Atomic Force Microscopy for Investigation of Ribosome-inactivating Proteins' Type II Tetramerization

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Abstract. Biology of the toxins violently depends on their carbohydrate-binding centres' organization. Toxin tetramerization can lead to both increasing of lectin-binding centres' number and changes in their structural organization. A number and three-dimensional localization of such centres per one molecule strongly influence on toxins' biological properties. Ricin was used to obtain the AFM images of natural dimeric RIPsII structures as far as ricinus agglutinin was used for achievement of AFM images of natural tetrameric RIPsII forms. It is well-known that viscumin (60 kDa) has a property to form tetrameric structures dependently on ambient conditions and its concentration. Usage of the model dimer-tetramer based on ricin-agglutinin allowed to identify viscumin tetramers in AFM scans and to differ them from dimeric viscumin structures. Quantification analysis produced with the NT-MDT software allowed to estimate the geometrical parameters of ricin, ricinus agglutinin and viscumin molecules.

INTRODUCTION

Ribosome-inactivating proteins type II (RIPsII) bind to carbohydrate residues of glycoproteins and glycolipids present in different clasters of cellular membrane. This property of RIPsII determines their binding to the variety of cell-surface receptors and probably participates in their endocytosis and retrograde transport [1].

Biology of the toxins violently depends on their carbohydrate-binding centres' organization. A number and three-dimensional localization of such centres per one molecule strongly influence on toxins' biological properties [2]. Many RIPsII were earlier shown to exist as dimers and under defined conditions some of them, for example viscumin, were observed to be tetramers. High concentration can be such a condition [3]. Toxin tetramerization can lead to both increasing of lectin-binding centres' number and changes in their structural organization. Such increase promotes changes in valency and avidity of toxin binding to cellular receptors. These changes result in multiple toxin binding to various both cell-surface and intracellular receptors and finally lead to changes in cytotoxic RIPsII action [2].

In spite of many investigations concerning biological properties of RIPsII it is not known if the toxins bind to their receptors in dimeric form or the binding requires

tetramerization of the proteins. Atomic force microscopy (AFM) techniques allow to investigate conditions of RIPsII tetramerization.

MATERIALS AND METHODS

Toxins' solutions were diluted in phosphate-buffered saline (PBS) to concentrations in the range of 0.5-5 μg/ml. A 10-μl drop of toxin solution was deposited onto freshly cleaved mica supports and incubated for 1 min. Then each sample was washed three times in deionized water and dried by N_2 gas.

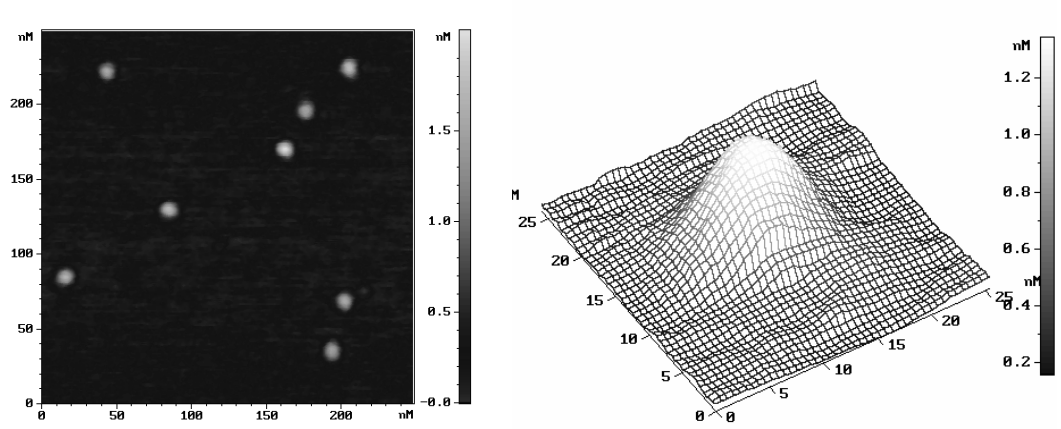
All the experiments were performed in air at room temperature and semicontact AFM mode was used. The advantage of semi-contact mode is decrease of lateral forces between the probe and the sample in comparison to contact mode of AFM. The normal forces can also be reduced by suitable choice of free and set-point amplitude of cantilever oscillations and by selection of force constant. Mentioned above advantages of semi-contact mode using are important when soft biological objects like adsorbed molecules weakly attached to substrate are studied.

Molecules were imaged with a scanning probe microscope Solver P47H (scanning-by-probe system configuration) equipped with attachment for atomic resolution measurements. High resolution "golden" silicon probes (NSG11S, NT-MDT, Russia) with two rectangular cantilevers with typical force constant 5.5 N/m and 11.5 N/m were used to perform the experiments. The amplitude of free cantilever oscillations was in the range of 5-15 nm and the set-point amplitude was in the range of 1-10 % of the free amplitude. These values were defined experimentally for each cantilever from amplitude curves.

RESULTS

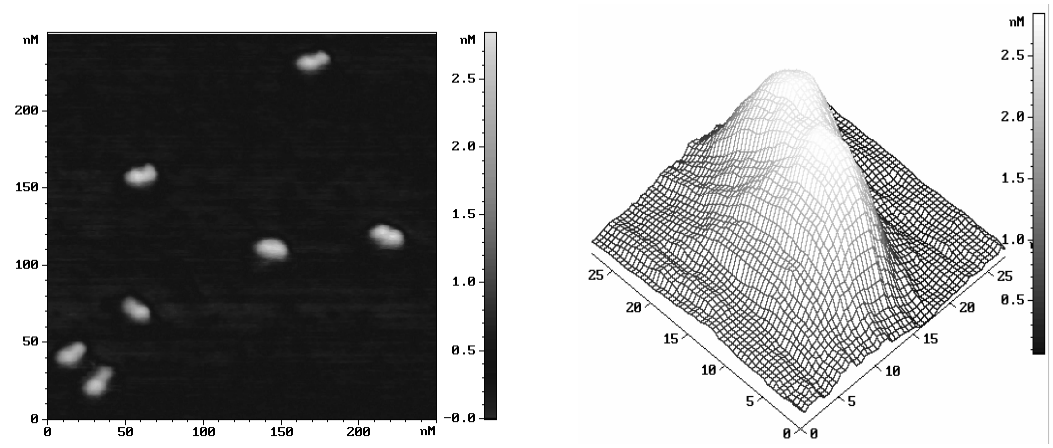
Using AFM techniques that allow to treat the object of investigation gently we obtained high-resolution images of RIPsII molecules sorbed on mica (Fig. 1 - 3).

Ricin was used to obtain the AFM images of natural dimeric RIPsII structures as far as ricinus agglutinin was used for achievement of AFM images of natural tetrameric RIPsII forms. It is well-known that viscumin (60 kDa) has a property to form tetrameric structures dependently on ambient conditions and its concentration. Usage of the model dimer-tetramer based on ricin-agglutinin allowed to identify viscumin tetramers in AFM scans and to differ them from dimeric viscumin structures. Figure 3a shows that the percent of tetrameric viscumin sorbed on mica in low density was no more than 20% from the total amount of structures in the single scan. Increase of concentration lead to the lessening the distance between the molecules sorbed on mica and the quantity of tetrameric viscumin structures raised (Fig. 3 b).



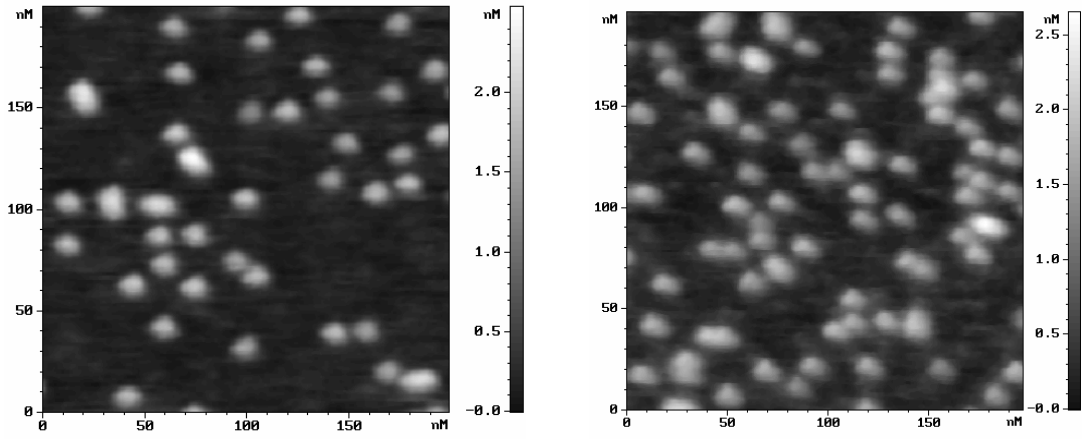
(a) **(b)**

FIGURE 1. (a) AFM image of dimeric ricin. Ricin is a plant ribosome-inactivating protein type II (RIPII). The toxin is present in the seeds of *Ricinus communis*. It consists of two subunits (A and B) joined by disulfide bond. Molecular mass of each subunit is about 30 kDa. Toxically active A-subunit can modify ribosomal RNA and thereby inhibits protein synthesis. Binding B-subunit is responsible for both toxin binding to the cellular membrane and toxin delivery to its intracellular target – ribosome. Toxin binding to the cell surface requires interaction between B-subunit lectin centres and carbohydrate part of cellular receptors. Ricin exists as dimer both in water solutions [4] and being sorbed on solid phase. **(b)** Three-dimensional AFM image of single molecule of ricin. Catalytic and binding subunits are not resolved. Diameter of ricin molecule is 12 nm and height is 1.2 nm.



(a) **(b)**

FIGURE 2. (a) AFM image of tetrameric ricinus agglutinin. Ricinus agglutinin is another RIPII from the seeds of *Ricinus communis*. In contrast to ricin this protein consists of two B- and two A-subunits. Thus, ricinus agglutinin has tetrameric structure formed by covalent interaction between two A-subunits. Tetramerization of ricinus agglutinin depends on disulfide bond between two A-subunits [5]. **(b)** Three-dimensional AFM image of single molecule of ricinus agglutinin. Molecule of agglutitin visualize as two ricin-like globular structures. Lateral dimensions of the agglutinin molecule are 23 and 12 nm; height is 2.5 nm.



(a) **(b)**

FIGURE 3. AFM image of viscumin. Viscumin is a RIPII from *Viscum album* leaves. Viscumin structure and properties are similar to those of ricin. The differences between vicumin and ricin lie in both specificity of their B-subunits to bind to different carbohydrates and ability of viscumin to form tetramer. Viscumin exists both in tetrameric and dimeric form depending on its concentration [3]. Viscumin concentration was **(a)** 1 μg/ml, **(b)** 3 μg/ml.

Quantification analysis allowed to estimate the geometrical parameters of particles. Software for the probe microscopes produced by NT-MDT company includes "***GRAIN ANALYSIS***" tool which is designed for statistical processing of the image of smooth plane containing projecting objects (for example pits of CD/DVD matrixes, microparticles and nanoparticles, biological objects etc.). We used this program to define the geometrical sizes of ricin, ricinus agglutinin and viscumin molecules (Fig. 4).

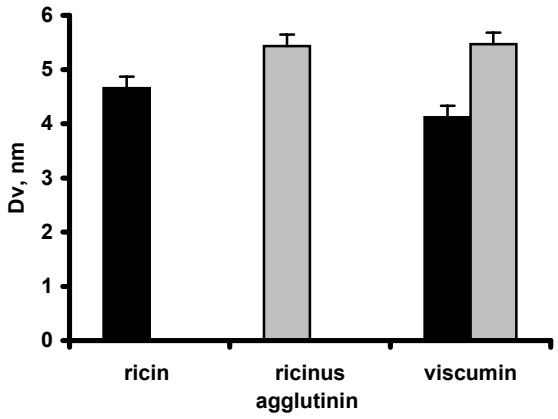


FIGURE 4. Results of quantity estimation of geometrical sizes of ricin and ricinus agglutinin molecules by "Grain analysis". **Dv**, the efficient size of the object above cutting plane level in the XYZ volume $(V^{1/3})$, was taken as estimated value. Calculations based on the number of images which were scanned with step of 10 angstroms and contained 35 molecules of ricin, ricinus agglutinin or viscumin were done.

Tetramerization has the great biological sense. Changes in the number and spatial organization of binding centres are crucial for biological properties of toxins such as binding to cell surface, endocytosis type, interaction with different intracellular receptors. All properties mentioned above result in strengthening or weaking the cytotoxic activity of RIPsII. We earlier showed by the confocal laser scanning microscopy that extra- and intracellular distribution as well as intracellular transport of ricin differed from those of viscumin and ricinus agglutinin [2]. One of the factors determining such differences is toxins' ability to form tetramers.

Employment of AFM techniques in combination with scanning electron microscopy, transmission electron microscopy, confocal laser scanning microscopy and fluorescence life-time imaging as well as immunochemical, biochemical and molecular biology approaches allows us to study intracellular events including such processes as receptor-mediated and fluid-phase internalization of macromolecules, their endocytosis, retrograde and anterograde transport, and translocation of proteins through biological membranes.

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