INFLUENCE OF OLIGOMERIC AND FIBRILLAR LYSOZYME ON PHYSICAL PROPERTIES OF MODEL MEMBRANES

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A pathological hallmark of more than 20 human diseases including Alzheimer’s disease, Parkinson’s disease, type II diabetes is the deposition in organs and tissues of insoluble highly ordered protein aggregates, called amyloid fibrils. It is becoming widely recognized that toxicity of amyloid species is related to their interactions with cell membranes. In the present study we focused our efforts on the examination of the influence of amyloid fibrils and their precursors (oligomeric aggregates) of lysozyme on the structural and physical properties of the model membranes composed of phosphatidylcholine and its mixture with cholesterol. For evaluating the extent of lipid bilayer modifications, we used fluorescence spectroscopy technique. The results of pyrene excimerization measurements showed that amyloid protein reduces membrane fluidity. Analysis of Laurdan emission spectra revealed the ability of lysozyme aggregates to produce bilayer dehydration. The most pronounced membrane-modifying effects were observed in the case of oligomeric lysozyme. Significantly less influence of pathogenic protein aggregates on the physical properties of cholesterol-containing vesicles confirmed the hypothesis on the preventive role of cholesterol in amyloid-related diseases.

KEY WORDS: amyloid lysozyme, oligomers, fibrils, pyrene, Laurdan, membrane fluidity, dehydration.

ВЛИЯНИЕ ОЛІГОМЕРНОГО ТА ФІБРИЛЯРНОГО ЛІЗОЦИМУ НА ФІЗИЧНІ ВЛАСТИВОСТІ МОДЕЛЬНИХ МЕМБРАН

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Характерною патологічною ознакою більш ніж 20 захворювань людини, включаючи хвороби Альцгеймера і Паркінсона, диabet II типу, тощо, є відкладення в органах і тканинах нерозчинних високопорядкованих білокових агрегатів, так званих амілоїдних фібрил. Загальноприйнятею є той факт, що токсичність амілоїдів обумовлена їх взаємодією з клітинними мембранами. У даній роботі досліджено вплив амілоїдних фібрил та їх попередників (олігомерних агрегатів) лізоциму на структурні та фізичні властивості модельних мембран, що складалися з фосфатидилхоліну і його сума з холестерином. Для оцінки ступеня модифікації ліпідного бішару був використаний метод флуорексцентної спектроскопії. Результати вимірювань ексимерізації пірену свідчать про те, що амілоїдний білок знижує текучість мембран. При аналізі спектрів випромінювання Лаурдана виявлено здатність агрегатів лізоциму викликати дегідратацію ліпідного бішару. Найбільш виражені ефекти модифікації мембран спостерігалися в випадку олігомерних лізоциму. Значно слабший вплив патогенних білокових агрегатів на фізичні властивості везикул, що містили холестерин, підтвердили гіпотезу про превентивну роль холестерину в захворюваннях, пов’язаних з амілоїдами.

КЛЮЧОВІ СЛОВА: амілоїдний лізоцим, олігомери, фібрили, пірен, Лаурдан, текучість мембран, дегідратація.

ВЛИЯНИЕ ОЛІГОМЕРНОГО И ФИБРИЛЯРНОГО ЛИЗОЦИМА НА ФИЗИЧЕСКИЕ СВОЙСТВА МОДЕЛЬНЫХ МЕМБРАН

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Характерным патологическим признаком более чем 20 заболеваний человека, включая болезни Альцгеймера и Паркинсона, диабет II типа и др., является отложение в органах и тканях нерастворимых высокоупорядоченных белковых агрегатов, называемых амилодными фибриллами. Общепринятым становится тот факт, что токсичность амилодов связана с их взаимодействиями с клеточными мембранами. В данной работе было исследовано влияние амилодных фибрилл и их предшественников (олигомерных агрегатов) лизоцима на структурные и физические свойства модельных мембран, состоящих из фосфатидилхолина и его смеси с холестерином. Показано, что лизоцин в виде олигомеров и фибрил уменьшает текучесть мембраны. Анализ спектров поглощения Лаурдана подтверждает способность агрегатов лизоцина вызывать дегидратацию бишлифера. Наиболее выраженные эффекты модификации мембран отмечались в случае олигомерных лизоцина. Значительно слабее влияние патогенных белковых агрегатов на физические свойства везикул, содержащих холестерин, подтверждают гипотезу о предупреждающем действии холестерина в патологических состояниях, связанных с амилодами.

KEY WORDS: amyloid lysozyme, oligomers, fibrils, pyrene, Laurdan, membrane fluidity, dehydration.

холестерином. Для оценки степени модификации липидного бислоя был использован метод флуоресцентной спектроскопии. Результаты измерений эксмеризации пирена показали, что амилоидный белок снижает текучесть мембран. При анализе спектров флуоресценции Лаурдана обнаружена способность агрегатов лизоцима вызывать дегидратацию бислоя. Наиболее выраженные эффекты модификации мембраны наблюдались в случае олигомеров лизоцима. Значительно меньшее влияние патогенных белковых агрегатов на физические свойства холестерин-содержащих везикул подтверждают гипотезу о превентивной роли холестерина в развитии заболеваний, связанных с образованием амилоидов.

**КЛЮЧЕВЫЕ СЛОVA:** амилоидный лизоцим, олигомеры, фибриллы, пирен, Лаурдан, текучесть мембран, дегидратация.

A pathological hallmark of more than 20 human diseases including Alzheimer’s disease, Parkinson’s disease, type II diabetes and other age-related neurodegenerative and systemic disorders is the deposition in organs and tissues of insoluble highly ordered protein aggregates, called amyloid fibrils [1]. It is becoming widely recognized that toxicity of amyloid species is related to their interactions with cell membrane [2].

Amyloid fibrils associated with different diseases were found to possess common structural features [3,4]. A defining characteristic of amyloids is the presence of cross-β structure as revealed by X-ray diffraction, circular dichroism, solid-state NMR and EPR measurements [5,6]. Protein aggregation into fibrils is also considered as a generic property of polypeptide chain, regardless of amino acid sequence [4,7]. Furthermore, extensive evidence supported the idea that protein aggregation and folding are competing pathways. Common structure of amyloid proteins may imply common mechanisms of their toxicity. However, the pathogenic influence of amyloids is not yet fully understood. In particular, there are some disagreements as to the nature of the most toxic aggregated species and molecular mechanisms of membrane disruption.

Numerous studies revealed that soluble pre-fibrillar aggregates (oligomers) but not mature fibrils are responsible for cell dysfunction via permeabilization of plasma membrane. Based on the experimental data, this effect was explained by pore formation [8] or reducing the dielectric barrier of lipid bilayer, domain formation and destabilization of membrane integrity [9,10,11]. Furthermore, oligomers were found to increase ROS production in differing cell lines [12]. However, memranotropich activity of mature amyloid fibrils has been demonstrated in a number of works [13-17]. Novitskaya and coauthors reported that both the β-oligomers and amyloid fibrils are highly toxic to hippocampal neurons and cerebellar neurons, inducing apoptosis [14]. Gharibyan and coworkers showed that lysozyme fibrils added to the cells produce necrosis-like death [15]. Another mechanism of cellular membrane disruption by amyloid lysozyme fibrils was reported by Huang and coworkers. Both the proteofibrils and mature fibrils induced hemolysis and aggregation of erythrocytes through intermolecular disulfide cross-linking [17]. Interestingly, the membrane plays the role of not only the primary target for amyloid proteins, but also can promote protein misfolding and favour formation of toxic aggregates [18,19]. Moreover, membrane integrity was damaged during fibril growth on lipid bilayer [19]. A number of recent studies are focused on the heterogeneity and polymorphism of protein aggregates originated from differences in the destabilizing conditions [20]. It was reported that specific molecular structure of amyloids regulates their interactions with the membrane [21,22].

Thus, in view of insufficient understanding of molecular toxicity mechanisms of amyloid-induced cell damage, further investigations of different aspects of amyloid-membrane interactions are still required. Elucidating the primary pathological events is of a great importance for more effective treatment of amyloid-related disorders.

In the present study we focused our efforts on the examination of the influence of amyloid fibrils and their precursors (oligomeric aggregates) of lysozyme, the protein whose
fibrillar forms are involved in molecular etiology of systemic amyloidosis, on the structural and physical properties of the model membranes composed of phosphatidylcholine (PC) and its mixtures with and cholesterol (Chol) (30 mol%). For evaluating the extent of lipid bilayer modifications, we used fluorescence spectroscopy technique. We tried to gain insights into three aspects of amyloid-membrane interactions; i) whether mature amyloid fibrils or pre-fibrillar aggregates are the most cytotoxic species: ii) what kind of structural alterations can amyloids produce in the lipid bilayer; iii) what is the role of cholesterol in amyloid-induced modifying effect of protein aggregates. To answer these questions, two fluorescent probes with different spectral properties and bilayer location, pyrene, distributing in the region of acyl chains and Laurdan locating at the lipid-water interface, have been employed.

MATERIALS AND METHODS

1. Materials. Egg yolk phosphatidylcholine (PC) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). Chicken egg white lysozyme and pyrene were obtained from Sigma (St. Louis, MO, USA). Laurdan (6-Lauroyl-2-dimethylaminonaphthalene) was from Invitrogen Molecular Probes, (Eugene, OR, USA).

2. Preparation of lysozyme fibrils. The reaction of lysozyme fibrillization was initiated in accordance with the method developed by Holley and coworkers [23]. The essence of their approach lies in obtaining amyloid fibers of lysozyme by protein incubation in 80% ethanol under continuous agitation during 30 days. The presence of fibrillar aggregates was confirmed by the increase in ThT fluorescence at 480 nm.

3. Preparation of lipid vesicles. Large unilamellar lipid vesicles were prepared by the extrusion technique from PC and its mixtures with CL (10 mol%) and Chol (30 mol%). The thin lipid film was obtained by evaporation of lipids’ ethanol solutions and then hydrated with 1.2 ml of 5 mM Na-phosphate buffer (pH 7.4) to yield final lipid concentration 2 mM. Lipid suspension was extruded through a 100 nm pore size polycarbonate filter.

4. Fluorescence measurements. Steady-state fluorescence spectra were recorded with LS-55 spectrofluorimeter (Perkin Elmer, Great Britain) equipped with magnetically stirred, thermostated cuvette holder. Fluorescence measurements were performed at 20°C using 10 mm path-length quartz cuvettes. Emission spectra were recorded with excitation wavelengths of 340 nm (pyrene) and 364 nm (Laurdan).

The excimer-to-monomer fluorescence intensity ratio (E/M) was determined by measuring fluorescence intensity at the monomer (391 nm) and excimer (465 nm) peaks.

The generalized polarization (GP) of Laurdan fluorescence was determined as [24]:

\[ GP = \frac{(I_B - I_R)}{(I_B + I_R)} \]  

where \( I_B \) and \( I_R \) are the maximum fluorescence intensities of the blue (440 nm) and red (490 nm) spectral components, respectively.

RESULTS AND DISCUSSION

1. Pyrene excimerization study into membrane effects of amyloid lysozyme

The modifications of lipid bilayer occurring at the level of acyl chains can be monitored with classical nonpolar fluorescent probe pyrene whose spectra have characteristic vibronic structure in the wavelength region 370-400 nm. A distinctive feature of this probe is an ability of excited pyrene molecule to form a complex (excimer) with an unexcited one. The process of pyrene excimerization is characterized by the appearance of a new fluorescent band at a longer wavelength (~460 nm) compared to the monomer fluorescence [25,26]. Excimer-to-monomer intensity ratio (E/M) is determined by the frequency of collisions between pyrene moieties in the lipid bilayer, being a function of the density of lipid molecular packing [27].
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Fig. 1. Pyrene excimer-to-monomer fluorescence intensity ratio in PC (A) and PC:Chol (30 mol %) (B) liposomes as a function of protein concentration for oligomeric, native and fibrillar lysozyme. Lipid concentration was 45 μM, probe concentration was 0.23 μM.

For evaluating the extent of membrane modifications, E/M values were calculated from the pyrene emission spectra in the neat PC and PC:Chol (30 mol %) liposomes without protein (control samples) and after addition of native, oligomeric and fibrillar lysozyme. As seen from Fig. 1, both pre-fibrillar lysozyme aggregates (withdrawn on the 10th day of protein incubation under denaturing conditions) and mature lysozyme fibrils induced the decrease of pyrene excimerization in all types of model membranes. In contrast, no significant effect of the native protein on the E/M value was observed. Remarkably, relative reduction of pyrene excimerization under the influence of oligomeric lysozyme reached about 20 % in PC liposomes and 10 % in cholesterol-containing vesicles (Table 1). Addition of the fibrillar aggregates resulted in the comparable decrease of this fluorescence parameter in both PC and PC:Chol (30 mol %) liposomes, by 12 and 14 %, respectively. This kind of alterations in pyrene emission spectra indicates that amyloid lysozyme gives rise to the reduction of lipid bilayer fluidity. Our pyrene excimerization data confirm the hypothesis that pre-fibrillar protein aggregates are the most toxic species.

Table 1. Relative decrease of pyrene excimer-to-monomer fluorescence intensity ratio (E/M, %) in PC and PC:Chol (30 mol %) liposomes under influence of oligomeric, native and fibrillar lysozyme.

<table>
<thead>
<tr>
<th>Lysozyme species</th>
<th>PC</th>
<th>PC:Chol (30 mol %)</th>
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<tbody>
<tr>
<td>Native</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Oligomeric</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Fibrillar</td>
<td>14</td>
<td>12</td>
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The ability of amyloid proteins to alter the lipid bilayer fluidity has been reported elsewhere [28-30]. Particularly, aggregated β-amyloid peptide was found to reduce the fluidity of unilamellar liposomes composed of anionic, cationic, and zwitterionic phospholipids as evidenced by the increased DPH fluorescence anisotropy [28]. Furthermore, single particle tracking revealed that Aβ oligomers incorporated into lipid monolayer produce the reduction of membrane fluidity coupled with the solid domains formation [29].

2. Membrane modifications at the interfacial layer as revealed by fluorescent probe Laurdan
To ascertain whether amyloid lysozyme aggregates can perturb polar membrane region we employed environmentally-sensitive fluorescent probe Laurdan. The emission maximum of this probe depends on the membrane phase state, being blue in the gel (440 nm) and green in the liquid-crystalline phase (490 nm). Above the phase transition the foregoing shift to the longer wavelength of the Laurdan emission maximum is observed with increasing temperature [24]. This spectral shift is attributed to dipolar relaxation processes. Since the reorientation of water molecules along the probe excited-state dipoles occurs only in the liquid–crystalline phase, red shift of Laurdan emission spectra cannot be observed in the tightly packed gel phase bilayers.

The spectral changes of Laurdan fluorescence can be quantitatively characterized by the steady-state fluorescence parameter known as generalized polarization (GP). The GP value was calculated according to the Eq. 1. This parameter depends on the dipolar relaxation processes, occurring in an excited state of Laurdan.

To evaluate, what forms of lysozyme display the highest membranotropic activity, Laurdan emission spectra were measured in PC and PC:Chol (30 mol %) vesicles in control samples and in the presence of native, pre-fibrillar or fibrillar protein, depending on the amyloid age- and protein concentration.

As seen from Fig. 2A, increasing concentration of oligomeric lysozyme in PC liposomes resulted in the rise of Laurdan GP. The most pronounced effect was observed at the 10th day (~140 %). The effects of mature lysozyme fibrils and native protein were comparable: GP increment was equal to 70 and 76 %, respectively (Fig. 3A and Table 2). However, in cholesterol-containing model membranes neither pre-fibrillar aggregates nor native or fibrillar lysozyme produced any remarkable changes of Laurdan generalized polarization (Fig. 2B, Fig. 3B). The growth of GP value indicates that aggregated lysozyme induces the decrease of bilayer hydration and increase of the lipid packing density.

Our results add fuel to the view that oligomeric aggregates but not mature amyloid fibrils are responsible for cytotoxicity. As evident from the presented data, the ability of pathogenic protein aggregates to modify physical properties of both hydrophobic and interfacial bilayer regions is hampered by cholesterol.
Fig. 3. Laurdan GP value in PC (A) and PC:Chol (30 mol %) (B) liposomes as a function of protein concentration for oligomeric, native and fibrillar lysozyme. Lipid concentration was 45 μM, probe concentration was 0.07 μM.

The interest in specific influence of cholesterol on amyloid-lipid interactions and its role in the pathogenesis of amyloid-related diseases has greatly increased. Numerous studies performed in this field yielded controversial findings. A number of works reported the preventive role of cholesterol in membrane disruption induced by oligomeric amyloid proteins [12,31,32]. Particularly, cholesterol was found to reduce membrane disordering effects of amyloid β-peptide in vitro [31]. Sponne and coworkers showed that cholesterol enrichment of plasma membrane of cortical neurons inhibited apoptotic events induced by oligomers of amyloid β-peptide. The increase of cholesterol content was reported to reduce the aggregation and fusion of liposomes induced by Aβ(1-40) peptide [32]. In addition, Cehhi et al. revealed that cell resistance to the toxic pre-fibrillar HypF-N aggregates was highly enhanced after the membrane enrichment with cholesterol [12].

Table 2. Relative alteration of Laurdan generalized polarization value (Δ GP, %) in PC and PC:Chol (30 mol %) liposomes under the influence of oligomeric, native and fibrillar lysozyme.

<table>
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<th>Lysozyme species</th>
<th>PC</th>
<th>PC:Chol (30 %)</th>
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<tbody>
<tr>
<td>Native</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>Oligomeric</td>
<td>140</td>
<td>2</td>
</tr>
<tr>
<td>Fibrillar</td>
<td>76</td>
<td>-3</td>
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Nevertheless, it was found that reduction of membrane cholesterol level inhibited the production of amyloid β-peptide in brain [33]. The ability of cholesterol to modulate Aβ generation in the pathway of Alzheimer’s disease is reported in the review [34]. The sensitivity of Aβ production to cholesterol content was explained by the fact that the activity of enzymes which cleave amyloid precursor protein to generate amyloid β-peptide depends strongly on cholesterol metabolism. Residing in cholesterol-rich lipid domains these enzymes require a high cholesterol level for their function [34]. Along with the influence on the Aβ production, cholesterol was reported to strengthen amyloid-membrane interactions and channel formation by amyloid β-peptide [35].

The results of our study suggest that the presence of cholesterol, which has the propensity to modify bilayer parameters such as thickness, molecular packing, conformational freedom of acyl chains, membrane fluidity, restricts the insertion of pre-fibrillar lysozyme aggregates into the membrane. The observed increase of Laurdan GP values is suggestive of the
penetration of oligomers into the neat PC bilayer leading to the increment of lipid packing and removal of adjacent water molecules.

CONCLUSIONS
The results of the present report can be summarized as follows:
1) Pre-fibrillar lysozyme aggregates but not mature amyloid fibrils possess the highest membranotropic activity;
2) Pyrene excimerization study and analysis of Laurdan emission spectra revealed that the toxic effect of oligomeric lysozyme may arise from the decrease of membrane fluidity and bilayer dehydration;
3) As judged from the fluorescence measurements, cholesterol has an ability to modify amyloid-lipid interactions and can play a preventive role in pathological processes of amyloid-related diseases.

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