Biochimica et Biophysica Acta 1828 (2013) 405–411

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

γ-Hemolysin oligomeric structure and effect of its formation on supported lipid bilayers: An AFM Investigation

Andrea Alessandrini [a,b,*,1], Gabriella Viero [c,1], Mauro Dalla Serra [c], Gilles Prévost [d], Paolo Facci [a]

[a] Centro S3, CNR-Istituto di Nanoscienze, Via Campi 213/A, 41125 Modena, Italy
[b] Department of Physics, University of Modena and Reggio Emilia, Via Campi 213/A, 41125 Modena, Italy
[c] CNR-Institute of Biophysics & Bruno Kessler Foundation, Via alla Cascata 56/C, 38123 Trento, Italy
[d] UR EA Physiopathologie et Médecine Translationelle, Institut de Bactériologie de la faculté de médecine, 3 rue Koeberlé, 67000 Strasbourg, France

ARTICLE INFO

Article history:
Received 26 June 2012
Received in revised form 5 September 2012
Accepted 25 September 2012
Available online 1 October 2012

Keywords:
γ-Hemolysin
Atomic force microscopy
Supported lipid bilayers
Membrane curvature

ABSTRACT

γ-Hemolysins are bicomponent β-barrel pore forming toxins produced by *Staphylococcus aureus* as water-soluble monomers, which assemble into oligomeric pores on the surface of lipid bilayers. Here, after investigating the oligomeric structure of γ-hemolysins on supported lipid bilayers (SLBs) by atomic force microscopy (AFM), we studied the effect produced by this toxin on the structure of SLBs. We found that oligomeric structures with different number of monomers can assemble on the lipid bilayer being the octameric form the stablest one. Moreover, in this membrane model we found that γ-hemolysins can form clusters of oligomers inducing a curvature in the lipid bilayer, which could probably enhance the aggressiveness of these toxins at high concentrations.

1. Introduction

Membrane proteins and lipid bilayers mutually interact to accomplish many biologically relevant tasks [1–3]. It is well known that the lipid environment can modulate the functional activity of membrane proteins in several ways: i) through specific interactions between the lipids and the proteins [4]; ii) affecting the mechanical properties of the lipid bilayer, considered as a structurally organized continuum medium, and affecting conformational transitions of membrane proteins [5]; iii) mediating membrane proteins aggregation by elastic interactions [6]. The last proposed mechanism is often related to the 2D spatial organization of membrane proteins. On the other hand, the lateral organization of transmembrane proteins can lead to a re-shaping of the membrane geometry [7]. This can be accomplished by both the formation of protein scaffolds, such as in the case of BAR domains, and the shape and oligomerization of integral membrane proteins [8]. A typical case of protein-induced membrane re-shaping is represented by the chromatophores of photosyntethic purple bacteria [9].

Pore-forming-toxins (PFTs) can be considered insightful model systems to study the interaction between lipid bilayers and membrane-interacting proteins. PFTs are released in the medium as soluble monomers able to reach the surface of a lipid bilayer and to oligomerize in complex structures which span the target lipid bilayer punching holes through the target membrane [10–14]. Among the different lipid model systems that can be exploited to study the assembly and the interaction of PFTs within lipid bilayers, solid supported lipid bilayers (SLBs) provide the great advantage of being prone to be investigated by imaging surface sensitive techniques such as atomic force microscopy (AFM) [15]. With regards to PFTs and membrane/protein interaction, AFM is potentially useful to study the dynamics of the oligomer formation, the subunit composition of the oligomer and the interaction of the oligomer with the membrane. All these investigations can be performed in liquid without any prior fixation or staining procedure, providing a unique physiologic-like environment.

In this work we study by AFM the assembling and the effect on the membrane of γ-hemolysins (γHLs) oligomers exploiting SLBs in nearly physiological conditions as model membranes. γ-Hemolysin (γHL) represents one of the several β-barrel pore-forming toxins (β-PFTs) produced by *Staphylococcus aureus* [10]. At variance with the well-known homoeptameric α-hemolysin (αHL) [16], γHL is a bicomponent structure that requires the assembly of two different polypeptides belonging to the class F and class S component. Whereas the crystal structure of the oligomeric αHL is known since many years [17], only recently a crystal structure of the γHL pore in a membrane-mimicking environment has been obtained [18], providing the first case of a β-PFT for which both the soluble monomers [19–21] and pore structure have been determined by X-ray crystallography. The case of bicomponent toxins involves also the problem of the stoichiometry of the two class components in the pore structure besides the number of subunits composing the pore. The obtained crystal structure points to a pore

* Corresponding author at: Department of Physics, University of Modena and Reggio Emilia, Via Campi 213/A, 41125 Modena, Italy. Tel.: +39 059 2055297; fax: +39 059 2055651.
E-mail address: andrea.alessandrini@unimore.it (A. Alessandrini).
[1] These authors contributed equally to the work.

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http://dx.doi.org/10.1016/j.bbamem.2012.09.027

composed by eight subunits, instead of the seven subunits involved in the αHL structure, with a 4:4 stoichiometry of the class S and class F subunits. In previous investigations different subunit compositions with varying stoichiometry were obtained [22,23]. Interestingly, in the case of αHL the presence of both heptamers [17] and hexamers [24] in different membranes was shown. As a matter of fact, the presence of γHL octameric stable structures does not exclude the possibility of other eventually less stable subunit compositions that may occur in physiological conditions. Nonetheless, functional data clearly show that only oligomers with an equal composition of the F and S component lead to functional species [23,25], leaning towards an even stoichiometry of the pore. In general, the presence of a different number of HlgA or HlgB subunits might depend on the physico-chemical properties of the bilayer or on metastabilities of the complex but, more importantly, might unravel still unknown intermediates or non-functional oligomers along the full pore formation process.

Here we present an AFM investigation of the assembly of the two γHL components on a SLB. The investigation shows the presence of oligomers with different subunit composition. The advantage of our approach is that we do not need a purification step after oligomer formation as for X-ray crystallography and we can obtain information on the native intermediate steps toward the formation of a complete and most stable structure. Moreover, we studied how the γHL oligomer formation process affects the stability of the lipid bilayer with respect to other PFTs such as αHL.

2. Materials and methods

2.1. Preparation of lipid vesicles

Large unilamellar vesicles (LUV) with two lipid compositions, eggPC or eggPC:Chol (1:1 molar ratio), were prepared by extruding multilamellar liposome preparations through polycarbonate filters (carrying 100 nm holes). Liposomes for atomic force microscopy measurements were prepared as a 3 mg/ml lipid suspension in 10 mM Tris/HCl, 20 mM NaCl, 0.1 mM EDTA pH 7.0.

2.2. Atomic force microscopy

Supported bilayers have been prepared on mica by the vesicle fusion method at 25 °C [26]. The lipid composition most prone to permeabilization by γ-hemolysin is PC:Chol (50:50 mol%) [27]. Thus, liposomes of 100 nm diameter comprised of egg-PC:Chol (50:50 mol%) and egg-PC (as control) were used. Briefly, a liposome suspension (0.25 mg/ml) in 20 mM NaCl, 20 mM Hepes, 0.1 mM EDTA, pH 7 was applied on freshly cleaved mica for 5 minutes. The sample was then washed with the incubation buffer to remove excess of unbound liposomes. 2 μl of solutions containing either of the two classes of protein (HlgA, HlgB) were added to the supported bilayers (buffer: 20 mM NaCl, 20 mM Hepes, 0.1 mM EDTA, pH 7 or PBS; protein concentration: 200 nM). The incubation time before gentle washing to remove unbound proteins was around 2 hours at room temperature. After two hours the formation of the pores should be almost at the steady state [25,28] even though intermediates can still be seen [23]. Samples were observed with a Multimode Nanoscope IIIa (Bruker Instruments) microscope in Tapping-Mode (TM-AFM) using Oxide-sharpened Bruker NP-20 tips with a nominal spring constant of 0.24 N/m. Typically, AFM images were obtained at a scanning line speed of 1–3 Hz and the force applied was kept at its smallest possible value enabling stable imaging.

2.3. Image averaging techniques

In order to increase the signal-to-noise ratio, AFM images were processed by XMIPP software using averaging techniques [29]. Single particle images of the pores were extracted manually from the AFM micrographs. The images were then 2D aligned. Before performing an average of the images, the oligomers were analyzed by classification methods in order to identify structurally heterogeneous image sets. The classification method we used is based on Self-Organizing-Maps of the rotational spectra. This method allows identifying different classes according to the rotational symmetry. The images corresponding to each identified class were then rotationally aligned and averaged.

2.4. Aggregation effects of γHL on liposomes by fluorescence resonance energy transfer (FRET)

The ability of γHL or αHL to induce changes to the liposome structure was investigated by the probe mixing assay based on NBD–rhodamine energy transfer. To discriminate between aggregation and fusion effects, we checked the ability of each toxin to cause changes in liposome fluorescence in a probe dilution assay [30,31]. In this case a suspension of double-labeled and unlabeled liposomes were mixed together (100 μM total lipid concentration) and subjected to toxin action, both γHL (up to 100 nM) and αHL (up to 200 nM). Two different protocols were used: 1) in the probe mixing assay, two liposome preparations of eggPC/Chol in the molar ratio 1:1 and containing either NBD-DOPE or rhodamine-DOPE (2% mol) as a donor and acceptor couple for FRET, were prepared as previously described; 2) In the probe dilution assay, a liposome preparation of eggPC/Chol in the molar ratio 1:1 and containing both NBD-DOPE and rhodamine-DOPE (0.6 mol %, each) was mixed with unlabelled liposomes (eggPC/Chol, 1:1). After addition of nanomolar concentrations of toxin to the liposome suspension (0.25 mM final lipid concentration) the fluorescence emission of the donor was monitored at 535 nm for 13 min; the excitation wavelength was set to 460 nm.

3. Results and discussion

The procedure for the assembly of the Supported Lipid Bilayer allows the formation of bilayers completely covering the mica substrate. Small defects may naturally occur, enabling the measure of bilayer thickness (Fig. 1). Firstly we studied the organization features of the complex formed by the addition of the two γ-hemolysin components (HlgB and HlgA) to an already formed SLB composed by eggPC and cholesterol in the 50:50 (mol%) proportion. After incubation and washing step to remove unbound proteins, circular structures appear on the lipid bilayer (Fig. 2a) and at high resolution imaging (Fig. 2b,c), circular structures emerge more evidently. The obtained signal-to-noise ratio for each single structure clearly prevents the possibility of addressing the number of subunits constituting the oligomer. As a consequence we exploited averaging techniques to increase the signal-to-noise ratio.

Due to the circular shape of the structure of interest, the alignment should include both a 2D lateral alignment according to the center of mass and a rotational alignment according to the presence of an *n*-fold symmetry. As a first check for the presence of oligomeric structures belonging to different classes, we measured the distribution of the observed diameters. The pore diameter measured by AFM is assumed to correspond to the largest distance between the two maxima along each line intersecting the circular structure, as indicated by the dashed line in Fig. 3a. The obtained value should fall between the internal and the external diameter of the oligomeric crystal structure. The distribution of the measured diameters is reported in Fig. 3b, where a total amount of 64 single images has been considered. The distribution points out the presence of three oligomeric structures characterized by a different number of subunits, as suggested by previous investigations for the γHL structure. In fact, according to different techniques exploited to study the oligomeric structure of γHL, hexameric [32], heptameric [33] and octameric [22] subunit stoichiometries have been reported. According to these results, here we found three classes of oligomeric organizations differing in the diameter value. It is to be stressed that the presence of different classes can