Ultrafast Bond Twisting Dynamics in Amyloid Fibril Sensor

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Received: December 5, 2009

The fundamental process of bond twisting that is responsible for the fluorescence sensing activity of the most extensively used amyloid fibril sensor, Thioflavin T, has been revealed using ultrafast time-resolved fluorescence spectroscopy. From the wavelength-dependent fluorescence decay kinetics and the subsequently constructed time-resolved emission spectra (TRES), the dynamic Stokes shift and the change in the spectral width were observed. These results are rationalized on the basis of the proposition that, following photoexcitation, Thioflavin T undergoes ultrafast bond twisting to form a twisted intramolecular charge-transfer state that is weakly emissive in nature. Formation of the twisted state from the local excited state was found to occur in the subpicosecond time domain (time constant $\approx$ 570 fs). Quantum chemical calculations support the proposition of the bond twisting process in the photoexcited Thioflavin T and suggest that the twisting around the central C−C single bond, rather than the C−N single bond, of the Thioflavin T molecule is mainly responsible for the observed ultrafast dynamics in the excited state. Detailed time-resolved fluorescence studies of Thioflavin T incorporated in amyloid fibril show substantial retardation in the bond twisting dynamics, suggesting the involvement of this process in the sensor activity of the dye.

Introduction

Thioflavin T (ThT), a benzothiazole-based cationic dye, is used as an extrinsic fluorescence sensor to monitor and estimate the formation of amyloid fibril, a filamentous protein form responsible for several neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases.[1−4] Although ThT in water is very weakly fluorescent,[5,6] ThT shows a remarkable enhancement in emission yield upon its association with amyloid fibril.[1−4,7−9] ThT is known to be very specific in its binding with the amyloid form of the protein with no significant interaction with other forms, namely, the folded or partially folded monomeric protein.[9,10] Although the underlying mechanism for the large enhancement of ThT emission upon association with amyloid fibril is still not clear, several hypotheses have been proposed to explain this phenomenon. Khurana et al.[11] suggested that the formation of ThT micelles in the amyloid fibril is mainly responsible for the observed emission enhancement. Further, formation of a ThT excimer in amyloid fibril was proposed by Groenning et al.[12] However, such an association of charged species is a matter of debate. Recently, an optical microscopic study clearly demonstrated the absence of any association of ThT in amyloid fibril and further established that the observed emission enhancement in fibril is due to the incorporation of monomeric ThT molecules within the fibril.[13]

Photophysical studies of ThT in different confined media, such as amyloid fibril,[14−18] glass matrix,[19] polymer,[20] nanoconfined water pool,[5] and so on, clearly demonstrate that a high local microviscosity is mainly responsible for the observed fluorescence enhancement of ThT in all of these systems. It is generally believed that, for ThT in bulk water, the benzothiazole group undergoes a very fast rotation with respect to the dimethylaminobenzene moiety and this bond twisting process introduces a fast nonradiative decay channel, resulting in a very low emission yield for the dye. In a viscous environment, such as that in amyloid fibril, the bond twisting is highly restricted, so it causes a large fluorescence enhancement for the dye.[21−23]

Although several research groups have focused on exploring the reason for the fluorescence enhancement of ThT in different chemical and biological environments, no effort has so far been made to identify the basic bond twisting process that is otherwise believed to be responsible for its fluorescence sensor activity. Knowledge of the bond twisting dynamics in ThT is of utmost importance in understanding its fluorescence sensor activity in different microenvironments. In the present article, we have thus undertaken a detailed study of excited-state dynamics of ThT in aqueous solution as well as in amyloid fibril to understand the fundamental process involved in the excited state of the dye that is expected to be responsible for the fluorescence sensor activity of ThT.

Materials and Methods

Thioflavin T was purchased from Sigma-Aldrich, St. Louis, MO. The dye was recrystallized twice from methanol solution. The purity of the recrystallized ThT was checked through NMR spectra. Nanopure water (conductivity less than 0.1 μS cm^{-1}), from a Millipore Milli Q system, was used for all sample preparations. All measurements were carried out using freshly prepared solution of the dye in water. Bovine insulin was purchased from Sigma-Aldrich and used as received. Bovine insulin is well-known to form fibril at low pH and elevated temperature.[24] Solutions of bovine insulin (2 mg/mL) in 20% acetic acid were freshly prepared and incubated at 70 °C for 24 h in a glass vial under stirring. Freshly prepared stock solution of ThT in nanopure water was added to fibril solution to make a final concentration of ThT of 5 μM, and the solution was incubated at room temperature for 1 h before final measurements.

Ground-state absorption measurements were carried out using a Shimadzu (Kyoto, Japan, model UV-160A) spectrophotometer. Steady-state fluorescence measurements were carried out using

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10.1021/jp911544r
Published on Web 01/22/2010

a Hitachi (Tokyo, Japan, model F-4500) spectrofluorimeter. All samples were excited with 410-nm light. The spectrum of the standard quinine sulfate solution was recorded and compared with the standard spectrum given in units of photon/nm,[25] to obtain the wavelength-dependent correction factors for the instrumental sensitivity. These correction factors were used to correct the observed fluorescence spectra for our sample. Because the measured spectra, $I(\lambda)$, were in the wavelength domain, they were converted to the frequency domain, $I(\bar{\nu})$, using the equation[25]

$$I(\bar{\nu}) = \lambda^2 I(\lambda) \quad (1)$$

Time-resolved fluorescence measurements in the subpicosecond time domain were carried out using a femtosecond fluorescence upconversion instrument (FOG 100, CDP Inc., Moscow, Russia), which was described previously.[26] Briefly, a second-harmonic laser pulse (410 nm, 50 fs, 88 MHz) of a Ti-sapphire oscillator was mainly used for the sample excitation. Fluorescence from the sample was collected using two parabolic mirrors and focused on to a 0.5-mm BBO (β-barium borate) crystal. The residual fundamental laser beam was mixed with the fluorescence light in the BBO crystal to generate the sum frequency light, which was detected by a photomultiplier tube (PMT) based photon counter after passing through a double monochromator. The instrument response function (IRF) of the present experimental setup was independently measured through the cross correlation of the excitation and the fundamental laser pulse. The IRF was found to have a Gaussian intensity profile with a full width at half-maximum (fwhm) of 220 fs. For the excitation wavelength dependent study, the Ti-sapphire laser output was tuned such that the second harmonic output was in the range of ~390−430 nm. All experiments were carried out at the magic angle condition to exclude the effect of rotational relaxation process on the measured decay traces.

The decay traces at each monitoring wavelength were collected at least two or three times to verify the reproducibility of the measurements. Samples were taken into a rotating cell of optical path length of 0.4 mm, to avoid the photodecomposition of the sample. At the monitoring wavelength of the fluorescence decay, the contribution of the scattering light (Raman scattering) from the medium was checked by using a sample cell filled with the solvent only. The contribution of such scattering at the monitoring wavelength was negligible compared to the sample intensity. All measured fluorescence decays were fitted with a triexponential function using the standard convolute-and-compare nonlinear least-squares procedure.[27] Fitting of the experimental data and corresponding weighted residuals at some representative wavelengths are shown in Figure S2 (see Supporting Information).

Because of the very long fluorescence lifetime of ThT in amyloid fibril media, time-resolved fluorescence measurement were carried out using a time-correlated single-photon-counting (TCSPC) based instrument from IBH, Glasgow, U.K. ThT in amyloid fibril was excited with a 408-nm diode laser (1 MHz,). The fluorescence from the sample was collected at right angles to the excitation source and detected using a microchannel plate (MCP) detector. The IRF of the TCSPC instrument was measured by collecting the scattered light from a TiO_2 suspension in water and was found be ~100 ps.

Quantum Chemical Calculations. The ground-state geometry optimization of the ThT molecule was performed using density functional theory (DFT). Becke's three-parameter hybrid exchange function with the Lee−Yang−Parr gradient-corrected

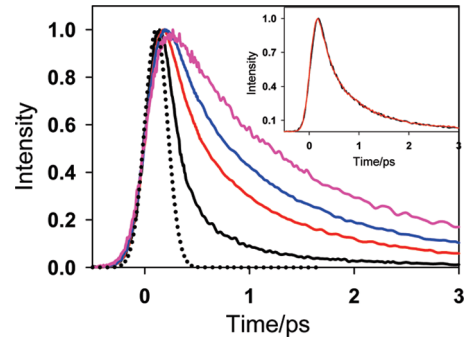

Figure 1. Fluorescence decay profile for ThT in aqueous solution at different emission wavelengths: (black) 440 nm, (red) 490 nm, (blue) 530 nm, (fuschia) 590 nm. The instrument response function (IRF) is also shown (• • •). Inset: Fluorescence decay profile for ThT in aqueous solution at different excitation wavelengths: (black) 390 nm, (red) 430 nm.

correlated functional (B3LYP)[28,29] was used in conjunction with the 6-311+G(d,p) basis set as implemented in the Gaussian 03 software package.[30] The conductor-like polarizable continuum model (CPCM)[31] was used to incorporate the effect of the bulk solvent (water). The time-dependent DFT (TDDFT) method using the B3LYP/6-311+G(d,p) basis set was used to calculate the energy in the excited state of the dye at different dihedral angles. The energy of the first excited singlet state (S_1) was determined as the sum of the ground-state (S_0) energy and the transition energy. The energy (expressed in electronvolts) is relative to the minimum of the ground-state energy.

Results and Discussion

Time-Resolved Fluorescence Studies in Aqueous Solution. The fluorescence decay profile for ThT in water was recorded at different emission wavelengths and is shown in Figure 1. At the emission maximum (490 nm), the decay was found to be very fast and to follow a nonexponential decay kinetics. From a multiexponential fitting, the average lifetime at 490 nm was found to be ~1 ps. The decays were also found to be strongly dependent on the monitoring wavelengths (cf. Figure 1). Thus, a fast decay, as compared to that at 490 nm, was observed at the blue edge, and a fast growth followed by a relatively slow decay was observed at the red edge of the emission spectrum. The observation that the transient decays at the blue edge of the spectrum (below 490 nm) were independent of the excitation wavelength (see inset of Figure 1) suggests no major contribution of the vibrational relaxation in the excited state to the observed fluorescence decays. Thus, the observed wavelength-dependent changes in the fluorescence decays could be due to either solvent relaxation dynamics or some ultrafast intramolecular process that takes place in the excited ThT molecule.

To reveal the reason behind the wavelength-dependent changes in the emission characteristics, fluorescence transient decays were converted to time-resolved emission spectra (TRES) following the standard procedure reported by Maroncelli et al.[32] The TRES thus obtained for ThT in water are shown in Figure 2 after normalization to the same peak intensity. It can be seen from this figure that there is a gradual red shift in the emission spectrum with time. Often such dynamic Stokes shifts are associated with solvent relaxation process around an excited fluorophore. The fact that for the present system the reason for the observed dynamic Stokes shift is not the solvent relaxation process is understood from the following considerations: First, in the case of solvent relaxation, the observed spectral shift should not be accompanied by any appreciable reduction in the

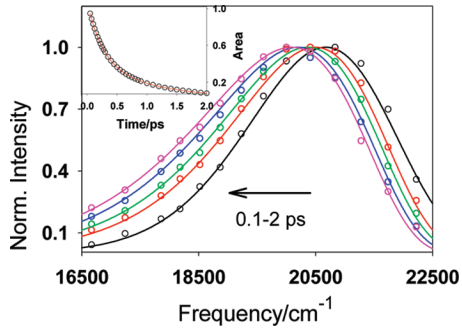

Figure 2. Time-resolved emission spectra (TRES) of ThT in water at different times following photoexcitation. The inset shows the variation in the integrated area under the emission spectra with time.

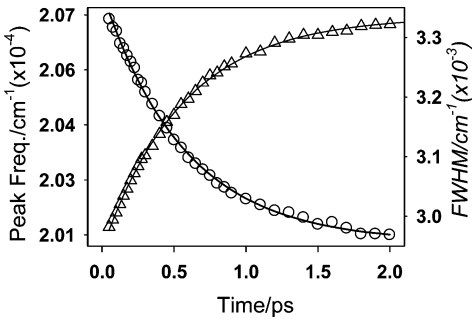

Figure 3. Variation in the peak frequency (○) and width (fwhm, Δ) of the emission spectrum with time.

fluorescence intensity (viz., excited-state populations). For the present system, however, contrary to the above expectation, there is a substantial decrease in the excited-state population within the small experimental time span (up to 2 ps) following photoexcitation (see Supporting Information). The variation in the integrated area under the emission spectrum with time is shown in the inset of Figure 2. It can be seen that the integrated intensity decreases by ∼90% of its initial value within a 2-ps time span following photoexcitation. This large reduction in the emission intensity clearly suggests that the observed dynamic Stokes shift in the present case is not related to the solvent relaxation process. The absence of any significant effect of solvent relaxation in the present system is also suggested by the fact that the steady-state emission maxima of ThT are almost the same in a nonpolar solvent (chloroform, $\varepsilon = 4.8$) and in a highly polar solvent (water, $\varepsilon = 78.5$) (see Supporting Information). The lack of any significant solvatochromism clearly indicates that there should not be any substantial amount of solvent relaxation in the present system.

It should be noted from Figure 2 that, along with the frequency shift in the TRES, there is a significant spectral broadening with time. A close examination of Figure 2 reveals that the spectral broadening primarily occurs at the red edge of the spectra, whereas the shape at the blue edge effectively remains unchanged. The observed changes in the spectral shape are also corroborated by the changes in the asymmetry parameter (b), obtained by fitting the experimental data in the TRES with a lognormal function (see Supporting Information). Thus, the asymmetry parameter is seen to change from −0.18 at 50 fs to −0.41 at 2 ps.

The peak frequency calculated from TRES is presented in Figure 3. A Stokes shift of about 600 cm^{-1} is observed within 2 ps following photoexcitation. Changes in the peak frequency with time are seen to follow an exponential kinetics with a time constant of ∼575 ± 25 fs. The change in the width (fwhm) of the spectra with time is also presented in Figure 3. Interestingly,

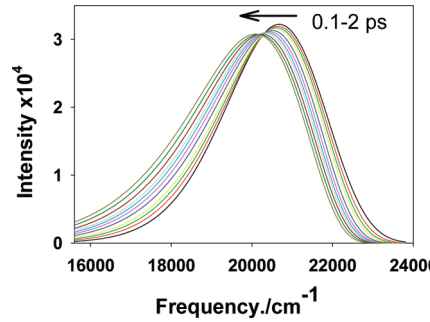

Figure 4. Time-resolved area normalized emission spectra (TRANES) of ThT in water at different times (0.1−2 ps).

the width of the emission spectra shows a very fast growth, with a time constant very similar (∼560 ± 30 fs) to that observed for the change in the peak frequency. These results thus suggest that the same process is responsible for both the observed dynamic Stokes shift and the changes in the spectral shape with time.

Further, to determine the reason behind the changes in the spectral shape, especially the asymmetric increase in the spectral width, we also constructed the time-resolved area-normalized emission spectra (TRANES),[33,34] which are shown in Figure 4. The presence of an isoemissive point is very clearly indicated in the TRANES. The appearance of an isoemissive point in the TRANES is generally considered as an indication of the presence of two emissive species in the system.[33,34] Thus, from the present observations, we propose that, following photoexcitation, the locally excited (LE) state of the ThT molecule undergoes an ultrafast intramolecular process with a time constant of ∼570 fs to generate a new species in the excited state that is also emissive in nature. To be mentioned here that similar changes in the spectral characteristics, namely, the increase in the spectral width and the dynamic Stokes shifts, have also been reported for hemicyanine[35] and benzothiazole dyes[36] and are explained on the basis of the formation of a new twisted emissive species from the locally excited state of the dye. Dynamic Stokes shift is also reported for the formation of the twisted conformer in the excited state of several dyes.[37,38] Drawing an analogy, we propose that, upon photoexcitation, an ultrafast bond twisting process takes place in the LE state of the ThT molecule to generate a twisted configuration in the excited state. It is interesting to note here that, in the literature, it has been suggested that the twisted configuration in the excited state of ThT is nonemissive in nature.[39] However, the present results clearly indicate that the twisted configuration of ThT is, in fact, also emissive in nature, although its emission yield could be much lower than that of the LE state, as suggested by a very fast decay in the integrated fluorescence intensity with time. The proposition of the involvement of a twisted configuration of the ThT dye in the excited state is also supported by the fact that the fluorescence quantum yield of ThT is highly dependent on the viscosity of the medium.[5,40] It is suggested that the ultrafast bond twisting process introduces an efficient nonradiative decay channel for the excited ThT molecules, resulting in a very low fluorescence quantum yield for the dye in water as well as in other less viscous solvents. A similar reduction in the emission yield due to the formation of the twisted configuration in the excited state is well-known for several dyes.[37,41−44]

Quantum Chemical Calculations. To further support our proposition of the bond twisting process, we carried out detailed quantum chemical calculations. The ground-state optimized geometry of the ThT molecule in water is shown in Figure 5.

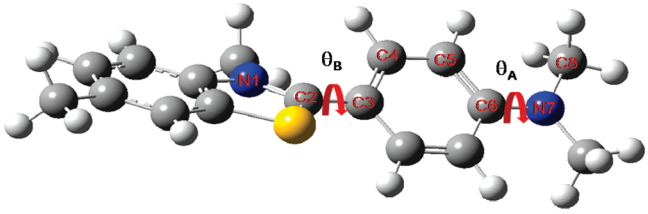

Figure 5. Ground-state optimized geometry for ThT in water.

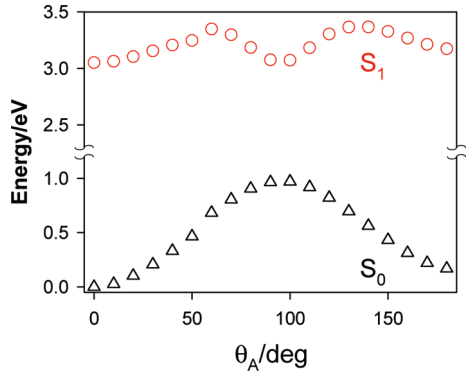

Figure 6. Potential energies of ThT in the ground (S_0) and excited (S_1) states as a function of the dihedral angle θ_A. The energy (expressed in eV) is relative to the minimum of the ground-state energy.

From this figure, it is evident that the ThT molecule in its ground state exists in a twisted configuration. Thus, the plane containing the benzothiazole ring makes an angle of ~39° with respect to the plane containing the dimethylanilino ring. This partially twisted configuration of ThT is in agreement with the results reported in the literature.[45,46]

In the ThT molecule, there are two possible single bonds, namely, the C−N bond between the benzene ring and the dimethylamino group (corresponding to dihedral angle θ_A in Figure 5) and the C−C bond between the benzothiazole moiety and the dimethylanilino ring (corresponding to dihedral angle θ_B in Figure 5), that can undergo twisting processes in the excited state. Thus, the potential energy surfaces for both ground and excited states were calculated as a function of the twisting angle for both of these bonds. Figure 6 shows the variations in the potential energies for both ground and excited state of ThT in water as a function of the dihedral angle θ_A. It is evident from the optimized structure of ThT in the ground state (cf. Figure 5) and also from Figure 6 that, in the ground state, the dihedral angle between the dimethylamino group and the benzene ring (θ_A) is 0°, that is, the two groups are in same plane. The excited-state potential energy surface indicates that the configurations with $\theta_A = 0°$ and 90° are of comparable energy but separated by a high energy barrier (~0.29 eV). Hence, the interconversion between the configurations with $\theta_A = 0°$ and 90° is highly prohibited. This result suggests that the twisting around the C−N bond in the excited state of ThT is not feasible and thus suggested not to be responsible for the ultrafast decay process observed in the excited state of the ThT molecule.

Ground- and excited-state potential energy surfaces were also calculated as a function of the dihedral angle (θ_B) between the benzothiazole moiety and the dimethylanilino ring, as shown in Figure 7A. As mentioned earlier and also as is evident from Figure 7A, the ground-state ThT molecule exists in a partially twisted configuration with $\theta_B = 39°$. The nature of the ground-state potential energy surface shown in Figure 7A is qualitatively quite different from that calculated for the ThT molecule in the

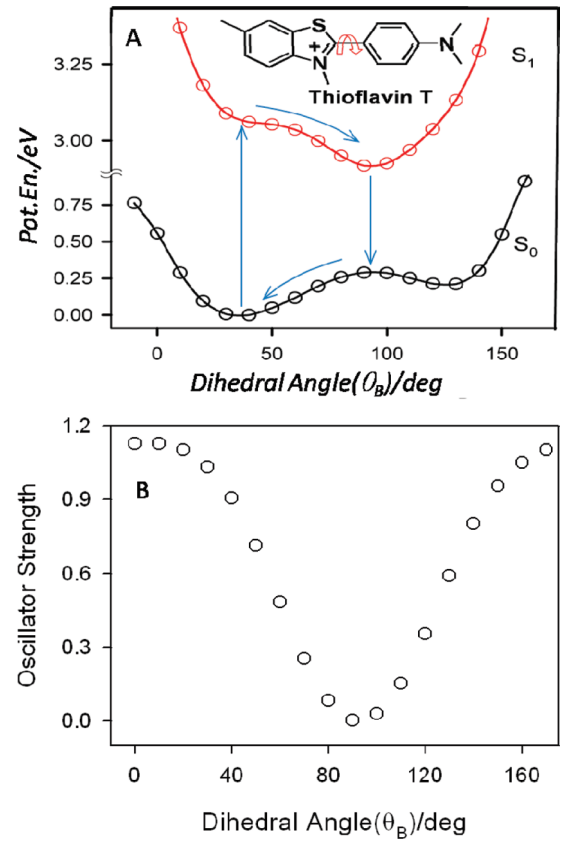

Figure 7. (A) Ground-state (S_0) and excited-state (S_1) potential energies of ThT as a function of dihedral angle θ_B. (B) Variation in the oscillator strength with the dihedral angle θ_B for the S_0-S_1 transition of ThT in aqueous solution.

gas phase using the relatively lower level of theory.[47] It is evident from Figure 7A that the LE state (with $\theta_B = 39°$) has a quasiminimum energy and that the most stable configuration in the excited state is the fully twisted configuration with $\theta_B = 90°$. Thus, it can be inferred that, following photoexcitation, the ThT molecule in the excited state undergoes a twisting around the C−C single bond to attain the fully twisted configuration (with $\theta_B = 90°$). It is also to be noted from Figure 7A that the transition from the LE state to the fully twisted excited state is a barrierless process. Because of this barrierless nature, the bond twisting process in the excited ThT molecule is expected to be ultrafast in nature. Thus, the corroboration of the results from quantum chemical calculations and femtosecond fluorescence upconversion studies suggests that the observed ultrafast process in the excited state of the ThT molecule is due to the twisting around the C−C single bond joining the benzothiazole moiety and dimethylanilino ring of the molecule. Figure 7B shows the variation in the oscillator strength for the transition between the excited state and the ground state for ThT as a function of dihedral angle θ_B. From this figure, it can be seen that there is a substantial decrease in the radiative rate as the molecule undergoes a twist around the C−C bond. For example the oscillator strength decreases from 1.1 at $\theta_B = 39°$ to 0.01 at $\theta_B = 90°$. This result clearly indicates that the twisted state is very weakly emissive as compared to the LE state. This result is also supported by the experimental observation that there is a substantial reduction in the emission intensity during the twisting process.

Excited-State Dynamics in Amyloid Fibril. To understand the fate of the observed ultrafast bond twisting process in the ThT molecule inside amyloid fibril, detailed time-resolved

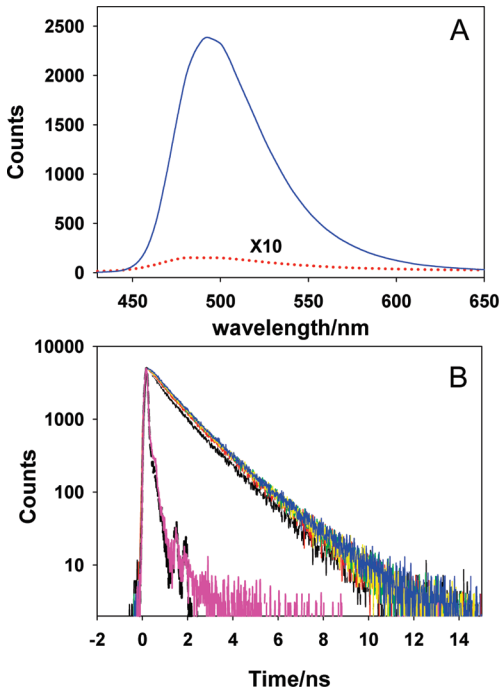

Figure 8. (A) Steady-state emission spectrum of ThT in aqueous solution (red dotted line) and in insulin amyloid fibril (blue solid line). (B) Emission decay traces for ThT in amyloid fibril at different emission wavelengths (450, 480, 500, 550, and 600 nm). The decay trace at 490 nm for ThT in aqueous solution (fuschia solid line) is also shown for comparison. The IRF is shown by the dotted curve.

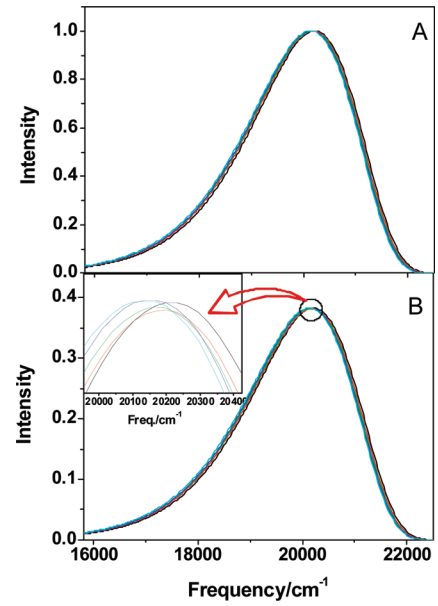

Figure 9. (A) Intensity-normalized TRES and (B) TRANES for ThT in amyloid fibril at different times (0.1, 0.3, 1, 2, and 6 ns).

emission studies were carried out in insulin fibril. Figure 8A shows the enhancement in the emission of ThT due to its incorporation in the amyloid fibril media. A large enhancement (~160 times) in the emission in the presence of fibrillar media indicates the incorporation of the dye inside the amyloid fibril. Fluorescence decay traces for ThT in amyloid fibril at different emission wavelengths were recorded and are shown in the Figure 8B. For comparison, the emission decay trace for ThT in bulk water measured at 490 nm by TCSPC instruments is also shown in Figure 8B. It is evident from Figure 8B that the lifetime of ThT in aqueous solution is instrument-limited. It is further to be noted from Figure 8B that the emission lifetime of ThT has significantly increased due to its incorporation inside the amyloid fibril. Thus, the average emission lifetime measured in amyloid fibril was found to be 1.3 ns, which is around 3 orders of magnitude higher than that in bulk water (~1 ps). The increase in the emission lifetime of ThT is also substantiated by the large enhancement in the emission intensity in amyloid fibril as compared to that in bulk water (cf. Figure 8A). Comparing the emission lifetimes of ThT in bulk water and in amyloid fibril, it can be inferred that the bond twisting process in the excited state of the ThT molecule is substantially retarded due to its incorporation in the latter medium. Further, it is to be noted from Figure 8B that, contrary to the case in bulk water, the emission decay was found to be almost independent of the interrogated emission wavelength. This result thus indicates the absence of any considerable extent of bond twisting process in the excited state of ThT in amyloid fibril.

TRES generated from the wavelength dependent decay traces for ThT in amyloid fibril are shown in Figure 9A after intensity normalization. It is clearly indicated from this figure that, unlike in bulk water, there is merely any dynamic Stokes shift present in the amyloid fibril. Further, the shape of the emission spectrum is seen to be invariable with time. Because of this fact, the width of the emission spectrum was also found to be independent of time. Thus, the absence of a dynamic Stokes shift and the absence of any variation in the spectral shape (i.e., width of the spectra) clearly indicates that, unlike in bulk water, the bond twisting process is largely retarded in the amyloid fibril. TRANES for ThT in amyloid fibril are also shown in the Figure 9B. It is evident from Figure 9B (cf. inset of Figure 9B), that there is no isoemissive point indicated in the present case. The absence of such an isoemissive point suggests that ThT does not form any twisted state, a weakly emissive state, when it is incorporated in the amyloid fibril medium. Thus, the present results clearly demonstrate that the bond twisting process in the excited state of ThT molecule is substantially retarded in amyloid fibril because of the very high microviscosity around the probe.

Conclusions

In conclusion, for the first time, it has been shown experimentally that the bond twisting process in ThT takes place on the subpicosecond time scale (570 fs). It is also confirmed that the twisting around the C−C single bond, not the C−N single bond, takes place in the excited state and that the process is barrierless in nature. The twisted state of ThT was found to be weakly emissive in nature. The bond twisting process was observed to be largely retarded in the amyloid fibril media. The fundamental information on the bond twisting dynamics of ThT obtained in the present study is very useful to understand its fluorescence sensor activity in different restricted media. This information might also help to develop a better fluorescence sensor based on the ThT chromophore. Based on the present results, the design and synthesis of ThT derivatives that could act as better sensors than ThT itself is currently underway.

Acknowledgment. The authors are thankful to Prof. A. Datta, Indian Institute of Technology, Powai, India, for his help in quantum chemical calculations.

Supporting Information Available: Ground-state absorption and steady-state emission spectra of ThT in water, fluorescence transient decay for ThT in water at three different emission

wavelengths, and time-resolved emission spectra (TRES) of ThT in water at different time following photoexcitation. This material is available free of charge via the Internet at http://pubs.acs.org.

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JP911544R