БІОФІЗИКА КЛІТИНИ

УДК 577.37

LIPID BILAYER MODIFICATION INDUCED BY FIBRILLAR LYSOZYME: FLUORESCENCE SPECTROSCOPY STUDY

A.P Kastorna, V.M. Trusova, G.P. Gorbenko

V.N. Karazin Kharkov National University, 4 Svobody Sq., Kharkov, 61077
Submitted October 19, 2010
Accepted November 10, 2010

The correlation between neurodegenerative diseases (Parkinson's, Alzheimer's diseases), type II diabetes, systemic amyloidosis, etc. and deposition of protein aggregates in brain and other tissues has long been established. A growing body of evidence has demonstrated that binding of amyloid proteins to the membrane may underlie their cytotoxic effect. It was shown that amyloid toxicity arises primarily from a soluble oligomeric form (pre-fibrillar aggregates) of the peptide rather than amyloid monomers or mature fibrils. The molecular basis of the amyloid protein toxicity is not sufficiently clear and requires further investigation. In view of this, the present study has been undertaken to ascertain the effect of fibrillar aggregates of lysozyme on the structural and physical properties of model membranes (liposomes) composed of zwitterionic lipid phosphatidylcholine and its mixture with cholesterol (30 mol%). To this end, two fluorescent probes with different properties and bilayer location, pyrene and Laurdan, have been employed. Pyrene spectra have characteristic vibronic structure in the region of 370-400 nm. Relative intensities of vibronic transitions exhibit dependence on solvent polarity. Excited species of pyrene can interact with non-excited ones thus forming excited state dimers – excimers. Excimer-to-monomer fluorescence intensity ratio reflects the extent of pyrene excimerization, which depends mainly on the rate of monomer lateral diffusion in lipid bilayer, being a function of the density of lipid molecular packing. Analysis of pyrene emission spectra revealed the absence of any influence of fibrillar lysozyme on the structural state of bilayer acyl chain region. Laurdan is an amphiphilic fluorescent probe, whose emission spectra are sensitive to the environmental polarity (hydration level). In the solvents of high polarity, Laurdan shows a considerable shift of its emission spectrum to longer wavelengths due to the dipolar relaxation processes. The changes in the emission spectrum of Laurdan were characterized by the generalized polarization value (GP). In all types of liposomes increasing concentration of fibrillar lysozyme resulted in the increment of GP, suggesting that amyloid fibrils cause the decrease in the lipid bilayer polarity.
KEY WORDS: amyloid, lysozyme fibrils, pyrene, Laurdan, model membranes.

ИЗУЧЕНИЕ МОДИФИКАЦИИ ЛИПИДНОГО БИСЛОЯ ПОД ДЕЙСТВИЕМ ФИБРИЛЛЯРНОГО ЛИЗОЦИМА МЕТОДОМ ФЛУОРЕСЦЕНТНОЙ СПЕКТРОСКОПИИ

А.П. Касторная, В.М. Трусова, Г.П. Горбенко

Харьковский национальный университет имени В.Н. Каразина, пл. Свободы, 4, Харьков, 61077

Связь между нейродегенеративными заболеваниями (болезнями Паркинсона, Альцгеймера), диабетом II типа, системным амилоидозом и отложениями белковых агрегатов в тканях организма уже давно установлена. Большое количество работ свидетельствуют о том, что в основе цитотоксического действия амилоидных белков лежат их взаимодействия с клеточными мембранами. Было показано, что наибольшей токсичностью обладают растворимые олигомерные формы полипептидов (предшественники фибриллярных агрегатов), по сравнению с мономерами или зрелыми фибриллами. Молекулярные механизмы, лежащие в основе токсичности амилоидных белков, недостаточно изучены и требуют дальнейших исследований. Ввиду этого, в данной работе было исследовано влияние фибриллярных агрегатов лизоцима на структуру и физические свойства модельных мембран (липосом), состоящих из фосфатидилхолина и его смеси с холестерином (30 мол %). Для достижения этой цели были использованы два флуоресцентных зонда с различными свойствами и локализацией в бислое – пирен и Лаурдан. Спектры пирена имеют вибронную структуру в диапазоне длин волн 370-400 нм. Отношение интенсивностей вибронных полос этого зонда зависит от полярности окружения. Возбужденные молекулы пирена могут взаимодействовать с невозбужденными, образуя димеры в возбужденном состоянии – эксимеры. Отношение интенсивности флуоресценции эксимеров к интенсивности флуоресценции мономеров (степень эксимеризации пирена) зависит от скорости латеральной диффузии

мономеров в бислое, которая является функцией плотности молекулярной упаковки липидов. Анализ спектров излучения пирена показал, что фибриллярный лизоцим не оказывает влияния на структурное состояние гидрофобной области липидного бислоя. Лаурдан – это амфифильный флуоресцентный зонд, спектры флуоресценции которого чувствительны к полярности окружения (уровню гидратации). В растворах с высокой полярностью наблюдается значительный длинноволновый сдвиг спектра флуоресценции Лаурдана. Это обусловлено процессами дипольной релаксации молекул растворителя. Изменения в спектрах флуоресценции Лаурдана были охарактеризованы величиной обобщенной поляризации (GP). В обоих типах липосом увеличение концентрации фибриллярного лизоцима привело к возрастанию GP. Полученные данные свидетельствуют о том, что амилоидные фибриллы вызывают уменьшение полярности на границе раздела липидный бислой-вода, где расположены молекулы Лаурдана.
КЛЮЧЕВЫЕ СЛОВА: амилоидные белки, фибриллы лизоцима, пирен, Лаурдан, модельные мембраны.

ВИВЧЕННЯ МОДИФІКАЦІЇ ЛІПІДНОГО БІШАРУ ПІД ДІЄЮ ФІБРИЛЯРНОГО ЛІЗОЦИМУ МЕТОДОМ ФЛУОРЕСЦЕНТНОЇ СПЕКТРОСКОПІЇ

А.П. Касторна, В.М. Трусова, Г.П. Горбенко
Харківський національний університет імені В.Н. Каразіна, пл. Свободи, 4, Харків, 61077

Зв'язок між нейродегенеративними захворюваннями (хворобами Паркінсона, Альцгеймера), діабетом типу II, системним амілоїдозом і відкладеннями білкових агрегатів у тканинах організму вже давно встановлений. Велика кількість робіт свідчать про те, що в основі цитотоксичної дії амілоїдних білків лежать їх взаємодії з клітинними мембранами. Було показано, що найбільшу токсичність у порівнянні з мономерами або зрілими фібрилами мають розчинні олігомерні форми поліпептидів (попередники фібрилярних агрегатів). Молекулярні механізми, що лежать в основі токсичності амілоїдних білків, недостатньо вивчені і потребують подальших досліджень. Дана робота була проведена з метою визначення впливу фібрилярних агрегатів лізоциму на структуру і фізичні властивості модельних мембран (ліпосом), з фосфатидилхоліну та його суміші з холестерином (30 мол %). Для досягнення цієї мети були використані два флуоресцентних зонда з різними властивостями і локалізацією в бішарі – пірен і Лаурдан. Спектри пірену мають характерну вібронну структуру в області 370-400 нм. Відношення інтенсивностей вібронних смуг цього зонду залежить від полярності розчинника. Збуджені молекули пірену можуть взаємодіяти з незбудженими, утворюючи димери у збудженому стані – ексимери. Відношення інтенсивностей флуоресценції ексимерів та мономерів (ступінь ексимеризації пірену) залежить від швидкості латеральної дифузії мономерів у бішарі, що є функцією щільності молекулярного пакування ліпідів. Аналіз спектрів флуоресценції пірену показав, що фібрилярний лізоцим не впливає на структурний стан гідрофобної області бішару. Лаурдан – це амфіфільний флуоресцентний зонд, спектри флуоресценції якого чуттєві до полярності оточення (рівня гідратації). У високополярних розчинах спостерігається значний довгохвильовий зсув спектрів флуоресценції Лаурдана. Це обумовлено процесами дипольної релаксації молекул розчинника. Зміни у спектрах флуоресценції Лаурдану були охарактеризовані узагальненою поляризацією (GP). В обох типах ліпосом збільшення концентрації фібрилярного лізоциму призвело до зростання GP. Отримані дані свідчать про те, що амілоїдні фібрили викликають зменшення полярності на межі розподілу ліпід-вода, де розташовані молекули Лаурдану.
КЛЮЧОВІ СЛОВА: амілоїдні білки, фібрили лізоциму, пірен, Лаурдан, модельні мембрани.

The correlation between neurodegenerative diseases (Parkinson's, Alzheimer's and Huntigton's diseases), type II diabetes, systemic amyloidosis, etc. and deposition of protein aggregates in brain and other tissues has long been established [1-3]. A vast number of recent studies were devoted to the protein misfolding and aggregation and to elucidating the molecular mechanisms of amyloid toxicity. A growing body of evidence has demonstrated that amyloid protein-membrane interactions may underlie the cytotoxic effects elicited by amyloid proteins [3-7]. The process of amyloid formation includes the misfolding of partially folded soluble proteins into oligomeric β-sheet structures, which further aggregate into protofibrils and form mature amyloid fibrils [4,8]. Such factors as specific mutations, environmental changes or chemical modifications reduce the conformational stability of the protein and may cause aggregation [2]. Numerous data from the recent studies indicate that fibril formation can be membrane-induced [4,9].

It was shown that amyloid toxicity arises primarily from a soluble oligomeric form (pre-fibrillar aggregates) of the protein rather than amyloid monomers or mature fibrils [1-7]. Several hypotheses, explaining the mechanisms of amyloid toxicity, have been suggested. It was demonstrated that the toxic aggregated species form non-specific pore-like channels in the membranes [2,4]. Another processes leading to membrane permeabilization under the influence of oligomeric proteins include decrease in membrane dipole potential between the highly hydrated lipid groups at the bilayer surface and polar exterior of a membrane [5], reduction of the hydrocarbon core packing density resulting in the increase of bilayer conductivity [6]. A number of studies revealed large increment of intracellular free calcium level and modification of the cell redox status upon oligomer addition [3,7]. Recent evidence demonstrated complete membrane degradation by lipid extraction and uptake into growing fibrils [4]. All the mechanisms mentioned here lead to the cell dysfunction and death. In spite of considerable progress achieved in understanding of amyloid protein-membrane interactions, molecular basis of this process is poorly understood and requires further investigation.

The present study has been undertaken to ascertain the effect of fibrillar aggregates of lysozyme, a protein possessing bactericidal and immunomodulatory functions, on the structural and physical properties of model membranes (liposomes) composed of zwitterionic lipid phosphatidylcholine (PC) and its mixtures with sterol cholesterol (Chol) (30 mol%). Using fluorescence spectroscopy, one of the most accurate and powerful methods for investigating the biological macromolecules, we made an effort to answer the question: what kind of modifications can fibrillar protein species produce in the lipid bilayer ? To this end, two fluorescent probes with different properties and bilayer location, pyrene (distributing in the region of acyl chains) and Laurdan (locating at lipid-water interface), have been employed.

MATERIALS AND METHODS

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). The reaction of lysozyme fibrillization was realized in accordance with the method developed by Holley and coworkers [10]. These authors revealed the ability of this protein to form amyloid fibrils in higly concentrated ethanol solution, suggesting that peptide backbone interactions play a basic role in fibril formation [10,11]. The essence of their approach lies in obtaining amyloid fibers of lysozyme by protein incubation in 80% ethanol under continuous agitation during 30 days. Large unilamellar lipid vesicles were prepared by extrusion from PC and its mixtures with 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. Fluorescence measurements were performed 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), 364 nm (Laurdan).

RESULTS AND DISCUSSION

Membrane modifications probed by pyrene

Pyrene is a classical nonpolar fluorescence probe localized in the region of acyl chains (Fig. 1). In excited state pyrene can form a complex with an identical unexcited probe molecule. Such a complex is called an excimer and is recognized by the appearance of a new

fluorescent band at a longer wavelength than the monomer fluorescence [12,13]. Excimer-to-monomer intensity ratio (E/M) reflects the rate of excimer formation and is determined by the frequency of collisions between pyrene moieties.

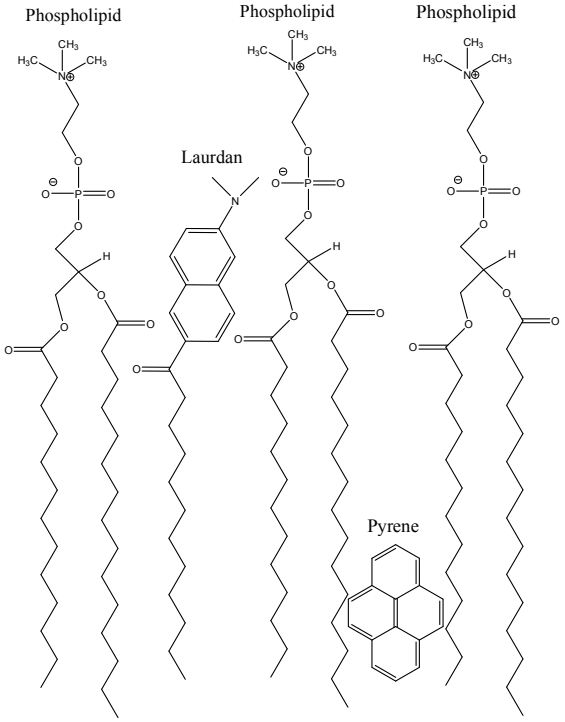

Fig. 1. Schematic representation of the location of Laurdan and pyrene in a lipid bilayer.

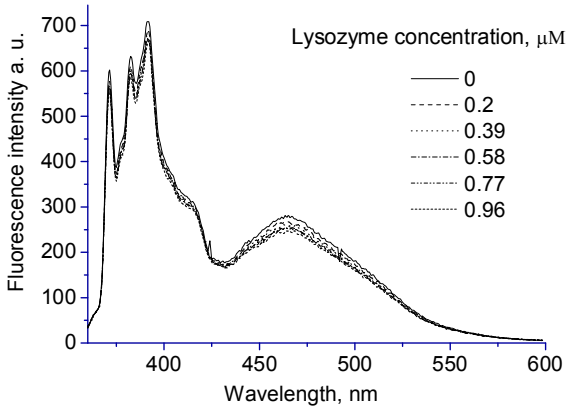

Fig. 2. Pyrene fluorescence spectra in PC liposomes with different concentration of fibrillar lysozyme. Pyrene concentration was 0.96 μM.

Generally, the results of pyrene excimerization studies are interpreted within the framework of free volume model. Membrane free volume is determined as a difference between effective and Van der Waals volumes of lipid molecules [14].

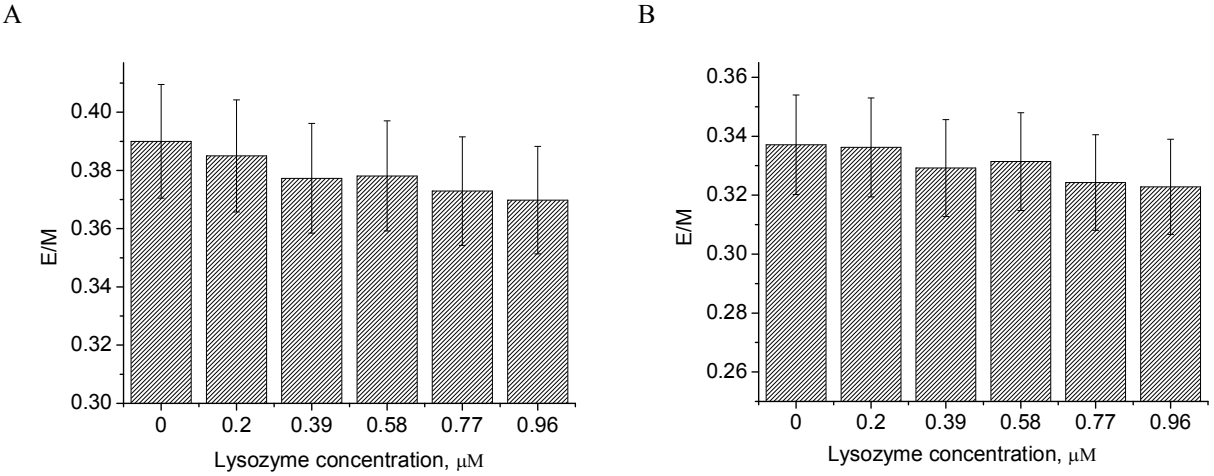

Fig. 3. Dependence of excimer-to-monomer fluorescence intensity ratio in liposomes on lysozyme concentration: A – PC , B – PC/Chol (30%).

Fig. 2 shows typical emission spectra of pyrene in the model membranes at varying concentrations of fibrillar lysozyme. Fluorescence intensities of monomers and excimers were measured at 391 nm and 466 nm, respectively. As seen in Fig. 3, excimer-to-monomer intensity ratio in both PC and PC/Chol lipid systems was not markedly influenced by fibrillar aggregates of lysozyme, suggesting that bilayer free volume remained practically unchanged. Another significant parameter of pyrene fluorescence specrtum is the ratio of intensity of the first (I_I at 371 nm) and third peaks (I_{III} at 382 nm), characterizing the polarity of the probe microenvironment. For example, I_I/I_{III} in hydrocarbon solvents has the value of about 0.6, in ethanol is ~ 1.1, and in water is ~ 1.96 [15]. Similarly to E/M value, the magnitude of I_I/I_{III} ratio was not affected by amyloid fibrils (Fig. 5), suggesting that lysozyme aggregates have no effect on hydration level in the acyl group region of the membranes.

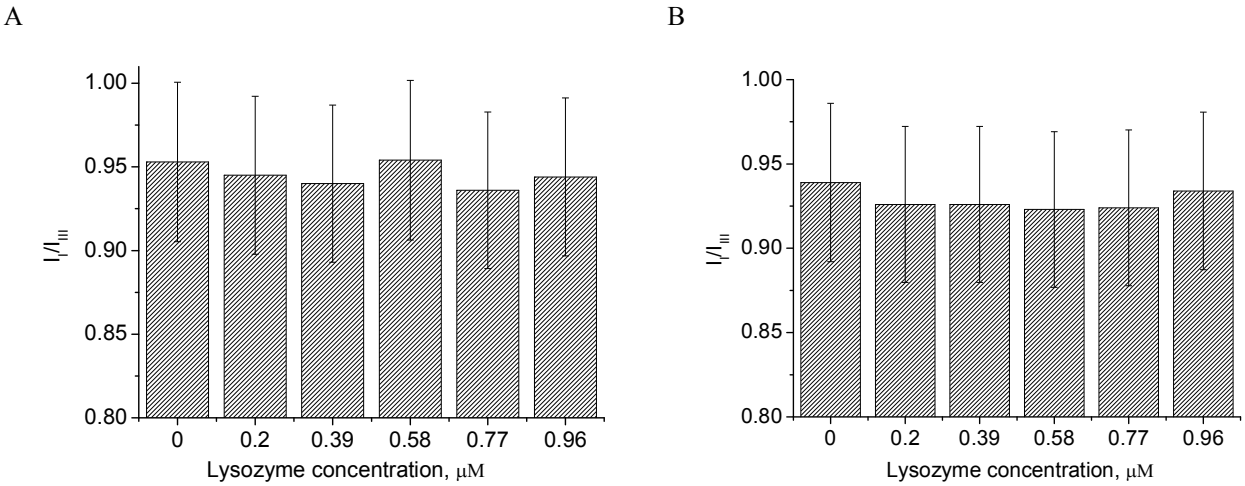

Fig. 4. Effect of lysozyme aggregates on vibronic structure of pyrene fluorescence spectra: A – PC , B – PC/Chol (30%).

Bilayer perturbations detected by Laurdan

Laurdan is an amphiphilic fluorescence probe, synthesized by GregorioWeber to study the processes of dipolar relaxation. In a lipid bilayer this probe is localized at lipid-water interface with the lauric acid tail anchored in the phospholipid acyl chain region (Fig. 1). Laurdan association with the lipid membranes is accompanied by the increase in fluorescence intensity. For quantitative description of Laurdan binding to liposomes of varying composition the results of fluorimetric titration were treated in terms of partition model. The partition coefficient, K_P, is defined as

$$K_P = (n_L / V_L)/(n_W / V_W), \quad (1)$$

where n_L and n_W are the molar concentrations of the probe in lipid and water phases respectively, V_L and V_W are the volumes of these phases. Based on fluorescence data, K_P can be calculated as:

$$\Delta I = I_L - I_W = (K_p V_L (I_{\max} - I_W))/(1 + K_P V_L), \quad (2)$$

where ΔI – fluorescence intensity change , I_L, I_W – fluorescence intensities in lipid and in water phases, respectively, I_{max} – limit fluorescence intensity of the probe in a lipid environment [17]. The volume of lipid phase was calculated as:

$$V_L = N_A C_L \sum v_i f_i \quad (3)$$

where C_L is the molar lipid concentration, f_i is mole fraction of the *i-th* bilayer constituent, v_i is its molecular volume taken as 1.58 nm^3 (PC) and 0.74 nm^3 (Chol). The experimental dependencies $\Delta I(C_L)$ (Fig. 5) were approximated by eq. 2. The recovered in such a manner partition coefficients are presented in Table 1. The results obtained are indicative of high Laurdan affinity for lipid bilayers. Addition of cholesterol to PC membranes resulted in K_P decrease of about 60%. This fact indicates that cholesterol prevents Laurdan partitioning into the phospholipid membranes. This observation can be explained by the condensing effect of cholesterol on the lipid bilayer, i.e. ordering of lipid molecules and tighter packing of hydrocarbon tails.

Laurdan is one of fluorescence probes widely used in the studies of biological and model membranes. It belongs to the group of so-called environmentally-sensitive probes that respond to the changes in their environment by the shift of emission spectra [18].

Table 1. Partition coefficients of Laurdan in lipid vesicles

Liposome composition	K_P
PC	$(1.4\pm0.54)*10^4$
PC/Chol (30%)	$(5.6\pm0.32)*10^3$

The fluorescent naphthalene moiety of this probe has a dipole moment caused by a partial charge separation between the 2-dimethylamino and the 6-carbonyl residues. This dipole moment increases upon excitation and in polar solvents may induce reorientation of the surrounding dipoles. The probe excited state energy is partially consumed on this reorientation that results in a continuous red shift of the probe steady-state emission spectrum. When the local environment of Laurdan is a phospholipid phase, the emission maximum depends strongly on the packing of the lipid chains. In the gel state emission maximum is near 440 nm and in the liquid crystalline state it is near 490 nm. With increasing temperature foregoing red shift of the emission maximum is observed only in the liquid–crystalline phase, originated from the increased concentration of water molecules in the bilayer at the level of the glycerol backbone and from their increased mobility.

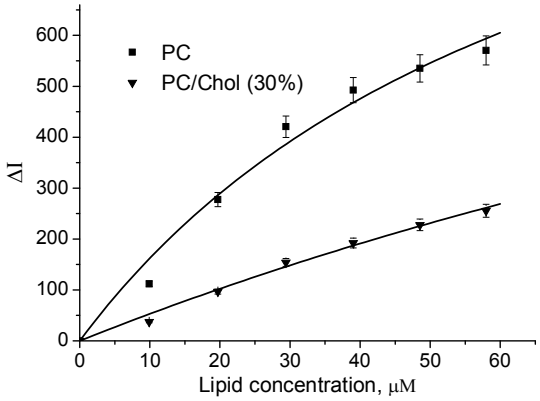

Fig. 5. Isotherms of Laurdan binding to PC and PC/Chol model membranes. Laurdan concentration was 0.17 μM.

The changes in the emission spectrum of Laurdan can be characterized by the generalized polarization value (GP). The generalized polarization was calculated according to the equation:

$$GP = (I_B - I_R)/(I_B + I_R), \quad (4)$$

where I_B and I_R are the maximum fluorescence intensities of the blue and red spectral components, respectively. The GP function also contains information on the dipolar relaxation process, which takes place while Laurdan is in an excited state, and, consequently, reflects the extent of bilayer hydration [16,18,19].

To calculate the value of this parameter, fluorescence intensities at 440 (I_B) and 490 (I_R) nm were used. The GP of Laurdan in two types of lipid vesicles as a function of amyloid protein concentration is shown in Fig. 6. As evident from this figure, the GP was negative (about -0.10) in PC liposomes, while it turned out to attain positive values in the Chol-containing vesicles (about 0.16). This finding may be explained by the fact, that in PC/Chol liposomes Laurdan located in a less polar environment than in PC membranes. Addition of fibrillar lysozyme resulted in the increase of the GP value. This effect may indicate that amyloid fibrils cause the decrease in bilayer polarity and increase of lipid packing density However, the magnitude of the effect was less pronounced in PC/Chol vesicles (18 %) than in PC vesicles (37 %). Thus, the ability of fibrillar aggregates to alter physical properties of the interfacial membrane region is prevented by cholesterol.

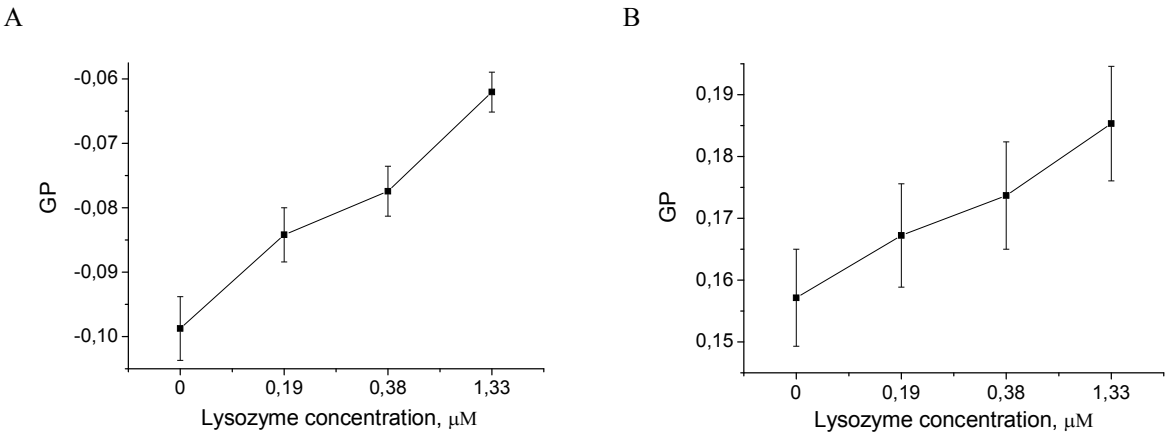

Fig. 6. Generalized fluorescence polarization (GP) of Laurdan in vesicles of different composition as a function of amyloid lysozyme concentration. A – PC, B – PC/Chol (30%).

CONCLUSIONS

To summarize, the present study provides evidence for modifying effect of fibrillar lysozyme on the physical properties of model membranes. Analysis of pyrene fluorescence spectra revealed that structure of hydrocarbon chains region of a lipid bilayer is not affected by lysozyme fibrils. In contrast, analysis of Laurdan spectra in terms of generalized polarization model showed that fibrillar aggregates of lysozyme brought about the reduction of bilayer polarity at the lipid-water interface originated, presumably, from the increased lipid packing.

ACKNOWLEDGEMENTS

This work was supported in part by the grant #4534 from the Science and Technology Center in Ukraine, Fundamental Research State Fund (project number F.28.4/007) and grant from European Social Fund (project number 2009/0205/1DP/1.1.1.2.0/09/APIA/VIAA/152).

REFERENCES

1. Ross C.A., Poirier M.A. Protein aggregation and neurodegenerative disease // Nat. Med. – 2004. – V. 10. – P. s10-s17.
2. Stefani M. Protein misfolding and aggregation: new examples in medicine and biology of the dark side of the protein world // Biochim. Biophys. Acta. – 2004. – V. 1739. – P. 5-25.
3. Cehhi C., Baglioni S., Fiorillo C., Pensalfini A., Liguri G., Nosi D., Rigacci S., Bucciantini M., Stefani M. Insights into the molecular basis of the differing susceptibility of varying cell types to the toxicity of amyloid aggregates // J. Cell Sci. – 2005. – V. 118. – P. 3459-3470.
4. Butterflied S.M., Laushel H.A. Amyloidogenic protein-membrane interactions: mechanistic insight from model systems // Angew. Chem. Int. Ed. – 2010. – V. 49. – P. 5628-5654.
5. Wang S. S.-S., Liu K.-N. Membrane dipole potential of interaction between amyloid protein and phospholipid membranes is dependent on protein aggregation state // J. Chin. Inst. Chem. Eng. – 2008. – V. 39. – P. 321-328.
6. Kayed R., Sokolov Y., Edmonds B., McIntire T., Milton S., Hall J., Glabe C. Permeabilization of lipid bilayers is a common conformation-dependent activity of soluble amyloid oligomers in protein misfolding diseases // J. Biol. Chem. – 2004. – V. 279. P. 46363-46366.
7. Glabe C.G. Common mechanisms of amyloid oligomer pathogenesis in degenerative disease // Neurobiol. Aging. – 2006. – V. 27. – P. 570-575.
8. Uversky V.N., Fink A.L. Conformational constraints for amyloid fibrillation: the importance of being unfolded // Biochim. Biophys. Acta. – 2006. – V. 1698. – P. 131-153.
9. Gorbenko G.P., Ioffe V.M., Kinnunen P.K.J. Binding of lysozyme to phospholipid bilayers: evidence for protein aggragation upon membrane association // Biophys. J. – 2007. V. 93. – P. 140-153.
10. Holley M., Eginton C., Schaefer D., Brown L. R. Characterization of amyloidogenesis of hen egg lysozyme in concentrated ethanol solution // Biochem. Biophys. Res. Commun. – 2008. – V. 373. – P. 164-168.
11. Goda S., Takano K., Yamagata Y., Nagata R., Akutsu H., Maki S., Namba K., Yutani K. Amyloid protofilament formation of hen egg lysozyme in highly concentrated ethanol solution // Protein Sci. – 2000. – V. 9. – P. 369-375.
12. Lakowicz J.R. Principles of Fluorescent Spectroscopy, 3rd ed. Plenum Press, New York. 2006.
13. Novikov E.G., Visser N.V., Malevitskaia V.G., Borst W., van Hoek A., Visser A. Molecular dynamics of monopyrenyl lipids in liposomes from global analysis of time-resolved fluorescence of pyrene monomer and excimer emission // Langmuir. – 2000. – V. 16. – P. 8749-8754.
14. Ioffe V., Gorbenko G. Lysozyme effect on structural state of model membranes as revealed by pyrene excimerization studies // Biophys. Chem. – 2005. V. 114. – P. 199–204.
15. Karpovich D.S., Blanchard G.J. Relating the polarity-dependent fluorescence response to vibronic coupling. Achieving a fundamental understanding of the *py* polarity scale // J. Phys. Chem. – 1995. – V. 99. – P. 3951-3958.
16. Bagatolli L., Gratton A.E., Fidelio G.D. Water dynamics in glycosphingolipid aggregates studied by Laurdan fluorescence // Biophys. J. – 1998. – V. 75. – P. 331-341.
17. Santos N.C., Prieto M., Castanho A.R.B. Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods // Biochim. Biophys. Acta. – 2003. – V. 1612. – P. 123-135.
18. Parasassi T., Krasnowska E.K, Bagatolli L., Gratton E. Laurdan and Prodan as polarity-sensitive fluorescent membrane probes // J. Fluorescence.– 1998. – V. 8. – P. 365-373.
19. Mukherjee S., Chattopadhyay A. Monitoring the organization and dynamics of bovine hippocompal membranes utilizing Laurdan generalized polarization // Biochim. Biophys. Acta. – 2005. – V. 1714. – P. 43-55.