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Direct Observation of Delayed Fluorescence from a Remarkable Back-Isomerization in Cy5

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Abstract: The direct observations of delayed fluorescence and phosphorescence from the cyanine dye Cy5 are reported. The delayed fluorescence is generated from the S_1 state of *trans*-Cy5 through a reserve intersystem crossing from the cis-triplet state T_1 to the trans-singlet state S_1 via thermal activation. The lowest cis-triplet state is evidenced to be involved in the formation of the isomer. The back-isomerization from cis-triplet state to trans-singlet state crossing, a remarkably back-isomerization pathway that has not been reported before, plays a significant role in this unusual delayed fluorescence.

Introduction

Cyanine dyes have been intensively studied by various photophysical and photochemical means,[1−14] owing to the interest for their widespread applications such as spectral sensitizers in photography,[15,16] in biomedical application,[17−20] and in nonlinear optics and laser physics.[21,22] The fluorescence and intersystem crossing properties, important for these applications, compete with the possible isomerization by carbon−carbon bond rotation, which takes place from the singlet states produced by visible excitation of the thermodynamically stable trans-ground state conformation of the cyanine dyes.[23−28]

Although much effort has been made to determine the photophysical properties of cyanine dyes such as Cy5, concerning the deactivation of the singlet state by internal conversion and fluorescence decay to the ground state, intersystem crossing to the triplet manifold, and trans−cis isomerization into a low fluorescent isomer,[25−30] the underlying mechanisms about the triplet-state properties and trans−cis photoisomerization still remain unknown to a large extent. For most investigators, intersystem crossing and isomerization are considered to be competing processes without any involvement of the triplet states in the formation of the isomers.[29,31] Furthermore, very few works have revealed information about the back-isomerization of cyanine dyes.[25−28,32,33] On a broader level, understanding the photophysical properties is of considerable importance for the Cy5, as it is one of a few commercially available single molecule fluorescence probes with an emission wavelength in the near-IR spectral region. Cy5 has recently become a very important probe and has been frequently used in ultrasensitive imaging and spectroscopy to characterize local

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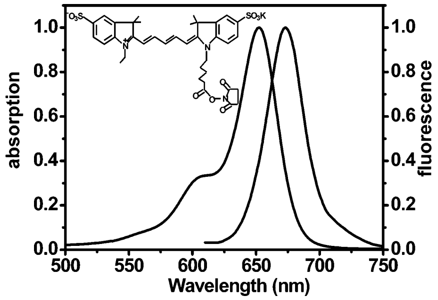

Figure 1. Absorption (left) and fluorescence (right) spectra of *trans*-Cy5 solution in ethanol (6.7 × 10^{-6} M). The inset shows the molecular structure of the Cy5 molecule.

environments in large biomolecules, such as proteins or nucleic acids.[32−35] The single molecule fluorescence experiments have revealed several unexpected photophysical phenomena of the Cy5 related to the cis−trans isomerization and triplet state formation.[25,26,28,33] In this article, we report the first observations of delayed fluorescence and phosphorescence from the Cy5. The unusual delayed fluorescence is generated from the S_1 state of *trans*-Cy5 through a reserve intersystem crossing from the cis-triplet state T_1 to the trans-singlet state S_1 via thermal activation, which indicates that the lowest cis-triplet state is involved in the formation of the isomer. The back-isomerization from the cis-triplet to the trans-singlet crossing, a remarkable back-isomerization pathway that has not been reported before, plays a significant role in this observed delayed fluorescence. The results presented in this article are of fundamental significance not only for general triplet states and trans−cis isomerization properties but also for the performance of the cyanine dyes in all applications.[1−22]

Materials and Methods

Cy5 was purchased from Amersham Biosciences. Absorption spectrum was recorded with a UV−vis spectrophotometer (model U-3010, Shimadzu). Fluorescence spectrum was measured with a fluorescence spectrophotometer (F4500, Hitachi). Phosphorescence and delayed fluorescence were measured with a high-quality emission spectrophotometer (Jobin Yvon TRIAX 320) with highly sensitive LN-CCD as detector (Jobin Yvon, CCD-3000 V) combined with a high-speed rotated sector wheel for chopping the laser and emission beams in turn. The sector wheel was revolved at a speed to allow observation of all delayed fluorescence and phosphorescence with a lifetime greater than 0.5 ms. No other prompt fluorescence and stray light from laser scattering were detected during delayed fluorescence and phosphorescence measurements. Cy5 samples (0.5 × 10^{-5} M in N_2-saturated ethanol solution) were excited at 632.8 nm with a CW He−Ne laser for phosphorescence and delayed fluorescence measurements. An exposure of about 50 s of the LN-CCD was used to accumulate the weak delayed fluorescence and phosphorescence.

Results and Discussion

Cy5 is believed to adopt an all-trans configuration in its ground states as its thermodynamically stable conformation.[23−28] Figure 1 shows the absorption and fluorescence spectra of the *trans*-Cy5 solution in ethanol. There is a broad absorption ranging from 600 to 660 nm, while the prompt fluorescence peak is around 673 nm. Figure 2a shows the phosphorescence spectrum of the Cy5 solution in ethanol at low temperature (77 K) excited at 632.8 nm. The emission maximizing near 775 nm is attributed to phosphorescence from *trans*-T_1 to *trans*-S_0 transition of the Cy5. Since the all-trans configuration in the ground state is the thermodynamically stable conformation of the Cy5, the relative energy of the *trans*-T_1 state is therefore determined to be about 1.60 eV (775 nm) from the phosphorescence spectrum of *trans*-Cy5 at low temperature (77 K). Unexpectedly, a stronger peak that is consistent with the prompt fluorescence peak (see Figure 1) also appears at about 673 nm during phosphorescence measurements at both low and room temperature as shown in Figure 2a. This stronger emission maximizing around 673 nm is assigned to the delayed fluorescence from the *trans*-Cy5 with the relative energy about 1.84 eV (673 nm).

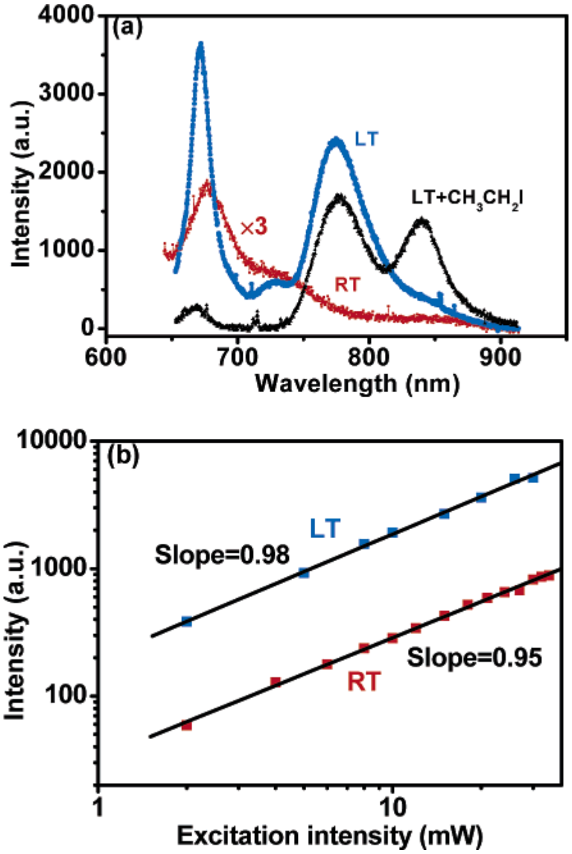

Figure 2. Phosphorescence and delayed fluorescence (DF) spectra (a) of Cy5 (0.5 × 10^{-5} M) at room temperature (RT) and low temperature (LT). DF intensity as a function of excitation energy (b) at RT and LT displayed on a logarithmic scale (λ_{ex} = 632.8 nm).

Three possibilities can cause delayed fluorescence. They are thermal-induced delayed fluorescence from T_1 to S_1 (E-type), T−T annihilation (P-type), and reverse intersystem crossing (RISC) from upper triplet levels (e.g., $T_1 \rightarrow T_N \rightarrow S_1 \rightarrow S_0$) following $T_1 \rightarrow T_N$ excitation.[27,29,30,35−38] Under the assumption that the observed delayed fluorescence is due to the RISC,[29,30] an additional excitation is required when the Cy5 molecule stays in its triplet state. In fact, during delayed fluorescence measurements with the help of the rotated section wheel, there is no

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additional excitation light with sample at the moment of detection. Furthermore, the lifetime of T_n is about 200 fs, and the time of the transition from T_n via S_n ($n \geq 1$) to S_0 is about 10 ns.[27,29,39−41] It is obvious that the lifetime of delayed fluorescence contributed from RISC is much shorter than the time resolution (around 0.5 ms) of rotated section wheel used. Therefore, the rotated section wheel can rule out the fast delayed fluorescence from RISC. Meanwhile, the analysis of power dependence of delayed fluorescence is a standard signature for obtaining information relevant to the mechanism of delayed fluorescence.[36,38] The delayed fluorescence caused from RISC is a typical two-photon excitation processes; the delayed fluorescence from RISC must obey the square-law dependence on excitation intensity. Similarly, the delayed fluorescence from T−T annihilation is also a typical bimolecular reaction, which must also obey the square-law dependence on excitation intensity. Before selecting a mechanism for the origination of the observed delayed fluorescence, the dependence of the delayed fluorescence intensity on the excitation intensity was measured at both room and low temperature as shown in Figure 2b. We did not observe the square-law dependence of the delayed fluorescence on excitation intensity. The mechanism for the delayed fluorescence of the Cy5 produced from RISC and T−T annihilation is ruled out.

The linear excitation power dependence of the delayed fluorescence intensity (see Figure 2b) indicates that the observed delayed fluorescence from the *trans*-S_1 state, therefore, originates from a transition from the triplet state T_1 to the singlet state S_1 via thermal activation. It should be emphasized that the energy difference of about 0.24 eV between the S_1 (1.84 eV) and the T_1 (1.60 eV) states of *trans*-Cy5 is about 10 times larger than the thermal activation energy $\boldsymbol{k}$T. The contribution from the *trans*-T_1 to the *trans*-S_1 crossing by thermal activation is expected to be minor. Since there is no cis-phosphorescence at this moment, this delayed fluorescence can originate from *trans*-S_1 state through the T_1 state of *cis*-Cy5 to the S_1 state of *trans*-Cy5 transition by thermal activation, rather than from the T_1 state to the S_1 state of *trans*-Cy5. To determine if there is a real *cis*-T_1 state formed upon excitation, we add ethyl iodide to the Cy5 solution in nitrogen-saturated ethanol, where the heavy atom effect is expected to increase the *cis*-T_1 state yield.[36,38] From Figure 2a, it is noted that an increased phosphorescence peak accompanied by a decrease of delayed fluorescence appears as expected at about 840 nm, which results from the *cis*-T_1 → *cis*-S_0 transition as cis-phosphorescence. The observation of delayed fluorescence and phosphorescence suggests that the lowest cis-triplet state is involved in the isomerization process. The back-isomerization from cis-triplet to trans-singlet states plays a significant role in this observed delayed fluorescence. This remarkable back-isomerization is much more efficient owing to a small activation barrier between the *cis*-T_1 and the *trans*-S_1 states with higher thermal activation energy $\boldsymbol{k}$T at room temperature, leading to the stronger delayed fluorescence. The fact that this unusual delayed fluorescence can be observed at room temperature may complicate the analysis of the observed single molecule behaviors, since the trans−cis isomerization has been proved to be involved in the on−off blinking characteristics of single Cy5 molecules and gives fast intensity fluctuations referred to the behavior as on/dim blinking.[25−28,34]

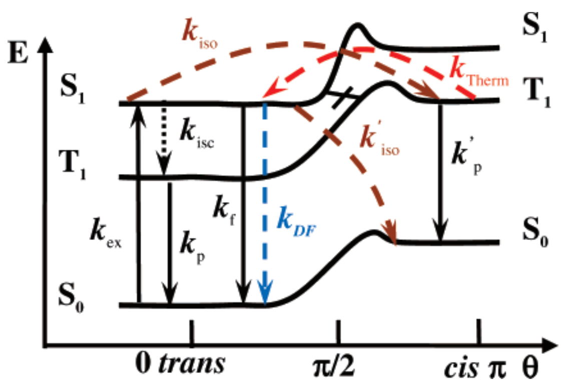

Figure 3. Diagram of the hypothetical potential energy surface of Cy5 molecule depicting the possible deactivation following the *trans*-S_0 → *trans*-S_1 excitation. The center cross symbol (×) means the degeneracy of the *trans*-S_1 and *cis*-T_1 states at the perpendicular minimum on the S_1 potential surface. In the diagram, S_0, S_1, and T_1 denote the ground singlet, the first excited singlet, and the triplet states of the Cy5, respectively. The results shown in Figure 2a further support this degenerated potential energy surface. The photoinduced isomerization from the *trans*-S_1 state to the *cis*-T_1 state (denoted as $k_{\rm iso}$) is directly evidenced from the phosphorescence measurement of the ethyl iodide-doped Cy5 solution in nitrogen-saturated ethanol. The remarkable back-isomerization from the *cis*-T_1 to the *trans*-S_1 state (denoted as $k_{\rm therm}$) is also characterized by the observed delayed fluorescence (denoted as $k_{\rm DF}$) at both low and room temperatures.

The small energy difference between the *cis*-T_1 and the *trans*-S_1 is also in accordance with the results predicted by the degenerated potential energy surfaces of the *trans*-S_1 and the *cis*-T_1 in the twisted conformation as speculated according to the CS-INDO calculations on a similar cyanine dye to Cy5.[30,42] Figure 3 shows the diagram of the hypothetical potential energy surface, where the center cross symbol (×) means the degeneracy of the *trans*-S_1 state and *cis*-T_1 state at the perpendicular minimum on the S_1 potential surface. The degenerated potential energy surfaces with minimal energy difference between the *trans*-S_1 and the *cis*-T_1 states in the twisted conformation make the unusual delayed fluorescence more efficient.

Conclusion

This study reports the direct observations of delayed fluorescence and phosphorescence in Cy5. A remarkably back-isomerization pathway in Cy5 was identified with a pathway from the *cis*-T_1 state to *trans*-S_1 state that leads to the unusual delayed fluorescence. The results presented in this article are of vital importance for understanding the complicated spectral characteristics of the triplet state and the photoisomerization of the Cy5 and therefore are of relevance for the performance of the cyanine dyes in all applications.[1−22]

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Supporting Information Available: Experimental geometries for the delayed fluorescence and phosphorescence measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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