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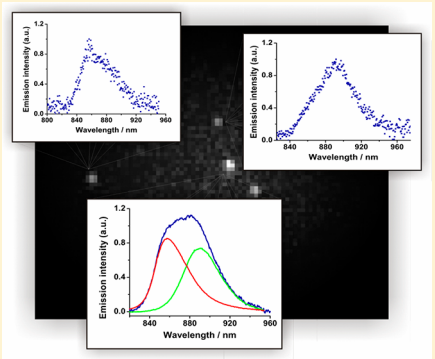
Fluorescence Micro-Spectroscopy Study of Individual Photosynthetic Membrane Vesicles and Light-Harvesting Complexes

Kristjan Leiger,[†] Liis Reisberg,[†] and Arvi Freiberg*[,†,‡]

[†]Institute of Physics, University of Tartu, Riia 142, Tartu 51014, Estonia

[‡]Institute of Molecular and Cell Biology, University of Tartu, Riia 23, Tartu 51010, Estonia

ABSTRACT: Single-molecule spectroscopy, by getting rid of unwanted ensemble averaging effects, has proved to be a very valuable tool in the research of individual photosynthetic light-harvesting (LH) complexes. However, to learn about real photosynthetic processes the minimal unit to study is a single photosynthetic membrane complete with all elements of its machinery. In the present work, the fluorescence spectra of excitons in lone intracytoplasmic (IC) photosynthetic membrane vesicles of the wild type purple bacterium *Rhodobacter sphaeroides* that involve peripheral (LH2) and core (RC−LH1−PufX) antenna pigment−protein complexes were investigated at ambient temperature under continuous-wave laser excitation into the Q_x absorption band of the bacteriochlorophyll-a (BChl) chromophores at 594 nm. In parallel, the spectra of mutant membrane vesicles occupied by just one type of complexes (either LH2 or RC−LH1−PufX) and the spectra of individual purified LH2 and RC−LH1−PufX complexes were measured. The fluorescence from full IC membranes shows a high sensitivity to excitation intensity, being varied over more than four orders of magnitude between 0.1 W/cm^2 and 2 kW/cm^2. At low to moderate excitation intensities, the spectra of IC membranes could be well reproduced by its component spectra, the ratio of the spectra related to peripheral and core complexes being the only adjustable parameter. The spectra of both intact chromatophores and individual membrane components recorded over 1−50 s experimental time frames are robust, strongly suggesting that large spectral fluctuations hardly play a role in the functional photosynthetic process. The significant, up to 14 times, variation of the LH2 and LH1 emission ratio observed in individual IC membranes could be related to variations in the stoichiometric ratio of the peripheral and core complexes. Evidence was found for the presence of LH2 parts that are detached from efficient energy transfer pathways. Upon strong and prolonged illumination, the membrane spectra reveal significant permanent modifications. These alterations, which mostly concern peripheral antenna complexes, were shown to be due to photo-oxidation of various numbers of BChl molecules in the B850 compartment of LH2.



INTRODUCTION

Recently, many photosynthetic pigment−protein complexes from plants and bacteria have become accessible by single-molecule spectroscopy (see refs 1−4 for reviews). The prime motivation behind this long-time effort has been the removal of ensemble averaging factors from spectroscopy data.[5,6] As a result, an improved comprehension of the fundamental nature of solar excitations[7] as well as of the details of their transport and energy trapping properties[8] is expected that eventually might lead to sustainable technologies of future solar energetics.[9−12]

In the context of bacterial photosystems, typically fluorescence spectra of single peripheral and core LH complexes have been studied. On experimental time scales of the order of 1 s, the spectra of these complexes measured at room temperature appear to be homogeneously broadened, since the widths of the spectra of single complexes and bulk solution almost coincide.[13−16] Occasionally, in about a tenth of the population, however, significant—within 5−10 nm—fluctuations of spectral positions have been observed.[16] The reported fluctuations, apparently correlated with the excitation power, are abrupt and occur toward the red (long wavelength) and blue (short wavelength) side of the mean spectral position with almost equal probability. Seldom, some really big blue jumps reaching 60 nm were also recorded.[16] The general reason for these spectral fluctuations is believed to be the modification of the local protein environment around the BChl chromophores.

It is widely speculated that the spectral fluctuations observed in individual LH complexes may have functional significance. However, to date, there has been no concrete evidence that the same effects are present in functioning photosynthetic membranes, leaving a possibility that we are dealing with artifacts of non-natural study conditions, which have no or little relevance to the biological operation of the bacteria. The study conditions that mostly deserve critical attention include the very high illumination intensity applied in the single-molecule experiments, usually by many orders of magnitude surpassing the bright daylight intensity (while the natural habitat of these bacteria is under dim light) and the surface immobilization of

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the proteins, which may lead to deformation and/or to surface- or linker molecule-induced denaturation of the samples.[17] Also, the removal of the integral membrane proteins like LH2 and LH1 from their native membrane environment is prone to destabilize the proteins[18,19] and change their functionality.[20] It might thus prove instructive to study the more integrated samples that are a step closer to native systems and under the much gentler handling. Such an attempt is undertaken in the present work where the fluorescence spectra of excitons in both bulk and single IC membranes (also called chromatophores) from the wild type (WT) purple bacterium *Rhodobacter (Rb.) sphaeroides* are thoroughly investigated over a broad excitation intensity range, which covers more than 4 orders of magnitude. No special immobilization of the single membranes other than spontaneous physisorption was used, as described in the Experimental Section.

The WT IC membranes from *Rb. sphaeroides* of spherical shape are usually populated by two types of LH pigment−protein complexes:[21−23] LH2 and RC−LH1−PufX (where RC stands for the reaction center chromoprotein). Together, they form a functional entity called the photosynthetic unit (PSU)[24] (see also ref 25 for a review). It is estimated that the chromatophores with a typical diameter of 50−60 nm contain up to 18 PSUs.[22,26] The success of bacterial photosynthesis thus depends on ultrafast events in which tens of LH chromoproteins are cooperating in a multistep process of solar energy funneling toward RC[27] (see ref 28 for a review).

Responsible for the strong fluorescence of these complexes are excitons due to tightly coupled arrays of BChl molecules, the main light harvesting pigment in this bacterium. In LH2, also named as the B800−850 complex, two cyclic BChl systems coexist, giving rise to two characteristic absorption bands at 800 and 850 nm. The former band in the complexes from *Rb. sphaeroides* belongs to 9 loosely bound BChl's, while the latter band, to the 18 strongly excitonically coupled BChl's. Emission from this lowest-energy state dominates the fluorescence spectrum of LH2 complexes peaking at 850−860 nm. The fluorescence around 800 nm related to the B800 absorption is usually rather weak due to ultrafast energy transfer between the B800 and B850 molecular arrays.[29] The core complexes absorb strongly at 875 nm; the fluorescence from B875 peaks around 890 nm. The architecture of photosynthetic membranes from purple bacteria has recently been intensely studied by means of atomic force microscopy.[21,30] Large variability of individual LH complexes as well as the structure of PSU was revealed. Regarding the photosynthetically grown cells of WT *Rb. sphaeroides*, there are two types and not just a single type of core complexes where open C- or S-shaped antenna structures encircle one or two RC complexes, respectively, in planar or nonplanar geometry.[22] Despite this complexity, in what follows, we will generally designate core complexes by LH1, if there is no risk of confusion.

The fluorescence spectra of chromatophores measured at ambient temperature generally exhibit contributions from both the LH1 and LH2 complexes. The apparent LH2/LH1 emission ratio is an elaborate function of the stoichiometric ratio of the LH1 and LH2 antenna pools, the intra- and interpool connectivity, i.e., existence of corresponding energy transfer pathways, the fluorescence quantum yield in separate antenna pools,[31] and other factors such as the equilibrium population of excited states in interconnected pools. Full experimental characterization of the antenna processes in PSU thus requires complex investigation that besides the measurements of fluorescence spectra and fluorescence decay times as a function of temperature includes recording of absorption and fluorescence-excitation spectra.

The previous fluorescence studies of individual chromatophore membranes performed at room temperature[8,32,33] established a considerable static[32] and dynamic[33] structural heterogeneity in protein assemblies, disturbing the degree of energy transfer between the proteins. Specifically in ref 32, major differences of the LH2-to-LH1 excitation energy transfer efficiencies in mature IC membranes and in the so-called precursor membranes at an early stage of the membrane development were recorded. While in IC membranes most of the excitation energy initially delivered to LH2 emerges from LH1, in precursor membranes, there is a greater contribution from LH2, indicating that a number of the LH2 complexes in this membrane is uncoupled from the LH1 complexes. It was further found in ref 8 that the fluorescence of purified LH2 complexes decays single-exponentially, whereas the quenching is biexponential for the LH2 packed into a membrane environment, suggesting involvement of the nonlinear excitation effects. In most of these studies, rather high intensity excitation into the strong B800 absorption band of LH2 was used, which might have obstructed the results. At cryogenic (nonphysiological) temperatures, energy transfer in single PSU reconstituted into an artificial lipid bilayer or self-aggregated in a non-membrane environment was studied in refs 34 and 35, respectively.

In the present work, the ambient-temperature fluorescence spectra of excitons in lone IC photosynthetic membrane vesicles of the WT purple bacterium *Rb. sphaeroides* were systematically investigated under continuous-wave laser excitation of varying intensity between 0.1 W/cm^2 and 2 kW/cm^2. The low-intensity border of this range corresponds to the bright daylight intensity. The utilized 594 nm laser line rather uniformly excites all the BChl's in PSU via the weak Q_x absorption band. To unravel structural and functional aspects of the photosynthesis process taking place in individual IC membranes, the fluorescence spectra of chromatophores with different stoichiometric ratio of peripheral and core complexes were studied. For comprehensiveness, the spectra of mutant membrane vesicles occupied by just one type of complexes (either LH2 or RC−LH1−PufX) and the spectra of individual purified LH2 and RC−LH1−PufX complexes were measured. Depending on the context, the title "individual" thus means a single LH protein complex (involving two different proteins) or a single membrane vesicle (three different membrane types). In all cases, the single-particle spectra were calibrated against the bulk solution spectra, which correspond to large ensembles of individual membranes or complexes.

EXPERIMENTAL SECTION

Materials. The purified into detergent micelles and self-assembled into native and mutant photosynthetic membrane vesicles LH complexes from *Rb. sphaeroides* were prepared as described earlier.[36,37] The *Rb. sphaeroides* DD13 deletion strain, manipulated to remove the genes encoding the LH2, LH1, and RC complexes, was complemented with plasmid-borne copies of the *puf BALMX* genes to produce membranes containing only the LH2 or dimeric RC−LH1−PufX complexes, correspondingly called LH2-only or LH1-only membranes in what follows. Two types of native chromatophores from WT *Rb. sphaeroides* 2.4.1 were used, one grown under low-intensity illumination with high stoichiometric ratio of the LH2 and LH1

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complexes (LLM in short) and another under high illumination (HLM) with low stoichiometric ratio. A rough estimation according to the methodology described in ref 23 and based on the peak heights in the absorption spectra gives a 4.2:1 ratio of LH2:RC in LLM and a 1.9:1 ratio in HLM samples.

For measurements, the samples were dissolved in a buffer solution (TEN in case of the native membranes; 20 mM Hepes at pH 7.5 in the case of the isolated LH2 and LH2-only membranes; 20 mM Hepes at pH 7.8 in the case of the isolated RC−LH1−PufX and RC−LH1−PufX-only membranes) to obtain a peak optical density of 1 cm^{-1} (in bulk measurements), 0.01 cm^{-1} (in single IC membrane measurements), or 0.001 cm^{-1} (in single LH complex measurements) in the Q_y absorption region (800−900 nm) of the BChl molecules. A value of 1 cm^{-1} here is roughly equivalent to 0.3 μM of isolated LH2 complexes (see ref 38 for calculations), or assuming that there are about 100 complexes on a membrane vesicle (most of which are LH2-s), 3 nM of membranes. In the case of the isolated samples, a detergent (1% β-OG for LH2; 0.03% β-DDM for RC−LH1−PufX) was also added to avoid aggregation of the proteins.

A number of photoinduced traps that quench fluorescence of the samples are known to be formed in the presence of oxygen. To remove dissolved oxygen, 1% w/v glucose, 100 units/mL of glucose oxidase, and 1000 units/mL of glucose catalase (all from Sigma) were added to the sample solution. As a result, the concentration of dissolved oxygen was reduced ∼50-fold compared with the equilibrium concentration, measured by a device using Pd-tetraphenylporphyrin/PMMA fluorescence decay to determine the oxygen concentration.[39] Other deoxygenating methods, such as bubbling with inert gases (nitrogen or argon), require much larger volumes of sample and, to our experience, generally provide less satisfactory results. For the bulk or single membrane measurements, the sample solution was dispensed inside a small cuvette formed between the microscope base glass and coverslip by a double-sided tape (∼0.17 mm thickness) with a central part cut out.

The IC membrane vesicles were usually immobilized by spontaneous physisorption on the coverslip. Alternatively, to exclude any interference with the substrate, the emission spectra of freely suspended particles were recorded while just diffusing through the excitation laser beam. These two approaches generally gave comparable results. The advantage of the latter, "on-flight", technique is the ease of implementation as well as larger sample statistics; however, the signal necessarily suffers from greater intensity fluctuations due to temporal defocusing and shorter average illumination time. Moreover, in spectral measurements, it does not allow following the time series of an individual sample. For a comparison with previous works, in the case of the single isolated complexes, we studied the samples that were immobilized on the coverslips treated with 0.1% poly-L-lysine (PLL) solution for a couple of minutes. The sample solution was then applied onto the coverslip for another couple of minutes before washing away with the deoxygenated buffer.

Micro-Spectroscopy Methods. The fluorescence spectra were recorded by a homemade micro-spectroscopy system based on an Olympus IX-71 inverted microscope, Andor Shamrock 303i spectrometer, and Andor spectroscopic camera (iDus 420). The spectral resolution of this system is about 0.5 nm/pixel. Another camera (Andor iXon 897 EMCCD) was used for imaging and focusing purposes. The fluorescence was collected by a 100× oil immersion objective (NA = 1.3, Olympus). A continuous wave He−Ne random polarized laser (Melles-Griot 25-LYR-173-230) was used as an excitation source at 594 nm. The excitation light was directed onto the sample from the opposite side through an objective (20×, Olympus), which resulted in an excitation spot of roughly 8−15 μm full width at half-maximum (fwhm). Two mixed-type long-pass filters (interference + colored glass, from Andor) with cutting edge 50% transmission wavelengths at 700 and 750 nm, respectively, were used to remove the excitation light from the fluorescence signal. In most experiments, also a dichroic mirror with reflection edge at laser wavelength (594 nm) at 45° was used for additional filtering, as it effectively deflected any passing-through excitation and prevented back-reflections from filters to appear in the image, as well as any possible filter fluorescence.

The excitation intensity at the sample varied from 0.1 W/cm^2 to 2 kW/cm^2, depending on the task. For estimating the excitation intensity profile, a slice of the background fluorescence from the coverslip was measured in a spectral region where no signal from the sample was present, and the result was fitted by a Gaussian function. According to the profile and laser power, the beam width and intensity were determined. For single isolated complexes and membrane vesicles, the positions within the beam were also taken into account to estimate the range of intensities used. In the case of bulk and freely suspended samples, generally the maximum intensity is indicated, while the actual fwhm of the beam on the focal plane was about 8−15 μm for the bulk samples and about 4 μm for the freely suspended particles.

The fluorescence images, as shown in Figure 1, were recorded in widefield mode. Imaged in Figure 1 with a spatial resolution of 0.13 μm per pixel are the mutant membrane vesicles containing just LH2 complexes (dubbed as LH2-only membranes), which are spontaneously immobilized on the microscope coverslip surface. The cross section of the fluorescent dots of 0.35 μm fwhm demonstrates spatial resolution near the diffraction limit. It can be seen that there are considerable differences of emission intensity from different vesicles, which do not follow the expected two-dimensional Gaussian exciting beam profile over the image plane. A common wisdom is that the size of the vesicles is rather constant.[32] However, the dynamic light scattering data[40] show a large range of vesicle diameters between 30 and 150 nm which, assuming a uniform coverage of the vesicle surface with LH2 complexes, could in principle explain up to 25 times intensity differences in Figure 1. Additional uncontrolled factors such as the presence of light-induced traps, as noted above, may contribute to the observed intensity variation.

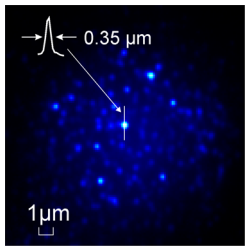

Figure 1. Fluorescence image of LH2-only membrane vesicles under 594 nm excitation. Indicated is a cross section of a fluorescent dot with a fwhm of 0.35 ± 0.05 μm obtained from a fit of the intensity profile.

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For the spectral measurements, the monochromator slit was used to pick out the selected sample areas. The spectra were usually acquired in time series (typically 50 spectra with 1 s acquisition time) to see the possible evolution of spectral characteristics in the 1 s time range. In many cases, spectral time averages (equivalent to 50 s acquisition time) are used, which increases the signal-to-noise ratio. The responses from four to eight adjacent rows of the camera pixels were summed up to obtain a raw spectrum. A further one to two rows from each side of the spectrum were averaged to obtain the background, which was then subtracted from the raw spectrum. The background-corrected spectra were further corrected for spectral sensitivity of the recording apparatus.

Data Processing. The corrected experimental spectra were generally fitted to a skewed Gaussian function[41] to characterize them in terms of four parameters: spectral position, fwhm, amplitude, and skewness. The spectra of WT membranes complete with peripheral and core LH complexes were fitted with a linear combination of independently measured bulk spectra of the RC−LH1−PufX-only and LH2-only membrane samples. In this case, the spectral positions and the ratio of emission intensities of the two components were the fitting parameters.

We will use common wavelength scale throughout the paper; in the actual spectral region (850−900 nm), a 1 nm difference corresponds to 12−14 wavenumbers in energy scale.

RESULTS AND DISCUSSION

Detergent-Isolated LH1 and LH2 Complexes. We began our studies with fluorescence measurements of single isolated complexes, LH1 and LH2, immobilized on a PLL-treated glass. This is partly done for calibration purposes, in order to be able to compare our data with the results of the previous measurements by others. As it was already briefly introduced in the Introduction, the fluorescence of LH1 complexes is mainly related to the B875 excitons, while that of LH2 complexes, to the B850 excitons. In LH2, also a weak signal due to the B800 states can be observed. This thermally activated fluorescence rapidly vanishes by cooling down the sample.[31,40]

In the case of LH1, the spectra of 13 different single complexes were studied. As can be seen from the comparison in Figure 2a, both the peak positions (889.5 and 888.1 nm) and the widths (45.0 and 42.3 nm) of the bulk and averaged single spectra match each other within the experimental error, which is ±0.6 and ±0.7 nm for the position and width, respectively. In the case of LH2 (Figure 2b), the observed differences are somewhat larger. The averaged spectrum of 14 different single complexes appears 3.7 nm broader (fwhm equal to 37.5 nm, instead of 33.8 nm in bulk) and also shifted by 1.9 nm to the red (from 853.3 to 855.2 nm) relative to the bulk solution spectrum.

The results for LH2 can be compared with the literature data[13−16] (to the best of our knowledge, single complexes of RC−LH1−PufX from *Rb. sphaeroides* have not been studied before). The LH2 complexes have been previously found to exhibit considerable fluctuations of intensity, spectral position, and width. Our data do not reflect any big changes, except for blinking, the fingerprint of a single-molecule excitation when the fluorescence occasionally vanishes and reappears at the same location.[14] Specifically, under the present experimental conditions, no significant fluorescence peak jumps toward either side of the spectrum were recorded. The leaning of the sum of single spectra toward longer wavelengths observed in Figure 2b can most probably be attributed to an incomplete averaging due to a small ensemble size.

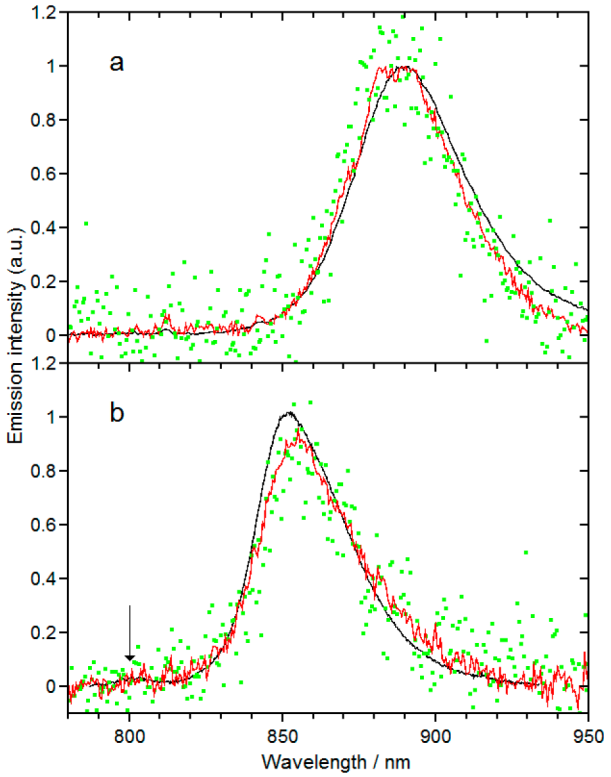

Figure 2. (a) Peak-normalized fluorescence spectra corresponding to the sum of 13 different isolated RC−LH1−PufX complexes (red line) and the bulk sample (black). Represented with scattered green dots is a typical single spectrum of the complex recorded with 5 s acquisition time. (b) Area-normalized fluorescence spectra corresponding to the sum of 83 spectra representing 14 different isolated LH2 complexes (red) and the bulk sample of isolated LH2 complexes (black). A typical single spectrum recorded with 2 s acquisition time is shown with scattered green dots. Indicated with an arrow is the fluorescence related to B800. The excitation intensity used to measure the various spectra was between 50 and 200 W/cm^2.

LH1-Only and LH2-Only Mutant Membranes. The gross spectroscopic data concerning the exciton fluorescence in individual LH1-only and LH2-only membranes spontaneously immobilized on a coverslip are presented in parts a and b of Figure 3, respectively. Also depicted in these figures for reference are the bulk spectra of the corresponding isolated complexes and membranes.

As seen, the spectra of LH1 complexes in various settings are very conservative; they hardly change when the complexes are surrounded by detergent micelles or embedded into a native lipid bilayer. Although the fluorescence band positions and widths fluctuate slightly from one vesicle to another, the averaged spectrum of single vesicles almost precisely overlaps with the bulk spectra of both isolated and membrane complexes.

This is rather different from the case of LH2 demonstrated in Figure 3b. While the two spectra of LH2-only membranes are fairly similar to each other, they are significantly (by ∼4 nm) shifted to the red with respect to the bulk spectrum of isolated complexes. The membrane spectra are also broader than the spectrum of isolated complexes. Both these characteristics, already noted earlier,[40,42] can be ascribed to a modified LH2

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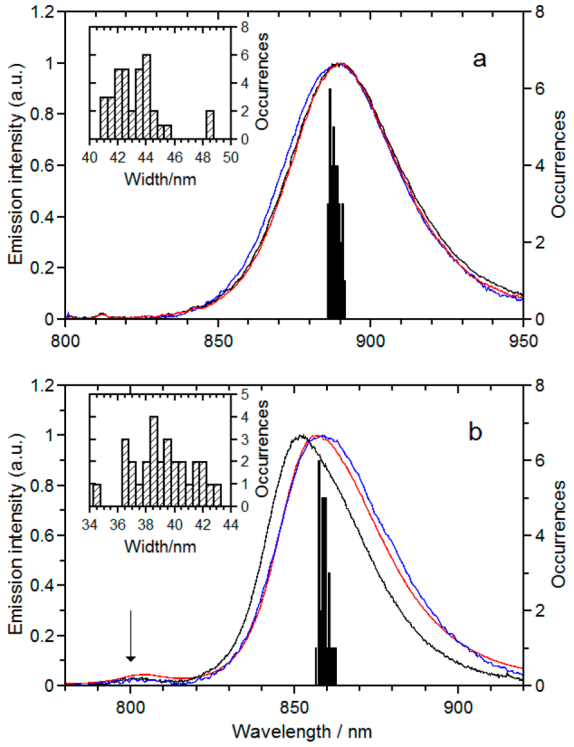

Figure 3. (a) Peak-normalized fluorescence spectra of RC−LH1−PufX-only membranes: red, bulk spectrum; blue, averaged spectrum of 35 single complexes. Shown in black is the reference bulk spectrum of isolated RC−LH1−PufX complexes (the same as in Figure 2a). Bars denote the histogram for single membrane peak positions (occurrences on the right scale) with a mean of 888.6 ± 1.5 nm (the respective number for the bulk spectrum is 889.1 nm). The inset shows the distribution of widths with a mean of 43.5 ± 1.7 nm (43.3 nm). (b) Peak-normalized fluorescence spectra of LH2-only membranes: red, bulk spectrum; blue, averaged spectrum of 29 single spectra. Shown with black is the reference bulk spectrum of isolated LH2 complexes (the same as in Figure 2b). Indicated with an arrow is the B800 fluorescence. Bars denote the histogram for single membrane peak positions (occurrences on the right scale) with a mean of 859.3 ± 1.5 nm (the respective number for the bulk spectrum is 857.7 nm). The inset shows the distribution of widths with a mean of 39.4 ± 2.0 nm (37.4 nm). The excitation intensity used to measure the various spectra was between 50 and 200 W/cm^2.

protein structure in detergent micelle relative to that in lipid membrane. Provided that in the native membrane the protein acquires the most compact structure corresponding to the maximum exciton coupling strength, the membrane spectra must be the most red-shifted as well as the broadest. Why then does a similar explanation not apply for the core complex (Figure 3a)? One obvious reason is the presence of RCs in core complexes, which by stiffening the LH1 structure makes it less responsive to the environmental forces.[43] A related stabilizing effect was revealed very recently by high-pressure spectroscopy.[44] The hydrogen bonds in core complexes that bind the BChl chromophores with their immediate protein surroundings are much easier to break if RCs are missing.

In membranes, it generally holds that under the conditions of moderate excitation intensity (≤100 W/cm^2) the fluctuations of the position, width, or intensity observed in the 1 s acquisition time spectra all remain within the experimental error margins. Only in rare cases, continuous, single-directional movements of the spectra toward either blue or red were observed with total shift ≤2 nm within the 50 s time series. At high excitation intensities, in contrast, the membrane spectra systematically tend to move toward the blue side of the spectrum. These latter changes may be caused by photoinduced modifications of the excitonically coupled groups of chromophores (or their immediate surroundings) in the presence of trace amounts of oxygen. We will return to the discussion of these issues shortly.

Full Membranes Complete with LH1 and LH2 Complexes. The fluorescence emission spectra for the two fully developed bacterial IC membranes, grown under different illumination conditions, are shown in Figures 4 and 5. Presented in Figure 4 are the data for LLM chromatophores. The high stoichiometric ratio of LH2 and LH1 complexes in this sample is clearly demonstrated by the absorption spectrum (seen in the left inset of this figure), which is dominated by the absorption of LH2 complexes. Exposed in Figure 5, on the other hand, are the data for HLM chromatophores. These samples are characterized by a relatively low stoichiometric ratio of the LH2 and LH1 complexes, which is obvious from the prominent absorption peak corresponding to LH1 complexes (the absorption spectrum is shown in the left inset of Figure 5).

The fluorescence spectra in Figure 4 for the bulk and the average of individual LLM chromatophores appear reasonably similar. At the same time, variations of the ratio of the emission intensities for the LH2 and LH1 pools in different chromatophores are considerable. This is very evident from the spectra of two individual samples represented by blue and green dots. To get a quantitative measure of spectral variations in terms of the LH2/LH1 emission ratio, the spectra of individual chromatophores were analyzed by fitting with the bulk spectra of two mutant membranes that contain just core or peripheral antenna complexes (shown in Figure 3a and b, respectively). The result for the studied population of 46 LLM chromatophores is shown in the right-hand side inset of Figure 4. It can be seen that the LH2/LH1 emission ratio in individual chromatophores varies as much as 14 times. At the same time, the means for the bulk (1.2) and individual chromatophore (0.8) ensembles quite satisfactorily match each other. Also, the fitted positions of the fluorescence spectra for the LH2 and LH1 pools, 858.8 ± 2.3 and 887.7 ± 3.9 nm, respectively, very well agree with those in the LH2-only and LH1-only membranes (see Figure 3). These data once again suggest that large spectral fluctuations of individual membrane components hardly play a role in the functional photosynthetic process.

A very similar situation is observed in HLM samples (Figure 5), except for the drastically diminished contribution of the LH2 complexes into the fluorescence spectrum, related to the reduced LH2/LH1 stoichiometric ratio. The variation of the LH2/LH1 emission ratio in the studied ensemble of individual HLM chromatophores is the same as that in LLM samples. Likewise, the means of the LH2/LH1 emission ratio are close in bulk (0.34) and individual HLM (0.40) samples. The visibly noisier averaged spectrum of individual chromatophores in Figure 5 as compared to that in Figure 4 is inherent to the applied "in-flight" recording method (see the Experimental Section).

The higher sensitivity of LLM spectra on excitation intensity compared with those of HLM spectra is of notice (Figure 6). While the HLM spectra obtained at low (1 W/cm^2) and at 1000 times higher (1 kW/cm^2) excitation intensities differ only

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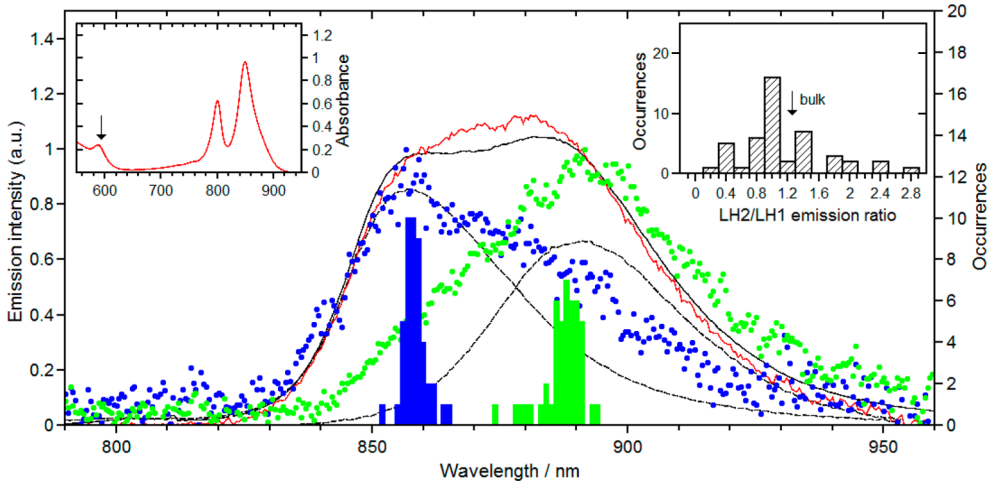

Figure 4. Fluorescence spectra of bulk (black) and averaged over 46 individually measured (red) LLM samples from *Rb. sphaeroides* recorded at excitation intensities of 1 and 50−200 W/cm^2, respectively. The spectra are normalized to the same area. Represented with scattered blue and green dots are the spectra from individual samples having extreme emission ratios within the studied population. Shown with dashed black line is the average fitting spectra of the LH2 and LH1 complements in individually measured chromatophores. The blue and green histograms (right scale) denote the deduced distributions of fluorescence peak positions for the LH2 and LH1 complexes, respectively. The left inset shows the absorption spectrum of the bulk solution (the arrow indicates the excitation wavelength in fluorescence measurements), and the right inset, the distribution of the LH2/LH1 emission ratio in individual chromatophores (the arrow indicates the ratio in bulk).

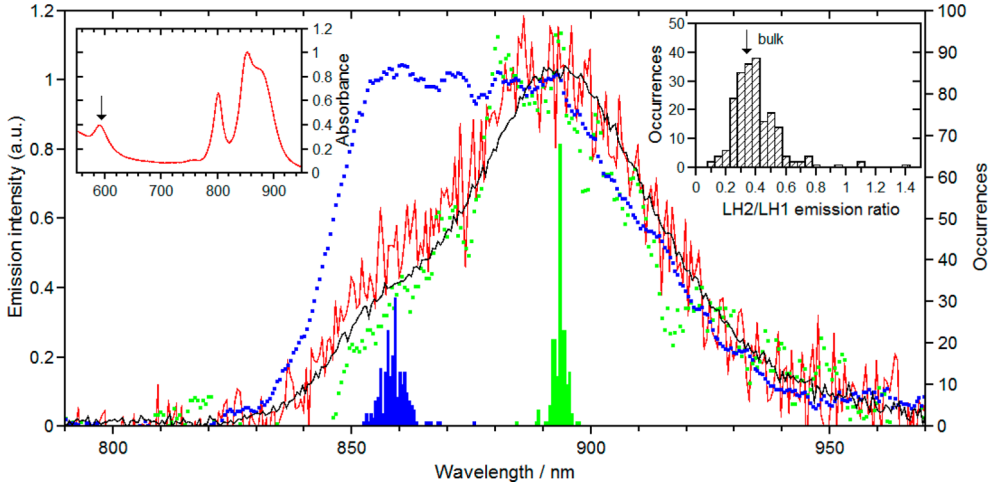

Figure 5. Fluorescence spectra of bulk (black) and averaged over 209 individually measured, freely suspended (red) HLM samples from *Rb. sphaeroides* recorded at excitation intensities of 1 and ∼400 W/cm^2, respectively. The spectra are normalized to the same area. Represented with scattered blue and green dots are the spectra from individual membranes having extreme emission ratios within the population. Bars denote the histogram (right scale) of peak positions for the LH2 (blue) and LH1 (green) components. The left inset shows the absorption spectrum of the bulk solution (the excitation wavelength is indicated by an arrow), and the right inset, the distribution of the LH2/LH1 emission ratios in individual chromatophores (the ratio in bulk is indicated by an arrow).

moderately (Figure 6b), the LLM spectra demonstrate large and characteristic variations (Figure 6a). The LLM spectrum that at the low excitation intensity range shows close amounts of the LH1 and LH2 emissions becomes at high intensity unexpectedly much more dominated by the fluorescence of LH2, as shown by the differential spectrum in the left inset of Figure 6a. This is followed by a large, ∼23 nm shift of the emission maximum toward the blue side of the spectrum (from 882 to 859 nm). In contrast, the shift in the case of HLM (Figure 6b) is just a few nm. The dependence of the LH2/LH1 fluorescence intensity ratio on excitation intensity across 4 orders of magnitude intensity change is for both samples demonstrated in the center inset of Figure 6. It is obvious that while in HLM the LH2/LH1 emission ratio is broadly constant over the whole excitation intensity range, in LLM it is reasonably constant only at relatively low intensities; past ∼100 W/cm^2, the ratio starts increasing rapidly.

The excitation intensity effects described cannot be explained by an increase of local temperature in the vicinity of the proteins. First, the temperature would generally affect the LLM and HLM spectra similarly. Second, there is a reasonably good overlap between the spectra of bulk and individual chromatophores in Figures 4 and 5, which were measured using 2 orders of magnitude different excitation intensities, as well as between the spectral bandshapes in Figure 6 that were recorded under 1000 times different excitation intensity. If temperature would directly follow excitation intensity, one would see a parallel equilibrium redistribution of intensity in favor of LH2, as well as significant broadening of the LH2 and LH1 spectra. None of these effects has been observed.

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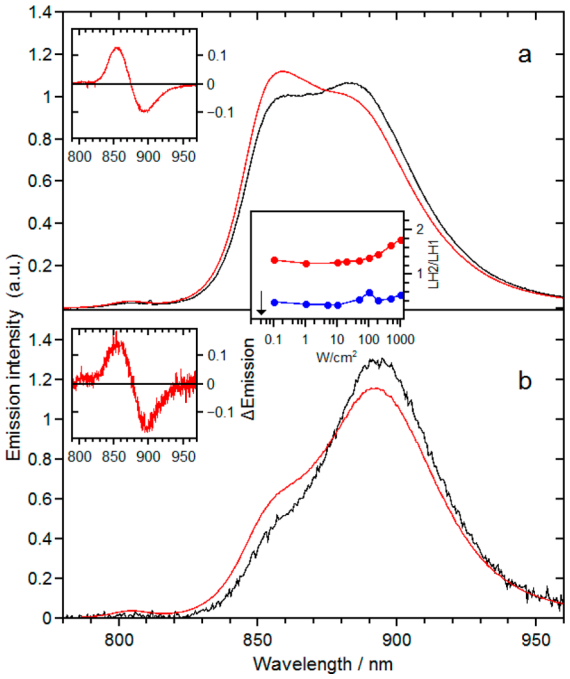

Figure 6. The area-normalized fluorescence spectra of bulk LLM (a) and HLM (b) samples at various excitation intensities: low of 1 W/cm^2 (black) and high of 1 kW/cm^2 (red). The left-hand insets in parts a and b demonstrate the difference between the spectra at high and low illumination for the LLM and HLM membranes, respectively. Shown in the center inset is the dependence of the LH2/LH1 emission ratio in the bulk LLM (red) and HLM (blue) membranes on excitation intensity. Lines connecting data points are for leading the eye. The arrow denotes the intensity of bright sunlight on the Earth's surface in the visible portion of the spectrum.

The modifications of the spectra demonstrated in Figure 6 are accompanied by emission quenching. Similar loss of yield and blue-shifting of the fluorescence spectrum was observed for the bulk chromatophores in ref 45, being explained[45,46] by annihilation between the short-living singlet and long-living triplet excited states in core complexes. A recent study[47] on reconstituted lipid membranes filled with just LH2 complexes of purple bacterium *Rhodopseudomonas acidophila* indicated that a major part of quenching in such network systems, as opposed to single antenna complexes,[38] comes from singlet−singlet annihilation. This is because a created B850 single excitation has a high mobility and can visit about 100 connected complexes during its lifetime, i.e., virtually all the LH2 complexes on a single membrane. The significance of the singlet-singlet annihilation process grows remarkably with excitation fluence.

Atomic force microscopy images of LLM membranes have revealed a highly heterogeneous buildup of PSUs.[8,21,32,48] Along with the well-connected PSUs, small lone domains of closely assembled LH2 complexes are present. The peripheral antenna structures develop late in the membrane remodeling process, during adaptation to the growth illumination conditions, and correlate with the extent of the observed fluorescence heterogeneity.[26,32] Excitation energy transport from the detached areas of LH2 to RC must be impaired, resulting in enhanced radiation of the LH2 complexes. The latter notion can be easily checked by recording fluorescence spectra at low temperatures. In the perfectly arranged PSUs, the fluorescence from LH2 should be extremely weak or altogether absent because of the very fast "downhill" excitation energy transfer from LH2 to LH1.[45,49,50] The actual measurements performed at 4.5 K revealed considerable LH2 signal (data not shown), thus validating that the studied chromatophores contain the LH2 complexes which are functionally detached from active LH2-to-LH1 energy transfer pathways.

Taking these leads, we tentatively assume that in LLM, and less so in HLM, there exists a number of LH2 complexes with poor functional contact with core complexes. At high excitation intensities, the fluorescence of this quasi-isolated peripheral complexes grows in prominence, because excitons in the major pool of well-contacted antenna complexes will be effectively

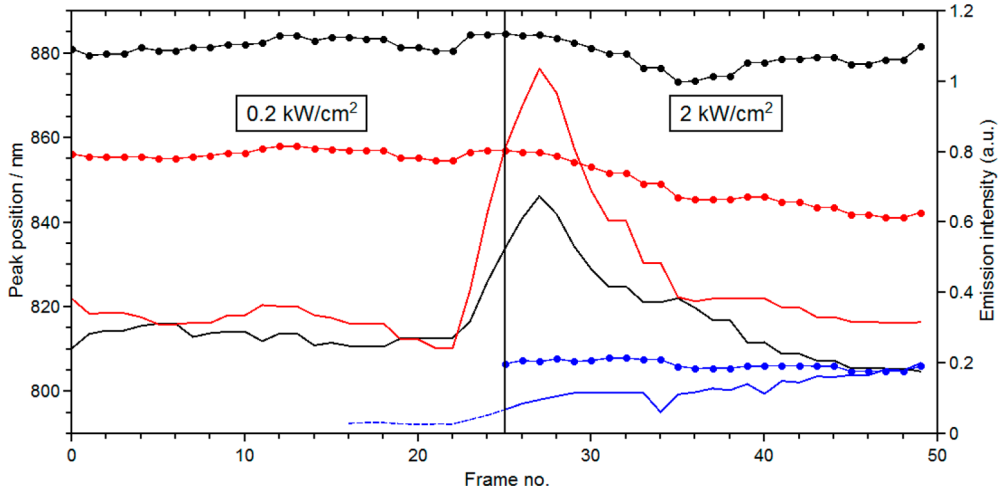

Figure 7. Time series of integral intensities (solid lines, right scale) and peak positions (connected dots, left scale) for the B875 (black), B850 (red), and B800 (blue) components in the fluorescence spectrum of an individual LLM sample. There is a 1.8 s gap of no excitation between every 1 s of laser exposure; one frame thus lasts 2.8 s. The excitation light is abruptly switched from the relatively low (0.2 kW/cm^2) to very high (2 kW/cm^2) intensity in the beginning of the 25th frame. The transition between the low and high intensity periods is a smoothing artifact, caused by averaging over five consecutive frames. The dashed line indicating the B800 fluorescence intensity in the low-intensity excitation region is illustrative, as the fitting results are not reliable in this range.

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quenched by singlet−triplet[45,46] and/or singlet−singlet[47] annihilation.

Permanent Spectral Modifications under Excess Light. The effects of excitation annihilation should largely be reversible; i.e., the low-intensity spectrum should recover upon reducing excitation intensity. This does not always happen. To solve this conundrum, individual and bulk LLM were studied under high-end excitation intensities reaching 2 kW/cm^2 over extended periods of time.

Shown in Figure 7 is a representative trace of such measurements where integral fluorescence intensity is followed together with peak positions for the B875, B850, and B800 fluorescence components of the spectrum, while modulating excitation intensity between the relatively low and high intensity limits. Only one low-intensity (0.2 kW/cm^2) and one high-intensity (2 kW/cm^2) period is reproduced in Figure 7. It should be noticed, though, that according to ref 45 both of these intensities are beyond the onset of singlet−triplet annihilation in bulk WT membranes.

A typical observation is that the B875 and B850 fluorescence intensity decreases over all measuring periods, albeit more rapidly over high-intensity intervals, and that recovery of the signal during dark (i.e., no illumination) periods is limited. The fact that upon switching excitation from low to high intensity their fluorescence is not proportionally increased is in agreement with the excitation annihilation mechanism, as discussed above. While the fluorescence bleaching in high-intensity sections is accompanied by gradual and lasting blue-shift of the B850 band of LH2, the position of the B875 band of LH1 shows only occasional, small (less than ±5 nm with an estimated error of about ±3 nm) shifts both toward blue and red. These data together with the recordings for isolated complexes (see Figure 2) evidence greater resistance of core antenna complexes against intense light as compared to peripheral complexes. Compared with the B850/B875 fluorescence quenching, the emerging behavior of the B800 emission is rather peculiar. The spectral position of this band peaking at ∼804 nm is also very stable.

Snapshots of the fluorescence band shape are shown in Figure 8. In correlation with the data in Figure 7, the spectrum varies significantly in time. The familiar B850 band of LH2 not only shifts to the blue but also broadens before it settles around 830−845 nm, depending on the waiting time and the sample. For convenience, this "equilibrated" band is in Figure 8 denoted as B840. Another significant observation is total absence of the fluorescence from free/solubilized BChl's that in the spectra of broken chromatophores show up around 774 nm.[19] The lack of solubilized BChl's importantly shows that in the present high-intensity illumination experiments the overall protein structure is not compromised.

To evaluate the origins of the B840 and B800 fluorescence bands of LH2, avoiding any interference with the LH1 protein, we continued experiments with the LH2-only membranes. The results of this study, illustrated by Figure 9, can be summarized as follows.

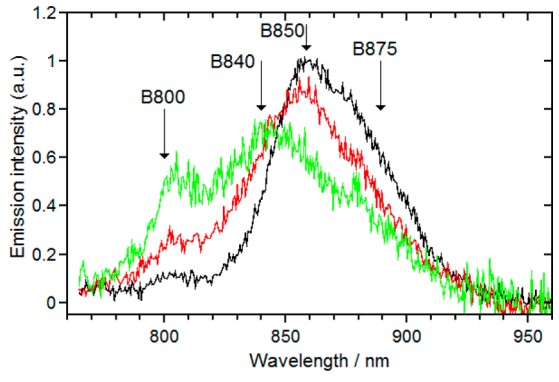

Figure 8. Fluorescence spectra of single LLM chromatophores recorded at different times under an excitation intensity of ∼1.7 kW/cm^2. The three area-normalized spectra (initial black, intermediate red, and final green) are recorded after about equal total excitation intervals of 12 s. In all spectra, 10 consecutive frames have been averaged to get a better signal-to-noise ratio. Spectral components mentioned in the text are denoted by arrows.

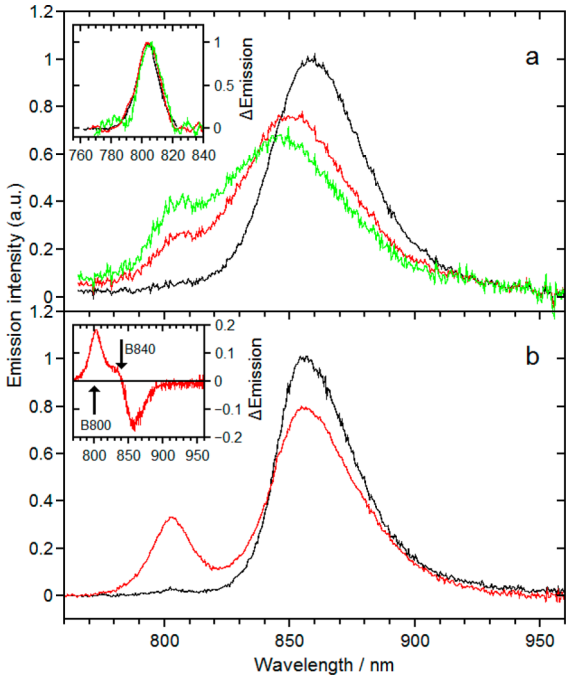

Figure 9. Area-normalized fluorescence spectra of a single LH2-only membrane (a) and bulk of the membranes (b). The three spectra shown in panel a are recorded at 25 s time intervals using ∼1.4 kW/cm^2 excitation intensity and with 10 consecutive frames averaged to get a better signal-to-noise ratio. The two spectra in part b are recorded with low (∼10 W/cm^2) excitation intensity in the presence of an oxygen scavenger (black) and with high intensity (∼1 kW/cm^2) without any precautions against oxygen taken (red). The inset in part a compares the peak-normalized B800 fluorescence bands, extracted from the spectrum of bulk LH2-only membranes at low illumination (black) with the spectra of single LH2-only membrane (red) and the single LLM membrane (blue) after prolonged illumination under strong light. The inset of part b shows the difference between the red and black spectra in Figure 9b, showing peaks at 804 nm and around 840 nm.

(i) There is no qualitative difference between the behavior of LH2 complexes under intense, prolonged illumination in LH2-only membranes and full IC membranes. This confirms that both the enhancement of the 804 nm emission and the blue-shift of the B850 band are properties of the LH2 complex only, with no role played by the LH1 complex in these processes.

(ii) The basis states for the enhanced 804 nm emission and the regularly very weak B800 fluorescence are identical. This notion follows from the perfect (within the experimental

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uncertainty) match between the B800 fluorescence at low-intensity excitation (shown by the black curve in the inset of Figure 9a) and the 804 nm emission spectra extracted from the spectra of full IC membranes and LH2-only membranes (represented by green curves in Figures 8 and 9a, respectively) upon prolonged illumination, and also from the steadiness of the position of the 804 nm emission spectrum under intense illumination (shown by blue dots in Figure 7).

(iii) Photoinduced enhancement of the B800 band and emergence of the B840 band goes in parallel with loss of the regular B850 fluorescence (see the inset of Figure 9b). This means that understanding the increase of the B800 fluorescence should go hand in hand with the comprehension of the mechanism(s) that transform the B850 band into the blue-shifted and broadened B840 band.

There are several options of such light-induced transformation. First, it may be due to the B850 rings that have lost a random number of H-bonds that stabilize BChl's in their protein binding pockets. It has been demonstrated[51] that the breakage of all the H-bonds coordinating 18 BChl molecules in the B850 ring of LH2 leads to a blue-shift of the B850 absorption band by 24 nm, i.e., to 826 nm. Taking into account that at ambient temperatures the Stokes shift between the B850 absorption and fluorescence spectra is 8−9 nm,[40] one might expect the newly formed exciton emission band to be found at 834−835 nm, the same region where the B840 band is located. Provided this is the case, the "H-bond breakage mechanism" would simply explain both the large width of the B840 band (due to the random number of broken H-bonds in multiple B850 rings) and the enhanced B800 emission (because of the reduced energy gap between the B800 and B840 exciton states and the equilibrium population of the former states).

There is an excellent innate model to examine the last point, which is the B800−820 LH complex from *Rhodopseudomonas acidophila*. This complex, also known as LH3, is developed when the bacterium is grown under stressed (e.g., insufficient light) conditions. Due to the decreased B800−B820 energy gap, the B800-related emission in LH3 is much stronger than that in the regular B800−850 LH2 complex. The massive B850 → B820 spectral shift is a consequence of restructuring the H-bonds that coordinate B850 BChl's.[52] This local bonding rearrangement, leaving the B800 system almost unaffected, changes not only transition energies of the B850 chromophores but also their exciton coupling.[53]

The H-bonds coordinating BChl's in LH complexes are weak, 4−6 k_BT,[44,54] meaning that they are relatively easy to break by external perturbation such as temperature. However, as we have already indicated, there are no clear signs of temperature rise under the present illumination conditions. Furthermore, high pressure spectroscopic studies of LH1 and LH2 complexes[44,55] have shown that the broken H-bonds have a tendency to recover whenever the physical perturbation causing the change is relaxed. The H-bond model thus falls short to explain the observed permanent spectral effects.

We, therefore, propose a complementary mechanism, related to photosensitized oxidation of BChl chromophores in the B850 assembly. Photo-oxidation of photosynthetic pigments is a well-known phenomenon, widely studied both in vitro[56] and in vivo[57] environments. Depending on the conditions, oxidation may tune the optical spectra of BChl chromophores either to the blue[56] or to the red,[58] whereas chemical[58,59] and electro-chemical[60] oxidation have been found to result in similar to photo-oxidation spectral effects. Antioxidants may partly reverse these oxidation phenomena.[61]

Very recently, it was observed[62,63] that LH complexes from phototrophic bacteria such as *Allochromatium minutissimum* contain 3-acetyl-chlorophyll (3-acetyl-Chl) as a major oxidation product of BChl. Photo-oxidation was only effective in the presence of oxygen (as well as of carotenoid) molecules. However, even with all efforts made to minimize oxidation of the BChl molecules, each LH2 complex contained approximately one molecule of 3-acetyl-Chl. The photo-oxidation in LH2 was observed to be accompanied by bleaching and blue-shifting of the B850 band, leaving the B800 band practically untouched. It was also established that the 3-acetyl-Chl molecules remain as a part of the LH complex, on the same place as BChl's before oxidation.

These same features are also apparent in the present work. Therefore, we primarily relate the B840 fluorescence band with excitons in the B850 ring where certain numbers of native BChl molecules are photo-oxidized. The reduced energy gap between the B800 and B840 exciton states then results in the enhanced B800 emission, just like explained above.

Photo-oxidation of BChl's also explains permanent bleaching of fluorescence observed already in the very first room-temperature studies of single LH complexes.[13,14] In refs 61 and 64, it was convincingly demonstrated that oxidation of only a very few BChl molecules in the LH1 ring is enough to completely quench its fluorescence. Although in our experiments the LH1 fluorescence decreases under prolonged illumination quite like the LH2 emission, its spectral position remains largely the same (see Figure 7). In this respect, the present observations confirm the findings in ref 65, where exceptional stability of LH1 complexes against photodamage was established. The different sensitivity of peripheral and core complex to deteriorating effects of oxygen is not quite understood. Only recently, it was recognized[66] that excited states of the carotenoids in the structure of LH2 are directly involved in photo-oxidation of the B850 BChl's. Removal of either carotenoids or oxygen results in a very stable B850 spectrum. It might thus be that the different photostability observed for LH2 and LH1 complexes is due to their different carotenoid content and/or conformation.

SUMMARY AND CONCLUSIONS

In the present work, fluorescence spectra of single photosynthetic IC membranes (chromatophores) from the WT bacterium *Rb. sphaeroides*, one of the model photosynthetic systems, and its LH complexes were studied. This fluorescence in principle reflects not only structural organization of photosynthetic membranes but also the complex interplay of various processes taking place in them. Since in single-particle investigations by necessity rather high excitation intensities are used compared with native solar illumination, one of the focuses of this study was the dependence of the spectra on excitation intensity, which varied over 4 orders of magnitude.

High sensitivity to excitation intensity was indeed revealed. At low to moderate (0.1−100 W/cm^2) excitation intensities, the spectra of excitons in full IC membranes can be well reproduced by a sum of the component mutant membrane spectra, the ratio of the spectra related to LH2 and RC−LH1−PufX complexes being the only adjustable parameter. This implies that the spectra of the components well reproduce those assembled into functional membranes. In experimental time frames of 1−50 s, we did not observe any noteworthy

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dynamical fluctuations of spectral shape, position, or intensity of the fluorescence for the LH2-only and RC−LH1−PufX-only mutant membrane samples. At the same time, steady and large intensity differences between the component antenna spectra, related to changed stoichiometric ratio of LH2 and RC−LH1−PufX complexes in individual samples, are common. In some chromatophores, primarily of LLM origin, it is concluded that parts of their peripheral antenna might be detached from efficient energy transfer pathways, confirming earlier deductions. At high excitation intensities (0.5−2 kW/cm^2), considerable permanent modifications of the LH2 emission yield and spectrum, which, however, occur without damaging the protein scaffold, are observed. All of these effects have been reasonably explained by photo-oxidation of a number of BChl's in the B850 compartment of LH2, possibly accompanied by breakage of H-bonds between the chromophores and the surrounding protein. Similar photoinduced damage to LH1 complexes was not established, confirming earlier findings.

AUTHOR INFORMATION

Corresponding Author

*Phone: +372 5645 3175. E-mail: arvi.freiberg@ut.ee.

Notes

The authors declare no competing financial interest.

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