peak at 9.05 kHz and the drive amplitude was chosen to maintain a RMS signal of 0.6–0.7 V at the maximum coupling distance between the tip and sample. The set-point after the engagement was manually regulated to ensure good image data with the minimum possible force.

The probes used were the standard silicon nitride (SI_3N_4) DI Nanoprobes with a nominal spring constant of 0.32 N m^{-1} and a length of 100 μm. They gave the lowest noise level in the image data.

Results

Depending on the concentration and adsorption conditions, good samples are not always obtained. The principal problem faced is the presence of aggregates. In fact, due to the wrong ionic conditions or too high a protein concentration, it is possible to find a large quantity of aggregates. These aggregates usually have dimensions that exceed that of the single molecule (as can be seen in Fig. 1) by 10–20 times and their formation can be induced during scanning by the tip (see Fig. 1). To avoid aggregation, it is necessary to scan with a very low force, as well as to work with the lowest possible protein concentration. On the other hand,

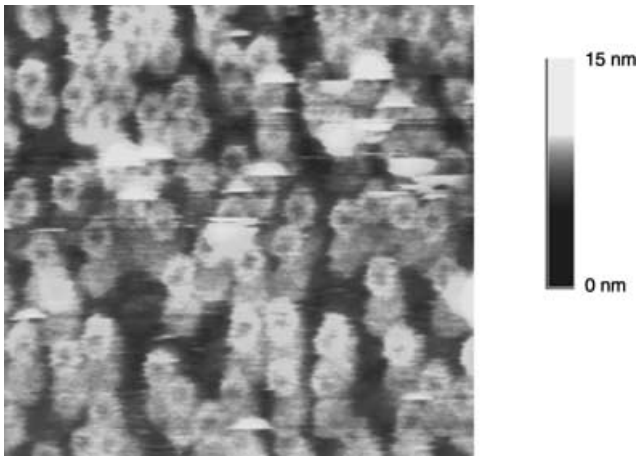

Fig. 3. TM-AFM image showing a film of proteins. It is possible to see well the typical toroidal structure of GroEL with the internal cavity where protein folding takes place. The scanned area is 225 nm × 225 nm.

the latter cannot be lower than a certain limit, because the surface coverage will be too sparse and it is then too difficult to find a protein because the tip can push them away.

When a good film with a low presence of aggregates is obtained, it is in any case important to pay attention to the force exerted by the tip on the proteins. In fact, if the adhesion of the molecules on the mica surface is not strong enough, large regions of the sample surface can be wiped clean of proteins by the scanning tip. This effect can be easily seen, when after the first scan of a surface region showing the presence of GroEL, immediately in the second scan they cannot be seen. After enlarging the scan size, it becomes evident that the tip has pushed away the GroEL molecules within a square equal in dimensions to the preceding scan size.

In Fig. 2, it is possible to see this effect of the proteins being pushed aside by the scanning tip. This effect was already described by Mou *et al*. (1996) concerning contact mode images of GroEL. They found it necessary to use completely fixed complex and poly ethylene glycol within the buffer to obtain a layer compact and rigid enough not to be displaced by the tip.

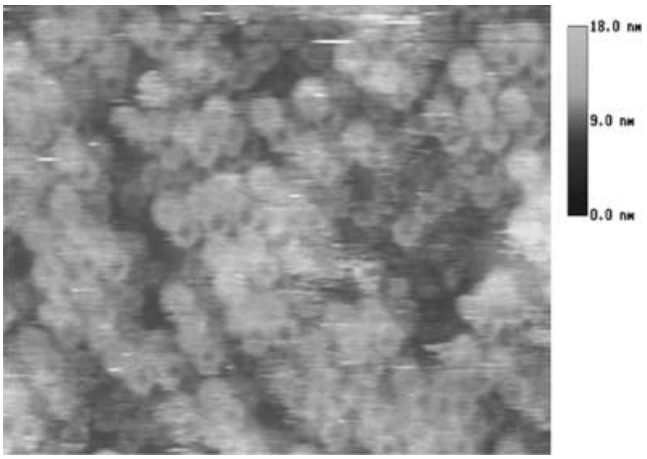

Fig. 4. TM-AFM image showing a multilayer film of GroEL molecules, all in an upright position. The scanned area is 300 nm × 300 nm.

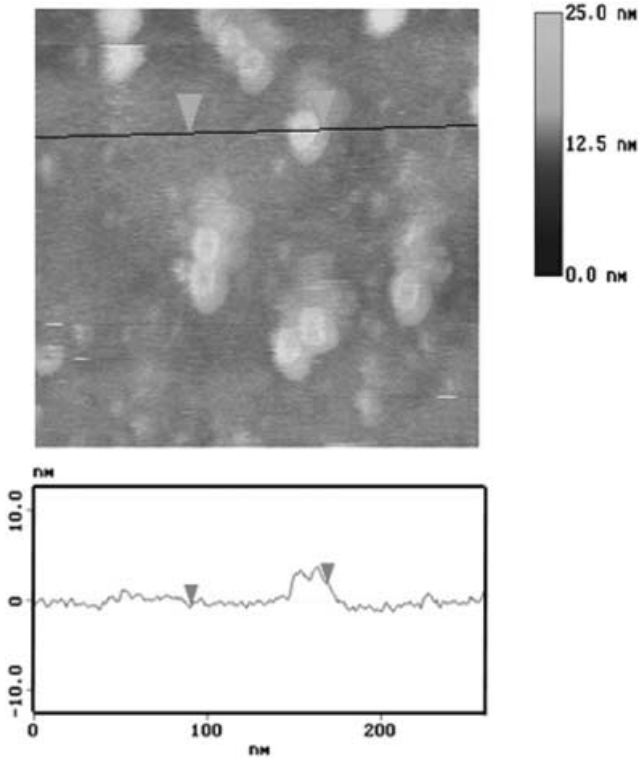

Fig. 5. TM-AFM image of single GroEL molecules directly adsorbed onto mica (top). Section analysis of an isolated complex (bottom). The scanned area is 250 nm × 250 nm.

For Mou *et al*. that effect was unavoidable because of the contact mode of scanning. We managed to change the ionic concentrations, especially of K^+, to obtain a stronger adsorption on the surface; after several attempts we achieved images that were stable after many scans. In Fig. 3, we show the results obtained with the conditions described in the sample preparation section. In the scanned area, proteins cover the surface nearly uniformly and, after going to higher magnifications, it is possible to see the structure of GroEL, a typical toroid with a central cavity where the protein folding takes place. It is striking that all 14-mers are adsorbed upright (pore visible).

A real monolayer film of GroEL has been found, albeit with some difficulties. In fact, despite the very good quality of images, in most experiments multilayer proteins films were found (see Fig. 4). When GroEL on mica was imaged, sometimes we found, unexpectedly, images of single molecules. This is the case for the image seen in Fig. 5 representing one complex on the surface and its cross-section. Lateral dimensions of the ring (15.3 ± 0.7 nm,

determined from the data for 40 GroEL molecules), measured at the top of each molecule, are very close to crystallographic measurements (Braig *et al.*, 1994), 14 nm, indicating that tip convolution is small when compared to results reported earlier concerning GroEL (Vinckier *et al.*, 1998) and other samples, such as colloidal particles (DeRose & Revel, 1997, 1999).

Conclusions

In this work, we have shown that it is possible to image GroEL without fixation under conditions which resemble those *in-vivo*. These conditions should allow the complex to remain active during imaging. Using tapping mode AFM, we can explore the dynamics of this molecular chaperone's function and activity. In fact, AFM can be used as a powerful tool for studying in detail both the structural conformations and functional activity of biomolecules.

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