Fluorescence Quantum Yield of Thioflavin T in Rigid Isotropic Solution and Incorporated into the Amyloid Fibrils

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Abstract

In this work, the fluorescence of thioflavin T (ThT) was studied in a wide range of viscosity and temperature. It was shown that ThT fluorescence quantum yield varies from 0.0001 in water at room temperature to 0.28 in rigid isotropic solution (T_H2O→0). The deviation of the fluorescence quantum yield from unity in rigid isotropic solution suggests that fluorescence quantum yield depends not only on the ultra-fast oscillation of ThT fragments relative to each other in an excited state as was suggested earlier, but also depends on the molecular configuration in the ground state. This means that the fluorescence quantum yield of the dye incorporated into amyloid fibrils must depend on its conformation, which, in turn, depends on the ThT environment. Therefore, the fluorescence quantum yield of ThT incorporated into amyloid fibrils can differ from that in the rigid isotropic solution. In particular, the fluorescence quantum yield of ThT incorporated into insulin fibrils was determined to be 0.43. Consequently, the ThT fluorescence quantum yield could be used to characterize the peculiarities of the fibrillar structure, which opens some new possibilities in the ThT use for structural characterization of the amyloid fibrils.

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Introduction

The deposition of proteins in the form of regular amyloid fibrils represents a pathological hallmark of several human diseases [1,2,3,4,5,6,7]. Depending on the disease, such proteinaceous deposits can be found in the brain, vital organs such as the liver and spleen, or skeletal tissue, depending on the disease [6,7]. The protein deposition diseases are among the most costly and debilitating health disorders. Many of them, such as Alzheimer’s and Parkinson’s diseases and late-onset diabetes, are age-related and are becoming increasingly prevalent in the modern world. Although fibrils from different pathologies display many common morphological and structural properties, the more than 20 proteins known to be involved in deposition diseases are structurally unrelated [6,7,8,9]. These amyloidogenic proteins may be well-folded proteins or intrinsically unstructured [9]. There is an increasing belief that the ability to fibrillate is a generic property of a polypeptide chain, and that all proteins are potentially able to form amyloid fibrils under appropriate conditions [8,9,10,11,12]. It has been established that protein aggregation involves a unifying mechanism where the structural transformation of a polypeptide chain into a partially folded or misfolded conformation represents a first crucial step [8,9]. Therefore, understanding the nature and structural features of different partially folded and misfolded conformations represents a crucial step in fundamental science, biotechnology and medicine.

Thioflavin T (ThT, Figure 1) is a common tool for diagnostics of the amyloid fibril formation [13,14,15,16,17,18,19,20]. Importantly, ThT interaction with amyloid fibrils is highly specific, as this dye does not interact with proteins in their folded, unfolded or partially folded monomeric forms or at least the formation of dye-monomeric protein complexes is not accompanied by the changes in the dye spectral properties. Therefore, due to these unique properties, ThT represents a useful and convenient diagnostic tool for the fast and reliable identification of amyloid fibrils in disease-affected tissues and organs. Furthermore, in in vitro fibrillation studies, the appearance of the specific ThT fluorescence is considered to be an indication of the amyloid fibril formation [21,22,23,24]. This approach is widely accepted and the number of studies based on ThT diagnostic capabilities is rapidly growing. The current status of ThT in the investigation of amyloid fibrils is given in a recent in-depth review [25]. Although it is of great importance for studies of amyloid fibrils, the molecular mechanisms of the specific ThT binding to these structures and the reasons underlying the characteristic increase in the ThT fluorescence quantum yield accompanying the incorporation of this dye into the fibrils are not yet fully understood. A model in which ThT incorporates into fibrils in its monomeric form [26] is in agreement with the explanation of the significant (several orders of magnitude) increase in the ThT fluorescence intensity induced by restriction of torsion oscillations of its fragments [27,28,29,30]. Further support for this model came
Results and Discussion

ThT fluorescence in solvents with different viscosity

The dependence of ThT fluorescence quantum yield and excited state lifetime on solvent viscosity and temperature was determined in water-glycerol mixtures. Glycerol content was varied from 13 to 99% by weight (wt) and solvent temperature ranged from 3 to 50 °C. The experimental data in the form of 1/q vs. T/η plot forms a straight line (Figure 2). The intercept on the ordinate axis is larger than 2.0 (Figure 2; Insert). Figure 3 shows the dependence of ThT fluorescence quantum yield and lifetime of the excited state on solvent viscosity and temperature in the coordinates 1/q vs. T/η. Solvent viscosity was changed by variation of glycerol content from 13 to 99% and solvent temperature from 3 to 50 °C. Insert. The section of the plot corresponding to solutions of high viscosity glycerol content from 96 to 99% and low temperature from 3 to 10 °C is given in the extended scale. Corresponding to energy minima at \( \varphi = 37 \) and 145° and at \( \varphi = 217 \) and 325°. Different approaches evaluate this barrier as 2.0 or 3.4–4.3 kcal mol\(^{-1}\). A ThT analog with a methyl group at the N5 atom of the benzothiazole ring replaced by a hydrogen atom has an energy minimum at \( \varphi = 0 \) (180°) separated by energy barriers of 11.4 kcal mol\(^{-1}\) at \( \varphi = 90 \) (270°).

Quantum-chemical calculations revealed that the isolated ThT molecule energy in the excited state is monotonously decreases with the \( \varphi \) increase from 0 to 90°, where it reaches its minimal value [29,30]. Thus, the conformation with the disturbed \( \pi \)-conjugated bond system became energetically favorable. Calculations also showed that for the isolated ThT molecule, there was no energy barrier preventing the molecule from transfer to the state, which, caused the non-radiative deactivation of the excited state as corresponded to energy minima at \( \varphi = 37 \) and 145° and at \( \varphi = 217 \) and 325°.
Taking into account that even in the ground state the zero, we have:

\[ T \]

where \( T \) is the absolute temperature and \( \eta \) is the solvent viscosity. As previously suggested \([29,30]\), such a deactivating process could be torsional oscillations of the benzothiazole and aminobenzyl rings relative to each other. As the minimum of ThT molecule energy in Figure 2; the data from \([33]\) are presented in terms of Figure 3. This provides strong support for the model of ThT non-radiative deactivation described above. A relationship similar to (7) was used to describe the ThT fluorescence quantum yield dependence on microviscosity in inverted AOT micelles with different ratios of \( \omega_0 = [\text{H}_2\text{O}] / [\text{AOT}] \) \([33]\).

The radiative lifetime, determined as an average value of the ratio of fluorescence quantum yield and fluorescence lifetime of the excited state of ThT in solutions with different viscosity and temperature \( \tau_r = \tau / q \) was estimated as \( \tau_r = 7.2 \) ns (Figure 4). The radiative lifetime for ThT in rigid environment was further evaluated based on the value of the fluorescence quantum yield at \( T/\eta \rightarrow 0 \) \([31]\). Using these parameters, the radiation lifetime was determined to be \( \tau_r = 7.8 \) ns (Figure 4, closed circle). This evaluation is close to the radiative lifetime value determined above \((7.2 \text{ ns})\) and the value derived from the data obtained for the ThT in inverted AOT micelles with different ratios \( \omega_0 \) of \( \text{H}_2\text{O} \) and AOT \([33]\). In Figure 4, the data from \([33]\) are presented in terms of \( \tau_r \) (open squares). Average \( \tau_r \) value was determined based on the data corresponding to \( \omega_0 \) in the range from 30 to 10. The \( \omega_0 \) decrease from 10 to 5 leads to the dramatic increase in the \( \tau_r \) values. This is probably due to the specific interaction of AOT with the dye, which makes the dye molecule more planar. The further decrease of \( \omega_0 \) leads to the \( \tau_r \) decrease practically to the values characteristic to the water solutions. Probably this means that ThT molecules do not incorporate into such micelles. Interestingly, the range of \( \omega_0 \) from 30 to 10 corresponds to the range of \( T/\eta \) values from 21 to 12 K-\( \text{cp}^{-1} \), whereas in experiments with water-glycerol mixtures,

\[
\tau = \frac{1}{k_f + k_g + k_{w0}}, \quad (3)
\]

where \( k_f = \frac{1}{\tau_r} \) is the rate constant of the deactivation process of the excited state with radiation, \( \tau_r \) is the radiative lifetime of the excited state; \( k_g \) is the rate constant of the ThT molecules reaching the non-fluorescent state with \( \varphi = 90° \) \((270)\); which usually called TICT (twisted internal charge transfer) and \( k_{w0} \) is the rate constant of the excited state deactivation when \( T \rightarrow 0, \eta \rightarrow \infty \), i.e. when \( k_g = 0 \). This state is known as the LE state (local excited). It is likely that deviation of the ThT fluorescence quantum yield from unity in the absence of torsional oscillations of the benzothiazole and aminobenzyl rings relative to each other is caused by the non-planar conformation of ThT molecules in Frack-Condon excited state just after excitation, with a maximum \( \varphi \) angle distribution close to \( \varphi_0 = 37° \) \([29]\). We suggest that the \( \varphi \) angle distribution of ThT molecules incorporated in amyloid fibrils can differ from that in rigid isotropic solutions and this can be an important factor in determining the fluorescence quantum yield of bound ThT. Within the frame of the given assumption for ThT in solution, we have:

\[
\frac{1}{Q} = 1 + a + b T/\eta \quad (4)
\]

Table 1. Emission Quantum Yield, Excited-State Lifetime and Radiative Lifetime of Thioflavin T in Solutions with Different Glycerol Content at Different Temperatures.

| Glycerol Content, % wt | T, °C | \( q \) | \( \tau_r \), ns | \( \tau, \) ns |
|------------------------|-------|---------|----------------|-------------|
| 99                     | 5     | 0.158   | 1.14           | 7.18        |
|                        | 7     | 0.156   | 1.02           | 6.51        |
|                        | 10    | 0.125   | 0.91           | 7.29        |
| 98                     | 20    | 0.066   | 0.48           | 7.27        |
| 96                     | 5     | 0.142   | 0.98           | 6.89        |
|                        | 7     | 0.131   | 0.91           | 6.96        |
|                        | 10    | 0.113   | 0.82           | 7.30        |
| 10                     | 5     | 0.119   | 0.90           | 7.54        |
|                        | 7     | 0.106   | 0.77           | 7.28        |
|                        | 10    | 0.091   | 0.73           | 8.05        |
| 20                     | 20    | 0.046   | 0.35           | 7.61        |

*The value was determined by extrapolation of the dependence given in Figure 2; the value was obtained for thioflavin T in 99% glycerol at 77 K \([31]\); the value was evaluated as \( \tau_r, q \), average \( \tau_r \) was taken as 7.8 ns.

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Fluorescence quantum yield of ThT incorporated into the insulin amyloid fibrils

The fluorescence quantum yield of ThT bound to the amyloid fibrils, \( q_b \), was determined using the following equation:

\[
q_b = \frac{I_{ThT} - I_{ATTO}}{I_{ATTO} - I_{b}} = q_{ATTO},
\]

(7)

where \( I_{ThT} \) is the ThT fluorescence in the solution containing amyloid fibrils, \( I_{ATTO} \) is the ATTO-425 fluorescence intensity in the fibrils, \( I_{b} \) is the ATTO-425 fluorescence intensity in the buffer, and \( q_{ATTO} \) is the ATTO-425 fluorescence quantum yield.

The activation energy of the process of non-radiative deactivation of ThT determined based on the temperature dependence of fluorescence quantum yield \( \Delta E_q \) is slightly larger than the activation energy of the solvent viscous flow \( \Delta E_v \) (Table 2). A possible explanation for this phenomenon is the existence of some other factors (along with viscosity) preventing transition of the ThT molecule from its fluorescent to non-fluorescent state with the disturbed \( \pi \)-conjugated system of bonds. One of these factors is the electrostatic interaction of ThT molecule in the excited state with the molecules of the polar solvent.

Table 2: Comparison of activation energy of the solvent viscous flow (\( \Delta E_v \)) with the activation energy of the non-radiative deactivation of excited state (\( \Delta E_q \)) (determined by the temperature dependence of fluorescence quantum yield at high glycerol content in solution).

| Glycerol content, % wt | \( \Delta E_v \) kcal \cdot mol\(^{-1} \) | \( \Delta E_q \) kcal \cdot mol\(^{-1} \) |
|------------------------|---------------------------------|---------------------------------|
| 96.1                   | 16.4                            | 20.3                            |
| 97.1                   | 16.7                            | 19.6                            |
| 97.8                   | 16.8                            | 24.2                            |
| 98.4                   | 17.0                            | 21.4                            |
| 98.8                   | 17.1                            | 22.7                            |

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the solution with the optical density determined at the excitation wavelength to be equal to $D_{ATTO} = D_0 + D_1$, where $D_0$ and $D_1$ are the optical densities of the bound and free ThT in solution containing amyloid fibrils. The quantum yield of the fibril-bound dye was measured in the solutions of amyloid fibrils and ThT prepared by the equilibrium dialysis. $D_0$ and $D_1$ values were determined from the absorption spectra of free and fibril-bound dye as described in legend to Figure 5. The fluorescence quantum yield of ThT incorporated into the amyloid fibrils was equal to 0.43. The noticeably higher values of the fluorescence quantum yield of ThT incorporated into the amyloid fibrils in comparison with the corresponding values measured for the dye in the rigid isotropic solution can be explained by the model where the ThT configuration became more planar at the dye embedding into the fibrils.

Conclusions

Earlier [31], the dependence of ThT fluorescence quantum yield on solvent viscosity was explained assuming the existence of the unique way of the ThT excited state deactivation, namely the existence of the ultrafast intramolecular twisting of the dye fragments relative to each other. According to this model, the ThT fluorescence quantum yield in rigid solution was expected to be equal to unity. In this study, we used the fluorescence dye ATTO-425 with spectral characteristics close to that of ThT as a reference. This permitted the determination of the ThT fluorescence quantum yield in a wide range of viscosity and temperature. The study demonstrated that the dependence $(1/g - 1)$ on $T/\eta$ is linear and extrapolation of this dependence allowed determination of the fluorescence quantum yield in water at room temperature ($\eta=0.001$) and showed that ThT fluorescence quantum yield in rigid solution is lower than unity: $q_{T/\eta=0} = 0.28$. This means that along with non-radiative deactivation of excited state by torsional oscillations of the ThT fragments relative to each other, leading to a twisted conformation ($\phi = 90^\circ$), there is some other cause of deactivation. We propose that this deactivation is due to the non-planar conformation of the ThT molecule in the ground state and consequently, in the Franck-Condon excited states. Determination of the fluorescence quantum yield and the lifetime of the excited state ($\tau$) for ThT in solutions of different viscosity and temperature allowed the determination of the radiative lifetime ($\tau_r = 7.8$ ns) and the calculation of excited state lifetime for ThT in water at room temperature ($\tau=0.001$ ns). Our results suggested that the fluorescence quantum yield of ThT incorporated into amyloid fibrils was determined not only by the steric restrictions of the ultra-fast twisting of the ThT fragments relative to each other, but also by the conformation of the bound ThT molecule.

It is evident now that fibrils formed by different proteins, or even by the same protein but at different conditions, are not structurally identical [35]. This potentially might result in the noticeable differences in the bound ThT fluorescence intensities. Furthermore, even in a single amyloid fibril, there could be several geometrically different binding sites [36,37,38] that are able to interact with different conformations of the dye and therefore resulting in differently bound ThT molecules having dissimilar fluorescence quantum yields. We showed that the ThT bound to the insulin amyloid fibrils is characterized by the fluorescence quantum yield which was noticeably higher than that measured in the rigid isotropic solution ($q=0.43$ vs. $q=0.28$). We believe that this difference is determined by the conformational difference between the dye molecule in the fibril-bound form and in the rigid isotropic solution, where the fibril-bound ThT is characterized by more planar structure. Consequently, the ThT fluorescence quantum yield can be used to characterize the peculiarities of the fibrillar structure. These observations clearly open new perspectives for the utilization of ThT in structural characterization of amyloid fibrils.

Materials and Methods

Materials

ThT from Sigma-Aldrich (USA) and Fluka (Switzerland) was purified by crystallization from 3:1 [v/v] acetonitrile with ethanol [27]. ThT “Ultra Pure Grade” from AnaSpec, (USA), glycerol from Merck, (Germany) and fluorescence dye ATTO-425 from ATTO-TEC, (Germany) were used without further purification.

The samples of insulin and buffer components from Sigma (USA) were used without additional purification. Insulin amyloid fibrils were generated according to the standard protocol described earlier [39]. The concentration of insulin in the fibrillar form was determined based on the concentrations of the protein prior to fibrillation. Viscosity of the water-glycerol mixtures was estimated on the basis of glycerol concentrations determined by Abbe refractometer, LOMO, (Russia) at 23°C. The temperature dependence of the viscosity of different water-glycerol mixtures was taken from the literature [40]. ThT concentration in solutions was $1.4 \times 10^{-5}$ M.
Thioflavin T Non-Radiative Deactivation

Figure 6. Absorption spectra of thioflavin T in water-glycerol mixtures. Curves 1–5 correspond to 13, 35, 56, 83 and 99% wt glycerol content, respectively. doi:10.1371/journal.pone.0015385.g006

Steady-state and time-resolved fluorescence

When determining fluorescence quantum yield, the dependence of the ThT molar extinction coefficient on the content of glycerol in the water-glycerol solution was taken into account (Figure 6). The recorded fluorescence intensity was corrected on a solvent refractive index [42]. The independence of the ThT fluorescence spectrum from glycerol content in water-glycerol mixtures suggested that the fluorescence quantum yield is proportional to the fluorescence intensity (\(I_{em} = 480 \text{ nm}\)). An aqueous solution of fluorescent dye ATTO-425 with known quantum yield (\(q = 0.9\)) was used as a reference. Fluorescence was excited at 435 nm and recorded at 480 nm.

Analysis of the fluorescence decay

Decay curves were fitted using the non-linear least-squares method. Minimization was accomplished according to Marquardt [43]. Experimental data were analyzed using the multieponential approach:

\[
I(t) = \sum_{i} a_i \exp(-t/\tau_i),
\]

where \(a_i\) and \(\tau_i\) are amplitude and lifetime of component \(i\), \(\sum a_i = 1\). The root-mean square value of fluorescent lifetimes, \(\langle \tau \rangle\), for biexponential decay is determined as:

\[
\langle \tau \rangle = \frac{\sum a_i \tau_i^2}{\sum a_i \tau_i}.
\]

Author Contributions

Conceived and designed the experiments: KKT IMK VNU. Performed the experiments: AIS AAM. Analyzed the data: AAM IMK KKT. Contributed reagents/materials/analysis tools: AAM KKT. Wrote the paper: IMK KKT VNU.
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