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Secondary structure and orientation of the pore-forming toxin lysenin in a sphingomyelin-containing membrane

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Abstract

Lysenin is a sphingomyelin-recognizing toxin which forms stable oligomers upon membrane binding and causes cell lysis. To get insight into the mechanism of the transition of lysenin from a soluble to a membrane-bound form, surface activity of the protein and its binding to lipid membranes were studied using tensiometric measurements, Fourier-transform infrared spectroscopy (FTIR) and FTIR-linear dichroism. The results showed cooperative adsorption of recombinant lysenin-His at the argon–water interface from the water subphase which suggested self-association of lysenin-His in solution. An assembly of premature oligomers by lysenin-His in solution was confirmed by blue native gel electrophoresis. When a monolayer composed of sphingomyelin and cholesterol was present at the interface, the rate of insertion of lysenin-His into the monolayer was considerably enhanced. Analysis of FTIR spectra of soluble lysenin-His demonstrated that the protein contained 27% β-sheet, 28% aggregated β-strands, 10% α-helix, 23% turns and loops and 12% different kinds of aggregated forms. In membrane-bound lysenin-His the total content of α-helices, turns and loops, and β-structures did not change, however, the 1636cm^{-1} β-sheet band increased from 18% to 31% at the expense of the 1680cm^{-1} β-sheet structure. Spectral analysis of the amide I band showed that the α-helical component was oriented with at 41° to the normal to the membrane, indicating that this protein segment could be anchored in the hydrophobic core of the membrane.

Keywords: Lysenin; Toxin; Sphingomyelin; Monolayer; Secondary structure determination by FTIR spectroscopy; FTIR-linear dichroism

1. Introduction

Lysenin is a 297-amino acid protein belonging to the group of cytolytic and bacteriostatic toxins isolated from the coelomic fluid of the earthworm *Eisenia foetida* [1,2]. The unique and the most interesting property of this toxin is its ability specifically to interact with sphingomyelin, a major plasma membrane lipid in animal cells [1–3]. Binding of lysenin to other sphingolipids, glycerophospholipids or cholesterol has not been observed [1,4]. Upon binding to sphingomyelin, lysenin forms oligomers that were first identified as SDS-resistant hexamers and visualized as hexagonal structures of 10-nm external diameter with a 3-nm pore inside [4,5]. Subsequent analysis using blue native gel electrophoresis revealed that trimer can be the functional unit of the protein associated with the membrane [5]. The lysenin's ability to oligomerize seems to be correlated with its lytic activity. After truncation of 160 N-terminal amino acids and a point mutation of the single tryptophan 20, both oligomerization and the cytolytic activity were abrogated despite unaltered sphingomyelin binding [5,6]. The lytic action of wild type lysenin relies on the formation of ion channels disturbing the ion balance in the cell, however, at higher concentrations of the protein membrane rupture takes place [5,7].

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Although not required for sphingomyelin binding, cholesterol facilities lysenin oligomerization [1,8]. Since a single lysenin molecule binds five sphingomyelin molecules and the toxin preferentially recognizes sphingomyelin when the lipid forms clusters [9,10], the effect of cholesterol could ensue from facilitated phase separation between sphingomyelin-rich liquid-ordered domains and the liquid-disordered glycerophospholipid-rich milieu. However, recent data indicate that cholesterol affects lysenin oligomerization by fluidizing sphingomyelin-rich domains rather than via facilitating phase separation. As a result, an interaction of sphingomyelin-bound lysenin molecules is promoted facilitating the protein oligomerization [8].

Apart from model membranes, sphingomyelin and cholesterol of the plasma membrane are thought to participate in the assembly of lipid microdomains, so-called rafts. These structures are recognized to play an important role in many cellular functions, especially in signal transduction [11–13]. The engagement of sphingomyelin in raft organization and cellular signalling has sparked interest in lysenin as a tool to study sphingomyelin distribution and function in the plasma membrane. Upon interaction with sphingomyelin, lysenin transforms from a water-soluble to a membrane-embedded form in an as yet not fully understood manner. Our earlier prediction of the secondary structure of lysenin posited that more than half of the residues tend to form β structures (53%), 23% form turns and loops, and 6% form α-helix. However, determination of the secondary structure by another method revealed no helical segments in the lysenin molecule, and the transmembrane domain of the protein also remained unidentified [2].

The monomolecular lipid layer studies presented in this report allowed a direct observation of insertion of recombinant lysenin-His into model lipid membranes composed of sphingomyelin and cholesterol. Fourier-transform infrared absorption spectroscopy (FTIR) was used to determine the secondary structure of lysenin-His, free and incorporated in lipid membranes, while orientation of the membrane-bound protein was examined by polarized IR radiation. The experiments allowed investigation of the structural changes of lysenin depending on its environment, especially in the course of the binding to the membrane and pointed to an α-helix as a possible membrane-inserting fragment of the protein.

2. Materials and methods

2.1. Materials

We synthesized the gene of lysenin fused with a polyhistidine tag at the N-terminus (lysenin-His) from DNA oligos by PCR as described [5], using the nucleotide sequence of lysenin cDNA cloned by Sekizawa et al. [14]. The recombinant protein was expressed in *Escherichia coli* and purified according to [5] with modifications (E. Czuryło, in preparation). For measurements, lysenin-His was equilibrated with 60mM NaCl and 10mM Tris-buffer (pH 7.7). Bovine brain sphingomyelin and cholesterol were purchased from Sigma-Aldrich Chemicals. Lipids were stored under argon at − 80°C. Lipid stock solutions were prepared in chloroform.

2.2. Monomolecular layer technique measurements

Monomolecular lipid layers were formed in a Teflon trough. Water used in all experiments was double distilled and deionized. In order to remove possible organic impurities the water was distilled for a third time with $KMnO_4$. Surface pressure was monitored by a NIMA Technology tensiometer, model PS3 (Coventry, UK). Monolayer experiments were carried out at 21 ± 1°C. Experiments were performed in darkness under argon atmosphere in order to avoid oxidative degradation of lipids. Two-component monolayer of sphingomyelin and cholesterol, at the molar ratio of 1:1, were deposited at the argon–water interface from chloroform solution. The water subphase was buffered with 10mM Tris–HCl (pH 7.7). A stock solution (20μl) of lysenin-His was injected to 12ml of the subphase. Final lysenin-His concentration in the subphase after single injection was 70nM.

2.3. FTIR measurements

The secondary structure of lysenin-His was estimated by analyzing FTIR spectra. Attenuated total reflection infrared (ATR-FTIR) spectra of lysenin-His deposited on the ATR crystal element by partial evaporation and of lysenin-His-containing lipid membranes were recorded with a Vector 33 spectrometer (Bruker, Germany). The internal reflection element was a Ge crystal (45° cut) yielding 10 internal reflections. Typically, 30 scans were collected, Fourier-transformed and averaged for each measurement. Absorption spectra at a resolution of one data point every $4cm^{-1}$ were obtained in the region between 4000 and $400cm^{-1}$ using a clean crystal as the background. The instrument was continuously purged with argon for 40min before and during measurements. The ATR crystals were cleaned with organic solvents. All experiments were done at 21°C. A wire grid infrared polarizer KRS-5 (Pike Technologies, USA) was used in the IR linear dichroism experiments. Spectral analysis was performed with OPUS (Bruker, Germany) and Grams 32 software from Galactic Industries (USA).

2.4. Bilayer preparation

The ATR-FTIR investigation of the binding of lysenin-His to lipid membranes required preparation of a lipid bilayer composed of sphingomyelin and cholesterol (1:1, mol:mol) at the surface of the Ge crystal. The "attach" technique was applied to deposit the first lipid monolayer, the so-called supporting lipid layer, directly on the crystal surface [15]. The surface pressure of the monolayer attached to the Ge crystal was adjusted to 22mN/m. The second monolayer was then deposited by approaching of the crystal (with the monolayer attached) to a lipid monolayer formed previously at the surface of the buffer in the Teflon dish, inside the ATR attachment. IR absorption spectra of the lipid bilayer were collected before and after the injection of lysenin-His into the aqueous subphase. For spectroscopic measurements of pure lysenin-His, directly before the measurements the protein stock solution prepared in the H_2O-based buffer was 100-fold diluted with D_2O and the protein was deposited onto the Ge support by evaporation under the stream of gaseous argon. Such a procedure yields removal of bulk water but retains so-called "structural water" tightly bound to protein. Short time of experiment prevents H/D exchange. Pronounced H/D exchange did not take place since a shift of the position of the amide II band of lysenin-His has not been observed.

2.5. Linear dichroism measurements

The FTIR technique with polarization of the IR beam provides information on the orientation of the protein secondary structure elements embedded in the lipid bilayer with the lipid acyl chains oriented perpendicular to the surface of the ATR plate.

The dichroic ratio (R) is defined as the ratio of integrated absorption bands or the absorbance values corresponding to the selected vibrational modes in the parallel ($A_{\|}$) and perpendicular ($A_{\perp}$) configuration [16]:

$$R = \frac{A_{\|}}{A_{\perp}} = \frac{\int A_{\|}(v)dv}{\int A_{\perp}(v)dv} \quad (1)$$

The order parameter S is a space averaged function of the θ angle between the molecular director and the molecular axis with respect to the axis perpendicular to the plane of the membrane (z axis). The order parameter (S) is defined as:

$$S = \frac{(3<\cos^2\theta> - 1)}{2} \quad (2)$$