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Thermal denaturation of fibrinogen visualized by single-molecule atomic force microscopy

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ABSTRACT

Fibrinogen denaturation is an important phenomenon in biology and medicine. It has been previously investigated with bulk methods and characterized by parameters, which refer to big protein ensembles. Here we provide a new insight into fibrinogen denaturation with a high-resolution single-molecule atomic force microscopy (AFM). The ultrastructure of individual fibrinogen molecules was studied after heating or extended contact with the highly oriented pyrolytic graphite surface (HOPG) modified with oligoglycine-hydrocarbon graphite modifier (GM). Fibrinogen heating to 65 °C and 90 °C resulted in the formation of various shapes containing fibrillar and globular structures, which were attributed to the monomers and small aggregates of fibrinogen. Fibrinogen unfolded by the extended (10 min) incubation on GM-HOPG surface in water revealed a different morphology. It contained fibrillar structures only, and their organization reflected the initial native structure of fibrinogen: typically, six polypeptide chains connected by multiple disulfide bonds were seen. A combination of two morphologies – globular aggregates with dense fibrillar networks – has been revealed for thermally denatured protein adsorbed on a GM-HOPG surface with extended (10 min) rinsing with water. The obtained results provide better understanding of fibrinogen unfolding induced by different factors and are important for improvement of biomedical applications, such as fibrinogen-based protein matrixes and carbon-based biomaterials.

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1. Introduction

Many physicochemical factors are known to induce protein unfolding and denaturation, such as addition of urea, different salts, organic acids, alcohols, detergents, change of pH, heating, mechanical stress, interaction with surfaces, etc. [1,2]. The terms "protein unfolding" and "protein denaturation" are very broad; they may refer to different states of a protein, which differ from its "native" states. Moreover, denatured proteins may populate an ensemble of multiple conformations, which are usually structurally not well defined. Information regarding protein unfolding is usually collected from a great variety of bulk methods including different spectrophotometric measurements, dynamic light scattering, nuclear magnetic resonance, X-ray scattering, chromatography, differential scanning calorimetry. These methods provide particular characteristics of a protein ensemble (e.g., molar ellipticity, intensity of a particular band on the spectra, hydrodynamic radius, diffusion coefficient, etc.), which reflect protein structure and change upon protein denaturation. They, however, do not supply with information concerning a denatured state of a single protein molecule in the population. Registration of configurations of individual unfolded molecules allows estimating an incidence of particular structures, e.g., globular/fibrillar ones, that may be characteristic to a particular denaturation pathway. Furthermore conformation of the denatured protein molecule may define its alternative folding pathway [3,4], including amyloid aggregation [5,6], and interaction with other molecules [7–9]. The latter problem is important, in particular, for pharmaceutical and biomaterial applications.

Investigation of mechanical unfolding of a single protein molecule has become very popular since 1990s, when atomic force spectroscopy methods [10,11], optical [12] and magnetic

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[13] tweezers have been developed. These methods allow stretching proteins to controllable length and estimating rupture forces, kinetic parameters of protein unfolding and mechanical stability [14]. At the same time, considerably less attention has been paid to high-resolution visualization of single denatured protein molecules. Such visualization is possible with high-resolution microscopy methods including scanning probe microscopy.

The single amino acid resolution level of denatured in formic acid cytochrome c and bovine serum albumin molecules was achieved with scanning tunneling microscopy under high vacuum: these molecules have demonstrated either unfolded or 2D-refolded state [15]. Different AFM studies have reported on the morphological changes of the adsorbed proteins [16–19] that may reflect surface induced denaturation, which is a well-known effect [2,20]. Recently using AFM operated in ambient environment we have visualized HOPG induced denaturation of several proteins of blood plasma [21]. HOPG modification with an amphiphilic oligoglycine-hydrocarbon graphite modifier (GM) eliminated the apparent denaturation effect of the surface on the protein molecules. The proteins adsorbed on GM-HOPG for a short period of time (1–10 s) retained their dimensions from solution much better than upon adsorption on the other substrates like mica or silica [21,22].

The latter result has provided the possibility for high-resolution AFM investigation of different unfolding states of a protein molecule, which do not interfere with surface induced protein denaturation. Such investigations are capable to illustrate and complement our knowledge on protein denaturation and may contribute to biotechnology applications, e.g., to the development of protein films [7,23].

The aim of this work was high-resolution AFM investigation of different denatured states of a fibrinogen molecule that are induced by heating or incubation in water on GM-HOPG surface. Fibrinogen was chosen as a model protein for the study of heat and GM-HOPG surface denaturation due to both its high biotechnological relevance (it is one of the most abandoned blood plasma proteins and a key protein of the coagulation system) and well defined characteristic shape, which is resolvable by AFM [17,24–30]. Investigation of fibrinogen unfolded states may be also important for understanding of the molecular origins of hereditary fibrinogen amyloidosis [31].

Fibrinogen is a large glycoprotein (Mw = 340 kDa), made up of three pairs of polypeptide chains (Aα, Bβ, γ)$_2$, which are organized in a symmetrical trinodular structure with a central globular region and two lateral globular regions connected by coiled coils [34]. Fibrinogen also has several unstructured regions including 390 residue-long C-terminal parts of Aα chains called αC regions. All six chains are held together by 29 disulfide bonds: 5 in the central nodule, 5 in each of the terminal globular regions, 6 in each of the coiled coils, and one in each of the αC regions [32,33].

Fibrinogen denaturation effects are important for biomedical applications. Fibrinogen heat denaturation can be used to separate fibrinogen from plasma and measure fibrinogen concentration [34,35]. Heat denatured fibrinogen may be useful as a basis for various matrixes to favor hemostasis [36], to culture cells [37] or to resist protein adsorption and platelet adhesion [38–40]. On the other hand, denaturation should be avoided during viral inactivation or sterilization of pure fibrinogen solutions or plasma.

Finally, fibrinogen adsorption and denaturation on a surface can define its biocompatibility [41]. In this regard, investigation of the peculiarities of fibrinogen denaturation upon extended contact with GM-HOPG may also contribute to the biomaterial applications, in particular, to the field of utilization of graphitic materials in biotechnological applications [42].

Mechanical unfolding of single fibrinogen molecules has been studied by atomic force spectroscopy and molecular dynamics simulations suggesting several molecular origins of mechanical fibrinogen unfolding including unfolding of coiled-coiled regions [43] and γ and β nodules [44,45]. Moreover, denaturation (or deformation) of fibrinogen upon adsorption to different surfaces has been studied on a single molecule level with AFM [16,17,26,28,46] and molecular dynamic simulations [47]. However, to the best of our knowledge, fibrinogen denatured states induced by heating or adsorption on modified HOPG surfaces (such as GM-HOPG) have never been investigated.

2. Experimental section

For HOPG modification, 10–20 μl of GM ($(CH_2)_n(NCH_2CO)_m$-NH_2, Nanotuning, Russia) solution in double distilled water at a concentration 0.01 mg/ml was deposited onto freshly cleaved HOPG (ZYB quality, NT-MDT, Russia) surface for 1 min. After that, the remaining droplet was removed by a nitrogen flow.

Freshly unfrozen fibrinogen from human plasma (Calbiochem, Germany) was diluted by 10 mM sodium phosphate buffer (pH 7.2) to a final concentration 2.3 μg/ml (for the study of untreated fibrinogen, fibrinogen heated to 45 °C and surface induced fibrinogen unfolding) or 12.5 and 23 μg/ml (for the study of fibrinogen heated to 65 °C and 90 °C). Fibrinogen stock concentration was determined by absorbance at λ = 280 nm using an extinction coefficient of 1.51 for 1 mg/ml in a 1-cm cuvette (Thermo Scientific NanoDrop Spectrophotometer, USA). For heating, a microtube containing 2 μl of diluted fibrinogen solution was placed for a desired period in the thermostat Gnome (DNA-technology, Russia) at a predefined temperature prior to deposition on the substrate. For protein deposition 0.5 μl of fibrinogen solution (either heated or not) was placed onto GM-HOPG surface for 10 s. After that, 100 μl of water was added on top of the sample and removed either immediately or in 10 min (for the study of surface induced unfolding) by a nitrogen flow. Rinsing of adsorbed proteins (or other samples) with deionized water prior to their AFM imaging is a standard procedure. It allows to remove the adsorbed salts from the surface, which may complicate AFM investigations. In this study, we have used a buffer with low ionic strength, therefore, significant alterations of protein conformation upon salt removal are unlikely.

AFM experiments were made using the multimode atomic force microscope Ntegra Prima (NT-MDT, Russia) equipped by ultrasharp tips (carbon nanowhiskers with a curvature radius of several nanometers grown at tips of commercially available silicon cantilevers with a spring constant of 5–30 N/m) [48] and operated in attraction regime of intermittent contact mode. The typical scan rate was 1 Hz, and a pixel resolution was typically ≈1 pixel/nm. For image processing we have used the following software: 1) FemtoScan Online (Advanced technologies center, Russia) for standard AFM image processing and presentation, as well as for automatic selection of objects and analysis of their projected surface area; 2) SPM Image Magic (http://spm-image-magic.software.informer.com) for height analysis.

Using FemtoScan Online software we have characterized the area of a projection of the selected objects (protein structures) on the surface (further referred to as "projected surface area" or "surface area"), which was calculated using an automatic procedure implemented in FemtoScan Online (Enumerate features). Briefly, edges of individual objects are selected by threshold which is set at the mean level of the background plus 2x RMSD (root mean square deviation of the signal from the mean level); the total area of the pixels within the object is then calculated. This parameter well characterizes the lateral size of adsorbed protein structures and allows to distinguish unfolded monomeric form of the protein from its aggregates. Standard deviation was used as an error value. Each experiment was reproduced at least three times.

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