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LETTERS

Direct Observation of Collective Blinking and Energy Transfer in a Bichromophoric System

Philip Tinnefeld, Volker Buschmann, Kenneth D. Weston,† and Markus Sauer*

Physikalisch-Chemisches Institut, Universität Heidelberg, Im Neuenheimer Feld 253, 69120 Heidelberg, Germany

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A bichromophoric model system—a short peptide labeled with tetramethylrhodamine (TMR) and the carbocyanine Cy5—embedded in poly(vinyl alcohol) (PVA) was used to investigate energy transfer and collective blinking effects in multichromophoric systems at the level of single molecules. Experiments using direct excitation of the acceptor show evidence of photoinduced reverse intersystem crossing ($T_1 \rightarrow T_N \rightarrow S_1 \rightarrow S_0$) in single Cy5 molecules. We observed that even when the Cy5 fluorophore is in the triplet state, it continues to act as an energy transfer acceptor. This demonstrates that singlet−triplet energy transfer occurs, and can lead to efficient quenching of the fluorescence of the whole bichromophoric system.

In recent years, single molecule studies have proven to be an excellent tool for the study of multichromophoric systems such as light-harvesting complexes.[1−3] To better understand natural antenna systems, synthetic model systems can be used. These offer the advantage that they can be synthesized in a controlled manner, enabling the variation of the parameters that influence the energy-transfer properties.[4−7] An artificial system that successfully imitates the energy-funneling behavior of natural light-harvesting complexes is dendrimers carrying a controlled number of chromophores at the rim.[7−10] One of the most intriguing features of these and other multichromophoric systems is collective blinking, i.e., when all the chromophores simultaneously enter a dark, nonemitting state, observed in single molecule experiments.[1,7,11] In the past, blinking was seen as a clear-cut criterion for a single chromophore. All models used to explain the phenomenon of collective blinking have in common that fast energy hopping among the chromophores to a local trap is assumed.[1,10−13] Local inhomogeneities might lead to slightly different energies of the individual chromophores. Consequently, the energy is funneled to the chromophore with the lowest excited-state energy. This chromophore acts as a trap for the excited-state energy and emits with the highest probability. Evidence for energy hopping and for the fact that the energetically lowest chromophore site acts as an energy trap came from the observation of a blue shift and a change in anisotropy of the fluorescence of individual constructs as a function of time as individual chromophores undergo photodestruction.[7] When a chromophore enters a dark state that exhibits transitions to higher excited states that are still in resonance with the $S_1 \rightarrow S_0$ transitions of the other chromophores, the whole aggregate might be quenched. Several processes might be responsible for the observation of collective dark states in multichromophoric systems: (i) intersystem crossing of one chromophore into the triplet-state, T_1, and subsequent resonant $T_1 \rightarrow T_N$ transitions,[7,9,10] (ii) resonant energy transfer to an oxygen adduct formed by one chromophore,[14] and (iii) the formation of a nonfluorescent radical cation or anion.[12,15] Recently, Hofkens et al. showed that there is indeed a spectral overlap between the emission spectrum and the triplet absorption spectrum of individual peryleneimide chromophores in a multichromophoric system.[10]

In this letter we report on the role of triplet traps and $T_1 \rightarrow T_N$ transitions of individual chromophores in multichromophoric

* Corresponding author. Phone: +49-6221-548460. Fax: +49-6221-544255. E-mail: sauer@urz.uni-heidelberg.de.

† Current address: Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306.

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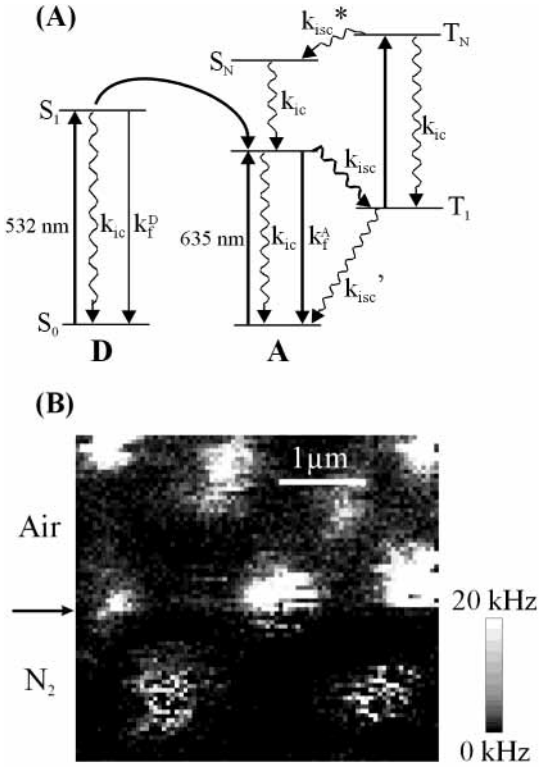

Figure 1. (A) Kinetic scheme of the bichromophoric model system used in this study. The donor TMR is excited at 532 nm and transfers its energy via dipole−dipole interactions to the acceptor Cy5. (B) Confocal fluorescence images of single Cy5 molecules on a dry glass surface. The sample was scanned from top to bottom and from left to right with a resolution of 50 nm/pixel at an excitation energy of 5 μW (7 ms integration time per pixel). The arrow indicates the point in time when the nitrogen purge was started. The appearance of the fluorescence spots representing single molecules changes dramatically upon the atmosphere changes due to an increase of the triplet lifetime.

systems by using a simple bichromophoric model system, a short peptide (Cys-$(Pro)_{10}$-Lys) labeled at one termini with a donor dye, tetramethylrhodamine (TMR), and at the other with an acceptor dye, carbocyanine (Cy5).[16] Ensemble measurements of TMR−Cys-$(Pro)_{10}$-Lys-Cy5 revealed a high-energy transfer efficiency of 0.85. The single-molecule measurements were performed with confocal fluorescence microscopes equipped with either a frequency-doubled Nd:YAG laser (532 nm) to excite the donor TMR or a diode laser (635 nm) to directly excite the acceptor Cy5.[16,17] Spectrally filtered signals were detected by avalanche photodiodes. Samples were prepared by diluting stock solutions in water containing 1% w/v poly(vinyl alcohol) (PVA) and spin casting onto glass substrates.

Since we use the dye pair TMR/Cy5, the energy transfer path is known beforehand. This is an advantage over dendrimer systems that have been studied in the past which hold chemically identical chromophores. The chromophores in those systems differ in energy only due to interactions with their local environment. Instead of a random lowest energy chromophore, the direction of the energy transfer is intrinsically controlled (Figure 1A). Energy transfer in this dye pair is known to occur and is well characterized.[16,18] Each chromophore can be excited selectively by choice of excitation wavelength, and detected independently using two spectrally resolved detection channels. These features facilitate the interpretation of energy transfer and collective blinking effects. Hence, resonant singlet−triplet energy transfer processes which might be responsible for the observation of collective blinking effects in multichromophoric systems[10] can be directly demonstrated.

The bichromophoric system used in this study has additional advantages: The carbocyanine dye Cy5 used as acceptor is a well characterized dye at the level of single molecules[17,19,20] and it is well established that the triplet lifetime of carbocyanine dyes is very sensitive to oxygen concentration. The triplet lifetime of carbocyanines dyes can be extended from about 50 μs in air to about 100 ms upon removing oxygen.[21−23] To remove oxygen to a sufficient extent, the dye is embedded in a poly(vinyl alcohol) (PVA) matrix that has low oxygen permeability. To verify that the observed dark states of Cy5 are in fact due to transitions to the triplet manifold, we varied the atmosphere from air to nitrogen while recording confocal fluorescence intensity images of Cy5 immobilized on cover slides (without PVA) (Figure 1B). The arrow indicates the point in time when the nitrogen flow was switched on. The nitrogen flow changes the image appearance dramatically. While only a few isolated dark states are evident within the point-spread function (PSF) in air, patchy spots with frequent off-states in the time range of several milliseconds are observed when the oxygen is removed. Long dark states were also observed for Cy5 molecules immobilized in PVA as the low oxygen permeability of PVA protects the Cy5 triplet states from being quenched. In contrast, when the rhodamine derivative TMR is imaged under the same conditions, the images are not altered by the reduction of oxygen either because the triplet states are not extended into the millisecond range (typical triplet lifetimes of rhodamines in solution are in the range of a few microseconds[24,25]) or the triplet yield is so low that even extended triplet states do not play a dominant role in the fluorescence images. Autocorrelation analysis of the fluorescence intensity trajectories of 45 Cy5 molecules immobilized in PVA yielded a triplet lifetime of 18 ± 10 ms and intersystem crossing yield, Y_T, of $\sim 10^{-4}$.[17]

For successful energy transfer from the donor to the acceptor triplet state, the $T_1 \rightarrow T_N$ transition of the acceptor must be in resonance with the $S_1 \rightarrow S_0$ transition of the donor. Recently, it was shown by single molecule spectroscopy that some chromophores can undergo photoinduced reverse intersystem crossing upon excitation with a second laser wavelength which is in resonance with the $T_1 \rightarrow T_N$ transition (Figure 1A).[26,27] In other cases, a single laser wavelength was sufficient to induce reverse intersystem crossing.[27,28] As depicted in the four-electronic-state scheme for the acceptor Cy5 in Figure 1A, there are two possible pathways available for the molecule in the excited triplet state, T_N, to return to the singlet regime: (i) by internal conversion, k_{ic}, and subsequent intersystem crossing, $k_{isc'}$, to S_0, or (ii) by reverse intersystem crossing, k_{isc}*, from $T_N \rightarrow S_N$ followed by relaxation to the first-excited singlet state S_1. Generally, a shortening of the triplet lifetime with increased excitation energy indicates photoinduced reverse intersystem crossing.[26] To determine if the Cy5 undergoes photoinduced reverse intersystem crossing when excited with the laser used for singlet absorption, $\lambda = 635$ nm, we studied the dependence of the triplet dark times on excitation intensity (Figure 2). Figure 2A shows a time trace of a single Cy5 molecule embedded in PVA. After about 8.5 s, the excitation intensity was increased from 1 μW to 10 μW. Simultaneously, a change in the blinking pattern occurred. The data were analyzed with both a duration histogram method and an autocorrelation approach[29] yielding comparable results. Figures 2B and 2C show the distribution of on-times and off-times, respectively, for the low and high excitation power regions of the time trace. The on-times shorten with

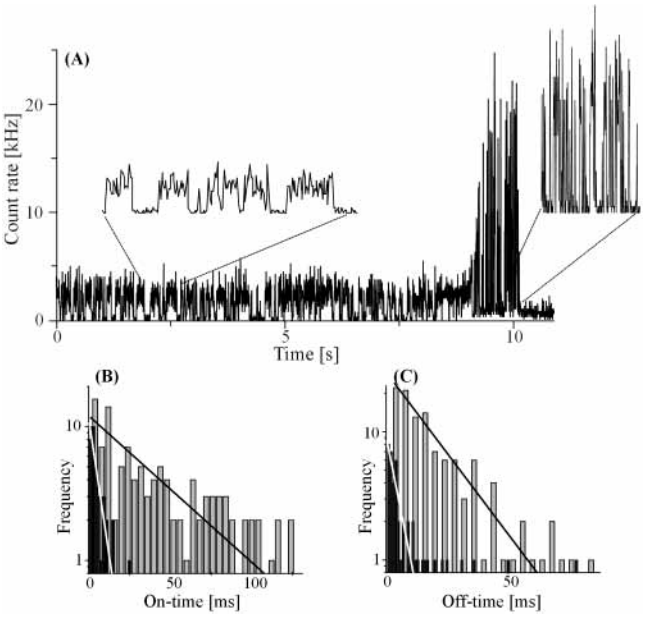

Figure 2. (A) Intensity time trace of a single Cy5 molecule immobilized in PVA (4 ms per bin). After 8.5 s the excitation intensity was increased from 1 μW to 10 μW. A change in the blinking pattern is evident. (B) Histogram of the on-times at 1 μW (gray) and at 10 μW (black). Average on-times are reduced from 39 to 5 ms at the higher excitation power. (C) Histogram of the off-times at 1 μW (gray) and at 10 μW (black). The off-times are reduced from 17 to 4 ms.

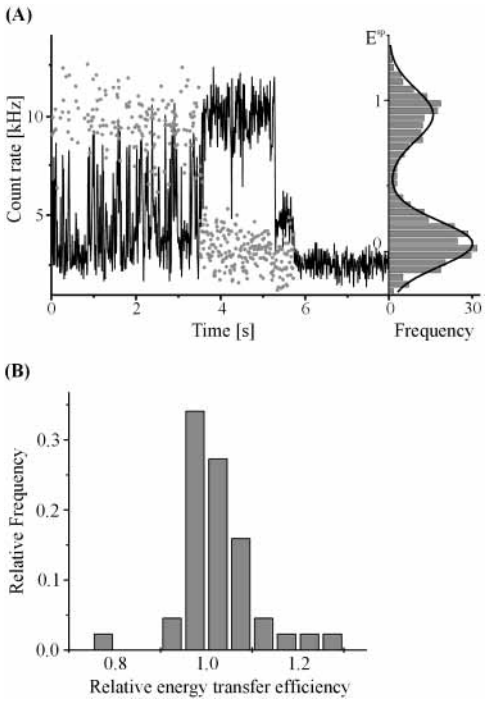

Figure 3. (A) Overall intensity time trace ($I = I_{donor} + I_{acceptor}$) of a single TMR−Cys-(Pro)$_{10}$-Lys-Cy5 molecule (black line) immobilized in PVA using an excitation energy of 5 μW (10 ms/bin). In addition, the calculated single-pair energy transfer efficiencies E^{sp} and corresponding intensity histograms with Gaussian fits are given. (B) Histogram of relative energy transfer efficiencies (quotient of mean donor intensities recorded during acceptor on- and off-states) comparing singlet state acceptor properties and triplet state acceptor properties for 44 bichromophoric systems.

increased excitation energy as expected for intersystem crossing to the triplet state, a photoinduced process. The average number of photon counts detected during on-times is constant with 75 $\pm$ 10 at 1 μW and 68 $\pm$ 12 at 10 μW. The off-times also decrease significantly at higher excitation power (from 17 to 4 ms), an indication of photoinduced reverse intersystem crossing. While fast photobleaching makes this experiment difficult, we observed a total of eight Cy5 molecules at low and high excitation power and observed that all showed the same result: shortened on-times and off-times with higher excitation power. We also observed large differences in photoinduced reverse intersystem crossing rates. This implies that besides the well-known heterogeneity of single-molecule absorption and emission spectra,[30] triplet absorption spectra or the coupling between the triplet and singlet manifold can also vary from molecule to molecule. Due to photoinduced reverse intersystem crossing of Cy5 molecules upon excitation at 635 nm, and the broadness of the tetramethylrhodamine (TMR) emission spectrum, singlet−triplet energy transfer between TMR and Cy5 can be anticipated. The results reported here are consistent with these hypotheses. Hence, intersystem crossing of one chromophore and subsequent resonant singlet−triplet energy transfer from the other chromophore(s) might cause the occurrence of collective blinking in multichromophoric systems.

By selectively exciting the donor, TMR, in TMR−Cys-(Pro)$_{10}$-Lys-Cy5, and detecting the fluorescence intensity of the donor and acceptor separately, it was possible to directly determine the efficiency of singlet−triplet energy transfer in individual bichromophoric systems. A representative example of the overall emitted fluorescence (sum of the intensities detected in the donor and acceptor channel) of a single TMR−Cys-(Pro)$_{10}$-Lys-Cy5 construct immobilized in PVA is shown in Figure 3A (solid line). The first 3.6 s of the trace is characterized by frequent blinking. Between 3.6 and 5.7 s the emission is more stable and only one intensity jump occurred before the construct photobleached. As the fluorescence of a bichromophoric system is monitored, the blinking in the first part of the trace can be denoted as "collective" because the fluorescence is temporary quenched, even though two chromophores are present. From this point of view, the system is comparable to multichromophoric systems that show collective on/off behavior. The background-corrected energy transfer efficiency, E^{sp}, was calculated ($E^{sp} = I_A^{corr} - C/(I_A^{corr} - C + I_D^{corr})$) for each 10 ms bin that had an overall intensity of 1 kHz above the average background level of 2 kHz. These calculated E^{sp}-values are also plotted in Figure 3A (gray dots). The cross talk, or contribution of TMR on the long-λ acceptor channel was 9%, so that $C = 0.09 \times I_D^{corr}$. In the first part of the trace, efficient energy transfer took place from the donor TMR to the acceptor Cy5. Although Figure 3 shows the overall fluorescence intensity recorded at the donor and acceptor channels, the first 3 s of the trace show strong fluorescence blinking similar to that observed from directly excited Cy5 in PVA (see Figure 2). This demonstrates that the TMR-fluorescence does not recover when the Cy5 molecule is in the nonfluorescent triplet state. The fact that the donor TMR remains quenched indicates that the triplet state T_1 of Cy5 is still an efficient energy acceptor. The fluorescence of the donor recovers only after the acceptor photobleached at ~3.5 s. The fluorescence intensity traces of TMR molecules are often characterized by several intensity levels. They also exhibit blinking on a slower time-scale than Cy5 with a few isolated transitions to dark states. Hence, the first part of the trace is dominated by the acceptor and reflects the typical behavior of a single Cy5 molecule in PVA while the second part of the trace represents a time trace of a single TMR molecule. For ≈80% of the 44 constructs studied, the emission of the donor TMR reemerges after the photobleach of the acceptor Cy5. In the other ≈20% of the traces, no donor

signal recovery was observed, indicating that the donor photobleached before the acceptor.[31]

The experimental data obtained from the biochromophoric system confirm that the $S_1 \rightarrow S_0$ transition of the donor is in resonance with the $T_1 \rightarrow T_N$ transition of the acceptor. Furthermore, we found that the energy transfer efficiency is similar for singlet−singlet and singlet−triplet energy transfer. According to Förster theory,[32] the difference between the rate constant for singlet−singlet and singlet−triplet energy transfer is controlled by the differences in absorption spectra and extinction coefficients of the $S_0 \rightarrow S_1$, and $T_1 \rightarrow T_N$ transitions of the acceptor, respectively. It has been shown that some cyanine dyes exhibit almost the same extinction coefficient, ϵ, for $T_1 \rightarrow T_N$ and $S_0 \rightarrow S_1$ transitions ($\epsilon_T = 1.1-1.6 \times 10^5$ M^{-1} cm^{-1}).[33] Furthermore, it is important to note that for fast energy transfer rates, i.e., for very short donor/acceptor distances such as in the peptide used, the extinction coefficient of the acceptor has only a minor influence on the energy transfer rate. To compare the singlet−singlet and singlet−triplet energy transfer efficiency, time traces of the double labeled peptides in PVA were analyzed in the following way. First, the acceptor-dominated part of the trace was selected, e.g., 0−3.5 s for the trace shown in Figure 3a. For this part of the trace the distribution of acceptor fluorescence intensities (5 ms time bins) was used to determine a threshold intensity level. The threshold level was used to decide, for each time bin, whether the Cy5 was in the triplet state (off-state) or in the singlet state (on-state). Hence, the difference between singlet−singlet and singlet−triplet energy transfer efficiencies of the constructs can be obtained by comparing the donor (TMR) fluorescence intensities recorded during the acceptor (Cy5) on- and off-states, respectively. The quotient of the mean values of donor intensities measured during the acceptor on- and off-states is a measure of the difference between the energy transfer efficiency to the acceptor singlet and triplet states, i.e., the relative energy transfer efficiency for each construct. The distribution of relative energy transfer efficiencies for the 44 constructs analyzed is shown in Figure 3B. The fact that the relative energy transfer efficiency is near one for all the molecules clearly demonstrates that the acceptor properties of the Cy5 triplet state are very similar to the acceptor properties for the Cy5 singlet state.

Some (7%) of the single-molecule traces exhibited a phenomenon different from that previously described. These molecules showed additional anticorrelated donor and acceptor fluorescence intensities. The observation of this phenomenon was previously reported for the same donor/acceptor pair covalently attached to a protein in an aqueous environment.[18,34] An example intensity trace showing anticorrelated donor and acceptor signals is shown in Figure 4. The acceptor shows fast intensity fluctuations with typical off-times of about 20 ms due to transitions to the triplet state and one pronounced off-state of ~100 ms duration after about 500 ms. In contrast to the short triplet dark times, the donor-fluorescence recovers fully during the longer off-state. These off-states occur very infrequently under our experimental conditions. It is known that Cy5 possesses a second nonfluorescent conformation in solution, i.e., the cis-conformation.[19] The fact that the isomerization is photoinduced in both directions[19] indicates that the cis-state absorbs in the same spectral region as the trans-state. Consequently, the donor should remain quenched when the acceptor Cy5 resides in the cis-state. The fact that the long off-state exhibits anticorrelated behavior of the donor and acceptor fluorescence means that the acceptor is not absorbing in this state. Hence, we conclude that the additional off-state that shows

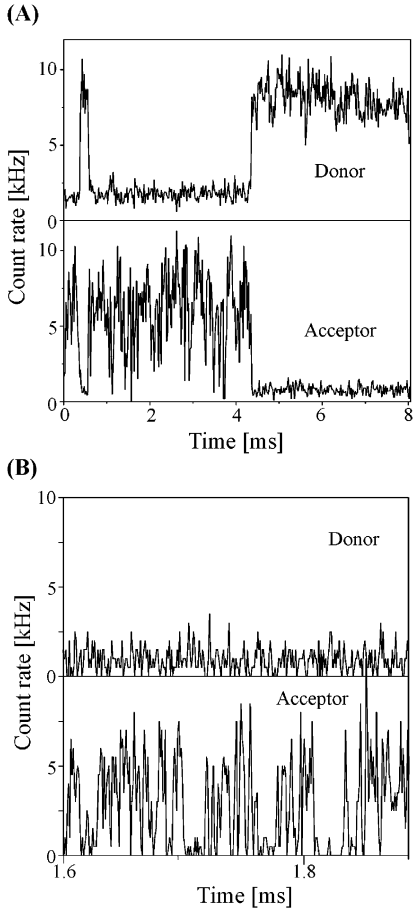

Figure 4. Fluorescence intensity time traces of donor (top) and acceptor (bottom) of a single bichromophoric model system immobilized in PVA at an excitation energy of 5 μW. (A) During the off state of the acceptor after ≈0.5 s the donor fluorescence fully recovered. In 7% of the donor/acceptor constructs studied, anticorrelated fluorescence intensities were observed for long acceptor dark times (in the range of 100 ms). (B) The expanded view of the same time trace directly demonstrates that within the shorter off-times of the acceptor (due to intersystem crossing to the triplet state) the donor emission does not recover. The data are binned 10ms/point and 1ms/point in (A) and (B), respectively.

anticorrelated fluorescence of donor and acceptor is not related to the cis-state of Cy5. More likely, it is related to an electron-transfer process (an oxidized or reduced acceptor) or to a reversible photoreaction with, e.g., oxygen, as was suggested to be the source of collective on−off blinking in conjugated polymers.[12]

The results presented here demonstrate that singlet−triplet energy transfer is an efficient pathway to quench the fluorescence of a multichromophoric system. Furthermore, our data show that singlet−triplet energy transfer should be taken into account for the dye pair TMR/Cy5 when interpreting spFRET experiments. Since the photophysics of the dyes used are influenced by the surrounding medium, e.g., the oxygen concentration and the experimental conditions (excitation intensity), the resulting FRET efficiencies might be affected.

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