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Triplet states as non-radiative traps in multichromophoric entities: single molecule spectroscopy of an artificial and natural antenna system

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Abstract

Energy transfer in antenna systems, ordered arrays of chromophores, is one of the key steps in the photosynthetic process. The photophysical processes taking place in such multichromophoric systems, even at the single molecule level, are complicated and not yet fully understood. Instead of directly studying individual antenna systems, we have chosen to focus first on systems for which the amount of chromophores and the interactions among the chromophores can be varied in a systematic way. Dendrimers with a controlled number of chromophores at the rim fulfill those requirements perfectly. A detailed photophysical study of a second-generation dendrimer, containing eight peryleneimide chromophores at the rim, was performed 'J. Am. Chem. Soc., 122 (2000) 9278'. One of the most intriguing findings was the presence of collective on/off jumps in the fluorescence intensity traces of the dendrimers. This phenomenon can be explained by assuming a simultaneous presence of both a radiative trap (energetically lowest chromophoric site) and a non-radiative trap (triplet state of one chromophore) within one individual dendrimer. It was shown that an analogue scheme could explain the collective on/off jumps in the fluorescence intensity traces of the photosynthetic pigment B-phycoerythrin (B-PE) (*Porphyridium cruentum*). The different values of the triplet lifetime that could be recovered for a fluorescence intensity trace of B-PE were correlated with different intensity levels in the trace, suggesting different chromophores acting as a trap as function of time.

Keywords: Single molecule detection; Multichromophoric dendrimers; Model systems; Energy transfer; Phycoerythrin

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1. Introduction

The absorption of photons by light harvesting complexes or antenna systems, followed by fast and efficient excitation transfer, is a key step in the photosynthesis. In general, the antenna systems consist of aggregates of protein-embedded chromophores. The chromophores are kept in a well defined, more or less ordered arrangement by the host proteins. It has been shown that the energy transfer (ET) process depends to a large extent on the strength of interactions among the chromophores [1,2]. These interactions in turn depend upon the relative orientations and distances between the chromophores in the antenna system. Moreover, in a recent publication the role of disorder (due to slow and fast fluctuations within the surrounding protein) on the ET process of antenna systems was pointed out [3].

The three-dimensional structure of a number of photosynthetic complexes has been worked out with high accuracy, e.g. the light harvesting (LH2) complex of purple bacteria by McDermott et al. [4]. The development of single molecule spectroscopy (SMS) allows studying individual antenna systems at cryogenic temperatures as well as at room temperature. It has been shown that SMS is an excellent technique to investigate spatial, conformational and temporal inhomogeneity of populations [5,6]. Linking these independently acquired data makes it possible to draw new conclusions concerning the structure of and processes in such antenna systems. Recently, this approach was applied to LH2 at cryogenic temperatures and resulted in new insights into the electronic structure of the LH2 complex [7,8]. Up to now, single molecule studies on immobilized light harvesting systems have been focussed on the highly symmetric LH2 system in which the binding sites of chromophores and the distances between them (within one of the two rings) are very similar [7–12]. In the light harvesting complexes of plants and algae, the symmetry in position of the chromophores is either much less pronounced or absent. The supramolecular light-harvesting antenna systems of cyanobacteria and red algae are called the phycobilisomes (attached to the stroma side of thylakoid membranes). These phycobilisomes consist of phycobiliproteins, which contain bilin-chromophores and linker-polypeptides. Several different chromophores are present in phycobiliproteins, e.g. phycoerythrobilin and phycocourobilin. Phycobiliproteins can be divided into three major classes: the phycoerythrins (PE), the phycocyanins (PC) and the allophycocyanins (APC) [13]. The structure of the light-harvesting complex reveals the direction of ET. APC is close to the reaction center, forming a core to which rods are attached. PC is present in the middle of these rods, whereas PE is present at the rim. The light energy is transferred from PE to APC via PC. Finally it reaches the reaction center. The overall ET efficiency approaches 100% (Fig. 1).

The mechanism of ET in PE is complicated because of the high number of chromophores present. Fluorescence polarization anisotropy has been used to investigate the ET process [14–16]. The energy migrates from bilin to bilin chromophore but the exact details remain to be solved.

The functional unit in B-PE, the antenna complex of *Porphyridium cruentum*, is an $(\alpha\beta)_6\gamma$ hexamer. Two trimers, formed by three $\alpha\beta$ -monomer units, are stacked and the open space is filled with a γ-subunit. In crystals, B-PE exists as stable hexameric aggregates [17]. It has been shown for phycoerythrin 545 [18–21] and for the related compound phycocyanin [22,23] that in solution, depending on the pH, concentration, type of buffer and ionic strength, the protein can be

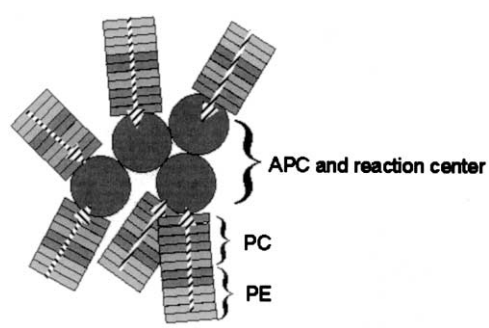

Fig. 1. General picture of the structure of phycobilosomes: the arrow indicates the direction of the ET from the phycoerythrin (PE) via phycocyanin (PC) and allophycocyanin (APC) towards the reaction center.

present as a monomer ($\alpha\beta$), trimer $(\alpha\beta)_3$, hexamer $(\alpha\beta)_6$ or another aggregated form. In B-phycoerythrin the α-subunit contains two phycoerythrobilins as chromophores and the β-subunit three phycoerythrobilins, whereas the γ-subunit of B-PE has four bilins, two phycoerythrobilins and two phycourobilins, respectively.

Single-molecule studies on B-PE reported in literature so far were carried out in solution at femtomolar concentrations [24–26]. Even at this low concentrations it was assumed that B-PE was present as an $(\alpha\beta)_6\gamma$ hexamer. However, Peck et al. stated that the use of 3-(pyridyldithio)propionic acid and *N*-hydroxysuccinimide ester leads to the formation of stable αβ-dimers at such concentrations [27].

The photophysical processes taking place in antenna systems, even at the single molecule level, are complicated. Therefore, we have chosen to focus on systems for which the amount of chromophores and the interactions among the chromophores can be changed systematically in order to unravel the photophysical processes, including ET, in multichromophoric systems. Dendrimers perfectly fulfill those requirements since fluorescent chromophores can be attached to the surface of the dendrimer [28–31]. Thus, dendrimer synthesis serves as a way to obtain a well defined number of chromophores in a confined volume element. Not only the number of chromophores can be controlled, but also the interactions among the chromophores can be governed by changing the structure of the branches to which the chromophores are attached or by attaching the branches to different cores. The chromophores in each branch of the dendrimer readily allow to probe interactions of the branches, conformational distortions as well as excitation ET or electron transfer among the chromophores.

In this contribution, a model for the collective fluorescence on/off jumps observed for individual immobilized molecules of a second-generation dendrimer, containing eight chromophores at the rim, is presented based on the comparison between the dendrimer and a model compound. The model is then extrapolated to explain the collective on/off jumps found in fluorescence intensity traces of individual αβ-monomers of B-PE (*P. cruentum*) immobilized in a polyvinylalcohol film.

2. Instrumental

A detailed description of the synthesis of the model compound (g0) and the second generation dendrimer containing eight chromophores in the rim (g2) was reported previously [32–35].

Absorption spectra were measured on a Perkin-Elmer Lambda 40. Fluorescence spectra were recorded on a SPEX Fluorolog 1680. The transient spectra were recorded with a setup, the details of which have been published elsewhere [36].

Samples for the single molecule measurements on the dendrimer were prepared by spin-coating solutions of g2 and g0 (5×10^{-10} M) in chloroform containing 3 mg/ml polyvinylbutyral (PVB) on a cover glass at 4000 rpm to yield thin (20–40 nm, measured by AFM) polymer films containing on average 0.2 molecules per μm^2.

The B-PE samples (B-Phycoerythrin from the red algae *P. cruentum*, lyophylized powder, Sigma) were prepared by mixing equal quantities of solutions containing 5×10^{-11} M PE in phosphate buffered saline (PBS, pH 7.5, Sigma) and solution containing 2 wt% polyvinylalcohol (PVA, Fluka, $M_w = 145\,000$) in PBS, respectively. The resulting solution was spincoated (1000 rpm) on a coverglass yielding polymer films of a few hundred nm thickness, containing on average 0.2 molecules per μm^2. In comparison to g0 and g2, lower concentrations of PE had to be used as similar concentrations resulted in a factor 5–10 more spots in an image then expected. This indeed points towards dissociation of the PE $(\alpha\beta)_6\gamma$ aggregate into monomer units. Spectra of individual spots were taken to ensure that the larger amount of spots was not caused by impurities. Transients were recorded of spots with equal intensity. The few spots that had a substantially higher intensity than the average spot have been attributed to trimers or intact $(\alpha\beta)_6\gamma$ aggregates and were not included in the analysis. Careful cleaning of the glassware as well as subsequent cleaning of the cover glasses by successive sonication in acetone, sodium hydroxide (10%) and milliQ water preceded sample preparation.

The fluorescence of single molecules was detected by means of a confocal microscope (Di-

aphot 200, Nikon) with an oil immersion lens (NA 1.4) equipped with an avalanche photodiode (APD) in single photon counting mode (SPCM AQ15, EG&G) as the detector. Suitable filters were placed in the detection path to suppress remaining excitation light. The fluorescence intensity transients were measured with dwell times ranging from 150 μs to 5 ms. Excitation sources were an Argon ion laser for 488 nm (Spectra Physics Stabilite 2017) and a Helium Neon (HeNe) laser (Melles Griot 05-LGR-193) for 543 nm. The fluorescence spectra were measured with a liquid nitrogen cooled, back-illuminated CCD camera (LN/CCD-512SB, Princeton Instruments) coupled to a 150 mm polychromator (SpectraPro 150, Acton Research Cooperation) using 5, 8 or 10 s integration time. The recorded spectra were corrected for the background, the response of the CCD-camera and the optics used. Determination of the peak position of each spectrum was done by calculating the first and second derivative. The resulting accuracy is $\approx \pm 1$ nm. Polarization measurements were performed by splitting the signal with a polarizing beam splitter cube (Newport 05FC16PB.3) and detecting *s* and *p* polarized components of the fluorescence light with two independent detectors. Modulation of the excitation was obtained by passing linear polarized laser light through a $\lambda/2$ plate rotating with a stable frequency.

3. Results and discussion

3.1. Dendrimer and model compound

The structure of the model compound g0, the dendrimer g2 and the chromophore are shown in Fig. 2(a), (b) and (d), respectively. As chromophore peryleneimide was introduced because of its photostability, its absorption wavelength (around 500 nm), its relatively high absorption coefficient ($\varepsilon = 38\,000\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$ at 490 nm) and its high fluorescence quantum yield ($\phi_f = 0.95$). The polyphenylene core of the dendrimer and model compound does not absorb at the excitation wavelength range of interest (above 450 nm). An important feature of the easily soluble 3D-polyphenylene dendrimers is the relatively high shape persistence [37]. A three-dimensional representation of the dendrimer with the chromophores in the rim is shown in Fig. 2(c).

As can be seen in the figure, both isolated and dimer-like interacting chromophores exist. The presence of both species within individual dendrimers was spectroscopically demonstrated [38]. Typical fluorescence intensity trajectories (transients), that is total fluorescence intensity of a single molecule as function of time, for g0 and g2 are shown in Figs. 3 and 4, respectively (molecules embedded in a 30 nm thin polyvinylbutyral (PVB) polymer film).

Sixty percent of the investigated transients for g0 show a one-step photobleaching (Fig. 3(a)) as can be expected for a single chromophore. In 35% of the transients the fluorescence intensity drops to the background level (Fig. 3(b)) for periods ranging from 5 to 1200 ms and returns to the initial level before irreversible photobleaching, an inevitable process in SMS, took place. These drops in fluorescence intensity (usually one or two per transient) are referred to as off times. Fig. 3(c) shows a zoom in one on/off event. Off times can originate from several processes. Often they are related to occupation of the triplet state [39,40]. As shown in literature, assuming a three-level system (S_0, S_1, T_1) triplet lifetimes can be calculated from the off-times [41]. Applying this model to g0, a fit of the off-times yields a triplet lifetime of 110 ms (inset in Fig. 3(c)). This value is considered as an upper limit since the bin time of 5 ms will hide short off-times. Triplet lifetimes in the millisecond range were reported for other immobilized single molecules [42,43]. In addition, for 5% of the investigated transients jumps between different emissive intensity levels were detected (results not shown).

Fig. 4(a) shows a transient for g2 excited with circular polarized light. On/off behavior as well as jumps between different emissive levels can be detected and are exemplified in panels b and c.

The transients of g2 show more levels and jumps and longer survival times (time of irradiation until irreversible photobleaching takes place) compared to those of the model under identical excitation conditions [35]. Using excitation light

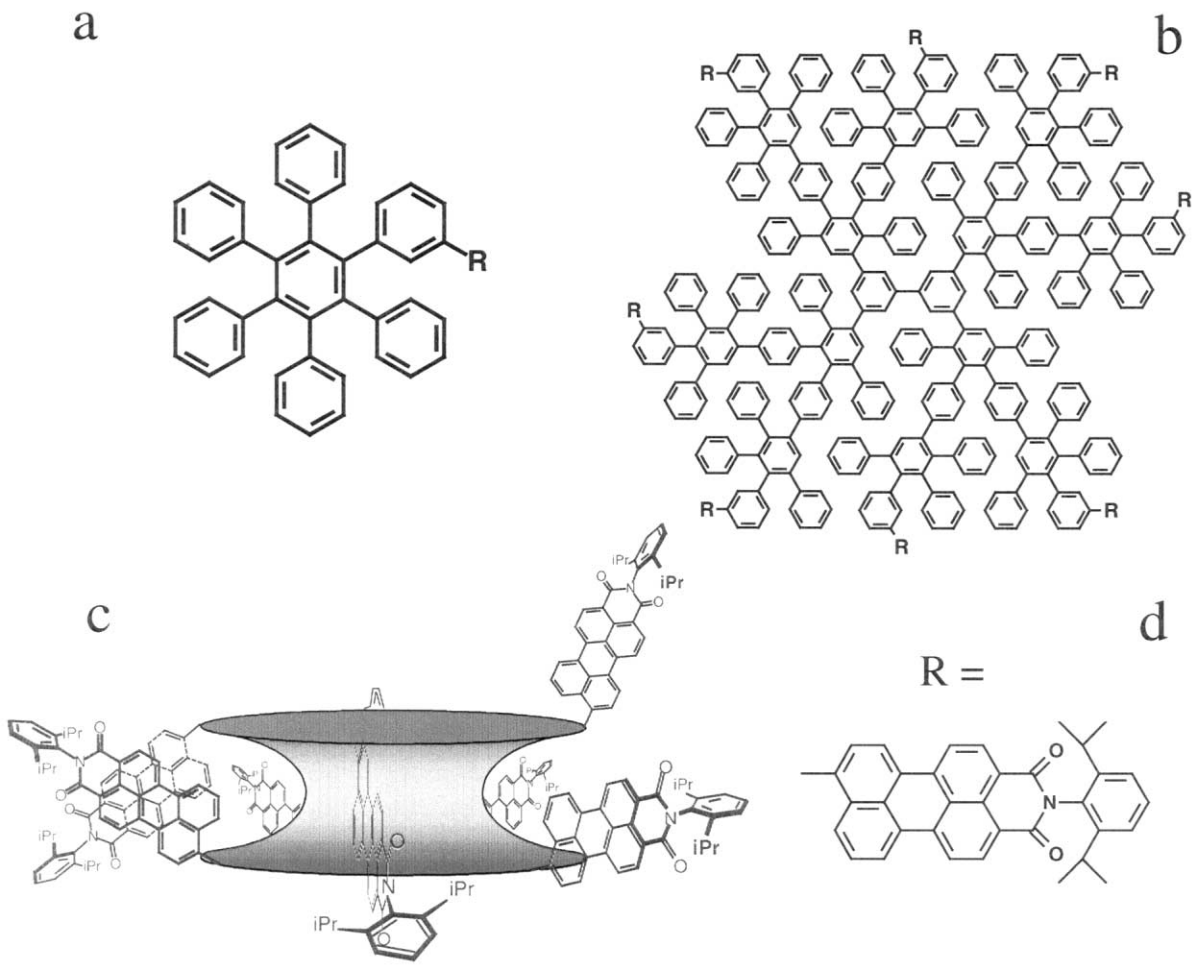

Fig. 2. Structures of (a) the model compound g0 and (b) the second generation dendrimer g2. The chromophores are represented by R. (c) Cartoon of the three dimensional structure of the dendrimer illustrating the presence of both isolated and interacting chromophores. (d) The structure of the chromophore R.

of 488 nm (350 W/cm^2 at the sample), g0 has an average survival time of 70 s and 1.5×10^5 photons are detected on average before the individual molecules photobleach (average of 84 molecules). Using circular polarized light at the same wavelength and with the same excitation power leads for g2 to transients containing on average 11×10^5 photons and showing a mean survival time of 450 s. Long survival times always correspond with low levels at the end of the transient, as demonstrated in Fig. 4(a). In general, the highest levels are found in the first part of the transient whereas low levels are observed in the end of the transient. If the eight chromophores of g2 absorbed and emitted independently from each other, one would expect mainly jumps between close lying intensity levels. All fluorescence transients of g2 show reversible jumps between a high level and a low level or the off level. The duration of the off state for the reversible jumps (back to the initial intensity level) in the beginning of the transient varies from a few ms to several hundreds. Off-states in later parts of the transient can last for several seconds or tens of seconds (Fig. 4(c)). The eight chromophores being simultaneously in the off state is an implausible explanation for the

observed collective on/off behavior in the transients of g2. At this point it must be emphasized that at the excitation powers used the formation of more than one singlet excited state at a time is very unlikely [44]. Another explanation could be that the eight chromophores are strongly coupled and hence act as one quantum system. If this system went to an off state, such as the triplet state, this would account for the collective phenomena observed for g2. The solution data do not support this model as the absorption spectra of g2 and g0 hardly differ [31]. However, coulombic interaction in the excited state is possible. Indeed there is a difference in the fluorescence properties as expressed in the reduced quantum yield of fluorescence, the emission spectra and the more complex time dependent behavior [31]. Calculations show that all chromophores are well within the Förster radius for singlet ET [31]. This implies that fluorescence will occur from the chromophoric site (either an isolated chromophore or

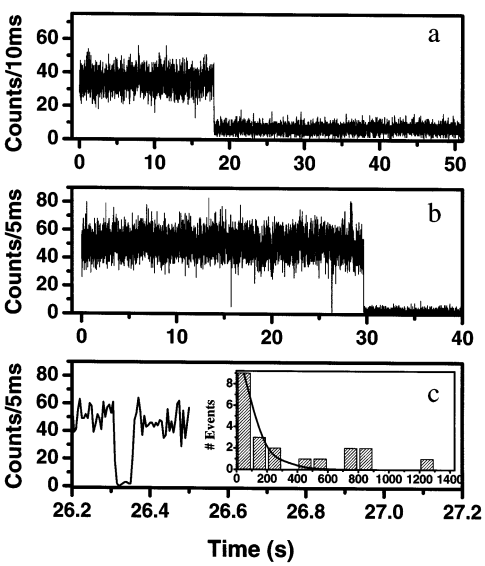

Fig. 3. Transients of the model compound g0 excited at 488 nm (intensity of 350 W/cm^2 at the sample) in a 30 nm thin PVB film. (a) 60% of the transients show a one-step photobleaching behavior and no other features. (b) 35% of the transients show one or more off-periods (intensity drops to the background level). (c) Zoom of the first off period of the transient in b. The inset shows the result of the analysis of the off-times of 80 investigated g0 molecules resulting in a triplet lifetime of 110 ms.

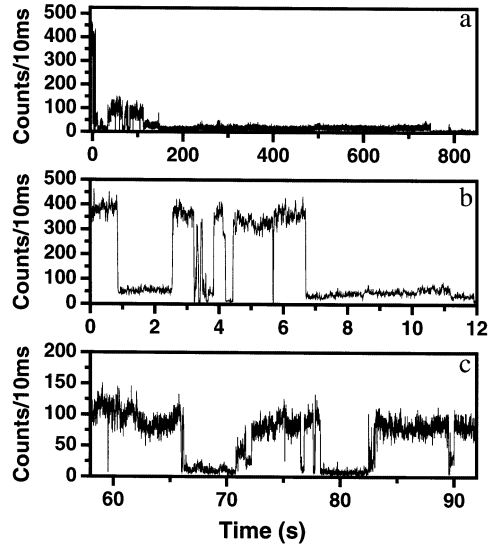

Fig. 4. Transients of the dendrimer g2 excited at 488 nm (intensity of 350 W/cm^2 at the sample) in a 30 nm thin PVB film. (a) Transient recorded with circular polarized excitation light, hence sampling all eight chromophores in the dendrimer. Several off-periods as well as levels can be seen in the transient. (b) Zoom in the high intensity level of the transient in a, exemplifying the short off-times. (c) Zoom in a later part of the transient in a, demonstrating the occurrence of long off-times.

dimer-like interacting chromophores) in the dendrimer that at a given point in the trajectory is lowest in energy and hence acts as a trapping site from which fluorescence will occur. Evidence for the fact that the energetically lowest chromophoric site is acting as a fluorescent trap was found in the gradual blue shift of the emission maximum for a number of individual g2 molecules as function of time [38]. Measuring polarized transients further substantiated the concept of one fluorescent trap. The polarization p was shown to change in discrete steps (values were found between -1 and 1) and a maximum of four different values for p was recovered for individual g2 molecules [38]. More evidence for one fluorescent trap was obtained by excitation of individual g2 molecules with modulated excitation light. Hereto, a 20 Hz modulation frequency was imposed on the excitation light by guiding linear polarized light through a rotating waveplate. This means for a single chromophore entity that the

superimposed frequency should be encountered in the acquired transient as a change in intensity, since the intensity should drop to the background level whenever the orientation of the excitation light and of the transition dipole moment are perpendicular with respect to each other [38].

For the multichromophoric system it cannot be expected that the modulated fluorescence intensity drops to the background level in the same way as for the model compound as the different chromophores have different orientations. Modulation pattern, depth of modulation and polarization characteristics vary from the one of single chromophore single molecules and strongly depend on the ET and emission processes taking place in the multichromophoric unit. As an example, Ying and Xie found no modulation in intact allophycocyanines (APCs) but modulation with different depth and phase depending on the number of emitting chromophores in APC with photoinduced trap states [13]. A separate study, in which we investigated extensively the behavior of individual g2 molecules when excited with modulated excitation polarization direction will be published elsewhere [45]. Two typical and distinctively different patterns were found, no modulation and in-phase modulation with small and medium modulation depths (Fig. 5). Comparison of modulation patterns and values of polarization with simulated data based on different photophysical models of g2 gave evidence for ET occurring in every single g2 molecule. The energy is transferred to the energetically lowest chromophore (or dimer-like-interacting state, [38]) from which emission occurs, further corroborating the 'one fluorescent trap' model. However, this is not a static situation since the energy levels of the chromophores can change as well as the location of the dimer-like state. The changes happen on a sub-millisecond to minute time scale. Details on the used procedures and simulating parameters can be found elsewhere [45].

The duration of the collective off periods in the beginning of the transients of g2 is very similar to the duration of the off states in g0. From this it can be deduced that off states are likely related to triplet formation in one of the chromophoric sites in the dendrimer, at least in the early stages of the transient. ET from the fluorescent trap to the chromophore in the triplet state will then result in the observed collective on/off behavior. Excitation ET from the first singlet excited state to the first triplet state resulting in the singlet ground state and a higher lying triplet state is a spin allowed process [46]. The mechanism is exemplified in Fig. 6.

This process can occur in multichromophoric dendrimer systems like g2, if the rate constant of ET from the singlet excited state to the triplet excited state is sufficiently high. The good overlap between the triplet absorption spectrum of g2, measured in solution by means of the transient absorption technique, and the emission spectra of

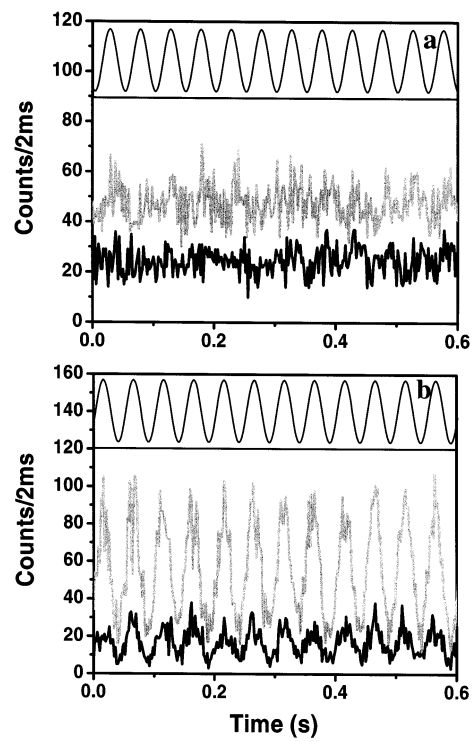

Fig. 5. Modulated transient trace for g2 molecules (excitation at 488 nm, 500 W/cm^2). The modulation frequency was 20 Hz (upper curve in a and b). Parallel and perpendicular-polarized components were recorded in different detection channels (gray for parallel-polarized light, black for perpendicular-polarized light, uncorrected signals). (a) Example of a molecule in which no modulation is seen. (b) Example of a molecule that shows modulation in both detection channels, although the intensity never drops to the background level.

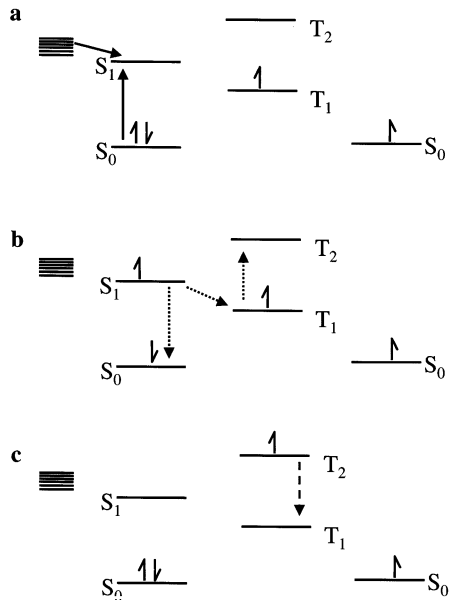

Fig. 6. Pseudo three-level scheme explaining the on/off intensity jumps in a multichromophoric system. (a) One chromophore in g2 is in the triplet state T_1, while the S_1 of the energetically lowest chromophore is either directly excited or populated via ET. (b) ET between S_1 and T_1 takes place resulting in repopulating of S_0 of the energetically lowest chromophore and population of T_2 of the chromophore carrying the triplet state. (c) T_2 relaxes back to T_1 via fast internal conversion.

both g0 and g2 in solution further supports this hypothesis as shown in Fig. 7. Note that the real overlap between both spectra will be larger as shown in the figure as the transient spectrum is truncated due to ground state depletion as an excitation wavelength of 532 nm is used in the experiment.

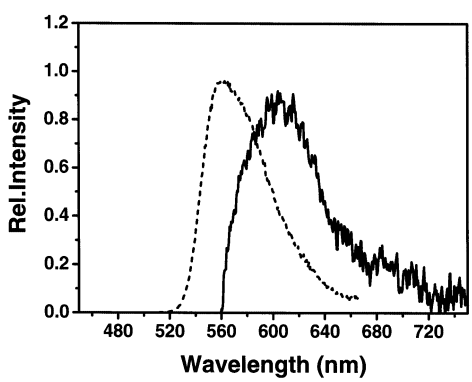

Fig. 7. Fluorescence spectrum (dashed line) and difference absorption spectrum of g2 in solution (toluene) at 45 μs after excitation.

The relaxation of the higher triplet state (T_n) to the first triplet state (T_1) is a very fast, spin allowed non-radiative process. The competition between singlet/triplet ET and fluorescence from S_1 might then account for the occurrence of both off levels and low levels within the binning time. A similar mechanism, involving singlet-triplet annihilation, was previously suggested in literature to play a role in the multichromophoric allophycocyanin system [13]. It is well known that O_2 is a quencher of the triplet state [47]. This was recently demonstrated on the single molecule level by a comparison of the triplet decay time of DiI immobilized in a polymer film under ambient conditions and in a rigorously degassed polymer film, sealed by aluminum coating. The latter sample yielded triplet decay times in the millisecond range, whereas the sample in ambient conditions gave a triplet lifetime of a few hundred microseconds [42,48]. If the triplet is involved in the collective on/off jumps the duration of off periods should increase upon lowering the oxygen concentration in the film. This is demonstrated in Fig. 8. The spot in Fig. 8(a) represents the fluorescence from a single g2 molecule recorded under ambient conditions. As can be seen, the fluorescence intensity is not uniformly distributed, dark pixels can be seen in the image. These pixels correspond to the above discussed collective on/off jumps. The images presented in Fig. 8 were recorded consecutively (15 s recording time per image). Purging of the polymer film with N_2 started at the end of the first image (Fig. 8(a)). As a result of the O_2 depletion in the film, a spot with much longer dark intervals (Fig. 8(b)) was obtained. The effect is even more pronounced upon longer purging in the third image (Fig. 8(c)). When the N_2 flow is interrupted, an image similar to the first one is obtained (Fig. 8(d)), suggesting that the triplet state is indeed involved in the process of collective on/off jumps.

As triplet lifetimes of several seconds, as observed in later parts of the transients of g2, are unlikely, other deactivation channels have to be

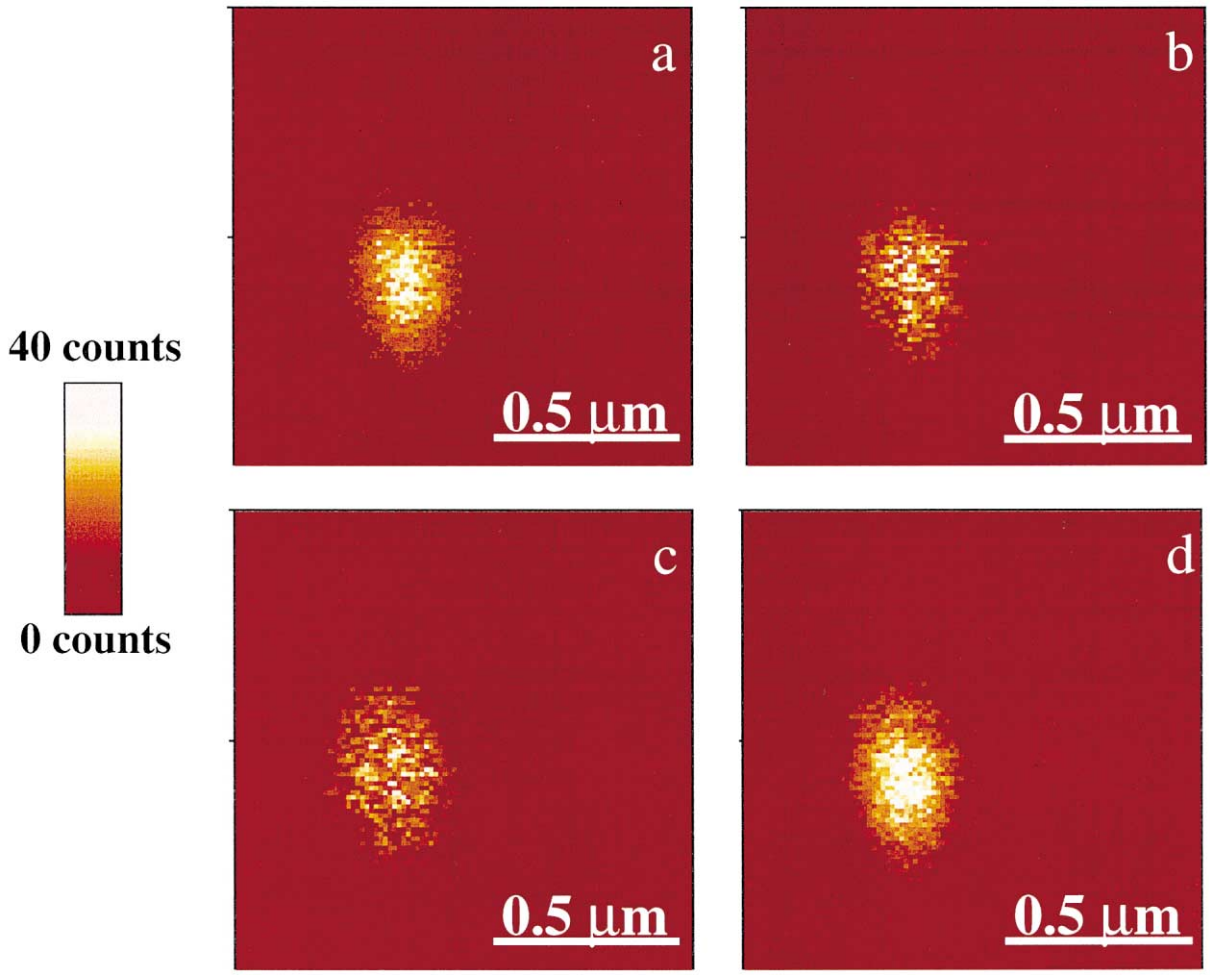

Fig. 8. Consecutive images of an individual g2 molecule in a thin PVB film. The scan time per image is 15 s. Immediately after recording image *a*, a N_2 flow was switched on in order to purge the sample while the recording of image *b* was started. Image *c* is also recorded under N_2 atmosphere but the N_2 flow is switched of after recording this image. Image *d* was obtained under ambient conditions again.

considered. The formation of a radical/cation or radical/anion pair was suggested for a different multichromophoric system and might play a role in this system as well [49].

3.2. B-Phycoerythrin

The structure of the bilin chromophores of B-PE and the positions within the polypeptide chain, based on crystallographic data [17], of an αβ-monomer unit are shown in Fig. 9. The chromophores are bound via the sulfur of cystein amino acids in the polypeptide chain. Due to the slightly different environments of the five chromophores, their energetic properties can differ slightly. Note that one of the chromophores (1β-50,61) is linked with two sulfur bonds. Interchromophore distances range from slightly more than 2 up to 6 nm [17].

From the overlap between absorption spectrum and emission spectrum a Förster radius for ET of $\pm$ 5 nm was calculated [17]. This means eventually that weak coulombic interactions in the excited state are present among the five chromophores

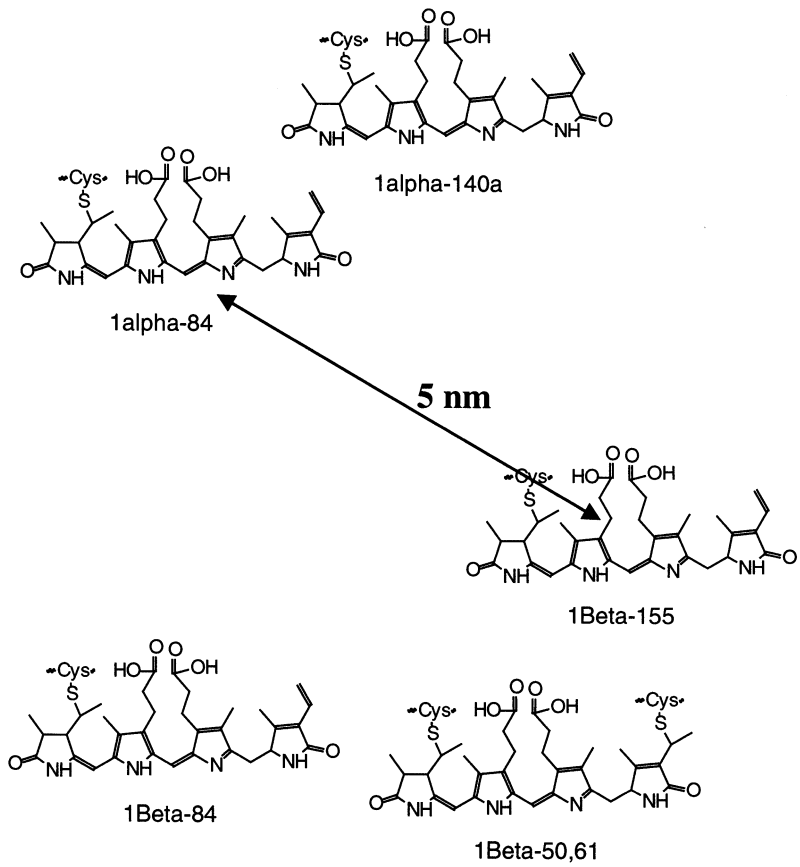

Fig. 9. The bilin chromophores present in an αβ-monomer unit of B-PE based on data from [17].

within individual αβ-monomer units (Fig. 10). However, it is obvious that they are not close enough to each other to have strong ground-state interactions as reported before by Wu et al. [26].

Typical transients recorded for B-PE immobilized in PVA are displayed in Fig. 11(a–c) (excitation wavelength 543 nm, circular polarized light, P is 200 W/cm^2 at the sample). A strong resemblance with the transients of g2 can be observed (Fig. 11(d)). Similar to the g2 transients, different intensity levels and jumps between the levels are present. Principally, the high intensity levels are located at early stages of the transient.

Long levels of low intensity are seen at the end of the transients. The main difference with g2 transients is the shorter survival time of the transients (although less excitation power was used) of about 80 s (450 s for g2). An average of 5×10^5 photons are emitted before the molecules photobleach (72 transients analyzed). Even though the extinction coefficient of a single bilin chro-

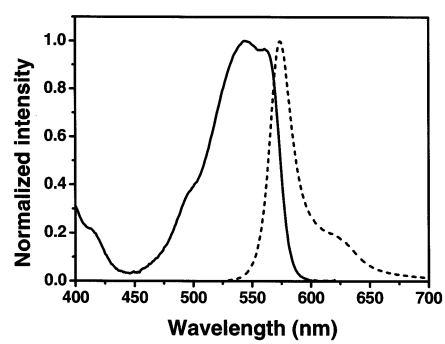

Fig. 10. Absorption spectrum (solid line) and emission spectrum (dashed line) of B-PE in PBS buffer.

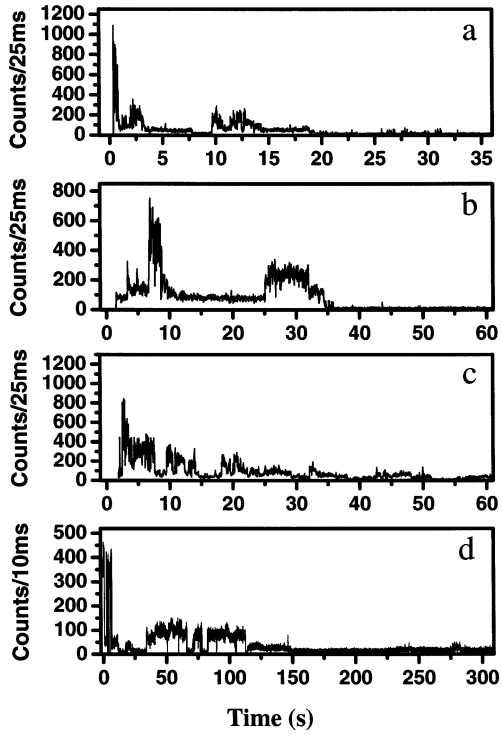

Fig. 11. (a–c) Typical transients of individual αβ units of PE in PVA excited at 543 nm (intensity at the sample 200 W/cm^2). High intensity levels are mainly located in the early parts of the transient. Low levels and long off periods can be seen in later parts of the transients. (d) A striking resemblance between the transients of g2 and PE can be seen. The main difference is the longer survival times of the g2 transients.

mophore is substantially higher than that of a perylenimide chromophore ($\varepsilon \cong 55\,000$ cm^{-1} M^{-1} versus $\varepsilon \cong 38\,000$ cm^{-1} M^{-1}) and both systems have a similar quantum yield of fluorescence ($\phi_{\text{f bilin}} = 1$, $\phi_{\text{f perylenimide}} = 0.9$), the number of emitted photons per chromophore (1×10^5 for bilin, 1.5×10^5 for peryleneimide) is lower. This indicates a lower photostability for the bilin chromophore as compared with the peryleneimide chromophore.

A detailed look into the transients of B-PE reveals numerous on/off jumps in all intensity levels. This is exemplified in Fig. 12. The transient in Fig. 12 is the same one as in Fig. 11(c) but plotted with different bin times (500 μs and 25 ms, respectively). The high level at the early stage of the transient is shown in detail in Fig. 12(b) and (c), clearly demonstrating collective on/off jumps of all 5 chromophores.

A model similar to the one for g2 can be assumed: energy hopping among the chromophores followed by emission from the energetically lowest chromophore and collective off jumps due to the creation of a triplet state in one of the chromophores. The large number of on/off jumps indicates that the process of intersystem crossing is more probable for bilin type of chromophores. The influence of O_2 on the duration of the off periods was tested in the same way as done for g2 (Fig. 8) by consecutive scanning and changing between ambient environment and N_2 atmosphere. Hardly any changes could be detected (results not shown). The reason for this difference is twofold: (1) much thicker films were used in the PE single molecule experiment as compared with

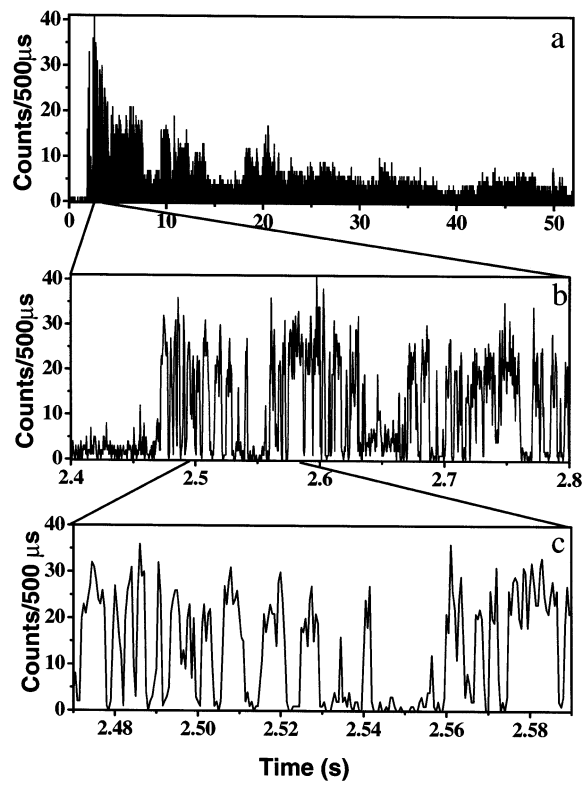

Fig. 12. (a) The transient from Fig. 11(c) plotted with a bin time of 500 μs. (b) Zoom in the first 400 ms of the transient. (c) Zoom in the first 100 ms of the transient. Distinct on/off steps can be seen.

the g2 single molecule experiment and hence the response to the change in conditions will be slower (2) PVA has a permeability for O_2 that is a factor 1000 lower than for polymers like polystyrene, resulting in already prolonged triplet lifetimes in PVA films [50].

Very few triplet transient absorption spectra of phycobiliproteins have been published in literature. Recently, the triplet transient spectrum of C-phycocyanin (C-PC) was presented [51]. A nearly perfect overlap between fluorescence spectrum and triplet transient absorption spectrum of C-PC can be seen. Due to the strong resemblance of all phycobiliproteins, a similar overlap can be expected for the fluorescence spectrum and triplet transient absorption spectrum of B-PE.

Wu et al. [26] also reported about one-step off-behavior of individual B-PE molecules in solution. The authors did not consider the equilibrium between the $(\alpha\beta)_6\gamma$ aggregate and the monomer units from which it is built up. Recovery of the fluorescence intensity was not reported and hence the authors call their observation a one-step photobleaching process of 34 chromophores. The transients in Fig. 12 clearly show that in our experiment photobleaching only occurs after many on/off steps. The differences in experimental results can be attributed to different experimental conditions. The excitation powers in both experiments are different (180 nW in our experiment versus 1 mW in the experiment of Wu et al.). As a result, there is a high probability to have simultaneously more than one excitation within one molecule leading to more complicated photophysics. Furthermore, the transit times of the molecules through the laser beam was varied from 6 to 40 ms while the integration time of the detected fluorescence is 1 ms. As the triplet lifetime in solution will be substantially shorter than in PVA, many on/off events will be missed and only an average intensity will be seen.

As stated above, the analysis of the duration of the off period yields information about the triplet lifetime, assuming a three-level scheme. Taking into account the model for the collective on/off behavior outlined above, the pseudo three-level scheme used in Fig. 6 can be used to analyze the duration of the off periods.

The duration of the dark periods was determined by selecting a signal level to discriminate between emission and dark periods. The 1% lower limit confidence level for the Poissonian photon noise distribution of the time-integrated fit of the intensity of the transient was taken as level to distinguishing between on and off levels [42]. A frequency histogram of the duration of all dark periods for a certain time interval of the transient yields an exponential decay with a typical triplet decay time (τ_T) for that interval. Usually, the full duration of the transient can be analyzed as being mono-exponential [48]. Non of the analyzed transients of B-PE (85 different molecules) yielded frequency histograms that could be fitted mono-exponentially. An example is given in Fig. 13(a). The transient displayed in Fig. 12 was analyzed from 1.85 s (corresponding to the moment where the laser was switched on) to 14 s (corresponding to the start of the first long off period). Even fitting with a 3-exponential decay yielded poor quality fitting parameters.

This implies that either the value of τ_T changes as function of time or that multiple decay channels are present [42]. As our on/off model implements that different chromophores can act as the non-radiative trap, the latter assumption can be valid in this system. Hence, the transients were not analyzed as a whole but in intervals of less than 1 s. Whenever consecutive blocks resulted in similar values of τ_T (within 10%), the blocks were summed up and reanalyzed. The result of the analysis of an interval (2.4–2.9 s) of the transient from Fig. 12 is shown in Fig. 13(b). A mono-exponential fit with good fit-quality parameters was obtained.

As can be seen in Fig. 14(a), τ_T varies from 1.5 ms to 500 μs as function of time. The different intensity levels in the transient have different corresponding triplet lifetimes. In Fig. 14(b) the correlation between the triplet lifetimes and the different intensity levels is visualized. This observation renders credibility to the extrapolation of the dendrimer-based model for fluorescence and collective on/off behavior to B-PE. Indeed, the model predicts that different chromophores in the multichromophoric system can act as the triplet trap. As the chromophores have different environ-

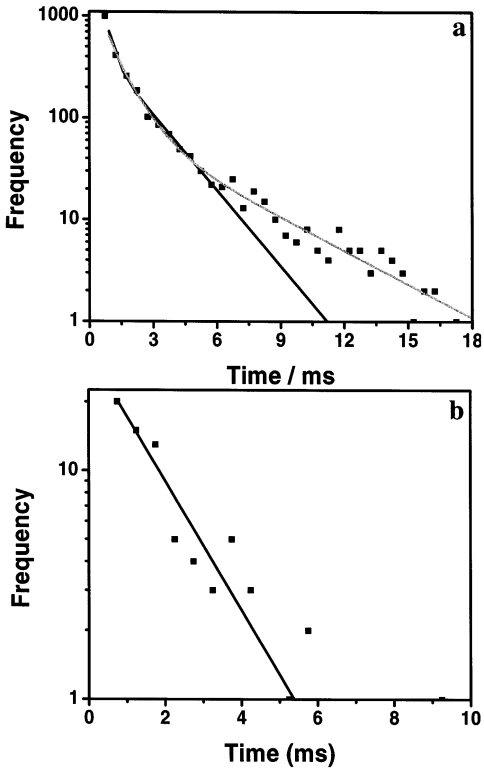

Fig. 13. (a) Frequency histogram of the duration of the off-times from the transient in Fig. 12(a) (from 1.85 to 14 s) and the fits to a 2-exponential (black line) and 3- exponential (gray line) function. The fit parameters for the 2-exponential function are: $\tau_{1T} = 0.3$ ms, $\tau_{2T} = 1.7$ ms, $\chi^2 = 38.8$, $R^2 = 0.99$. The fit parameters for the three-exponential function are: $\tau_{1T} = 0.2$ ms, $\tau_{2T} = 0.9$ ms, $\tau_{3T} = 3.9$ ms, $\chi^2 = 15.2$, $R^2 = 0.99$. (b) Frequency histogram of the duration of the off-times from the transient in Fig. 12(a) (from 2.5 to 2.9 s) and the fits to a mono-exponential (black line) function. The fit parameters for the mono-exponential function are: $\tau_T = 1.5$ ms, $\chi^2 = 1.49$, $R^2 = 0.97$.

ments, different triplet lifetimes are to be expected.

The analysis was stopped after 14 s as the triplet lifetime became of the same length as the duration of the bin time (500 μs) and reliable parameter estimation became difficult. In this situation either shorter bin times are required or special analysis procedures have to be developed. New analysis methods dealing with low number of photocounts per detection interval were reported elsewhere [52,53].

4. Conclusions

In this paper, the single molecule behavior of two different multichromophoric systems, a synthetic dendrimer and an antenna complex, is reported. Investigation of single molecules shows for both systems collective on/off jumps in their fluorescence intensity traces as function of time. The very photostable dendrimer system allowed to construct a model that explains the observed spectral, temporal and polarized fluorescence be-

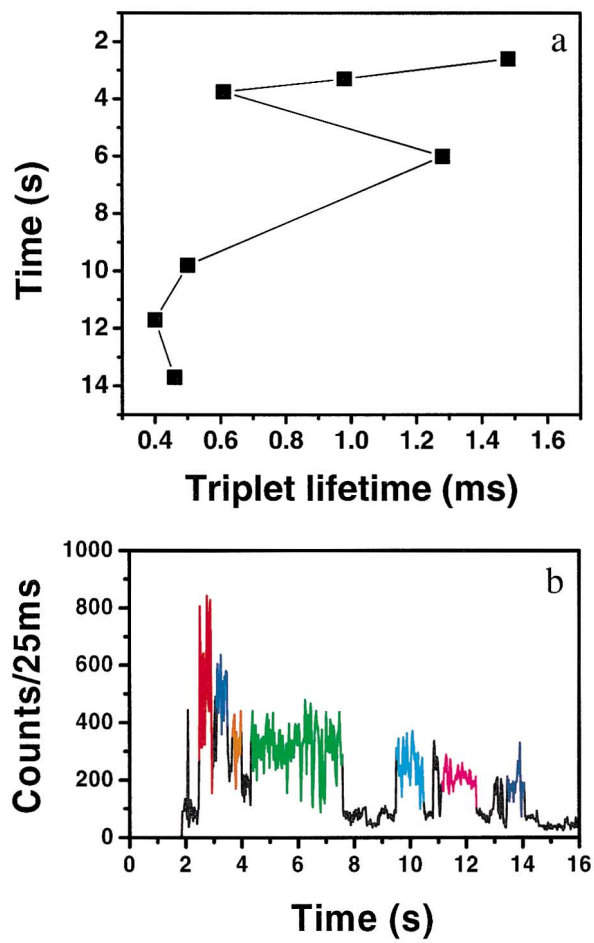

Fig. 14. (a) The triplet lifetime τ_T as a function of time. (b) Transient from which the time dependent triplet lifetimes in *a* were calculated: red 2.35–2.9 s, $\tau_T = 1.5$ ms; blue 3.15–3.45 s, $\tau_T = 1$ ms; orange 3.65–3.95 s, $\tau_T = 0.65$ ms; green 4.4–7.4 s, $\tau_T = 1.3$ ms; cyan 9.5–10.4 s, $\tau_T = 0.5$ ms; magenta 11.2–12.3 s, $\tau_T = 0.4$ ms; dark blue 13.5–14 s, $\tau_T = 0.5$ ms.

havior as well as the collective on/off jumps. The model includes both a radiative trap (energetic lowest chromophore) and a non-radiative trap (triplet state of one of the chromophoric sites). This model was extrapolated to the antenna complex B-PE. The latter shows fast on/off behavior in all intensity levels of the recorded fluorescence intensity traces. The introduction of a pseudo three-level model allowed to extract triplet lifetimes from the duration of the off periods. Detailed analysis of a transient reveals that the triplet lifetimes can be correlated with intensity levels in the transient.

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