БІОФІЗИКА КЛІТИНИ

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LIPID-MEDIATED LYSOZYME AGGREGATION: FÖRSTER RESONANCE ENERGY TRANSFER STUDY

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Aggregation of proteins into insoluble complexes is intimately linked to pathogenesis of several neurodegenerative diseases. Protein aggregation is commonly regarded as nonspecific coagulation of incompletely folded or partially denaturated polypeptides, driven by interaction between the exposed hydrophobic patches. Accumulating evidence indicates that protein self-association can be induced by protein-lipid interactions. The present study addresses a problem of aggregation behavior of lysozyme (Lz) bound to model membranes composed of phosphatidylcholine (PC) and phosphatidylglycerol (PG) (8:2). The formation of Lz assemblies in lipid environment has been monitored by measuring steady-state resonance energy transfer (FRET). Several donor-acceptor pairs have been employed: tryptophan (Trp) – pyrene, pyrene – fluorescein 5'-isothiocyanate (FITC) and FITC – rhodamine-isothiocyanate (RITC). Fluorescence spectra of pyrene maleimide-labelled Lz (Lz-PM) recorded at different lipid-to-protein molar ratios (L:P) with excitation wavelength of 296 nm were featured by three bands corresponding to Trp, pyrene monomer and excimer emission. Analysis of the shape of emission spectra showed that the ratio of PM to Trp intensity rises with increasing Lz-PM concentration and decreasing L:P values from 379 to 77. This effect is most probably to arise from the enhanced FRET between Trp and pyrene. The finding that the magnitude of this effect depends on protein concentration suggests that FRET enhancement is caused by the formation of protein aggregates. The same result was obtained with Lz-attached pyrene as donor and Lz-attached FITC as acceptor – the efficiency of energy transfer increased with increasing total protein concentration and decreasing L:P. Notably, the most pronounced increase of energy transfer efficiency was observed at surface coverage *ca.* 38 lipids per one protein molecule suggesting that this L:P value is critical for formation of Lz self-associates. The assumption that Lz forms aggregates in membrane environment is also corroborated by the quantitative analysis of FRET between FITC and RITC. The distance between FITC and RITC was found to be *ca.* 8 nm which exceeds the dimensions of Lz molecule by 2-2.5 times, lending additional support to the idea about Lz self-association in lipid surroundings.

KEY WORDS: lysozyme, lipid bilayer, aggregation, Förster resonance energy transfer

ЛІПІД-ОПОСЕРЕДКОВАНА АГРЕГАЦІЯ ЛІЗОЦИМУ ЗА ДАНИМИ ІНДУКТИВНО-РЕЗОНАНСНОГО ПЕРЕНОСУ ЕНЕРГІЇ

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Агрегація білків з утворенням нерозчинних комплексів пов'язана з патогенезом деяких нейродегенеративних захворювань. Олігомеризація білків розглядається зазвичай як неспецифічна коагуляція частково розгорнутих поліпептидів, яка контролюється взаємодією між експонованими гідрофобними ділянками. Все більшого підтвердження набуває гіпотеза про те, що самоасоціація білків може бути викликана білок-ліпідними взаємодіями. У даній роботі була досліджена агрегаційна поведінка лізоциму, зв'язаного з модельними ліпідними мембранами, що складалися з фосфатидилхоліну (ФХ) та фосфатидилгліцерину (ФГ) (8:2). Утворення агрегатів білка детектувалося за допомогою методу індуктивно-резонансного переносу енергії (ІРПЕ). Було використано три донорно-акцепторні пари: триптофан – пірен, пірен – флуоресцеїн 5'-ізотіоціанат (ФІТЦ) та ФІТЦ – родамін-ізотіоціанат (РІТЦ). Спектри флуоресценції лізоциму, міченого піреном, характеризувалися трьома піками, які відповідали флуоресценції триптофану, мономерів та ексимерів пірену. Аналіз форми спектрів показав, що відношення інтенсивності флуоресценції пірену до інтенсивності флуоресценції триптофану зростає зі зменшенням молярного відношення ліпід:білок (Л:П) з 379 до 77. Цей ефект було пояснено посиленням ІРПЕ між триптофаном та піреном. Той факт, що величина цього ефекту залежала від концентрації білка, дозволяє

припустити, що посилення ІРПЕ викликається утворенням агрегатів білка. Аналогічний результат було отримано з використанням донорно-акцепторної пари пірен – ФІТЦ – ефективність ІРПЕ зростала зі збільшенням загальної концентрації білка та зменшенням Л:П. Примітно, найбільша ефективність ІРПЕ спостерігалась при поверхневому покритті у 38 молекул ліпідів на одну молекулу білка, вказуючи на те, що ця величина Л:П є критичною для утворення самоасоціатів лізоциму. Припущення щодо агрегації білка у мембранному оточенні підтверджується також кількісним аналізом ІРПЕ між ФІТЦ та РІТЦ. Відстань між донором та акцептором дорівнювала 8 нм, що у 2-2.5 рази перевищує розміри молекули лізоциму.
КЛЮЧОВІ СЛОВА: лізоцим, ліпідний бішар, агрегація, індуктивно-резонансний перенос енергії

ЛИПИД-ОПОСРЕДОВАННАЯ АГРЕГАЦИЯ ЛИЗОЦИМА ПО ДАННЫМ ИНДУКТИВНО-РЕЗОНАНСНОГО ПЕРЕНОСА ЭНЕРГИИ

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Агрегация белков с образованием нерастворимых комплексов связана с патогенезом некоторых нейродегенеративных заболеваний. Олигомеризация белков обычно рассматривается как неспецифичная коагуляция частично развернутых полипептидов, которая контролируется взаимодействием между экспонированными гидрофобными участками. Все большего подтверждения получает гипотеза о том, что самоасоциация белков может быть вызвана белок-липидными взаимодействиями. В данной работе было исследовано агрегационное поведение лизоцима, связанного с модельными липидными мембранами, состоящими из фосфатидилхолина (ФХ) и фосфатидилглицерина (ФГ) (8:2). Образование агрегатов белка детектировалось с помощью метода индуктивно-резонансного переноса энергии (ИРПЭ). Были использованы три донорно-акцепторные пары: триптофан – пирен, пирен – флуоресцеин 5’-изотиоцианат (ФИТЦ) и ФИТЦ – родамин- изотиоцианат (РИТЦ). Спектры флуоресценции лизоцима, меченого пиреном, характеризовались тремя пиками, которые соответствовали флуоресценции триптофана, мономеров и эксимеров пирена. Анализ формы спектров показал, что отношение интенсивности флуоресценции пирена к интенсивности флуоресценции триптофана увеличивается с уменьшением молярного отношения липид:белок (Л:П) с 379 до 77. Этот эффект был объяснен усилением ИРПЭ между триптофаном и пиреном. Тот факт, что величина эффекта зависела от концентрации белка, позволяет предположить, что усиление ИРПЭ вызвано образованием агрегатов белка. Аналогичный результат был получен с помощью донорно-акцепторной пары пирен – ФИТЦ – эффективность ИРПЭ увеличивалась с возрастанием концентрации белка и уменьшением Л:П. Примечательно, самая высокая эффективность ИРПЭ наблюдалась при поверхностном покрытии в 38 молекул липидов на одну молекулу белка, указывая на то, что эта величина Л:П является критической для образования самоассоциатов лизоцима. Предположение об агрегации лизоцима в мембранном окружении подтверждается также количественным анализом ИРПЭ между ФИТЦ и РИТЦ. Расстояние между донором и акцептором составляло 8 нм, что в 2-2.5 раза превосходит размеры молекулы белка.
КЛЮЧЕВЫЕ СЛОВА: лизоцим, липидный билсой, агрегация, индуктивно-резонансный перенос энергии

During the last decade the phenomenon of protein aggregation attracts ever growing attention due to its involvement in the etiology of a number of the so-called conformational diseases, including Alzheimer's, Parkinson's, Huntington's diseases, type II diabetes, rheumatoid arthritis, spongiform encephalophaties (prion diseases) [1-3]. A good wealth of evidence provides strong grounds for believing that the formation of abnormal protein aggregates *in vivo* can be driven by destabilization of the protein structure upon its adsorption at interfaces, formed by cellular membranes [2]. Lipid bilayer, a basic structural element of biological membranes, provides a unique environment favoring the structural transformation of polypeptide chain into partially folded conformation, protein accumulation at lipid-water interface, screening of the protein surface charge, modifications in hydrogen bonding capability of the adsorbed molecules, aggregation-favoring orientation of the bound protein, the processes which can ultimately lead to the protein polymerization [2,4]. For these reasons increasingly growing efforts are currently focused on the accurate detection and characterization of lipid-assisted protein aggregation, since timely identification of oligomers

may help to prevent their conversion into pathogenic species. A great number of experimental techniques, including circular dichroism, atomic force, electron and fluorescence microscopy, Förster resonance energy transfer, electron paramagnetic and nuclear magnetic resonance, Fourier transform infrared spectroscopy, etc. are employed successfully to trace the formation of protein aggregates in a lipid environment [5-12]. Of these, Förster resonance energy transfer (FRET) represents one of the most promising analytical tools for detection and precise characterization of oligomeric species. The uniqueness of this spectroscopic technique lies in elegant combination of distance-dependent manner of radiation-less energy transfer between donor and acceptor dipoles with attractive advantages of fluorescence spectroscopy – high informativity, relative simplicity, non-invasive nature, potential for real-time in-vivo cellular applications, and experimental convenience. In the present study intra- and intermolecular FRET have been utilized to analyze the oligomerization of small cationic protein lysozyme (Lz) bound to the model lipid membranes composed of zwitterionic lipid phosphatidylcholine (PC) and anionic lipid phosphatidylglycerol (PG).

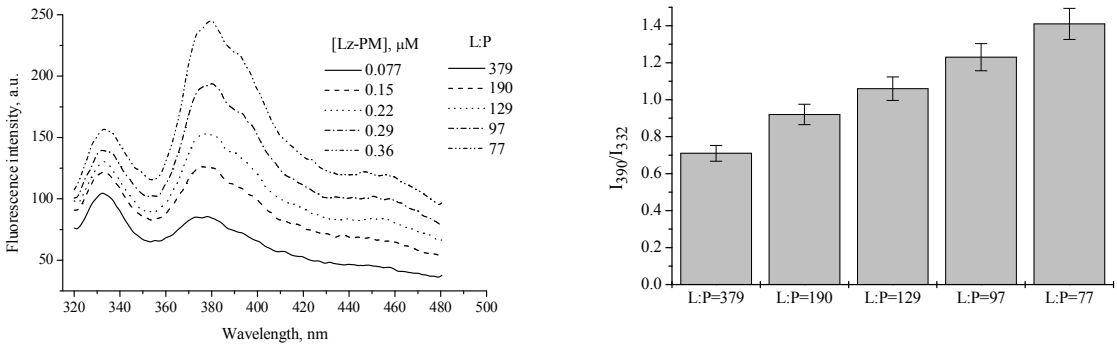
MATERIALS AND METHODS

Chicken egg white lysozyme, fluorescein 5'-isothiocyanate, rhodamine-isothiocyanate and pyrene-maleimide were purchased from Sigma (St. Louis, MO, USA). 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (PG) were from Avanti Polar Lipids (Alabaster, AL). Large unilamellar lipid vesicles composed of PC and its mixtures with PG in molar ratio 4:1 were prepared by the extrusion method [13]. Steady-state fluorescence spectra were recorded with LS-50B spectrofluorometer equipped with a magnetically stirred, thermostated cuvette holder (Perkin-Elmer Ltd., Beaconsfield, UK). Fluorescence measurements were performed at 25 °C using 10 mm path-length quartz cuvettes. Excitation wavelengths were 296, 340 and 490 nm for Lz, pyrene and fluorescein, respectively. Excitation and emission slit width were set at 5 nm.

RESULTS AND DISCUSSION

Intra- and intermolecular FRET have been employed to analyze lysozyme aggregation behavior in lipid environment. FRET is highly informative technique for detection of macromolecule aggregation since it can be used not only as quantitative, but also as qualitative method. Very often the fact of existence or absence of FRET or changes in energy transfer upon proper factors may be a hallmark of protein oligomerization [14].

The strategy used for analysis of aggregation of membrane-bound lysozyme by means of intramolecular FRET was based on fluorescent labeling of lysozyme cysteine residues by *N*-(1-pyrene)maleimide. Donor-acceptor pair was represented by tryptophan and pyrene. Fig. 1 represents fluorescence spectra of pyrene-labelled lysozyme (Lz-PM). As seen from this figure, the spectra are characterized by three bands which correspond to the emission of Trp ($\lambda_{max} = 332$ nm), pyrene monomers ($\lambda_{max} = 390$ nm) and excimers ($\lambda_{max} = 460$ nm). Quantitative analysis of FRET efficiency for this donor-acceptor pair seems impossible because intramolecular FRET interferes with intermolecular energy transfer. However, increasing concentration of Lz-PM is followed by the increase in the ratio of PM to Trp intensity (I_{390}/I_{332}) (Fig. 1, B).



A B

Fig. 1. Lz-PM emission spectra (A) and I_{390}/I_{332} ratio (B) at different L:P. Liposomes contain 20 mol% PG. Excitation wavelength was 296 nm. Lipid concentration was 28 μM. Experiments were done at ionic strength 20 mM and pH 7.4.

This effect most probably arises from the enhanced FRET between Trp and pyrene. The finding that the magnitude of this effect depends on protein concentration suggests that FRET enhancement is caused by the formation of protein aggregates. In contrast to I_{390}/I_{332}, E/M ratio was virtually unchanged at varying protein concentration. This finding suggests that pyrene excimerization is intramolecular process. According to the data available in literature, intermolecular excimers may adopt stacked or non-stacked configuration depending on protein conformation and its local environment (Fig. 2). If the loop, which separates pyrene monomers, is rather elastic, stacked conformation will prevail. Such conformation is stabilized by hydrophobic interactions between pyrene monomers in ground state.

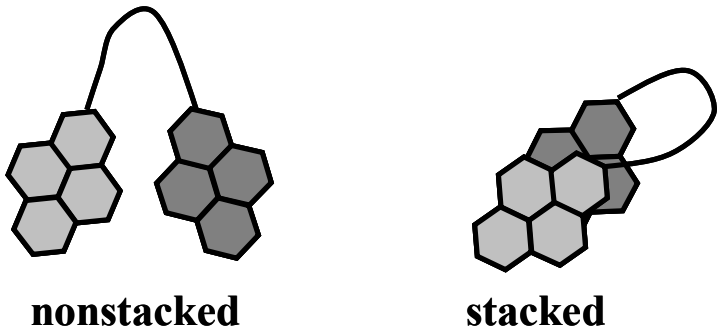


Fig. 2. Possible configurations of pyrene intramolecular excimers.

However, if the loop is represented by the elements of secondary and tertiary protein structure, which hamper the interactions between pyrene molecules, then the equilibrium would shift towards the nonstacked configuration. Spectroscopic detection of one or another configuration is possible by detecting existence/absence of excimer fluorescence – only excimers in stacked conformation emit. Appearance of excimer band in Lz-PM fluorescence spectra (Fig. 1, A) may be indicative of stacked configuration of fluorophore aggregates.

Analysis of aggregation state of membrane-bound lysozyme was done also using such donor-acceptor pairs as pyrene – fluorescein 5'-isothiocyanate (FITC) and FITC – rhodamine-isothiocyanate (RITC) which was covalently attached to lysozyme. While analyzing the obtained results quantitatively, it was assumed that donors and acceptors are distributed in one plane. As seen in Fig. 3, A, efficiency of energy transfer between pyrene- and FITC-labeled lysozyme was nearly unchanged at L:P ranging from 380 to 98. However, at L:P=77 significant increase in this parameter was observed.

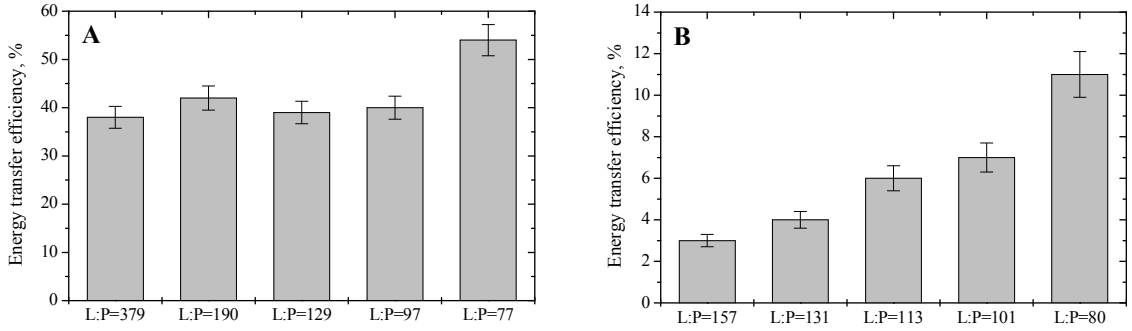


Fig. 3. Efficiency of energy transfer between pyrene and FITC (A) and FITC and RITC (B) at different values of of L:P. Lipid concentration was 46 μM. Liposomes contain 20 mol% PG.

Obviously, such FRET enhancement is the result of decrease in donor-acceptor separation distance due to the protein aggregation. This L:P value is critical for formation of Lz self-associates. The assumption that lysozyme forms oligomers in membrane environment is also corroborated by the quantitative analysis of RET between FITC and RITC (Fig. 3, B). The distance between FITC and RITC was found to be *ca.* 8 nm which exceeds the dimensions of Lz molecule by 2-2.5 times, lending additional support to the idea about Lz self-association in lipid surroundings (Fig. 4).

The results obtained strongly suggest that Lz aggregation is a coverage-dependent process occurring upon protein binding to lipid bilayer. Both electrostatic and hydrophobic protein-lipid interactions may favor the formation of Lz aggregates.

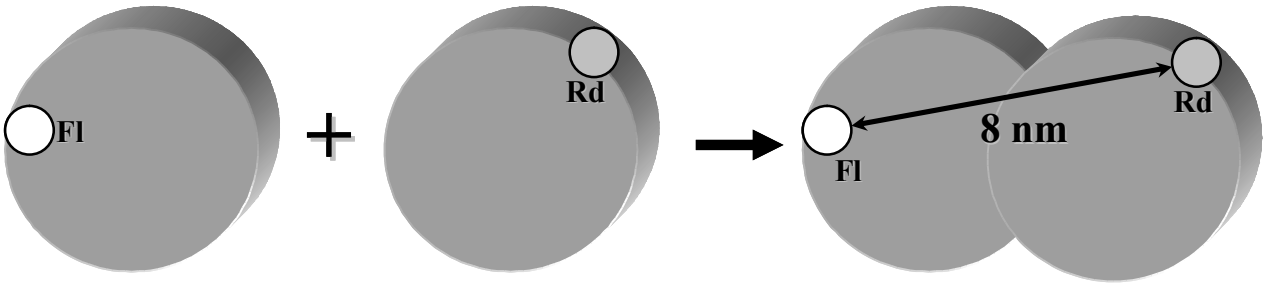


Fig. 4. Schematical representation of lysozyme aggregation as revealed by FRET between FITC and RITC, covalently attached to the protein. Lysozyme is depicted by grey disks.

Self-association of membrane-bound lysozyme is likely to involve protein conformational transitions, increase of its local concentration at lipid-water interface, partial neutralization of the protein surface charges by anionic lipid headgroups, and particular arrangement of the inserted and solvent exposed segments of the protein molecule. Besides these membrane determinants of lysozyme aggregation, it seems important to analyze also the following aspect. According to the theoretical predictions based on mean-field chain packing theory peptides containing amphipathic α-helices have stronger tendency to adsorb onto the membrane surface and thereafter to self-associate in membrane-bound state.

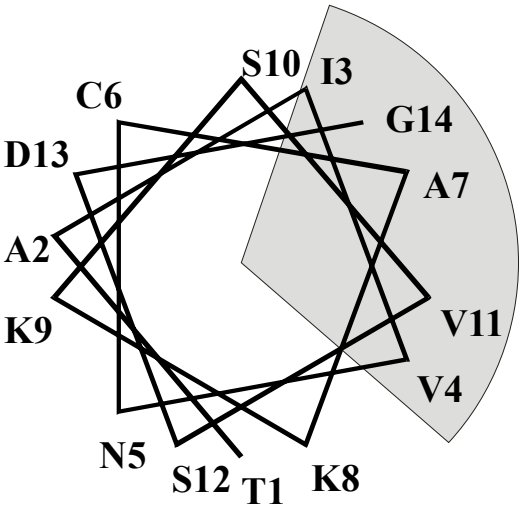


Fig. 5. Helical wheel diagram of lysozyme α-helix (residues Thr89-Gly102). Segregated hydrophobic residues in α-helical conformation are highlighted by grey shading. Radius of the unshaded sector subtends the protein polar angle.

Hydrophobic mismatch between membrane thickness and protein segment incorporated into bilayer is believed to lie behind this process. Analysis of lysozyme four α-helices showed that the helix including the residues Thr89-Gly102 is amphipathic (Fig. 5). Interestingly, this helix belongs not only to HLH domain which is responsible for protein-membrane association but also to protein fragment which has the highest aggregation propensity (residues 49-101). Therefore, amphipathy may also be among the most important factors which lead to the increase in aggregation properties of the protein in membrane environment.

CONCLUSIONS

Overall, the results presented here strongly suggest that Lz binding to the lipid membranes induces the formation of protein oligomers. The factors controlling Lz aggregation were found to involve surface electrostatic potential of lipid bilayer, coverage of membrane surface with protein molecules, and amphipathy of Lz α-helix. Importantly, Lz aggregation at lipid/water interface may prove essential for its assembly into amyloid-like fibrillar structures. Likewise, enhanced membrane permeabilization ability of the aggregated protein species may account for bactericidal properties of lysozyme.

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