распределения длины петельных доменов, размером до 100 тысяч пар нуклеотидов, согласуются со структурой фрактальной глобулы. Таким образом, такая модель организации хроматина является справедливой также и для меньших масштабов – на уровне нескольких тысяч пар нуклеотидов.

Ключевые слова: фрактальная глобула, петельне домены ДНК, кометный электрофорез.

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ASSOCIATION OF NOVEL BENZANTHRONE DYES WITH AMYLOID FIBRILS: A RESONANCE ENERGY TRANSFER STUDY

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Quantitative analysis of resonance energy transfer between amyloid-specific fluorophore Thioflavin T and novel benzanthrone dyes showed that the binding sites of fibrillar lysozyme, insulin and apolipoprotein A-I variants for the novel probes are represented by the fibril grooves separated from those of Thioflavin T by the distances falling in the range 2.1–6.2 nm. It is demonstrated that RET technique may prove suitable for both detection and characterization of amyloid fibrils.

Keywords: amyloid fibrils, benzanthrone dyes, resonance energy transfer, Thioflavin T.

Amyloid fibrils are protein aggregates sharing a common cross-β conformation, in which β-sheets are parallel to fibril axis, with β-strands running perpendicular to this axis. In view of the well recognized pathogenic and functional role of this kind of protein aggregates, there exists a

necessity of developing the powerful techniques for detection, characterization and controlling of amyloid fibril formation [9]. Förster resonance energy transfer (RET) has been successfully employed to study protein structural transformations due to its high sensitivity to the distance between donor and acceptor [10]. Specifically, RET between intrinsic (Trp) and extrinsic (fluorescent dyes) fluorophores has been used to selectively identify the aggregates from α-synuclein [11], determine the degree of Aβ fibrillization [6], oligomerization [14] and morphological differences in fibril structure [13], the inter-peptide arrangement of fibrillar transthyretin [3], etc. In general, RET analysis of protein fibrillization offers significant advantages over common Thioflavin T (ThT) assay, since it allows deeper structural characterization of amyloid fibrils. In the present study RET between ThT as a donor and novel benzanthrone dyes as acceptors [7, 15] was employed to obtain information on the fibrillization of lysozyme, insulin and apolipoprotein A-I (apoA-I) variants.

Materials and methods

Hen egg white lysozyme (Lz), bovine insulin (Ins) and Thioflavin T were purchased from Sigma. Single Trp variants of human apolipoprotein A-I 1-83 fragment and benzanthrone dyes (BD) (Fig. 1, A) have been recently synthesized by Adachi et al. and Kirilova et al., respectively [7, 1]. Amyloid fibrils of lysozyme were prepared by the protein incubation at 60°C, pH 2 for two weeks. Fibrillization of insulin (apoA-I variants) was induced by continuous shaking of the protein solution at 37°C, pH 2 (7.4) for 10 (18) days. The amyloid nature of the resulting aggregates was confirmed by ThT assay. Fluorescence spectra of ThT were recorded at CM-2203 spectrofluorimeter with excitation and emission wavelengths 420 and 482 nm, respectively. The efficiency (E) of RET from ThT to BD was estimated by measuring the extent of donor quenching upon addition of acceptors (Fig. 1, B). Emission spectra of fibril-bound ThT in the presence of BD were corrected for dilution and inner filter effect, and the calculations of the E were performed at the point, where ThT fluorescence intensity reached its minimum value (at the concentrations of donor (D), acceptor (A) and protein given in Table 1). The evaluation of Förster radii and orientation factor (κ^2) limits was performed to obtain the range of possible D-A distances (R_{min}, R_{max}), according to approach described in [10].

Results and discussion

As seen in Fig. 2, A, the energy transfer between ThT bound to Lz fibrils and BD occurs with lower efficiency than that of ThT associated with Ins fibrils, pointing to larger D-A distances in lysozyme fibrils (Table 1). Notably, ThT-IAH, ThT-IBH and ThT-ISH D-A pairs turned out to be more appropriate for RET studies than ThT-A6, ThT-ABM and ThT-A8 pairs, due to more pronounced separation between the D and A excitation spectra [10]. Thus, taking advantage of the former probes, we further estimated E and R values for these dyes in the presence of fibrillar apoA-I

variant, 1-83G26R/W@8 (M3). It appeared that the binding sites for BD at M3 fibrils reside at the closest distances from ThT, while maximum separation between BD and ThT is observed in Lz fibrils (Table 1).

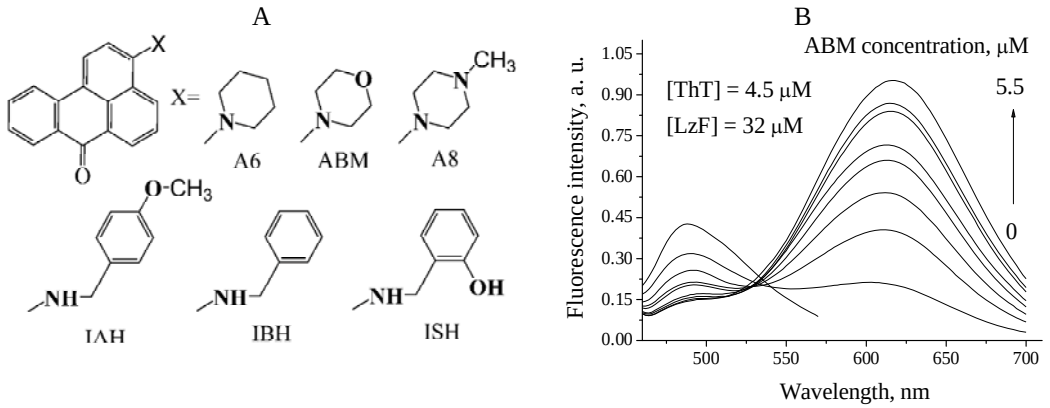

Fig.1. Structural formulas of benzanthrone dyes (**A**) and emission spectra of lysozyme fibril-bound ThT (left fluorescence band) and ABM (right fluorescence band) recorded in the presence of increasing concentrations of ABM (**B**).

Assuming that BD, similarly to ThT, are located in the grooves abundant in fibril structure, the recovered distances 2.1–6.2 nm turned out to correspond to the width of ca. ~3–9 grooves (table 1). Two lines of evidence support this hypothesis: i) BD display strong affinity for amyloid aggregates indicative of specific fibril binding centers for these probes [15]; ii) the length of the core β-strands in fibrillar Lz [4], Ins [8] and M3 [5] was found to be ca. ~7, 5.6 and 5–10 nm, respectively, allowing the large separation between the different fibril grooves in a single β-sheet. Furthermore, parallel in-register arrangement of the β-strands of each β-sheet predominantly observed in amyloid fibrils, eliminates the possibility of resonance energy transfer between D and A from the adjacent laterally associated β-sheets of the "wet" steric zipper interface, which should result in much lower D-A distances [12]. Notably, competitive binding between ThT and BD is unlikely to occur, since it should lead to very low R values [13]. Additionally, the observed wide range of D-A separations may arise from the different binding modes and heterogeneity of BD binding sites, which can be further specified using, e.g., molecular docking approach. Taking into account the preference of ThT for the fibril grooves involving aromatic amino acid residues [6], it can be supposed that the fluorophore is associated with the grooves formed by the side chains of the following solvent-exposed residues: T51_Y53_I55 (Lz), A14_Y16_V18 (insulin B chain), T16_Y18 (M3) [5].

Table 1

Quantitative parameters of resonance energy transfer between Thioflavin T and benzanthrone dyes bound to fibrillar lysozyme, insulin and G26R/W@8 variant of apolipoprotein A-I 1-83 fragment

Lysozyme (insulin) fibrils								
Dye	[Dye], μM	[ThT], μM	[Protein], μM	E, %	R_0, nm κ^2=0.67	R, nm κ^2=0.67	R_{min}, nm	R_{max}, nm
A6	2.5	3.4	1.1 (9.5)	11 (30)	3.4 (3.5)	4.8 (4)	3.5 (3.1)	6.2 (5.3)
ABM	5.5 (12)	4.5 (3.4)	32 (9.5)	65 (78)	3.3 (3.4)	3 (2.8)	2.3 (2.1)	3.9 (3.6)
A8	2.5	3.4	1.1 (9.5)	26 (62)	3 (3.1)	3.6 (2.9)	2.6 (2.1)	4.5 (3.7)
IAH	2.5 (4.9)	3.4	1.1 (9.5)	42 (58)	3.5 (3.8)	3.7 (3.6)	2.7 (2.7)	4.7 (4.6)
IBH	4.8	3.4	1.1 (9.5)	35 (69)	3.6 (3.7)	4 (3.2)	3 (2.5)	5.1 (4.2)
ISH	12 (9.6)	3.4	1.1 (9.5)	68 (79)	3.8 (3.9)	3.4 (3.1)	2.5 (2.3)	4.3 (4.1)
M3 (G26R/W@8) fibrils								
IAH	2.3	3.1	0.6	73	3.5	3.0	2.2	3.8
IBH	2.3	3.1	0.6	82	3.8	3.0	2.2	3.8
ISH	4.9	4.7	0.5	84	3.8	2.9	2.2	3.7

The above part of the insulin β-strand is the only one, containing aromatic residues, while in lysozyme negatively charged D52 and aromatic W62 residues can also be responsible for ThT binding. However, the side chains of the latter residues may locate on the “dry” steric zipper interface of the β-sheet, which does not bind ThT due to steric restrictions [2]. Based on these considerations and the distance estimates presented in Table 1, BD can be assumed to associate with the grooves formed by the core residues Asn59_R61_W63_N65_G67_T69 (Lz), Q4_L6_G8_H10_V12 (insulin B chain) and L22_D24_R26_D28_V30 (M3).

It is noteworthy that RET between ThT and BD is supposed to occur only in the presence of fibrillar aggregates, while in monomeric protein there are no prerequisites for energy transfer as was demonstrated, particularly, for Lz [15]. As a result, this technique can be used for the quantitative analysis of amyloid fibril formation *in vitro* [11, 13]. Inspired by such unrivalled potential of RET, we preformed ThT titration with IAH and IBH in the presence of different variants of apoA-I, referred to here as 1-83G26R/W@50 (M1), 1-83G26R/W@72 (M2), 1-83G26R (G26) and 1-83 fragment of apo A-I (A83) (fig. 2, B). The observed E values appear to correlate positively with the ThT fluorescence response, i.e. with the extent of protein fibrillization. Among the above apoA-I mutants, M1 and A83 did not formed fibrillar aggregates, while G26 had different fibril morphology, compared to that of M3. Accordingly, no

energy transfer was observed between D-A pairs incorporated into M1 and A83, while about twofold lower efficiency and larger distance constrains were found for M2 and G26 compared to those of M3. In conclusion, the present study demonstrated that RET between ThT and BD can be employed for: i) identifying fibril binding sites for the novel probes, ii) amyloid fibril detection and structural characterization. This work was supported by the grant from Fundamental Research State Fund of Ukraine (project number F.54.4/015) and CIMO Fellowship (KV).

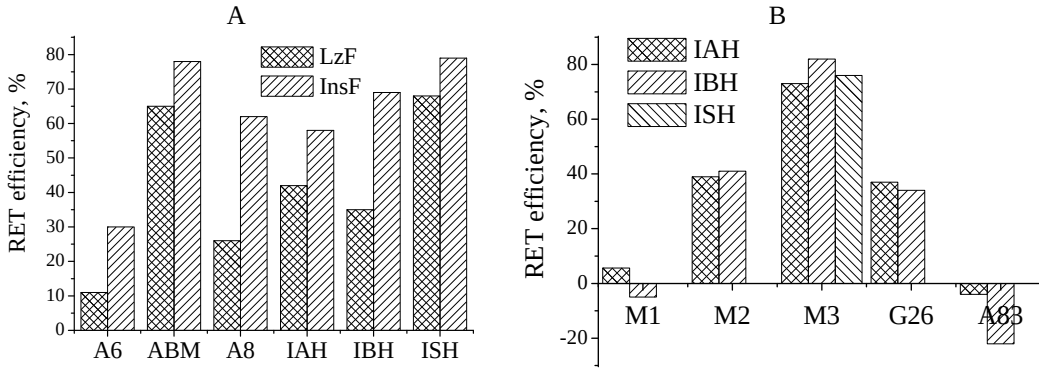

Fig. 2. The efficiency of resonance energy transfer between ThT and benzanthrone dyes bound to lysozyme (insulin) fibrils (**A**) or fibrillar variants of apolipoprotein A-I (**B**).

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АСОЦІАЦІЯ НОВИХ БЕНЗАНТРОНОВИХ ЗОНДІВ З АМІЛОЇДНИМИ ФІБРИЛАМИ: ДОСЛІДЖЕННЯ МЕТОДОМ РЕЗОНАНСНОГО ПЕРЕНОСУ ЕНЕРГІЇ

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Аналіз резонансного переносу енергії між амілоїд-специфічним барвником Тіофлавіном Т і новими бензантроновими зондами показав, що сайти зв'язування фібрилярних лізоциму, інсуліну та варіантів апоА-І для бензантронів представлені жолобками, віддаленими від жолобків Тіофлавіну Т на 2,1–6,2 нм. Продемонстровано, що метод переносу енергії придатний для детектування амілоїдних фібрил і визначення їх структури.

Ключові слова: амілоїдні фібрили, бензантронові зонди, резонансний перенос енергії, Тіофлавін Т.

АССОЦИАЦИЯ НОВЫХ БЕНЗАНТРОНОВЫХ ЗОНДОВ С АМИЛОИДНЫМИ ФИБРИЛЛАМИ: ИЗУЧЕНИЕ МЕТОДОМ РЕЗОНАНСНОГО ПЕРЕНОСА ЭНЕРГИИ

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Анализ резонансного переноса энергии между амилод-специфическим красителем Тиофлавином Т и новими бензантроновыми зондами показал, что сайты связывания фибриллярных лизоцима, инсулина и вариантов апоА-I для бензантронов представлены желобками, отдаленными от желобков Тиофлавина Т на 2,1–6,2 нм. Продемонстрировано, что метод переноса энергии пригоден для детектирования амилоидных фибрилл и определения их структуры.

Ключевые слова: амилоидные фибриллы, бензантроновые зонды, резонансный перенос энергии, Тиофлавин Т.