Spectroscopy on Single Light-Harvesting Complexes at Low Temperature

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The fluorescence of individual light-harvesting 2 complexes from *Rhodopseudomonas acidophila* has been observed by confocal microscopy in a temperature range between 300 and 7 K. Under ambient conditions, changes in the polarization of the fluorescence emission of single complexes on a time scale from milliseconds to seconds are found. In the temperature range between 250 and 100 K most complexes emit fluorescence with a temporally stable linear fluorescence polarization. At temperatures below 70 K, spectral diffusion is found to dominate the dynamics of the fluorescence intensity and polarization. The increase in photostability of single complexes at low temperature allows the detection of fluorescence emission spectra of single complexes. A marked variation in the shape and the position of the spectra is found. The results are discussed by considering static and dynamic disorder within the B850 aggregate of the light-harvesting complexes.

1. Introduction

Photosynthetic purple bacteria are among the simplest photosynthetic units. In the past years numerous investigations including a variety of biochemical and spectroscopic techniques have been applied to such systems.[1−3] Recently, the atomic structure of the peripheral antenna (light harvesting system 2: LH2) of the purple bacteria *Rhodopseudomonas (Rps) acidophila* was resolved.[4,5] The knowledge of the high resolution structure and the availability of ultrafast laser sources in the spectral region of the LH2 absorption bands has made these photosynthetic systems one of the most studied objects in the current photosynthetic research.[6] Recently, single molecule spectroscopy has been reported on LH2 complexes under ambient conditions and at low temperature.[7] Near-infrared excitation in combination with confocal microscopy allows an efficient suppression of background luminescence. Consequently, single LH2 complexes can be observed with high signal-to-noise ratio despite their low quantum efficiency for fluorescence emission. At low temperature, the photostability of the complexes is increased considerably and hence more detailed investigations on the energy transfer characteristics of such systems can be performed. In addition the homogeneous linewidth of optical emission and excitation lines depend on temperature. Differences among complexes in their position of energy levels and hence in their optical absorption and emission profiles thus become more clearly visible at low temperature. This is of particular interest in the present work since inhomogeneous distributions are known to be important for the understanding of the photophysical parameters of LH2.[3] Single molecule spectroscopy presents an alternative to techniques aimed at the removal of inhomogeneous distributions, and we shown in this paper that it is of special value in the study of macromolecular systems such as LH2 at low temperature.

The basic structural unit of the LH2 is a heterodimer of two short peptides (trans membrane helices) referred to as α- and β-apoproteins. The two helices noncovalently bind the bacteriochlorophylls (Bchl) and carotenoids.[4] Whereas the Bchl are the dominant chromophores responsible for capturing sunlight, the carotenoids quench the chlorophyll triplet state to prevent photooxidation and capture sunlight in those spectral regions not covered by the Bchls. The Bchls are arranged in two coplanar and concentric rings. In *Rps. acidophila* ring 1 comprises 9 Bchl and ring 2 18 Bchl molecules.[8] Molecules in ring 1 show a large intermolecular spacing and are arranged such that they are coplanar with the plane of the ring. The intermolecular spacing of the 18 Bchl molecules in ring 2 are much smaller compared to those in ring 1. Molecules in ring 2 thus form a strongly interacting supermolecular aggregate.[6] Moreover their orientation is nearly perpendicular to the orientation of the molecules in ring 1. LH2 of *Rps. acidophila* has two distinct absorption bands around 800 and 850 nm termed B800 and B850, respectively, according to the two Q_y transitions of the two types of Bchl pigments.[2] Molecules in ring 1 are thought to absorb around 800 nm, whereas molecules in ring 2 are responsible for the absorption around 850 nm. Steady-state fluorescence polarization studies together with subsequent pump−probe experiments have been interpreted in terms of an energy transfer between molecules in B800 with a time constant of 400 fs.[9,10] Similar time resolved experiments found an excitation energy transfer from B800 to B850 within 0.7 ps at room temperature.[11−14] At temperatures from 4 to 77 K the value of this time constant is found to be 2.4 ps.[15,16] The excitation is delocalized among the molecules in ring 2 on a time scale of around 100 fs[17] similar to the relaxation of population among the excitonic manifold of the B850 aggregate.[18] Because of the energy transfer and subsequent population relaxation, excitation around 800 nm results in fluorescence emission at around 850−900 nm with a fluorescence quantum yield of roughly 10%.[19]

2. Materials and Methods

The procedure for growing cells of *Rps. acidophila strain 10050* and subsequent isolation of LH2 has been described in ref 20. Isolated LH2 complexes were dissolved in buffer (pH = 8.0) containing 20 mM Tris and 0.05% LDAO and stored at 20 °C until required. For sample preparation this solution was diluted with pure buffer by a factor of 10^6. A drop (20 μL) of the solution was deposited on the substrate.[7] After 10 min, the drop was removed and the substrate was rinsed three times

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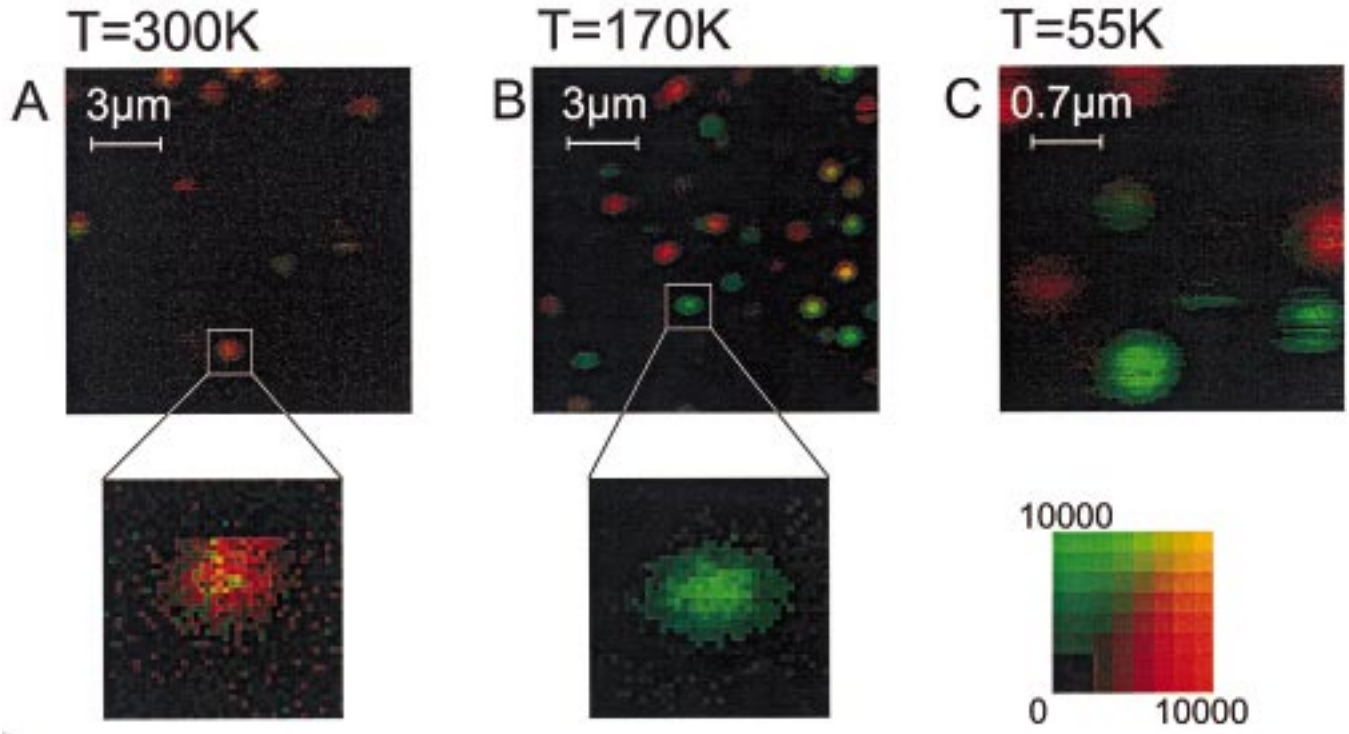

Figure 1. Confocal fluorescence images of single LH2 complexes. Parts A−C represent images where two orthogonal polarization directions have been detected simultaneously. The fluorescence intensity of those two channels is shown in red and green. Images A−C have been recorded at different temperature as indicated. Because the scan range of our device reduces considerably at lower temperature, images of single complexes in C appear bigger.

with pure buffer before it was mounted on the sample holder. Rapid cooling below 250 K prevented denaturation of the complex. Individual complexes could be observed in the same sample for a month when it was kept below 250 K. The instrument for monitoring single complexes is a home-built sample scanning confocal optical microscope capable of operating in a temperature range between 1.5 and 300 K. The sample is scanned via an *x*,*y* scan unit (scan range at 4K: 8 μm) in front of a microscope objective (63X, N.A. 0.85). The whole probe head is mounted inside a cryostat. Excitation of the sample has been carried out via the 799.3 nm line of a Kr^+-ion laser. Fluorescence is passed through a polarizing beam splitter cube for simultaneous detection of two orthogonal linear polarization directions. A spectrometer equipped with a charge-coupled device camera is used for detection of the fluorescence emission spectra of individual complexes. Correlation measurements have been carried out with a logarithmic hardware correlator (ALV 5000).

3. Experimental Results

Room temperature single molecule spectroscopy is complicated by rapid photobleaching of organic species.[21] Although carotenoids provide efficient triplet quenching and hence protection against photobleaching, LH2 complexes prove to be quite susceptible to photodestruction.[7] Our first task thus was to explore the photostability of this molecule as a function of temperature. Figure 1 shows a series of images taken at three different temperatures. The increase in photostability upon reduction of temperature is apparent from the images. At 200 K a more than 2 orders of magnitude increase as compared to room temperature is found. At still lower temperatures no substantial further reduction in the quantum efficiency for photobleaching occurs. However, below 70 K a marked increase in fluctuations of the fluorescence intensity takes place, which does not affect the overall photostability. This behavior is illustrated in the fluorescence intensity traces in Figure 2A, C, and D. Figure 2B shows a linescan where the laser is scanned across one of the single complexs in Figure 1A while recording the fluorescence intensity. Except for Figure 2A, each part contains two traces per complex. They represent the fluorescence intensity of two orthogonal fluorescence polarization directions of the complex. The polarization of the fluorescence is also given in Figure 1 via a color code.[22] From Figure 1 it is apparent that while most of the complexes emit light randomly polarized in the plane of the B850 aggregate at room temperature, at lower T (T < 200K) they emit linearly polarized light. In this temperature range the few sudden jumps in the fluorescence intensity mostly affect both polarization channels simultaneously. The traces in Figure 2C shows one of the rare examples in this temperature range where an intensity fluctuation affects only one channel (around t = 100 s). At T < 70 K, however, the increase in fluorescence intensity fluctuation is accompanied by a random change in relative intensities in the two detection channels (see Figure 2D).

In order to investigate the dependence of the polarization of the low temperature fluorescence emission on the polarization of the excitation light, images of single complexes with a laser polarization rotated by 90° were studied and are displayed in parts A and B of Figure 3. It is obvious from those figures that there is no such dependence.

Because of the much increased photostability of single complexes at low temperature, it becomes possible to record their fluorescence emission spectra. Accumulation times around 300 seconds produce fluorescence spectra with sufficient signal-to-noise ratio for quantitative analysis (see Figure 4). As expected, the fluorescence emission occures in the range 850−900 nm. Apparently, individual complexes show a considerable dispersion in the center of gravity of their emission spectra.

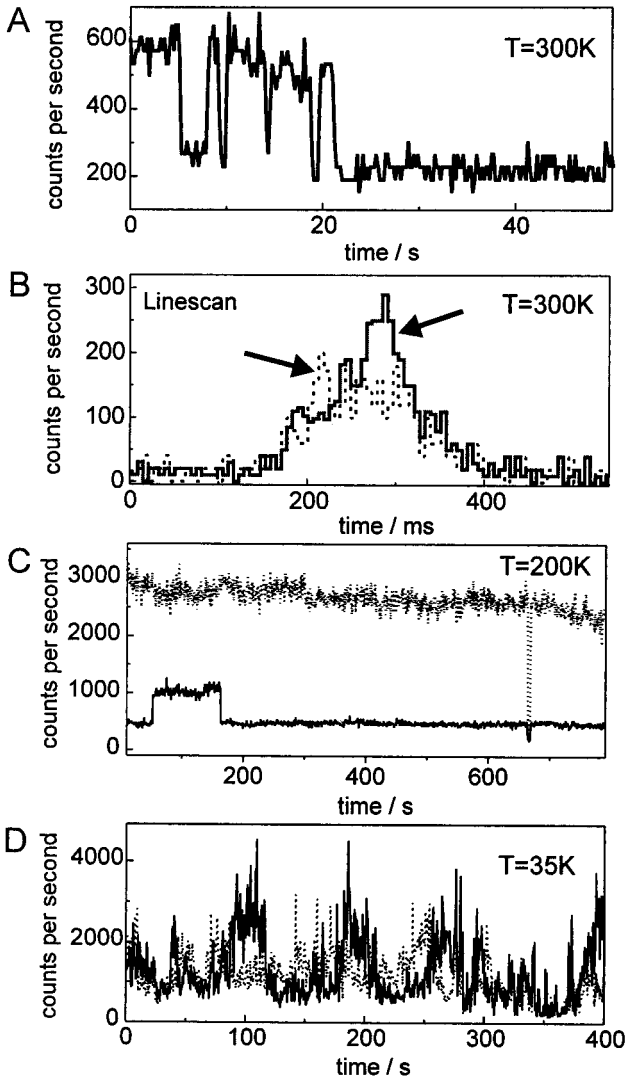

Figure 2. Parts A, C, and D show fluorescence intensity traces as a function of time at different temperature. The traces have been recorded for different complexes respectively. Parts B, C, and D contain two traces representing the fluorescence intensity of two orthogonal polarization of the complex under study. Due to piezo hysteresis the reposition accuracy of our scanner is poor. Hence lengthy adjustment of the sample position under the microscope objective is necessary while observing the fluorescence intensity of a single complex. At low temperature this is feasible because of the high photostability of LH2. However at room temperature rapid photobleaching hinders any adjustment. That is why part B shows a linescan across one of the single complex images from Figure 1A. The arrows mark points in the linescan which have been used to identify deviations from isotropic fluorescence polarization (see text).

Moreover, the spectral shape varies markedly from complex to complex. Also the spectral width of the fluorescence emission spectra gets narrower when the temperature is further reduced, as shown in Figure 5.

4. Discussion

The basic energy transfer steps in LH2 together with the position of the energy levels involved have been investigated intensely.[6] For an intact complex, fluorescence emission almost exclusively relates to physics of the B850 aggregate, i.e., the molecules in ring 2. The photophysics of B850 is determined by the degeneracy of the energy levels of the monomer subunits, the coupling between monomers and the symmetry of the aggregate. Any variation of these parameters either dynamic or static, influences the fluorescence properties, such as the spectral shape and polarization of the emission, substantially.

The optical properties of the B850 pigments have been described by considering a circular aggregate of n Bchl molecules.[23,24] As a starting point we shall assume perfect symmetry. A realistic model of LH2 takes into account that the n molecules in the aggregate ($n = 18$ for *Rps. acidophila*) are formed into N groups of M molecules. Then the N exciton levels are further grouped into M branches. The X-ray structure of LH2 suggests $M = 2$ because the spacing between Bchl molecules alternates. If we use a coupling matrix element $U_{\alpha\beta}$ between adjacent Bchl molecules within a $\alpha\beta$-unit and $U_{\alpha\beta-\alpha\beta}$ between adjacent monomers in different $\alpha\beta$-units, we find the lowest energy level at $E_{\mathrm{Bchl}} - U_{\alpha\beta} - U_{\alpha\beta-\alpha\beta}$ and the highest level at $E_{\mathrm{Bchl}} + U_{\alpha\beta} + U_{\alpha\beta-\alpha\beta}$. Here E_{Bchl} is the transition energy of the uncoupled Bchl molecule. The exciton level structure of such an aggregate is well-known.[23] In the energy level diagram, all kth states are doubly degenerated except the lowest and highest state. According to this simple model the energetically lowest state ($k = 0$) carries no dipole moment for decay from the first excited to the ground state.[19,23] Only the $k = \pm 1$ and $k = \pm 8$ show allowed transitions to the ground state. However, for the configuration present in LH2 (alternating orientation of transition dipole moments) transitions forming the $k = \pm 8$ state are only weakly allowed. On the basis of this idealized description of the system, one would expect fluorescence emission only from the $k = \pm 1$ state and hence a marked decrease in fluorescence quantum yield upon reduction of temperature.[19] This is in contrast to previous experimental findings[19] and also to our single molecule data. Hence the model used so far must be an oversimplified one. For example, disorder present in the B850 aggregate will influence its photophysical parameters significantly. Theoretically such disorder may be introduced into the complex by replacing E_{Bchl}, $U_{\alpha\beta}$, and $U_{\alpha\beta-\alpha\beta}$ by a distribution of energies. In this case the spectrum gets more complicated because degeneracies are removed and transitions from all exciton states gain oscillator strength. The increasing oscillator strength of the $k = 0$ state[19] as a consequence of disorder may be a possible cause for the nonzero emission quantum efficiency of single complexes at low temperature.

A. Photostability, Intensity Fluctuations, and Fluorescence Polarization at Low Temperature. A marked increase in photostability on a lowering of temperature is found for most organic systems. This can partly be attributed to a reduction in oxygen diffusion and hence in the photooxidation probability.[25] Other yet undefined mechanisms which depend on temperature may play an important role in our case since a thorough removal of oxygen from the thin buffer film covering our samples did not result in a noticeable increase in photostability. A remarkable experimental observation is that the overall stability of the fluorescence intensity is highest in the temperature range 100−250 K as compared to both lower and higher temperatures as shown in Figure 2. The increase in fluorescence intensity fluctuation at lower temperatures maybe attributed to spectral diffusion. At $T < 100$ K the optical absorption line width of B800 of a single complex starts to get considerably smaller than the inhomogeneous distribution of transition energies (see, e.g., ref 26 and references therein). Consequently, spectral diffusion becomes noticeable[7] and results in a fluctuating temporal detuning between excitation laser and the molecular transition frequency with a concomitant variation in fluorescence intensity.

In case of a perfectly symmetric B850 aggregate, the two states $k = \pm 1$ carrying nearly all of the oscillator strength for radiative relaxation have transition dipole moments which are

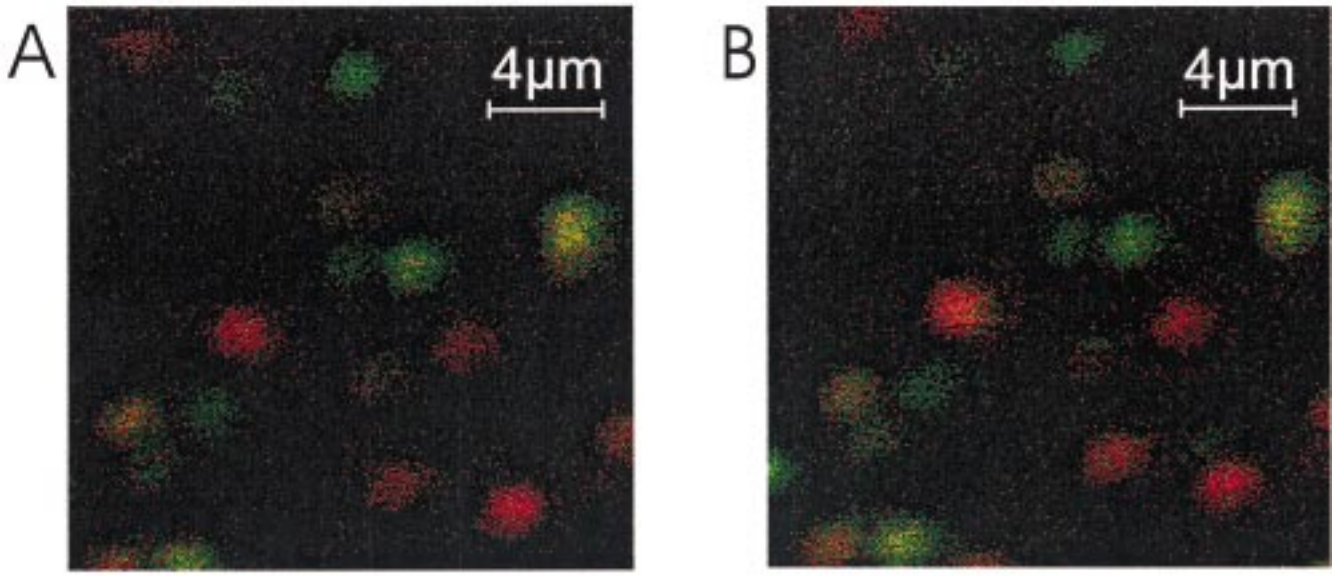

Figure 3. Confocal images of single LH2 complexes with different polarization of the excitation laser. In image B the polarization of the laser has been rotated by 90°. The images were recorded at 50 K.

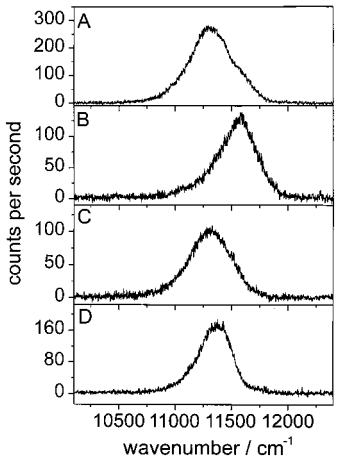

Figure 4. Fluorescence emission spectra of different LH2 complexes. The spectra have been recorded at 200 K. The aquisition time was 180 s.

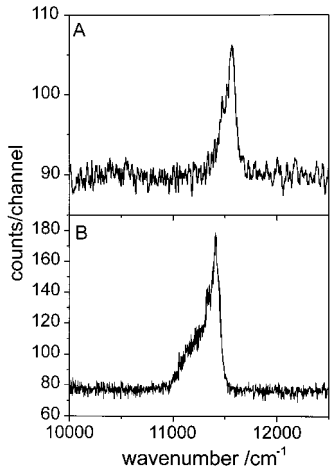

Figure 5. Fluorescence emission spectra of two different complexes at low temperature. In part A the acquisition time was 60 s, and the temperature $T = 19$ K. For part B the acquisition time was 300 s, and $T = 9.5$ K.

oriented in the plane of the ring and are mutually orthogonal.[23] Indeed, at room temperature most of the complexes emit fluorescence randomly polarized in the plane of the B850 aggregate (see Figure 1A). A closer analysis based on line scans of single molecule images in Figure 1A (see Figure 2B) reveals that during short time intervals τ abrupt changes in the polarization occur. Such events become noticeable as changes in the relative intensities of the two detector channels marked with arrows in Figure 2B. With highest probability (48%) for τ values were in the region of 10−50ms. At room temperature only few complexes (roughly 10%) show permanently linear polarized fluorescence emission. These complexes are characterized by rapid photobleaching.

Between 250 and 100 K only a few events are detected which alter the fluorescence polarization (see Figures 1B and 2C). A statistical analysis of the single complex images in Figure 1B reveals that around 80% of the complexes show linearly polarized fluorescence emission at temperatures below 250 K. The polarization of the fluorescence does not depend on the polarization of the excitation as demonstrated in Figure 3.

Measurements on LH2 (*Rhodobacter sphaeroides*) have shown isotropic fluorescence emission under ambient conditions[24] similar to the present results. Averaged over some seconds the fluorescence emission of single complexes is isotropic in most cases. A deviation from isotropic fluorescence emission of single complexes is found only during short time intervals. Our experiments may be explained by dynamic changes in E_{Bchl} of monomer units in the B850 aggregate (dynamic diagonal disorder) or by variations in the coupling $U_{\alpha\beta}$, $U_{\alpha\beta-\alpha\beta}$ between the molecules in the ring (dynamic off-diagonal disorder) which occur on a millisecond time scale. Reasons for such dynamic disorder in the B850 aggregate may be protein dynamics of the α- and β-apoproteins of the complex because the pigment−protein interaction can influence the electronic states of individual pigments via specific interactions such as hydrogen bonding and ligation or the polarizable Coulombic field it forms around the pigment. In the energy level picture of the highly symmetric B850 aggregate even minute pertubations may cause a lifting of the degeneracy of the $k = \pm 1$ levels with a concomitant anisotropic fluorescence emission.

Dark intervals where the intensity in both detector channels drop to the background level are also found at $T < 250$ K. However, the probability for such processes is considerably lower than at room temperature and also their duration is much longer (seconds as compared to millisecond at $T = 300$ K).

Previously, the creation of a trap state in the aggregate was discussed as a possible reason for the observation of dark intervals.[7,27] The sudden reduction in fluorescence intensity then is due to the photophysical parameters of the trap state. Such a mechanism may also explain anisotropic fluorescence emission. If the creation of the trap state is reversible, as it is the case for a charge transfer state,[7] then different monomers or subgroup of monomers of B850 may form a trap for the excitation energy in the course of the experiment with a concomitant change in the polarization of the fluorescence emission. However, changes in the polarization of the fluorescence of single complexes are not always accompanied by significant changes in the overall fluorescence intensity. In those cases the underlying physical processes for the creation of dark intervals and the polarization properties of B850 may be different.

The presence of static disorder of the B850 aggregate at low temperature has been found from an analysis of ensemble averaged measurements (see, e.g., refs 24 and 28). The detection of permanent anisotropic fluorescence emission of single complexes between 250 and 100 K may just reflect the effect of such static disorder. Below 70 K, the behavior of the fluorescence polarization gets surprisingly complex. The relative intensities in the two polarization channels change in a much more pronounced way than at higher temperature (see Figures 1C and 2D). Slow changes (tens of seconds) together with sudden ones (<50 ms) are observed without any observable auto-[29] or cross correlation.[30] We believe that this observation is related to minute structural changes in the complex and its environment which result in slightly different energetic configurations of the B850 ring. At low T the excitation energy gets more easily trapped in a local minimum of the energy hypersurface describing the B850 aggregate. This may correspond to an alteration of the fluorescence polarization of the LH2 complex.

B. Fluorescence Emission Spectra of Single LH2 Complexes. Since disorder may play an important role in the photophysics of the B850 aggregate it is interesting to get a quantitative measure of the transition energy between ground and excited state for different single complexes. At $T < 200$ K single complexes emit sufficient photons before photobleaching to allow the acquisition of fluorescence emission spectra with high signal-to-noise ratio. Typical examples for such spectra are shown in Figure 4A−D. Most complexes exhibit asymmetric emission lines. On average the emission spectra are centered around 11400 cm^{-1}, close to the value found in other low temperature studies.[31] Peak positions are distributed between 11200 and 11750 cm^{-1} and the width of the spectra (full width half, maximum) is 400 cm^{-1}. Upon lowering temperature the width of the fluorescence emission spectra reduces as expected. The smallest line widths found presently are around 120 cm^{-1} (see Figure 5A). This value is similar to those found in experiments on light harvesting complex 2 (LHC2) of green plants.[32] Some complexes also show broader asymmetric lines (see Figure 5b) with no resolved structure. The average peak position of the fluorescence emission at $T = 7$ K is 11430 cm^{-1}. Intra- and intermolecular contributions to fluorescence emission spectra have been investigated for light-harvesting complexes and isolated Bchl. From hole-burning and high-resolution fluorescence emission spectra on LHC2 it is known that electron-phonon coupling is weak in pigment−protein complexes. Thus in case of the LHC2 complex well-resolved vibronic zero phonon lines have been found in fluorescence line narrowing experiments at temperatures below 40 K.[32] No clear indication of such vibronic zero phonon lines is found in our spectra. Molecular vibrations of the Bchl in the B850 ring have been investigated by resonance Raman spectroscopy. These experiments have shown that there is little dispersion in the vibrational frequencies of chromophores in the ring.[28] Probably the considerable dispersion in spectral shape and position found among different complexes are not due to differences in the vibrational modes of the complex but are determined by differences in the electronic structure of the aggregate.

5. Conclusion

The increase in photostability of single LH2 complexes at low temperature is not accompanied by a substantial decrease of the fluorescence quantum yield. At $T < 250$ K most of the LH2 complexes emit anisotropically polarized fluorescence. Within signal-to-noise ratio the polarization is stable for minutes with no detectable cross correlation between the two orthogonal polarization channels on a time scale from 100 seconds down to 10 μs. This is in contrast to room temperature experiments where most of the complexes emit fluorescence isotropically polarized in the plane of the B850 aggregate. Only during short time intervals preferentially linear polarized emission is observed. For an ideally symmetric B850 aggregate with rapid intermolecular energy transfer isotropic in-plane fluorescence emission is expected. This is indeed found at room temperature when an average is taken over some seconds. Already at $T <$ 250 K we find a surprisingly large number of complexes with anisotropic fluorescence emission. The fluorescence emission of these complexes cannot be described by a perfectly symmetic B850 aggregate without disorder. A static random variation of the eigenenergies of the monomers forming the aggregate or the coupling between them may prevent a delocalization of the excitation over the whole B850 ring. A localization of the excitation energy on a certain subgroup of molecules in the ring would then explain the observed preferential linear polarization of the fluorescence of most of the complexes. We would like to add however that no clear relation between fluorescence polarization and degree of exciton delocalization is known yet and that no estimation of the number of Bchl monomers over which the exciton is delocalized can be derived from the present work. Comparison of the fluorescence emission spectra of individual complexes reveals a distribution in center of gravity of $\approx$300 cm^{-1}. This is evidence for the substantial disorder present in the aggregate.

Besides giving insight into the physics of the energy level scheme of the B850 aggregate our experiments also provide interesting insight into the physics of the energy transfer within LH2. The fluorescence emission of most of the complexes is linearly polarized at $T < 250$ K. The polarization of fluorescence emission remains unaltered when the polarization of excitation light is rotated by 90° although this results in the excitation of different monomers is the B800 ring and hence in a different energy transfer route to the B850 ring. There are two mechanisms explaining our experimental finding: (1) The energy is rapidly delocalized in the B850 ring once the energy transfer B800-B850 occurred. Subsequently the exciton always gets trapped in the same global energy minimum of the B850 ring from where fluorescence occurs. (2) There is a rapid energy transfer between B800 molecules prior to B800-B850 energy transfer. Both mechanisms are known to exist and can explain the above mentioned observation.

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