Single Molecule Spectroscopy on the Light-Harvesting Complex II of Higher Plants

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ABSTRACT Spectroscopic and polarization properties of single light-harvesting complexes of higher plants (LHC-II) were studied at both room temperature and $T < 5$ K. Monomeric complexes emit roughly linearly polarized fluorescence light thus indicating the existence of only one emitting state. Most probably this observation is explained by efficient triplet quenching restricted to one chlorophyll *a* (Chl *a*) molecule or by rather irreversible energy transfer within the pool of Chl *a* molecules. LHC-II complexes in the trimeric (native) arrangement bleach in a number of steps, suggesting localization of excitations within the monomeric subunits. Interpretation of the fluorescence polarization properties of trimers requires the assumption of transition dipole moments tilted out of the symmetry plane of the complex. Low-temperature fluorescence emission of trimers is characterized by several narrow spectral lines. Even at lowest excitation intensities, we observed considerable spectral diffusion most probably due to low temperature protein dynamics. These results also indicate weak interaction between Chls belonging to different monomeric subunits within the trimer thus leading to a localization of excitations within the monomer. The experimental results demonstrate the feasibility of polarization sensitive studies on single LHC-II complexes and suggest an application for determination of the Chl transition-dipole moment orientations, a key issue in understanding the structure-function relationships.

INTRODUCTION

The light-harvesting complex II (LHC-II) is the most abundant chlorophyll *a/b* binding protein, embedded in the thylakoid membranes of higher plants and green algae. This antenna associated with photosystem II accounts for half of the entire chlorophyll (Chl) content in green plants. Within this photosynthetic unit the LHC-II complexes collect light like an antenna system and funnel it to the reaction centers. There the primary charge separation process forms a proton gradient over the thylakoid membrane, thus driving all later processes of the photosynthetic metabolism and energy conversion. The excitation energy transfer is performed by exchange of excitation among the pigment molecules according to the strength of their electrodynamic interactions. Characterized by high efficiency and rare regulatory abilities, this energy transfer process has become a topic of enormous interest (Agarwal et al., 2000; van Grondelle et al., 1994).

Electron microscopy of LHC-II trimers reveals the structure at 3.4-Å resolution (Kühlbrandt et al., 1994). Trimers are thought to be the native form of LHC-II in the photosynthetic membrane and, also, in mild detergent solution the trimeric form is preserved. Monomers can be generated from the native trimeric form by the removal of the lipid phosphatidyl-glycerol, which is directly involved in the formation of trimers (Nussberger et al., 1994).

A monomeric subunit of LHC-II comprises one polypeptide chain of 232 amino acids forming three *trans*-membrane α-helices and one membrane associated α-helix. From the structural data, the binding positions of 7 Chl *a*, 5 Chl *b*, and 2 lutein molecules are known (although a slightly higher pigment content is confirmed biochemically by Jansson (1994)). However, the resolution is neither sufficient enough to distinguish between Chl *a* or Chl *b* molecules nor to determine the exact orientation of the porphyrin dipoles within their molecular plane. The seven chlorophylls closest to the luteins have been assigned as Chl *a* molecules (Kühlbrandt et al., 1994) due to considerations about their necessity for efficient triplet quenching.

Later results obtained by studies on LHC-II mutants lacking one of the Chl molecules confirm this assignment in general, but suggest permutations in one or two of the pairs of chlorophyll *a*/*b* molecules (Remelli et al., 1999; Rogl and Kühlbrandt, 1999; Yang et al., 1999).

The first available structural data led to a wealth of spectroscopic investigations addressed to the clarification of the complicated interplay among the considerable number of Chls apparent in monomers and even more in trimers of the complex (Conelly et al., 1997; Reddy et al., 1994). The Q_y region of the bulk absorption spectrum of LHC-II is relatively unstructured, showing two broad bands around 650 nm and 675 nm attributed to the absorption of Chl *b* and Chl *a*, respectively. However, several room and low-temperature techniques (i.e., linear/circular dichroism (Hemelrijk et al., 1992) and nonlinear polarization spectroscopy in the frequency domain (Schubert et al., 1997)) reveal up to

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11 spectral subbands within the Q_y absorption region. Nevertheless, any satisfying interpretation of this substructure as a result of Chl protein and/or Chl-Chl interaction is still a matter of extended debate (Leupold et al., 1999). Femtosecond spectroscopy revealed that ultrafast Chl *b* → Chl *a* energy transfer occurs only within the monomeric subunit of LHC-II trimers (Kleima et al., 1997). This is consistent with the assumption of weak interaction between Chl molecules belonging to different subunits (Voigt et al., 1995), owing to the relative large distance between them. The coupling strength between Chl molecules of different monomers is thought to be $<5\ cm^{-1}$ (Pieper et al., 1999).

In the last few years, several groups have begun to apply single molecule spectroscopy to pigment-protein complexes (Bopp et al., 1997). Especially the light-harvesting complex 2 of purple bacteria has been studied extensively (van Oijen et al., 1999, 2000; Tietz et al., 1999; Bopp et al., 1999). Recently this approach was successfully applied to photosystem I (Jelezko et al., 2000).

In this paper we present the first single-molecule spectroscopy experiments on the plant antenna complex LHC-II, in particular application of polarization sensitive studies on single complexes. In our opinion, this technique is ideally suited to solve the long-standing problem of the exact orientations of the Chl *a*/*b* molecules within their protein frame. Here we present the initial step toward the goal.

EXPERIMENTAL

LHC-II was prepared from freshly harvested pea leaves (*Pisum sativum*) according to the procedure described elsewhere (Krupa et al., 1987). The complexes were stored in buffer (10 mM Tricine, pH 7.8) at 77 K until used. Samples were prepared by dilution of the stock solution in buffer containing 1% detergent (n-octyl β-D-glucopyranoside; Sigma Chemical Co., St. Louis, MO). One drop (100 μl) of the diluted solution was mixed with 1 ml of a 1% PVA solution and immediately spin coated on a clean cover glass to obtain thin LHC-II doped films. At room temperature these samples were stable for more than an hour (as determined by fluorescence detection). At temperatures <77 K, the stability increased dramatically to >1 day. The extremely low final concentration of LHC-II allows the spatial selection of individual complexes. To minimize photooxidation of the samples in room temperature experiments, the samples were deoxygenated by an enzymatic system (1 μg/ml glucose oxidase, 20 μg/ml glucose, and 50 ng/ml catalase; Sigma), which was added to the PVA solution directly before the last step of dilution.

Monomers were obtained by incubation of 1 mg/ml of the trimeric complex with 0.1 units/ml phospholipase A_2 (Sigma; P-6534) for 2 h at room temperature. Monomers were separated from trimers using sucrose gradient centrifugation. The further handling of monomeric samples was the same as in the case of trimers.

For all optical investigations, a home-built beam-scanning confocal microscope was used, operating in the temperature range of 300–1.8 K. The objective (63, 0.85 NA; Melles Griot) is mounted inside the cryostat, thus enabling polarization studies as well as fluorescence emission spectroscopy. Excitation was performed via the 647-nm line of a Kr^+-ion laser. Fluorescence excitation spectra were recorded using a narrow band dye laser (Coherent 699–21, Santa Clara, CA; line width, 1MHz) operating with DCM dye. Fluorescence light was separated from stray light using holographic notch filters (Kaiser Optical Systems, Ann Arbor, MI) and detected by an avalanche photodiode (SPCM-AQR-16; Perkin Elmer, Woburn, MA) or focused on a spectrograph (0.25 m; Acton Research, Trenton, NJ) equipped with a back illuminated CCD camera (Spec-10: 100B; Princeton Instruments, Trenton, NJ).

RESULTS

Polarization study

The investigations on polarization properties of the light emitted by single LHC-II trimers or monomers were performed to obtain information about the number of emitting states. For better separation between excitation and fluorescence, the complexes were excited in the Chl *b* region at 647 nm. The degree of polarization of the fluorescence was determined using a continuously turning polarizer (Glan-Thompson-Prism) in front of the detector. To minimize the error due to distribution of background signal we measured the background signal in close vicinity to every spot of a single LHC-II complex. The degree of polarization (p) is defined as

$$p = \frac{I_{max} - I_{min}}{I_{max}},$$

where I_{max} and I_{min} are maximum and minimum fluorescence intensities of the $\cos^2$-fitted intensity traces. Hence, one obtains the two cases $p = 0$ for nonpolarized emission and $p = 1$ for linearly polarized emission.

At room temperature, the investigation of single light-harvesting complexes is complicated by the effect of photobleaching, resulting from degradation of pigments due to irreversible effects of excess energy in the system. Nevertheless, observation times up to minutes allow determination of the degree of polarization with sufficient accuracy. Fig. 1 *A* shows a typical polarization modulation trace of a LHC-II monomer. The emitted light is linearly polarized until the complex is irreversible photo-bleached in a single step after 51 s. The histogram in Fig. 1 *B* shows that most of monomers emit linearly polarized light. Photo-bleaching of most complexes occurs in one step as typical for single quantum systems. Only a few monomers are characterized by a two-step photo-bleaching behavior.

Polarization modulation traces of LHC-II trimers are more complicated. For that reason the investigations of

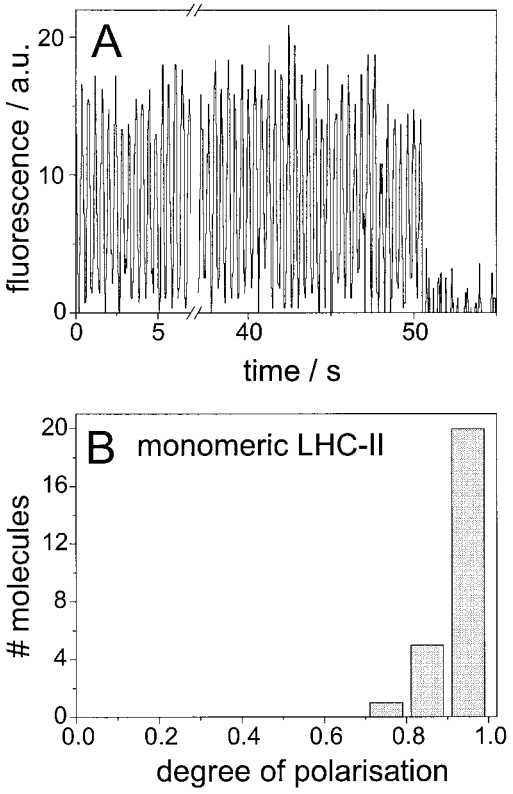

FIGURE 1 (*A*) Polarization modulation trace of a single LHC-II monomer at room temperature. Complexes are immobilized in a PVA matrix and excited via the Chl-*b* absorption band at 647 nm. (*B*) Histogram of the degree of polarization. Most monomers show linearly polarized emission.

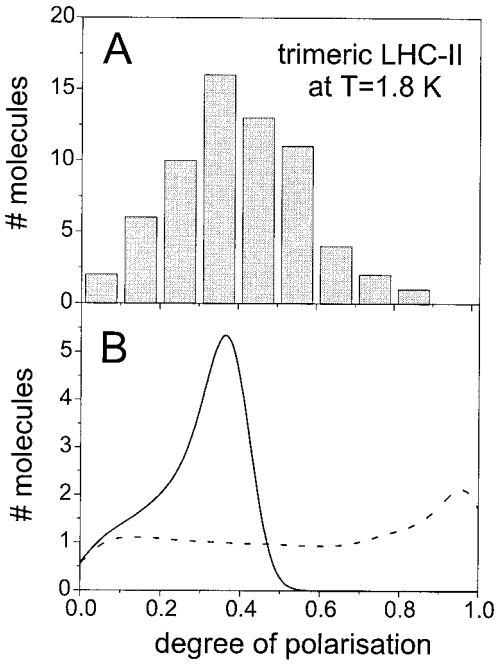

FIGURE 2 (*A*) Histogram of the degree of polarization of trimers at $T = 1.8$ K. Most complexes show p values around 0.4. (*B*) Simulation of the histogram assuming a tilt angle of 0° (*dashed line*) and 25° (*solid line*) between the symmetry plane (membrane plane) and the orientation of the dipole moments of the trimer.

fluorescence polarization has been extended to low temperature. Fig. 2 contains the histogram of p from 70 single LHC-II trimers at $T = 1.8$ K. As obvious in the histogram, most of the LHC-II complexes emit light with a degree of polarization around 0.4. Moreover, there is no significant effect of the temperature on p in the temperature range 1.8 K $< T <$ 100 K. However, at room temperature, intensity jumps in the traces can be observed, also changing the degree of polarization. Most of the trimers exhibit a two- or three-jump behavior. In a couple of cases we also found trimers showing more than three jumps, probably caused by either slight aggregation or intensity fluctuations within the monomeric subunits. The histograms in Fig. 3 summarize the evolution of p in the typical case of three-step photobleaching. It is also worth to mention that most of the trimeric complexes emit linearly polarized light before the last bleaching step (like monomers).

Emission spectra

A typical emission spectrum of a single LHC-II trimer at low temperature ($T = 1.8$ K) is shown in Fig. 4 *A*, recorded with an acquisition time of 120 s. The emission spectrum represents the convolution of a number of (barely resolved) emission lines with a resulting spectral width of about 100 cm^{-1}. Different trimers show differences in spectral width and shape. The complex spectral shape is caused by spectral diffusion as illustrated by reduction of the acquisition time (see Fig. 4 *C*). For a more detailed investigation of spectral diffusion, we recorded series of 500 spectra as shown in Fig. 4 *B* using acquisition times of 3 s per spectrum. Each row of the image represents a spectrum like the one shown in Fig. 4 *C*. The number of lines per spectrum varies in principle from 1 to 5, but usually three lines are apparent. Spectral jumps occur in the range of $\Delta E = 40$–100 cm^{-1}. Typical fluorescence emission spectra of LHC-II monomers and trimers with short acquisition times are compared in Fig. 5. In general, monomeric LHC-II complexes show fewer fluorescence emission lines than trimers. Typically we find one only line in monomers, whereas trimers frequently show three lines separated by 20–100 cm^{-1}.

DISCUSSION

LHC-II monomers represent an interacting system of 12 Chls with fast energy transfer toward the Chl *a* molecule with the most red-shifted Q_y transition (Kleima et al., 1997; Pieper et al., 1999). The detailed analysis of ensemble absorption spectra revealed a relatively high density of states in the red wing of the absorption spectrum. Current models rely on the assumption that 4 to 5 Chl absorption

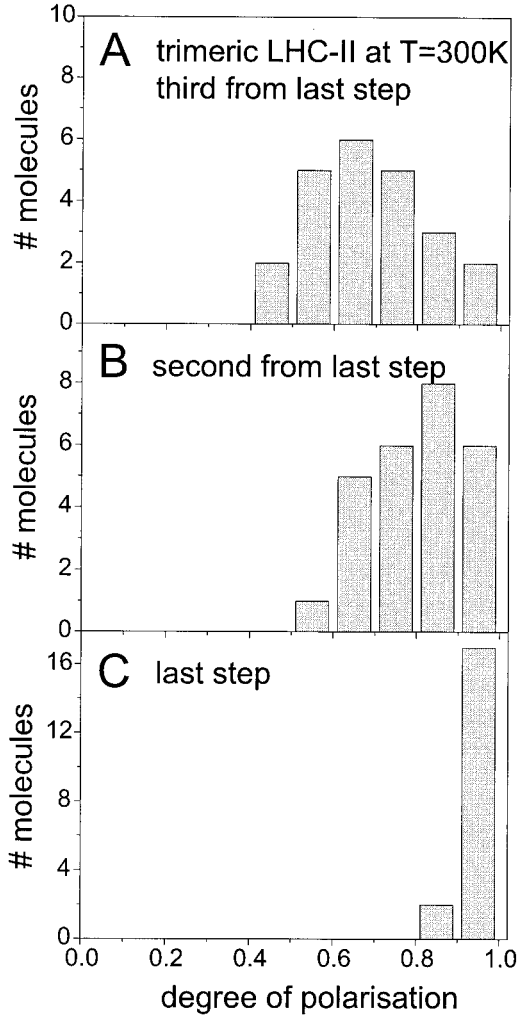

FIGURE 3 Bleaching behavior of trimers at room temperature. The histograms show the distribution of the degree of polarization before the third from last step (*A*), the second from last step (*B*), and the final step (*C*).

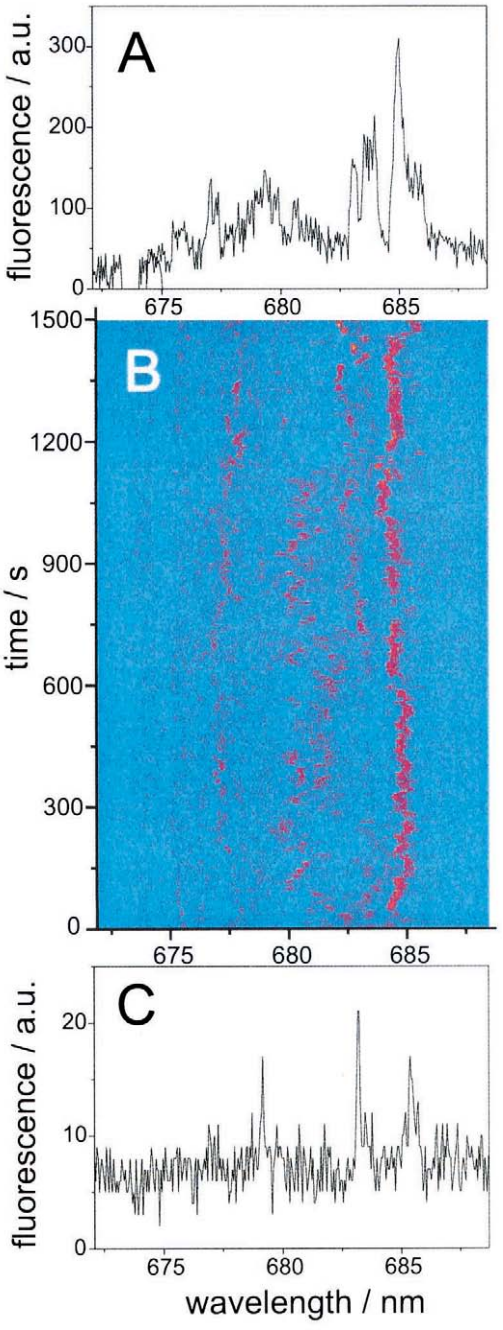

FIGURE 4 Fluorescence emission spectra of trimeric LHC-II at 1.8 K. (*A*) Acquisition time, 120 s. (*B*) Series of 500 spectra recorded using an acquisition time of 3 s per spectrum. The intensity is given via color code. (*C*) Example of a emission spectrum with a acquisition time of 3 s.

bands are centered within an interval of 250 cm^{-1} from the energetically lowest Chl *a* molecule (Hemelrijk et al., 1992). Assuming that the population of these molecules is distributed in a Boltzmann equilibrium, all of them should contribute considerably to the total fluorescence at room temperature. Our observation that LHC-II monomers emit linearly polarized fluorescence is , thus, rather surprising. We discuss the following possible explanations: 1) the transition dipole moments of all Chl molecules contributing to the fluorescence are parallel; 2) there is no thermal equilibrium between the Chls absorbing at the red edge of the Q_y-spectrum, possibly because of irreversible energy transfer between the Chl *a* molecules; 3) the five molecules have highly different photo-physical parameters, i. e. one Chl has a triplet quenching efficiency considerably higher than the others.

Structural data reveal the binding sites of Chl molecules and also determine the orientation of their molecular planes, but do not uncover the orientation of the transition dipole moments within this plane. Thus, it cannot be excluded that the transition dipole moments of a number of emitting molecules are oriented in parallel. However, considering the rather random orientation of Chls within the structure (Kühlbrandt et al., 1994), a completely parallel alignment seems to be unlikely.

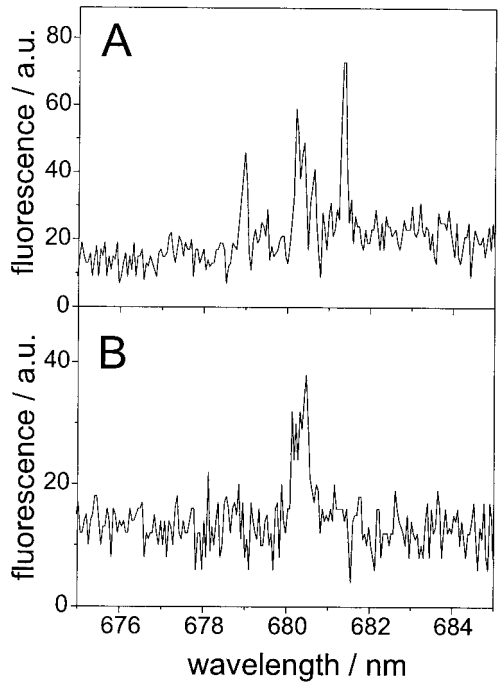

FIGURE 5 Typical emission spectra of a trimeric (*A*) and monomeric (*B*) LHC-II at low temperature (T = 1.8 K). Acquisition time in both cases was 10 s.

Time-resolved optical spectroscopy shows that the slowest time constants in the energy transfer among Chl *a* is on the order of 1–10 ps (Gradinaru et al., 1998). This is consistent with the assumption that the energy transfer within the Chl *a* region is caused by the Förster hopping mechanism. Hence, the transfer time is determined by the spectral overlap between donor and acceptor. Asymmetries in the line shape due to the vibrational components of the wave function can thus cause considerable differences between downhill and uphill energy transfer. Although the Chl absorption and emission lines in LHC-II are rather broad and characterized by a small Stokes shift, the influence of asymmetric line shapes on differences in energy transfer rates can not be excluded.

Detection of single Chl fluorescence depends on an effective triplet-state quenching e.g., by attached carotenoids. Unquenched molecules are trapped most of the time in the long-living triplet-state, thus fluorescing weakly. In general, the saturated fluorescence intensity for a single molecule containing a metastable triplet state can be expressed as (Ambrose et al., 1991)

$$I_{sat} = \frac{(k_{12} + k_{32})\Phi_F}{(2 + k_{32}/k_{13})},$$

where Φ_F is the fluorescence quantum yield, k_{12} is the inverse lifetime of the first excited singlet state, k_{32} is the population rate of the triplet state and k_{13} is the decay rate of this state. Inserting the values obtained for Chl in solution (without quenchers) yields a maximum fluorescence intensity of I_{sat} = 500 photons per second. With detection efficiency in the experiment of <1%, the resulting number of photon counts per second do not exceed the noise level of the detector. The high inter system crossing probability of Chl (high k_{32}) in combination with a large lifetime of the triplet state (low k_{13}) is responsible for this low intensity leading to a bottleneck in the transition between $|3\rangle$ and $|1\rangle$ (Fig. 6 *A*). However, with efficient triplet quenching, the rate k_{13} may be increased considerably and, hence, I_{sat} will increase by the same factor. In a recent paper (Schödel et al., 1998) an upper limit of the inverse triplet-quenching rate of 0.5 ns could be determined. In this case, signal intensity would be determined by the deactivation rate of the carotinoids (k_{Car}, typically μs), i.e., the bottleneck in the combined system Chl and Car becomes the decay rate of the Car triplet state (Fig. 6 *B*). Hence, triplet quenching of Chl would increase the signal by three orders of magnitude. The fluorescence signal of one Chl possessing an extremely efficient quenched triplet state can thus be much higher than that of the other Chls. Highest triplet quenching efficiency for only the most red-shifted Chl absorption transition makes also sense from the physiological point of view. Situated at the end of the energy transfer chain, this molecule has the highest probability for conversion to the triplet state (intersystem crossing) due to its considerably longer lifetime.

The p values for trimers at low temperatures are distributed between 0 and 1, with a marked peak around 0.4, thus indicating more than one emitting state per trimer. Previous (low temperature) ensemble measurements have been inter-

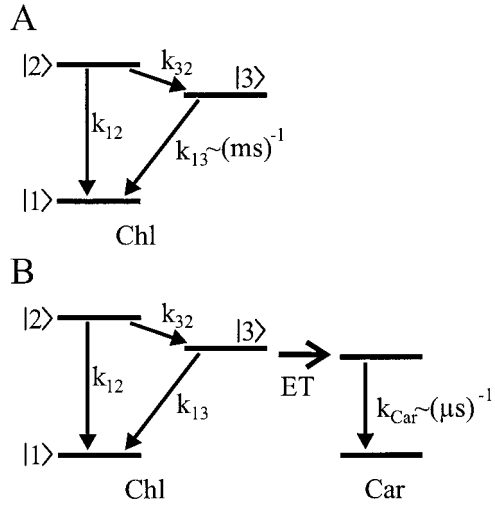

FIGURE 6 Energy level diagram of isolated Chl (*A*) and Chl in close contact with a Car molecule (*B*). The triplet decay rate of Chl *a* is in the order of ms building the bottleneck of the excitation cycle. A Car molecule in van der Waals contact with the Chl molecule causes a fast (~ns) energy transfer from the Chl triplet to the Car triplet. The bottleneck of the Chl/Car system is now the decay time $(k_{Car})^{-1}$ of the Car triplet state (~μs).

preted in the way that three states per trimer contribute to the fluorescence of LHC-II (Pieper et al., 1999). The same evidence can be obtained from our fluorescence emission spectra as shown in Fig. 4. With the assumption of three emitters per trimeric LHC-II and the trimers randomly oriented in the polymer film, we attempted to simulate the p histogram (Fig. 2 *B*). If one further assumes that the transition dipole moments of the fluorescing Chls are oriented within the symmetry plane of the LHC-II trimer (membrane plane), one obtains a broad distribution of p values between 0 and 1 (Fig. 2 *B*, *dashed line*). To achieve a distribution reflecting the pronounced peak around $p = 0.4$, it is necessary to tilt the dipoles out of the plane of symmetry. In the case of an isotropic distribution of orientations within the PVA matrix, the best fit is obtained assuming an angle of either 25 or 47° between plane of symmetry of the complex and the transition dipole moments of the Chls, as shown in Fig. 2 *B* (*solid line*). The discrepancy between the simulation and the measured histogram could be due to a distribution of orientations that is not completely isotropic, and is caused by the spin coating procedure. Such a behavior was found in spin-coated samples of light harvesting complexes of purple bacteria (van Oijen et al., 1999).

In contrast to the monomeric samples, trimers bleach in multiple steps. Such a behavior would be expected in the case of extreme low coupling between the monomeric subunits which only leads to very slow ET times, slower than the fluorescence lifetime (~ns). At least at non-cryogenic temperatures this is not the case. The closest distance between Chl molecules belonging to different monomeric subunits is ~20 Å (molecules a4 and a5 in Kühlbrandt et al. (1994)), and the coupling between these molecules has been estimated to be around 5 cm^{-1} (Pieper et al., 1999). This value justifies to calculate the energy transfer rate using the Förster equation. Because most of the parameters to calculate the spectral overlap integral are unknown, the integral was estimated using the absorption and emission data of Chl in solution (Gradinaru et al., 1998). Hence, the inter-monomeric energy transfer times can be estimated to be in the range 10 to 100 ps. In a recent work, an estimation of the equilibration time within the trimer was found to be ~30 ps (van Amerongen and van Grondelle, 2001). Considering this, one would expect that during one excitation cycle all three subunits should be reached by the exciton due to a hopping process. The fact that we see a multiple-step bleaching behavior indicates that no trap state is created that would lead to a non-fluorescing decay of the excitation. Such a trap state would cause a one-step bleaching behavior, as is found in light harvesting complexes of purple bacteria (Bopp et al., 1997). Obviously, in LHC-II the monomeric subunits bleach without the creation of a trap state containing an excited state at lower energy than the states of the complex. Another possible explanation of the three step bleaching behavior would be a very low coupling strength between Chl *a* molecules of different subunits due to disturbance by the PVA matrix. To reach energy transfer times in the ns range a coupling strength conspicuously smaller than 1 cm^{-1} is needed. Using the same parameters as above this would lead to an enlargement of the intermonomeric Chl *a* distance by a factor of two. Because LHC-II trimes are remarkable stable, it is hard to believe that the PVA matrix effects such a deformation of the trimeric complex.

One would expect to find the same histogram of p values for trimers at low temperature (Fig. 2) and for nonbleached trimers at room temperature (Fig. 3 *A*), whereas the histogram at room temperature is shifted to higher p values. As mentioned above, a couple of monomers show two-step bleaching, and even more trimers show a bleaching behavior in more than three steps. Difficulties in distinguishing between bleaching of a monomeric subunit and a step within a subunit, respectively, could lead to the observed shifting to higher p values.

Fluorescence emission spectra of monomers and trimers show a number of well-resolved lines. The linewidths of the emission spectra in Figs. 3 and 4 are limited by the spectrometer resolution ($\Delta\bar{\nu} \approx 3$ cm^{-1}). Fluorescence excitation spectra with higher resolution show a linewidth of the most red-shifted Chl *a* states of ~0.02 cm^{-1} (S. Schuler, F. Jelezko, U. Gerhen, B. Götze, C. Tiek, J. Wrachtrup, H. Wolf-Klein, H. Paulsen, manuscript in preparation). Without dephasing processes at $T = 4.2$ K, the line width of Chl transitions should be determined by the exited state lifetime. This corresponds to a width of ~0.001 cm^{-1} considering a fluorescence decay time of 4.3 ns (Schödel et al., 1998) for LHC-II. Most probably, the measured value is larger than the theoretical value because of spectral diffusion processes remaining even at lowest excitation intensities. Hence, it is possible that spectral diffusion occurs with the Chl molecule in the ground state, e.g., the spectral diffusion is partly not photo-induced as shown in other systems (Boiron et al., 1999). From this point of view, the obtained linewidth is rather comparable to that measured by hole-burning spectroscopy (0.037 cm^{-1}) for the energetically lowest band at 680.4 nm (Pieper et al., 1999).

The fact that the same mean emission wavelength of 681.3 nm is found for monomers and trimers indicates a comparable protein environment for the fluorescing Chls in both species. This behavior confirms the assignment made earlier (Rogl and Kühlbrandt, 1999) where Chl *a*2 (Kühlbrandt et al., 1994) is attributed to the fluorescing state of the complex. For this pigment molecule, the environment in trimeric and monomeric arrangement is not changed.

CONCLUSION

Here we present the first results of single molecule spectroscopy on single LHC-II complexes in trimeric and monomeric form. The experiments provide various lines of evidence that the monomeric subunits within a trimer are acting rather independently, and three fluorescing states per

trimer (one per monomer) were found. The center wavelength of the emitting transition could be determined as 681.3 ± 0.1 nm (at cryogenic temperatures). The line width of those transition bands measured by fluorescence excitation spectroscopy is around 0.02 cm^{-1}. Meanwhile, we recorded excitation spectra up to 673 nm (data not shown). Those spectra revealed a couple of lines belonging to higher energetic Chl *a* transitions. Thus, by combining polarization sensitivity and fluorescence excitation spectroscopy, determination of the relative orientations of several transition-dipole moments becomes possible. This might clarify the complexity of orientations of Chl molecules within their molecular planes and provide a basic knowledge necessary for theoretical description of energy transfer processes within the LHC-II system.

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