THE JOURNAL OF PHYSICAL CHEMISTRY B

ARTICLE

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Interaction of Thioflavin T with Amyloid Fibrils: Stoichiometry and Affinity of Dye Binding, Absorption Spectra of Bound Dye

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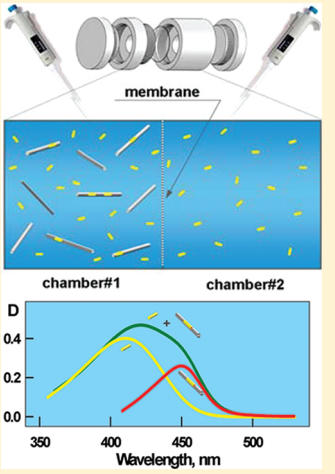
ABSTRACT: The fluorescence of the benzothiazole dye thioflavin T (ThT) is a well-known test for amyloid fibril formation. It has now become evident that ThT can also be used for structural investigations of amyloid fibrils and even for the treatment of amyloid diseases. In this case, one of the most urgent problems is an accurate determination of ThT−amyloid fibril binding parameters: the number of binding modes, stoichiometry, and binding constant for each mode. To obtain information concerning the ThT−amyloid fibril binding parameters, we propose to use absorption spectrophotometry of solutions prepared by equilibrium microdialysis. This approach is inherently designed for the determination of dye−receptor binding parameters. However, it has been very rarely used in the study of dye−protein interactions and has never been used to study the binding parameters of ThT or its analogues to amyloid fibrils. We showed that, when done in corpore, this approach enables the determination of not only binding parameters but also the absorption spectrum and molar extinction coefficient of ThT bound to sites of different binding modes. The proposed approach was used for the examination of lysozyme amyloid fibrils. Two binding modes were found for the ThT−lysozyme amyloid fibril interaction. These binding modes have significantly different binding constants (K_{b1} = 7.5 × 10^6 M^{-1}, K_{b2} = 5.6 × 10^4 M^{-1}) and a different number of dye binding sites on the amyloid fibrils per protein molecule (n_1 = 0.11, n_2 = 0.24). The absorption spectra of ThT bound to sites of different modes differ from each other ($\varepsilon_{b1,max}$ = 5.1 × 10^4 M^{-1} cm^{-1}, $\varepsilon_{b2,max}$ = 6.7 × 10^4 M^{-1} cm^{-1}, λ_{max} = 449 nm) and significantly differ from that of free ThT in aqueous solution (ε_{max} = 3.2 × 10^4 M^{-1} cm^{-1}, λ_{max} = 412 nm).



INTRODUCTION

Fluorescent dye thioflavin T (ThT, Figure 1) is a common tool for diagnostics of amyloid fibril formation in diseases associated with disturbances in protein folding, such as Alzheimer's and Parkinson's diseases, type II diabetes, and prion diseases.[1] This approach is based on the unique property of ThT to form highly fluorescent complexes with amyloid and amyloid-like fibrils.[2−4] Other than with acetylcholinesterase[5] and serum albumins,[6] ThT does not interact with globular proteins in a native state. It also does not interact with molten globule and unfolded states or amorphous aggregates of proteins. The study of the spectral properties of ThT[7] and the dependence of its fluorescence quantum yield on solution temperature and viscosity,[8−10] together with quantum-chemical calculations of the dye molecule in the ground and excited states,[11] have led us to believe that a significant increase in the fluorescence quantum yield when the dye is incorporated into amyloid fibrils is caused by a restriction of the rotational motion of ThT fragments relative to each other in the excited state.[8−10] The Krebs model of ThT binding to amyloid fibrils[12] suggests that the dye inserts itself into the grooves formed by side chains of amino acids forming β-sheets.

Investigations of amyloid fibrils formed by different fibril-forming proteins (globular proteins, intrinsically unstructured, rich in β-sheet, α-helix, or containing both β-sheets and α-helixes)

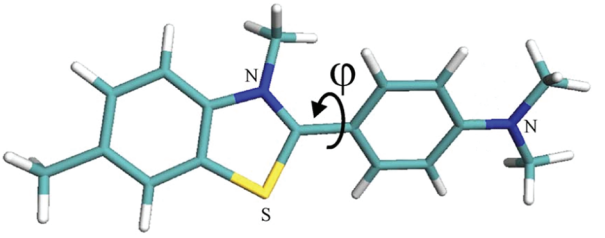


Figure 1. Thioflavin T molecule.

showed great similarity in their structures. All of them contain a cross-β spine with β-strands perpendicular to the fibril axis.[13,14] At the same time, later investigations showed that amyloid fibrils formed by different proteins or even one protein under different conditions are not identical.[15,16] Thus, the spectral characteristics of ThT bound to amyloid fibrils formed by different proteins and ThT bound to different binding modes may also be different.[17−19] Given this effect, an urgent problem is the examination of dye binding parameters and the characterization of binding sites. In addition, the determination of the ThT−amyloid fibril binding

Received: July 26, 2011
Revised: August 23, 2011
Published: August 24, 2011

parameters is utterly important in connection with new data about the therapeutic effect of ThT.[20,21] Nearly all of the research on ThT−amyloid fibril interaction binding parameters has been based on the assumptions that the fluorescence intensity is proportional to the concentration of bound dye and that the plateau of the dependence of fluorescence intensity on the dye concentration is caused solely by the occupation of all binding sites. We have shown that both assumptions are not true and that, in principle, fluorescence alone cannot be used for the determination of dye−receptor binding parameters. To obtain information concerning ThT−amyloid fibril binding stoichiometry and binding constants, we propose to use absorption spectrophotometry of solutions prepared by equilibrium microdialysis: a method that is inherently designed for the determination of dye−receptor binding stoichiometry and binding constants.[22−26] In the present work, this approach was used for the examination of lysozyme amyloid fibrils.

EXPERIMENTAL METHODS

The samples of ThT from Sigma (USA) and Fluka (Switzerland) were used after purification by crystallization from a mixture of acetonitrile with ethanol in the ratio 3:1.[1] The samples of ThT "UltraPure Grade" from AnaSpec (USA) were used without further purification. ThT was dissolved in 2 mM Tris−HCl buffer (pH 7.7) with 150 mM NaCl. The samples of lysozyme and buffer components from Sigma (USA) were used without further purification. Lysozyme amyloid fibrils were prepared as described earlier.[27] Absorption spectra were recorded by a U-3900H spectrophotometer (Hitachi, Japan). Fluorescence measurements were done by a homemade spectrofluorimeter[4] and a Cary Eclipse spectrofluorimeter (Varian, Australia). Fluorescence was excited at the wavelength 435 nm. Equilibrium microdialysis was done with a Harvard Apparatus/Amika (USA) device.[28] It consists of two chambers (500 μL each) separated by membrane (MWCO 10 000) that is impermeable to particles larger than 10 000 Da.

RESULTS AND DISCUSSION

In the case that ThT binding sites in amyloid fibrils are identical and independent of each other, the binding constant of the dye to amyloid fibrils (K_b) is determined as follows:

$$K_b = \frac{C_b}{(nC_p - C_b)C_f} \quad (1)$$

where C_f is the concentration of free ligand; C_b is the concentration of the dye bound to fibrils, that is, the concentration of occupied binding sites; C_p is the concentration of the protein that forms fibrils; and n is the number of sites where ThT binds to amyloid fibrils per protein molecule. Thus, nC_p is the total concentration of the binding sites and $(nC_p - C_b)$ is the concentration of free binding sites. We want to pay attention to the fact that the value of n can be less than unity because several protein molecules can participate in the formation of one binding site. This means that the dependence of bound dye concentration on the concentration of free dye in solution is a saturation curve as follows:

$$C_b = \frac{nC_pC_f}{K_d + C_f} \quad (2)$$

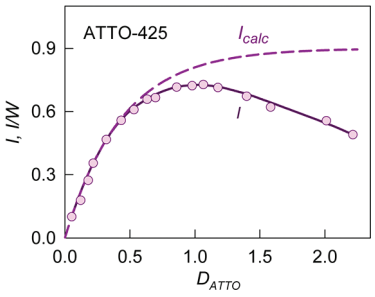


Figure 2. The dependence of fluorescence intensity on optical density of the fluorophore and on total optical density of the solution. The dependences of fluorescence intensity on the optical density (D_{ATTO}) of the fluorescence dye ATTO-425 with a known quantum yield ($q = 0.9$), calculated as $I_{calc} = (1 - 10^{-D})q$ and experimentally recorded I. Fluorescence measurements were conducted using a homemade spectrofluorimeter.[39]

where K_d is the dissociation constant ($K_d = 1/K_b$). This relation can be presented in two equivalent forms known as a Klotz plot:

$$\frac{1}{C_b} = \frac{1}{nC_p} + \frac{K_d}{nC_p}\frac{1}{C_f} \quad (3)$$

and a Scatchard plot:

$$\left(\frac{C_b}{C_p}\right)/C_f = nK_b - K_b\left(\frac{C_b}{C_p}\right) \quad (4)$$

Nearly all existing data on the parameters of ThT binding to amyloid fibrils have been based on the assumptions that the recorded fluorescence intensity is proportional to the concentration of bound dye ($I = kC_b$) and that the dependence of fluorescence intensity on C_b reaches a plateau when all binding sites are occupied ($I_{max} = knC_p$).[1,29,30] Both of these assumptions are not true. Fluorescence intensity of any fluorophore is connected with solution optical density D ($D = \varepsilon Cl$, where ε is the molar extinction coefficient, C is fluorophore concentration, and l is the length of the optical pathway) as follows:

$$I_{calc} = kI_0(1 - 10^{-D})q = \begin{cases} 2.303k\, I_0Dq & \text{at } D \to 0 \\ kI_0q & \text{at } D \to \infty \end{cases} \quad (5)$$

where I_0 is the intensity of the excitation light, k is the proportionality coefficient, and q is the fluorescence quantum yield of fluorophore. The dependence of I_{calc} on D is the curve with saturation, and it significantly differs from linearity even at small values of D (Figure 2). In reality, the dependence of fluorescence intensity on the optical density of the fluorescent substance (I) differs from the calculated one (I_{calc}) because an increase in the total optical density of the solution results in an increased absorption of excitation light by the solution layers adjacent to the front wall of the spectrofluorometer cell, whereas the detection system of the spectrofluorometer "sees" only the central part of the cell, which is reached by a respectively smaller amount of excitation light. Because of this effect, the recorded fluorescence intensity begins to diminish after optical density reaches a certain value (Figure 2).

Thus, the experiments based on fluorescence intensity measurements, in principle, cannot provide information about the concentrations of bound and, consequently, free dye. Nonetheless, for the determination of binding constants, researchers

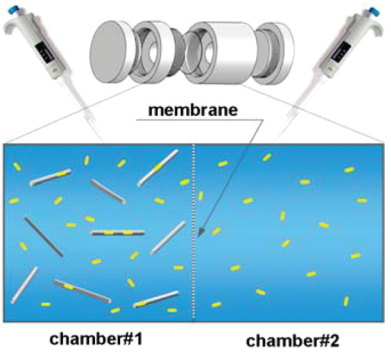


Figure 3. Principle of microdialysis experiment. Details are in the text.

have used eqs 2 or 3, replacing the value of free dye concentration with the value of introduced (total) dye concentration without any explanation of the change (see refs 3, 29, and 31−33). Only in the supplement to the work by Sutharsan et al. was this problem mentioned, though not solved.[33] We believe that the replacement of the concentration of free dye with the concentration of total dye in eqs 2 and 3 contradicts the physical meaning of the current task.

To overcome the difficulties, which cannot be solved by fluorescence, and to obtain information concerning ThT−amyloid fibril binding parameters, we propose to use absorption spectrophotometry of solutions prepared by equilibrium microdialysis. This approach is inherently designed for the determination of dye−receptor binding parameters.[22−26] However, it has been very rarely used in the study of dye−protein interactions (and even then, not all of its opportunities were used) and has never been used to study the binding parameters of ThT or its analogues to amyloid fibrils. We have shown that, when done in corpore, this approach enables us to determine not only the binding parameters but also the absorption spectrum and molar extinction coefficient of ThT bound to sites of different binding modes.

Preparation of Sample and Reference Solutions for Determination of the Concentrations of Free and Bound Dye at Equilibrium by Absorption Spectrophotometry. Equilibrium microdialysis implies allocation of two interacting agents, a ligand and receptor, in two chambers (2 and 1, respectively) divided by a membrane permeable to the ligand and impermeable to the receptor (Figure 3). In our case, the amyloid fibrils in the buffer solution were placed in chamber 1 and the ThT solution in the same buffer, with an initial concentration C_0, was placed in chamber 2. After equilibration, the ThT concentrations in chambers 1 and 2 become equal (C_f), while the total ThT concentration in chamber 1 is greater than that in chamber 2 by the concentration of the bound dye (C_b). Thus, taking into account that chambers 1 and 2 have identical volumes:

$$C_b = C_0 - 2C_f \quad (6)$$

The initial concentration of ThT in chamber 2 (C_0) and the dye concentration in this chamber after equilibration (C_f), which equals the concentration of free dye in chamber 1, can be determined by absorption spectrophotometry. The concentration of the dye bound to fibrils (chamber 1) can be determined on the basis of eq 6, and, consequently, the parameters of ThT binding to amyloid fibrils can be evaluated. Furthermore, the use of equilibrium microdialysis also allows for the measurement of spectral characteristics of the dye incorporated into amyloid fibrils.

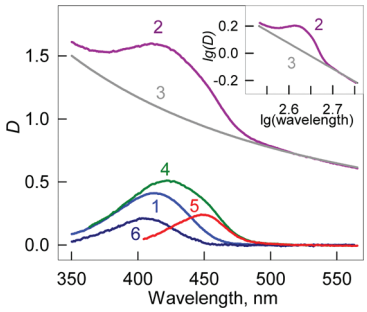


Figure 4. Absorption spectra of thioflavin T (ThT) incorporated in lysozyme amyloid fibrils. Curves 1 and 2 represent the absorption spectra of ThT in chamber 2 (free ThT in concentration C_f) and in chamber 1 (superposition of absorption spectra of free ThT in concentration C_f, ThT bound to fibrils in concentration C_b, and apparent absorption caused by fibril light scattering) after reaching equilibrium. Curve 3 is the optical density determined by fibril light scattering: $D_{scat} = a\lambda^{-m}$. Coefficients a and m were determined from the linear part of curve 2 (where there is no active dye absorption) plotted in logarithmical coordinates $\log(D_{scat}) = f(\log(\lambda))$, inset, curve 3. Curve 4 is the total absorption of free and bound dye after light-scattering subtraction $(D(\lambda)_1 - D_{scat})$. Curve 5 is the absorption spectra of ThT incorporated in amyloid fibrils $D_b(\lambda) = D(\lambda)_1 - D(\lambda)_{scat} - D(\lambda)_2$ (the difference between 4 and 1 spectra). Curve 6 is the absorption spectra of free dye in a concentration equal to that of bound dye $(D(\lambda)_0 - 2D(\lambda)_2)$.

Absorption Spectrum of ThT Bound to Amyloid Fibrils. At equilibrium, the absorption spectrum of the solution in chamber 2 represents the absorption spectrum of free ThT at a concentration of $C_f(D_f(\lambda))$, and the absorption spectrum of the solution in chamber 1 represents the superposition of the absorption spectra of free ThT at a concentration of $C_f(D_f(\lambda))$, ThT bound to fibrils at a concentration of $C_b(D_b(\lambda))$, and the apparent absorption determined by light scattered by the fibrils ($D_{scat}(\lambda)$). The contribution of light scattering can be eliminated as described earlier.[7] Thus, in chambers 1 and 2, we have a sample and optimal reference solutions for the determination of the absorption spectrum of ThT bound to fibrils. The analysis of these spectra (Figure 4) shows that the absorption spectra of ThT incorporated into lysozyme amyloid fibrils (λ_{max} = 449 nm) are red-shifted in comparison to that of free ThT (λ_{max} = 413 nm). It was also shown that the true absorption spectra of the bound ThT coincide with the excitation spectra of fluorescence recorded at 480 nm. The significantly shorter wavelength of the absorption spectrum of free ThT in solution, in comparison to that of ThT incorporated into amyloid fibrils, can be explained by the orientational dipole−dipole interaction of the dye molecules with a polar solvent (see refs 7 and 9).

The value of the binding parameters (K_b and n) can be determined on the basis of the experimental dependence of C_b on C_f (or C_0) by nonlinear regression using appropriate software (e.g., SigmaPlot or GraphPad Prism). The failure to find appropriate parameters means that the chosen model does not correspond to the experimental data. In particular, this mismatch can be attributed to the existence of two or more binding modes (i) with different binding constants (K_{bi}). In this case, the binding sites are assumed to be independent of each other, $C_b = \Sigma_i C_{bi}$, and C_{bi} is characterized by an equation similar to that in eq 3. Thus, in the case of i modes, we have the following:

$$C_b = \sum_i \frac{n_i C_p C_f}{K_{di} + C_f} \quad (7)$$

dx.doi.org/10.1021/jp207118x |J. Phys. Chem. B 2011, 115, 11519–11524

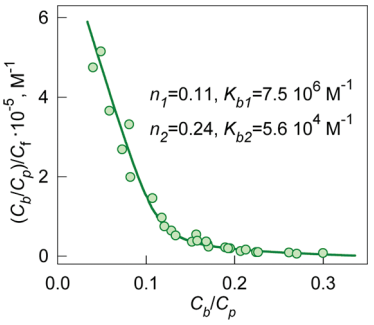


Figure 5. Scatchard plots for thioflavin T (ThT) interaction with lysozyme amyloid fibrils. Experimental data (circles) and best-fit curve with binding constants (K_{bi}) and number of binding sites (n_i) are given.

The nonlinearity of Scatchard plots obtained for ThT binding to lysozyme fibrils (Figure 5) and the failure of eq 2 to describe the experimental data suggest that amyloid fibrils of this protein have more than one binding mode with significantly different binding constants. To adequately describe the experimental data, a value of $i = 2$ was assumed, and the values of K_{bi} and n_i were found by fitting the data to eq 7 using GraphPad Prism 5. These values are given in Figure 5. Our results indicate that 4 and 10 protein molecules are needed for the formation of binding sites with low and high affinity, respectively. It is quite natural, because fibrils consist of a large number of proteins, and several protein molecules can participate in the formation of one ThT binding site. According to the model proposed by Krebs, ThT inserts itself into the grooves (channels) formed by side chains of amino acids, making up β-sheets, in parallel to the long axis of amyloid fibrils. It must be at least five β-sheets to form a binding site of ThT.[12]

Lysozyme amyloid fibrils have at least two dye-binding modes, which are characterized by different binding constants and the number of binding sites. Dye molecules bound to these modes have different spectral characteristics. The existence of several dye-binding modes for amyloid fibrils has been shown in many works (see the review by Groenning[1]). The possibility of several binding modes for ThT interactions with Aβ amyloid fibrils has also been shown by molecular dynamics simulations.[18,19]

Molar Extinction Coefficient of ThT Bound to Amyloid Fibrils. In the case of one binding mode, the measured absorption spectrum can easily be presented in the units of the molar extinction coefficient as follows:

$$\varepsilon_b(\lambda) = \frac{D_b(\lambda)}{C_b l} \tag{8}$$

where l is the optical pathway length. If there are two binding modes, then the concentrations of dye bound to each mode can be calculated on the basis of the K_{d1}, K_{d2}, n_1, and n_2 values:

$$C_{b1} = \frac{n_1 C_p C_f}{K_{d1} + C_f} \quad \text{and} \quad C_{b2} = \frac{n_2 C_p C_f}{K_{d2} + C_f} \tag{9}$$

Figure 6a shows the decomposition of C_b into two components, C_{b1} and C_{b2}, for lysozyme amyloid fibrils. Taking the following into account:

$$D_b(\lambda) = D_{b1}(\lambda) + D_{b2}(\lambda) = \varepsilon_{b1}(\lambda) C_{b1} l + \varepsilon_{b2}(\lambda) C_{b2} l \tag{10}$$

the values of $\varepsilon_{b1}(\lambda)$ and $\varepsilon_{b2}(\lambda)$ can be determined using the known values of $D_b(\lambda)$, C_{b1}, and C_{b2} by multiple linear regression

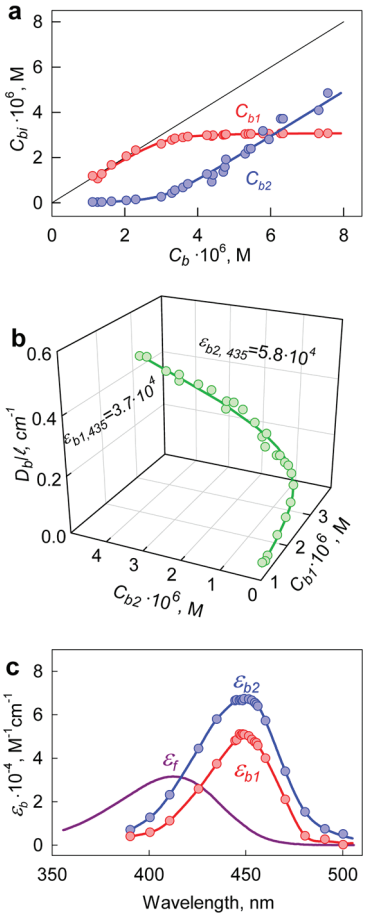


Figure 6. Determination of the molar extinction coefficient of thioflavin T (ThT) bound to amyloid fibrils. (a) Concentration of ThT bound to amyloid fibrils (C_b) as a superposition of the dye concentrations bound to mode 1 (C_{b1}) and mode 2 (C_{b2}). (b) The dependence $D_b = D_{b1} + D_{b2} = \varepsilon_{b1} C_{b1} l + \varepsilon_{b2} C_{b2} l$ on C_{b1}, C_{b2}. In the panel, experimental data, best-fit curve, and the values of molar extinction coefficients ε_{b1} and ε_{b2} obtained by multiple nonlinear regression are presented. (c) Absorption spectra of ThT bound to mode 1 and mode 2 in the units of the molar extinction coefficient.

(e.g., using SigmaPlot). Figure 6b shows the relation between D_b at 435 nm and C_{b1} and C_{b2} values for ThT bound to lysozyme amyloid fibrils. Similarly, the values of ε_{b1} and ε_{b2} can be determined at the other wavelengths. In maximum of absorption spectrum (λ_{max} = 449 nm), $\varepsilon_{b1,max} = 5.1 \times 10^4$ M^{-1} cm^{-1} and $\varepsilon_{b2,max} = 6.7 \times 10^4$ M^{-1} cm^{-1}. Figure 6c shows the absorption spectra of the dye bound to each of the two modes of lysozyme amyloid fibrils in units of the molar extinction coefficient. These data show that the molar extinction coefficient of ThT bound to fibrils depends on the binding mode and can be significantly greater than that of the free dye in solution. This result is in good agreement with quantum chemical simulations that predict the dependence of the value of the oscillator strength corresponding to the ThT transition from the ground to excited state on the φ angle value between the benzothiazole and aminobenzoyl rings[11] and with the assumption that the conformation of ThT molecules bound to amyloid fibrils (value of φ) may differ from that of free molecules in solution.[10]

Can Equilibrium Microdialysis Be Replaced by Centrifugation? It would seem that, for the determination of the parameters

of ThT binding to amyloid fibrils, microdialysis can be replaced by centrifugation, as was done by Groenning et al.[30] However, sedimentation is not equivalent to microdialysis because the equilibriums between concentrations of free and bound ThT molecules before and after centrifugation are different. Fibril sedimentation is equivalent to diminishing the volume of the chamber containing fibrils in a microdialysis experiment. In sedimentation, the fibril concentration in the lower part of the centrifuge tube will increase, and that will lead to an increase in the amount of bound dye and, consequently, to a decrease in the concentration of free dye (see eq 1). Thus, sedimentation will lead to coprecipitation of ThT together with fibrils. Therefore, the values of K_{bi} evaluated on the basis of C_f values measured using sedimentation will be overestimated in comparison to their real values. That is why sedimentation cannot be used in the experiment for the evaluation of the binding parameters of the dye bound to amyloid fibrils at equilibrium. An interesting consequence of the aforementioned effect is that local increases in fibril concentration because of aggregation will also lead to a shift of the equilibrium between free and bound dye, resulting in an increase in bound dye concentration.

CONCLUSION

In conclusion, we have shown that binding parameters, namely, the number of binding modes, stoichiometry, and affinity of ThT binding to amyloid fibrils, can be obtained by absorption spectrophotometry of solutions prepared by equilibrium microdialysis. Surprisingly, this approach, which is inherently designed for determining dye−receptor binding stoichiometry, has rarely been used for the determination of dye−protein interactions, and it has never been used for the determination of the binding parameters of ThT or its analogues to amyloid fibrils. The proposed approach also permits one to obtain absorption spectra and molar extinction coefficients of ThT bound to each binding mode of amyloid fibrils

The obtained results show that the use of equilibrium microdialysis enables new ThT applications for the investigation of amyloid fibril structure. The proposed approach is universal for determining the stoichiometry of any dye binding to a receptor, such as 8-anilino-1-naphthalenesulfonate (ANS) binding to proteins in a molten globule state or amorphous aggregates. Accurate determination of the binding stoichiometry and affinity of amyloid fibrils to neutral analogues of ThT, which can penetrate the hematoencephalic barrier, could have considerable consequences for the diagnosis and therapy of neurodegenerative diseases.[32−38] The proposed approach can be used for the analysis of interactions of chemical compounds (including nonfluorescence substances) that are assumed to inhibit amyloid fibril formation and can even be used for therapeutic purposes.[20,21]

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ACKNOWLEDGMENT

This work was supported in part by the Program "Molecular and Cell Biology" of the Russian Academy of Sciences, Russian Foundation of Basic Research, Grant 10-04-90038_Bel (K.K.T.) and Dmitry Zimin's Russian Charitable Foundation "Dynasty" (A.I.S.).

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