БІОФІЗИКА КЛІТИНИ

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THIOFLAVIN T BEHAVIOR IN LYSOZYME – LIPID SYSTEMS

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The applicability of thioflavin T (ThT) to detection of amyloid-like aggregates formed in membrane environment was evaluated using lysozyme-lipid model system. It was found that ThT is capable of partitioning into lipid bilayers composed of zwitterionic (1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC)) and anionic (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG)) phospholipids. The ability of ThT to associate non-specifically with lysozyme in its native state was uncovered. These properties of ThT may impose limits on the use of this dye for identification of membrane-induced fibrillar structures.

KEY WORDS: amyloid fibrils, thioflavin T, liposomes, lysozyme

A number of so-called conformational diseases including neurological disorders (Parkinson's, Alzheimer's and Huntington's diseases), type II diabetes, spongiform encephalopathies, systemic amyloidosis, *etc*., are associated with the deposition in tissue of highly ordered aggregates of specific proteins [1]. These aggregates are composed largely of misfolded proteins polymerized into amyloid fibrils sharing a core cross-β-sheet structure, in which polypeptide chains are oriented in such a way that β-strands run perpendicularly to the long axis of the fibril, while β-sheets propagate in its direction [2,3]. Despite extensive research efforts, the mechanisms of fibrillogenesis remain obscure. Accumulating evidence substantiates the idea that formation of fibrillar structures can be initiated and modulated by protein-lipid interactions [4,5]. Membrane-related prerequisites for fibrillization are supposed to include conformational changes of the protein, increase of its local concentration at lipid-water interface, specific orientation of aggregating species, neutralization of the protein surface charges by anionic lipid headgroups, particular arrangement of the inserted and solvent exposed segments of the protein molecule, *etc*. While examining lipid-induced fibrillogenesis it is important to correctly differentiate between amyloid fibrils and other types of protein or protein-lipid aggregates. One widespread criterion for amyloid fibril identification both *in vivo* and *in vitro* is based on the property of benzothiazole fluorescent dye thioflavin T (ThT) to form complexes with fibrillar structures [6]. Despite indisputable advantages of this criterion, application of ThT to amyloid detection in protein-lipid systems may be hampered by the dye lipid-associating ability. In view of this the present study was undertaken to assess the magnitude of such limitation. Using fluorescence spectroscopy technique we explored ThT spectral properties in the model systems whose protein and lipid components were represented by lysozyme and SOPC:POPG lipid vesicles, respectively. The choice of experimental system was dictated by the recently reported lysozyme property to form amyloid-like fibers in membrane environment [7].

MATERIALS AND METHODS

Chicken egg white lysozyme and thioflavin T were from Sigma (St. Louis, MO, USA). SOPC and POPG were from Avanti Polar Lipids (Alabaster, AL). Large unilamellar lipid vesicles composed of SOPC and its mixture with 20 mol% POPG were prepared by the extrusion method [8]. The thin lipid films were obtained by evaporation of lipids' ethanol solutions and then hydrated with 1.2 ml of 20 mM HEPES (pH 7.4). Lipid suspension was extruded through a 100 nm pore size polycarbonate filter. Phospholipid concentration was determined according to the procedure of Bartlett [9]. Fluorescence measurements were performed at 20 °C with PerkinElmer Life Sciences LS50B spectrofluorometer. ThT emission spectra were excited at 440 nm. Excitation and emission slit widths were set at 10 nm. Analysis of lysozyme crystal structure (Protein Databank entry 1HEW) was performed by means of WebLab ViewerPro Trial37 software.

RESULTS AND DISCUSSION

Binding of ThT to β-sheets, abundant in amyloid fibrils, is followed by characteristic 120 nm red shift of its excitation spectrum up to 450 nm, resulting in a fluorescence maximum at 482 nm. This unique property makes ThT an amyloid-specific probe [10]. One explanation for such specificity of ThT involves its peculiar location in amyloids. It has been proposed that ThT binds to fibrils via insertion into channels formed between every other row of side chains, with the dye long axis being parallel to the fibril axis (Fig. 1) [6]. This results in significant restriction of the dye mobility, and, as a consequence, enhancement of its fluorescence by the orders of magnitude. Since the channels running perpendicularly to the strands are characteristic of all β-sheets, any protein having β-sheets in its native state contains binding sites for ThT [11]. Yet, interaction of ThT with native β-sheets seems to be non-specific because the binding channels are commonly shorter, distorted and irregular.

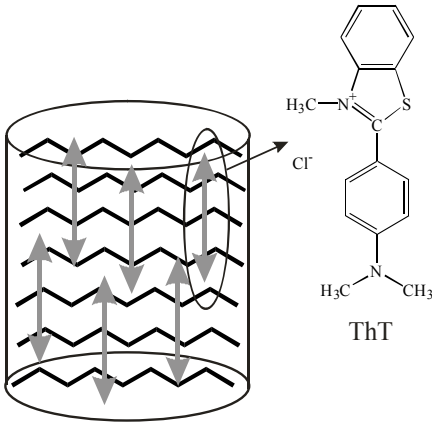
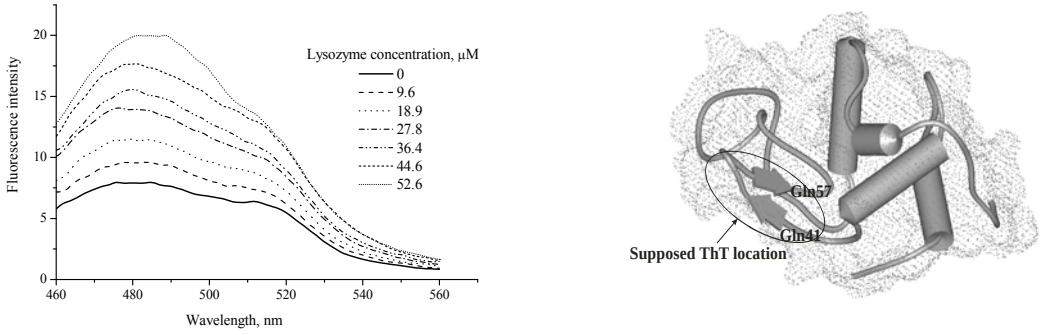


Fig. 1. Schematic representation of ThT location in amyloid filament [6]. Cylinder represents the plaque, each β-strand is depicted as black zig-zag lines. For the clarity β-strand organization into β-sheets is not drawn. ThT molecules are shown as grey double-headed arrows. The inset is the chemical structure of ThT.

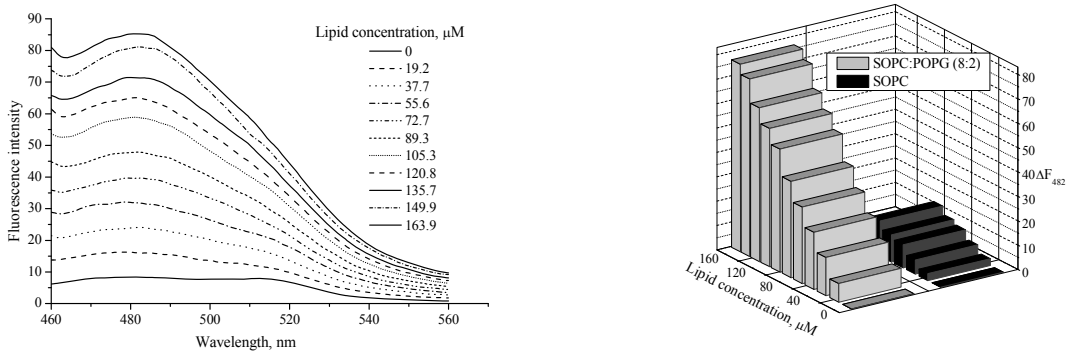
Analysis of lysozyme crystal structure revealed the existence of β-sheet-region in the protein native state. In keeping with this fact, we observed enhancement of ThT fluorescence on the dye titration with lysozyme (Fig. 2, A). The β-sheet segment of lysozyme embodies 16 amino acid residues (from Gln41 to Gln57) located near the protein active site (Fig. 2, B). It is tempting to suppose that this β-sheet-region located at the surface of protein molecule accounts for ThT complexation with lysozyme. This assumption is confirmed by the absence of the shift of ThT emission maximum upon protein binding (Fig. 2, A). Solvent exposed location of lysozyme β-sheets provides their accessibility for ThT and results in the invariability of the dye emission maximum.



A B

Fig. 2. ThT fluorescence spectra in lysozyme solution (A), and schematic representation of lysozyme molecule and putative location of ThT (B). Helical structures are represented as wide cylinders, β-sheets as arrows pointing in the direction of the C-terminus, and irregular structures are shown as tubes.

Next, it seemed of interest to ascertain whether ThT would display non-specific partitioning into lipid phase. As seen in Fig. 3, A, in the presence of liposomes ThT fluorescence intensity markedly increases suggesting that the dye is capable of associating with the model membranes.



A B

Fig. 3. ThT emission spectra in SOPC:POPG (8:2) lipid vesicles (A) and increase in ThT fluorescence intensity in the suspension of SOPC and SOPC:POPG (8:2) liposomes (B). The concentration of ThT was 8.2 μM.

The observation that fluorescence maximum remains virtually unchanged upon increasing lipid-to-dye molar ratio can be explained by the dye inability to embed deeply into hydrophobic region of the lipid bilayer. Nevertheless, hydrophobic interactions are likely to contribute to ThT association with model membranes because fluorescence increase was observed not only for charged, but also for neutral (SOPC) lipid vesicles (Fig. 3, B).

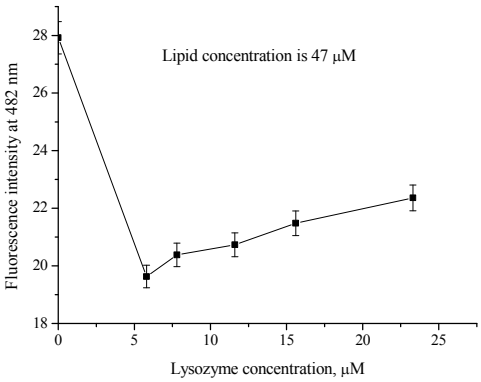


Fig. 4. Changes in fluorescence intensity of ThT incorporated into SOPC:POPG liposomes at varying lysozyme concentration.

At the next step of the study we examined ThT behavior in the system liposomes + lysozyme. As shown in Fig. 4, at minimum employed concentration of lysozyme (lipid-to-protein molar ratio (*L/P*) about 9) ThT fluorescence decreases by *ca*. 30 %. This effect is most likely to arise from the competition between ThT and lysozyme for the membrane binding sites containing anionic phospholipids. Further slight increase of fluorescence intensity may be a consequence of ThT binding to lysozyme free in solution, because at $L/P < 9$ membrane surface is expected to be fully covered with the protein. Note that theoretical saturation coverage of the liposome corresponds to *L/P* ratio *ca*. 40, or $L_a/P \sim 20$ (L_a is the concentration of lipids in outer monolayer accessible to lysozyme binding), with the protein cross-section and mean area phospholipid headgroup taken as 13.5 nm^2 and 0.65 nm^2, respectively.

CONCLUSIONS

The use of ThT for detection of fibrillar aggregates in lysozyme-lipid systems is limited by the dye capacity for non-specific association with both liposomes and lysozyme containing β-sheet structures in its native state. It was found that ThT is capable of partitioning into neutral and negatively charged lipid bilayers. Lysozyme, whose charge at neutral pH is *ca*. +8, can compete with ThT for the membrane binding sites. These properties of ThT must be taken into account while monitoring the process of fibrillogenesis in protein-lipid systems where proportion of amyloid aggregates can be relatively low and incorrect interpretation of the dye spectral responses may lead to false positive result.

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