Apoprotein heterogeneity increases spectral disorder and a step-wise modification of the B850 fluorescence peak position.

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Abbreviations.

Bchl, bacteriochlorophyll; B800, 800nm-absorbing Bchl; B850, 850nm-absorbing Bchl; LH, light-harvesting; FLP, fluorescence peak; fwhm, full width at half maximum

Highlights

- Apoprotein heterogeneity in LH2 increases fluorescence spectral disorder
- Fluorescence emission studies show the presence of multiple sub-classes in LH2
- Multiple LH2 ‘ring’ structures are present in low-light adapted membranes

Abstract.

It has already been established that the quaternary structure of the main light-harvesting complex (LH2) from the photosynthetic bacterium *Rhodopseudomonas palustris* is a nonameric ‘ring’ of PucAB heterodimers and under low-light culturing conditions an increased diversity of PucB synthesis occurs. In this work, single molecule fluorescence emission studies show that different classes of LH2 ‘rings’ are present in “low-light” adapted cells and that an unknown chaperon process creates multiple sub-types of ‘rings’ with more conformational sub-states and configurations. This increase in spectral disorder significantly augments the cross-section for photon absorption and subsequent energy flow to the reaction centre trap when photon availability is a limiting factor. This work highlights yet another variant used by phototrophs to gather energy for cellular development.

Introduction

Net primary carbon production on earth is essentially derived from the light reactions of photosynthesis, of which purple photosynthetic bacteria are noteworthy contributors. In these phototrophs, the primary goal of the light-harvesting (LH) proteins, LH1 and LH2, is to capture the solar photons and subsequently channel the resulting excitation energy to the reaction centres (RCs), where it is transformed into potential chemical energy [1]. The LH proteins maximise the efficiency of exciton energy transfer towards the RC within the photosynthetic unit by tuning the near-IR absorption properties of their non-covalently bound bacteriochlorophyll (Bchl) molecules; in LH2 this corresponds to approximately 900 cm^{-1}, *i.e.* between *ca*. 790 and *ca*. 850 nm.

The LH2 proteins display a variety of annular structures but are all based on the same basic minimal structural unit: a pair of membrane-spanning apoproteins, termed α and β, binding three Bchls and one carotenoid (Car) molecule. The α and β apoproteins can also be named after their *pucAB* tandem gene pair and are thus called PucA and PucB, respectively. Although there are exceptions, the LH2 structure is nonameric. Nine Bchl molecules are located in the space between PucAB, and form a weakly coupled ring that is responsible for the absorption at 800 nm (B800). A second ring of 18 strongly coupled Bchl molecules are responsible for the absorption at 850 nm (B850). Excitation energy resulting from photon absorption by the Car molecules present in LH2, or by Bchls from the B800 ring, is rapidly transferred to the B850 ring from which it is either emitted [2, 3] or, *in vivo*, within a few ps transferred to an adjacent ring (LH2 or LH1) and finally to the reaction centre [4-6]. The electronic properties of the B850 'ring' are governed by the electronic coupling between its constitutive pigments and interactions between each Bchl and the surrounding PucAB apoproteins [6-11].

The natural variants of the B850 'ring' in LH2 tend to have their lowest energy absorption peaking at *ca.* 850 nm or, due to the expression of alternative PucAB peptides, it can be blue-shifted to *ca*. 830 nm (*e.g*. *Rbl. acidophilus* [12]). These blue-shifted LH2 variants are sometimes termed LH3, or B800-830. The molecular origins of this 20nm absorption shift is due to altered pigment site energies *via* the replacement of residues Trp_{44} and Tyr_{45} that H-bond to the Bchl-B850 molecules with non H-bonding side chains [7, 13]. Moreover, there are a number of species, which include *Roseobacter denitrificans* [14, 15] and *Rhodopesudomas (Rps.) palustris* [16-18], where the B850 'ring' is so blue-shifted such that the room-

temperature absorption spectrum may appear to lack a significant “850 nm” contribution, and consequently they exhibit a (broadened) 800nm-absorption band.

Applying a strict exciton model to the LH2 ring as observed in crystallography would result in a spectrum with the lowest energy transition (almost) dark and all the oscillator strength accumulated in the two next higher transitions [19]. However, it is well known that the LH2 major transitions are inhomogeneously broadened [20], beautifully demonstrated by the first single molecule fluorescence experiments on LH2 [21-24] due to energetic disorder [3]. As a consequence of this anisotropic structure of LH2 the complex provides a flexible environment for the B850 Bchls (*viz.* competition between energetic disorder and excitonic coupling) due to which the lowest transition of the LH2 ring is no longer forbidden and even superradiant [2, 25, 26]. Furthermore, since the Bchl-protein and Bchl-Bchl interactions are time-dependent the Bchl site energies and Bchl-Bchl excitonic couplings fluctuate with time which in turn will modulate the fluorescence emission wavelength. Hence the spectral dynamics between individual LH2 proteins can be directly related to intrinsic differences between individual B850 ‘rings’. The dynamic disorder of LH2 has largely been documented by studying the fluorescence dynamics of individual proteins at ambient temperature transition [21, 27-33]. These studies instinctively used antennae where presumably only one type of PucAB is expressed and thus able to compare experimental data with exciton models [30, 34-36]. From these works, the minimal exciton model capable to explain the LH2 spectral dynamics includes one coordinate with two conformational states, shifting the site energies by 190 cm^{-1} (small blue or red jumps mostly within 10 nm), and a second coordinate with two more conformational states, creating bigger blue/red shifts up to 440 cm^{-1} (the so-called four-state model [35]).

The question now arises, What happens to the fluorescence dynamics of individual proteins when the bulk LH2 sample is known to contain multiple types of Puc apoproteins? Perhaps the best studied system where natural expression of multiple types of LH2 PucAB occurs is found in the metabolically versatile photosynthetic bacterium *Rps. palustris*, whose genome has been sequenced [37], and apoprotein expression carefully linked to the steady-state near-IR bulk absorption [38] as well as a battery of other spectroscopic and structural studies [17, 38-44]. From these works it was concluded that when the culture conditions change from a high-light (HL) regime to a low-light (LL) one it induces assembly of LH2 with a heterogeneous composition of Puc apoproteins [40-42]. Furthermore, in *Rps. palustris* membranes that are low-light adapted the backward energy transfer process is reduced

relative to high-light ones, suggesting that directionality to the reaction centre is actively controlled by the bacteria [44].

In this present work, a series of single molecule fluorescence emission measurements were conducted on *Rps. palustris* LH2 proteins isolated from HL, IL (intermediate-light) and LL growth regimes. Hence, we directly probe the role played by increased PucAB heterogeneity on the spectral properties of LH2 fluorescence and by inference on the quaternary structure.

Materials and Methods.

Protein purification.

Rps. palustris, strain 2.6.1, was grown photoheterotrophically in Böse medium [45] at 28 ± 2 °C in glass bottles located between banks of incandescent lamps at three light intensities termed high-light (HL), intermediate-light (IL) and low-light (LL) which corresponded to irradiation levels of 10 Wm^{-2}, 0.02 Wm^{-2} and 0.01Wm^{-2}, respectively. Cultures were regularly transferred to ensure a constant low optical density as previously described [40]. This minimized self-shading caused by the cells themselves which would precipitate an uncontrolled "low-light" regime. This ensured that the final inoculums and harvested cells contained HL, IL or LL adapted intracytoplasmic membranes [46]. Cells were harvested and membranes prepared essentially by the method described by [17]. The LH2 pigment-protein complexes were isolated and purified in the presence of the zwitteronic detergent *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO) (Fluka) as described previously [17, 46, 47]. The purified LH2 complexes from HL, IL and LL adapted membranes were termed $LH2_{HL}$, LH_{IL} and $LH2_{LL}$, respectively. The purified antennae were stored in 100 mM NaCl, 0.05% (*w/v*) LDAO, 20 mM Tris.HCl, pH8.5. The same (deoxygenated) buffer was used for the fluorescence measurements.

Spectroscopy.

Spectrometer. Fluorescence images and spectra were acquired with an inverted confocal microscope (Nikon, Eclipse TE300). The excitation source was a constant power and random polarization He-Ne laser (Melles Griot, 05SYR810-230). The excitation wavelength of 594 nm permits direct excitation of the Q_x transition of all the bacteriochlorophyll molecules in LH2. A dichroic beam splitter (Chroma Technology Corp., 605dcxt) reflects the laser beam

into the objective lens (Nikon, Plan Fluor 100x, 1.3 NA, oil immersion), focusing the excitation light onto the glass-water interface in the sample cell to a diffraction-limited spot (fwhm of ~ 600 nm). The intensities used in these experiments represent the values at this interface. The emission is focused through a 100 μm pinhole and filtered using the long-pass glass filter RG715 (Edmund Optics (York, UK), 46065). The sample cell is mounted on a closed loop two-dimensional piezo stage (Physik Instrumente, P-731.8C) controlled by a digital four-channel controller (Physik Instrumente, E-710.4LC). To obtain images, emission is detected with a Si avalanche photodiode (APD) single photon-counting module (SPCMAQR- 16, Perkin-Elmer) and counter timer board (National Instruments, PCI-6602). Spectra are acquired by dispersing the fluorescence onto a liquid nitrogen-cooled back-illuminated CCD camera (Princeton Instruments, Roper Scientific, Spec10: 100BR). Pixel binning yields a resolution of < 0.8 nm.

Images and Spectra. A FL image is acquired by continuously sweeping the piezo stage over the laser focus with a frequency of 3 Hz while its position in the perpendicular direction is changed by 100 nm for each line; the FL signal is concomitantly detected with an APD. Images are then constructed by associating the piezo stage coordinate with the corresponding intensity. The scanning covers a 10 μm x 10 μm area. After the coordinates of bright particles are determined, the piezo stage is positioned to bring the particle into the focus of the objective, and after the mode is switched to a spectroscopic one, a series of FL spectra are collected for 30 s, or longer, with an integration time of 1 s per spectrum. Efforts were made to ensure that no sample degradation occurred during each experiment, *viz* that no temporal evolution of the distribution of the fluorescence peak position. (see supplementary figure S1).

Data analysis.

Each measured FL emission spectrum in the time series was fitted with a skewed Gaussian function as previously described by applying a least squares fitting procedure [27, 48-50] that closely reproduces the bulk spectrum (see supplementary figure S2) The expression for the skewed Gaussian function is

$$F(\lambda) = \Delta + A \exp\{-\ln(2)/b^2 \ln[1 + 2b(\lambda - \lambda_m)/ \Delta\lambda) \}^2$$

where Δ is the offset, A the amplitude, λ_m the fluorescence peak (FLP) wavelength, $\Delta\lambda$ the width, and b the skewness. The fwhm of the spectrum is calculated from the width and the skewness. Consequently, by fitting each spectrum from a series, we obtain the time traces of

the amplitude, the fwhm, and the FLP with the corresponding confidence margins. In some cases two skewed Gaussian functions were required to fit the temporally active FL spectra as described previously [50].

Results and Discussion

Shown in Figure 1 are the room-temperature absorption spectra for the LH2 antennae isolated from cells adapted to high-light (HL), intermediate-light (IL) and low-light (LL) culture regimes hereon termed $LH2_{HL}$, $LH2_{IL}$ and $LH2_{LL}$, respectively. The absorption properties of the LH2 antennae are similar in the $Bchl_{(Soret)}$-Car-Bchl Q_x (*ca.* 320 – 625 nm) range. The most striking differences between the $LH2_{HL}$ and $LH2_{LL}$ antennae are the position and shape of the Q_y transitions of the Bchl-B800 and Bchl-B850 bacteriochlorophyll molecules. As we go from HL to LL the Bchl-B850 molecules show a blue-shift in the Bchl-Q_y peak position which is concomitant with an apparent increase of the half width half maximum (hwhm, on the low energy side) of the absorption band: 857 nm (174 cm^{-1}), 852 (243 cm^{-1}) and 850 nm (257 cm^{-1}), respectively. The increase in inhomogeneous broadening, as well as the lack of an isobestic point in the absorption spectra of the bulk sample, and under the growth regimes presented in this work, has been ascribed to the presence of multiple Qy-Bchl-B850 transitions in $LH2_{LL}$ [17, 38-40] and is a direct result of increased expression of PucAB peptides other than $PucAB_{ab}$ [38]. The $LH2_{HL}$ and $LH2_{LL}$ bulk absorption spectra shown in Fig. 1 are fully consistent with those reported previously by Brotosudarmo and co-workers [38] who also derived an averaged X-ray crystal structure of the nonameric $LH2_{LL}$ to a resolution of 5.6 Å. The expression of multiple PucAB peptides also explains the complex resonance Raman spectra observed in bulk $LH2_{LL}$ [38, 40].

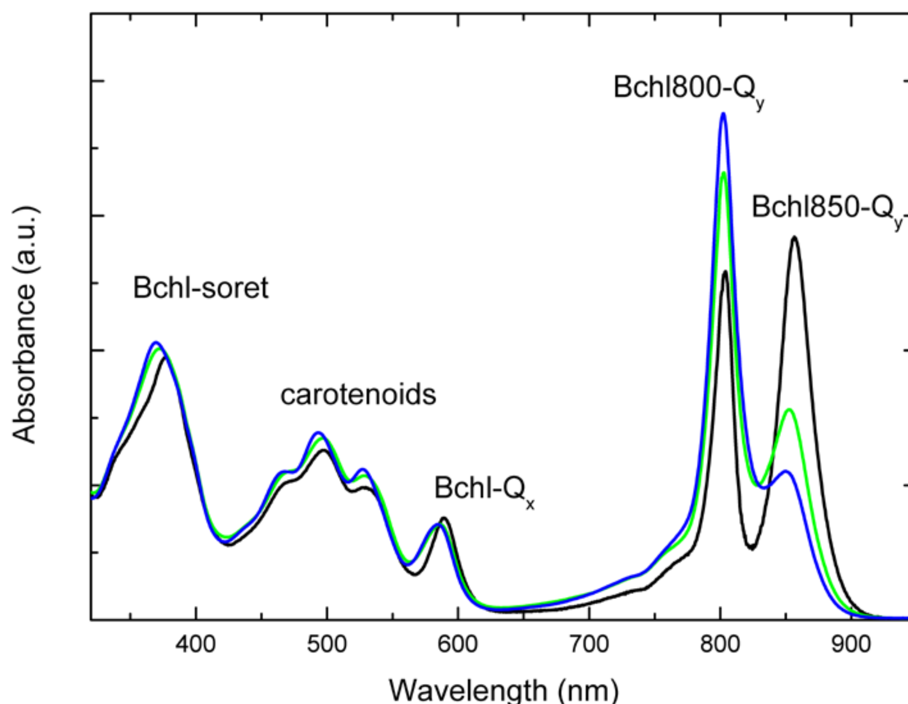

Figure 1. Vis-NIR absorption spectrum of the HL (black trace), IL (green trace) and LL (blue trace) LH2 complexes isolated from *Rps. palustris* 2.6.1. The main electronic transitions of the Bchl molecules are indicated. The absorption in the *ca*. 410-550 nm region is attributed to the carotenoid molecules.

In this present work, a series of single molecule fluorescence emission measurements were conducted on the above LH2 samples after optimising the incident power level to minimalize spectral jumping in $LH2_{HL}$, which is often attributed to localised heating [28, 51]. Hence, we directly probe the role played by increased PucAB heterogeneity on the spectral properties of LH2 fluorescence, as we migrate from $LH2_{HL}$ to $LH2_{LL}$ 'rings'. Our results present no evidence of sample degradation or (partial) denaturation of the quaternary structures of $LH2_{LL}$, as this would result in increased fluorescence emission at ca 760-780 nm as well as a general blue-shift in the FLP position during the course of the measurements (see supplementary figure S1).

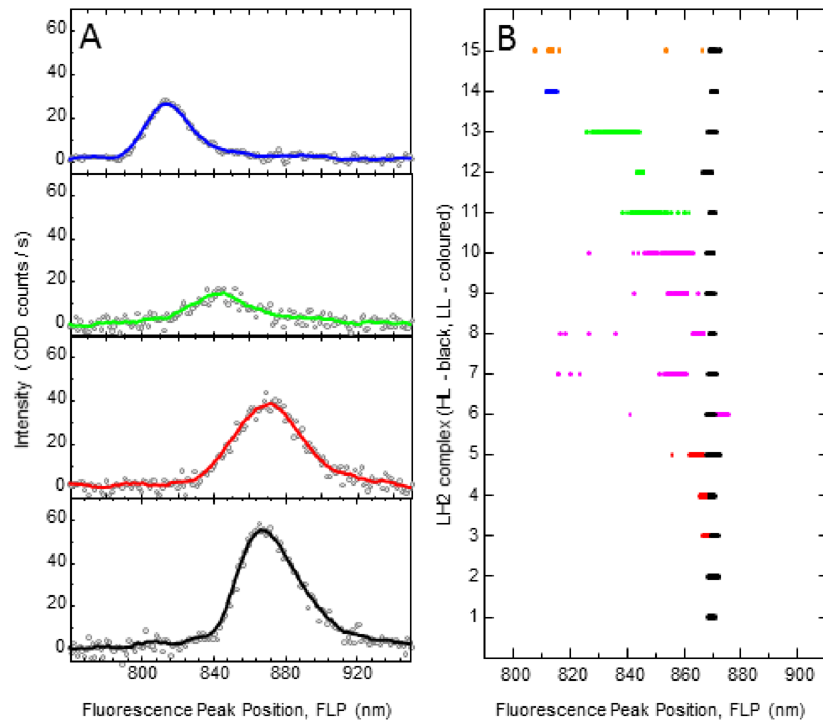

Figure 2. Variation of the fluorescence emission spectra of LH2. (A) Averaged fluorescence spectra from three $LH2_{LL}$ (blue, green and red traces) complexes compared with a typical $LH2_{HL}$ (black trace) protein. (B). Variance of the fluorescence peak position in 15 $LH2_{LL}$ (coloured dots) and 15 $LH2_{HL}$ (black dots) proteins.

Displayed in Figure 2A are fluorescence spectra obtained from three different single $LH2_{LL}$ molecules. A representative $LH2_{HL}$ spectrum is also plotted for comparison. In each case the spectrum is an average of 120 individual spectra (which corresponds to 2 min of data collection per complex). Although none of these LH2 molecules undergo major spectral jumps (not shown) it is evident that the FLP positions and fwhm values are different. However, many of the $LH2_{LL}$ complexes do exhibit spectral jumping. Shown in Figure 2B are the FLP positions of 15 $LH2_{LL}$ molecules sorted as a function of decreasing averaged FLP position (coloured dots) and superimposed on them are an equal number of complexes from the $LH2_{HL}$ data set (black dots). In general, the $LH2_{HL}$ complexes occupy a tight FLP domain situated at 870 nm ± 3 nm. It is evident that multiple classes of $LH2_{LL}$ are present: some molecules have a range of FLPs localised at *ca.* 860-880 nm (red dots), those that have FLP positions in the same region but also exhibit significant spectral jumping to bluer wavelengths (*ca.* 820 nm, magenta dots), yet other $LH2_{LL}$ proteins have FLP positions situated only in the 830-850 nm range and are usually associated with a broad fluorescence band (green bars).

There is a subset of $LH2_{LL}$ molecules that fluoresce only at *ca.* 816 nm (blue bars) while some complexes have fluorescence peak positions that cover the entire spectral range (orange bars).

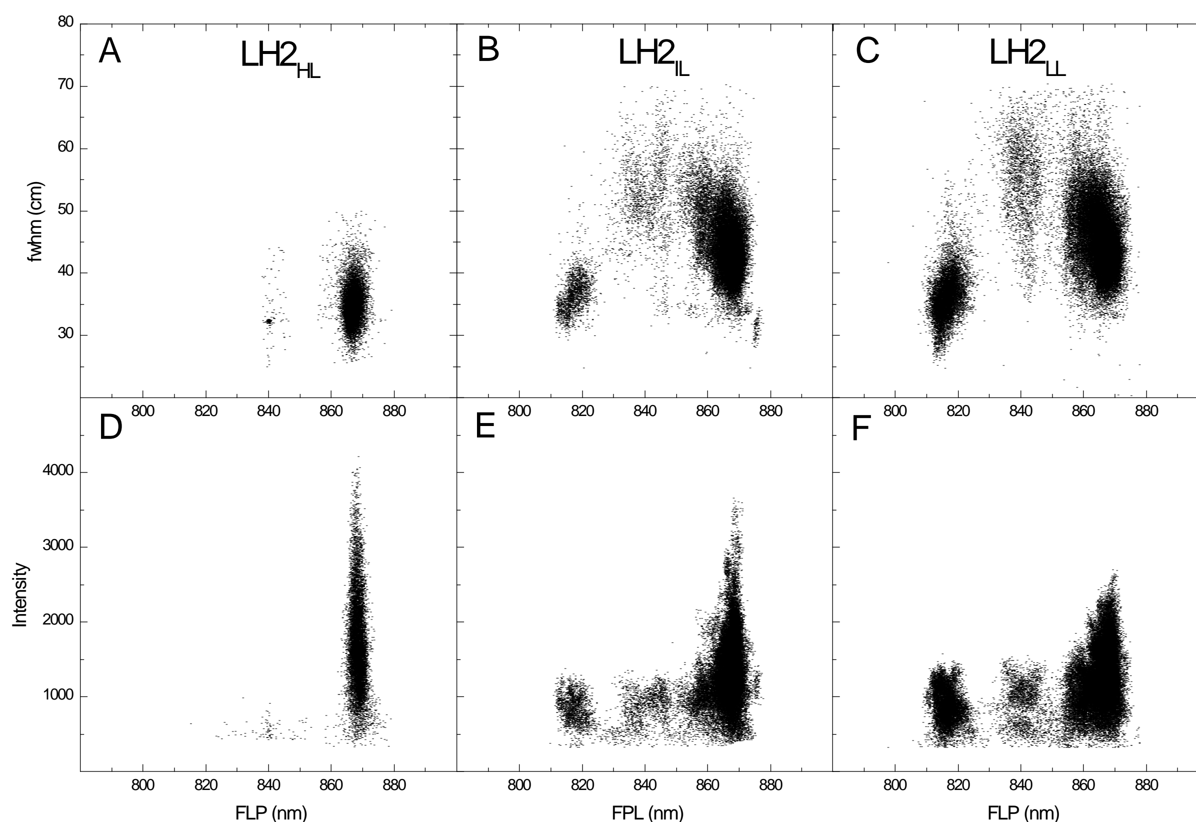

Figure 3. Distribution of the FLP position for each analysed fluorescence spectrum of the $LH2_{HL}$ (left column), $LH2_{IL}$ (middle column) and $LH2_{LL}$ (right column) data-sets plotted against FLP peak fwhm (A, B, C) and intensity (D, E, F).

The FLP positions, intensity and fwhm for the $LH2_{HL}$, $LH2_{IL}$ and $LH2_{LL}$ proteins are plotted in Figure 3. As in figure 2B, where a selection of LH2 molecules were randomly chosen, the full $LH2_{LL}$ data set exhibits a large distribution of FLP positions (Figure 3C) ranging from 811 to 880 nm, and three clusters can be clearly distinguished, centred at *ca*. 816, 840 and 865 nm. In contrast, the HL sample has a single FLP cluster situated at *ca*. 870 nm with an averaged fwhm of *ca.* 37 nm (Figure 3A). The spread of $LH2_{HL}$ data points in the sub 860 nm region is primarily due to small spectral jumps (less than 10nm) from the most frequent FLP value and is consistent with premise that localised heating has not induced spectral jumping in this sample [28]. Hence, as we migrate from HL to LL we directly probe the influence of mixed apoprotein composition in our fluorescence measurements. Very occasionally in the HL sample (less than 1%), larger spectral jumps peaking at *ca*. 840 nm occur. Compared to

the $LH2_{HL}$ proteins, the fwhm of the FLPs in the three $LH2_{LL}$ sub-classes are broader. Furthermore, going from the red-most FLPs there is a general increase in the observed fwhm value except for the cluster situated at *ca*. 816 nm. Only this sub-class has fwhm values of *ca*. 35 nm and is somewhat similar to that of the HL sample which, in the vast majority of cases, does not undergo significant spectral jumping. Comparing the $LH2_{IL}$ sample (Figure3 B and E) with the others it is evident that it is intermediate between the high-light and low-light data sets.

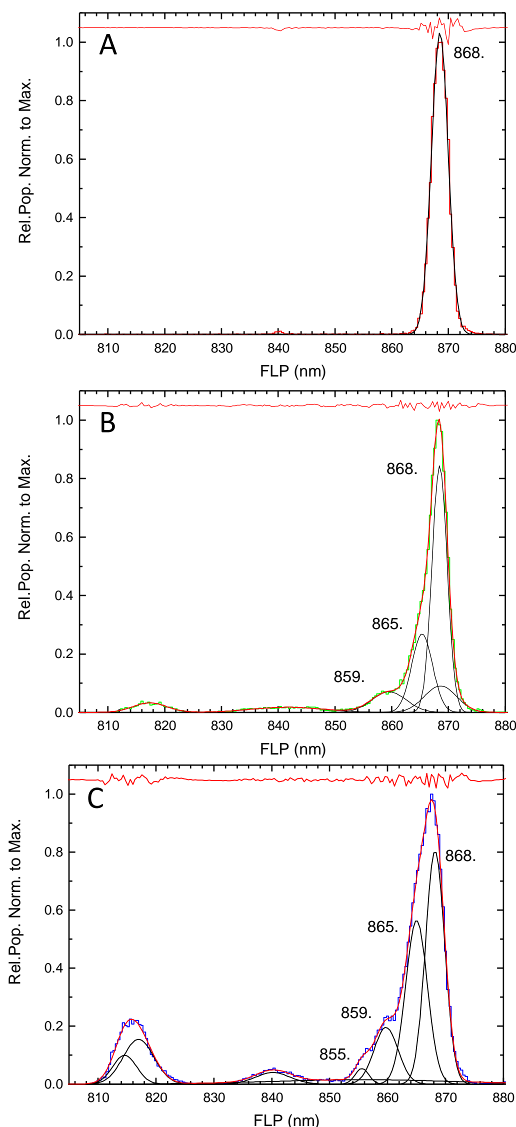

Figure 4. Histogram of the relative population of FLP position as a function of light-regime: (A) high-light (black), (B) intermediate-light (green), and (C) low-light (blue) regimes. The relative FLP abundances are normalised to the most frequent value. The HL, IL and LL data sets were fitted with Gaussian distributions (black, green and blue traces, respectively) and overlaid by the combined fit (red traces); residuals are offset for clarity.

In order to further investigate the presence of different fluorescence clusters the data were plotted as a function of relative FLP abundance and these results are represented in figure 4. The $LH2_{HL}$, which contains $PucAB_a$ and $PucAB_b$ heterodimers [38], has only one major tight cluster which was fitted by a simple Gaussian distribution, giving a fwhm of 3 nm centred at 868.5 nm (Fig. 4A). Based on the same Gaussian analysis, $LH2_{LL}$ (Fig. 4C), which is known to also contains $PucB_d$ apoproteins [38], we obtain four distinct sub-clusters centred at 868.2, 865.0, 859.7, and 855.6 nm with similar widths of 3.3, 3.8, 4.2 and 2.3 nm, respectively. Interestingly, these appear to be increments of about 5nm, half the value associated with a single H-bond breakage between a Bchl-B850 molecule and its proteotic bath - if spread over the entire B850 'ring' [3, 7, 13, 52]. The central cluster of fluorescing LH2 complexes located at 840 nm has no apparent multifaceted structure and may be represented by a single Gaussian distribution with a width of 5.9 nm. In contrast the blue-most cluster can be represented by two Gaussian populations located at ca. 816 nm (fwhm= 5.3 nm) and 814.6 nm (fwhm = 4.1 nm). Applying the initial Gaussian distributions obtained for the $LH2_{HL}$ and $LH2_{LL}$ samples, $LH2_{IL}$ also exhibits a similar 5 nm step in FLP peak position, albeit with less overall structure and appears to lack the small cluster that is centred at 855 nm (Fig.4B).

An alternative approach is to plot the experimental data only using the maximum red-shifted FLP position (FLP_{max}) obtained for each individual LH2 complex. Again, multiple $LH2_{LL}$ sub-populations are present in the 870 nm region (fig 5). At least 3 'ring types' are present as the FLP_{max} is again separated by increments of *ca.* 5 nm (870.6, 866.7 and 862.5 nm) which indicates that these sub-populations have slightly different tertiary/quaternary structures. The 840 nm cluster, now centred at 846.8 nm, is broad (11.3 nm) and the *ca.* 816 nm cluster can now only be represented by a single broad Gaussian with a fwhm of 6.1 nm. It is evident from Fig 5 that there are some $LH2_{LL}$ 'rings' that only fluoresce in the 820 nm spectral region. Neither this present work nor other publications [17, 39-41] on similarly grown LL cultures obtained an 800 nm-only LH2 fraction after biochemical purification. Nonetheless, it is evident from this work that there is a sub-population of $LH2_{LL}$ where the FLP maxima are unable to jump more than a few nanometres to the blue (at most *ca.* 10 nm) from *ca.* 816 nm. This implies that these '816 nm-rings' contain enough $pucB_d$ polypeptides to disallow any formation of a 'red' B850 exciton manifold, *via* the well documented H-bonding network of Bchl molecules in LH2 [7, 13, 52, 53]. There is no reason to assume that the $LH2_{LL}$ '816 nm-rings' are artefacts as it is well known that is effectively impossible to separate LH2 (or B800-

850) from LH3 (or B800-830) from the same species (*e.g. Rhodoblastus acidophilus*) as their physico-chemical properties (size, shape, surface charge, etc,..) are fundamentally identical – the differences are located deep within the interior of the proteins. Indeed, if mixed antennae are observed in the *in vivo* membrane the usual protocol is to reinitiate the culture in order to obtain a spectroscopically pure antenna spectrum of distinct proteins (either LH2 or LH3). In general, if another LH is present as a minor component in the *in vivo* membrane it will be co-purified with the protein of interest. Moreover, as no attempt was made here to preferentially purify any individual LH2 sup-population the biochemical preparations will closely represent the antennae content present in the native membranes. Very recently, a deletion mutant of *Rps. palustris* has been constructed that only contains the $pucA_d$ and $pucB_d$ genes [54, 55] and it expresses a $LH2_{LL}$ complex where the B850 band is blue-shifted by *ca.* 40 nm, resulting in a 'single' absorption peak at about 810 nm. Thus the '816 nm-rings' observed here are considered to be antennae complexes identical to, or very similar to, those reported by Ferretti and co-workers [54, 55].

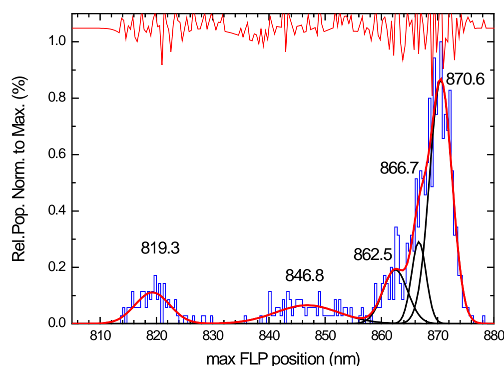

Figure 5. The relationship between the distribution of the relative abundance of FLP_{max} and maximum spectral jumping in $LH2_{LL}$. The relative abundances of the FLP_{max} position. The data set (blue trace) is fitted with Gaussian distributions (black traces) and overlaid by the combined fit (red trace) with its residual, which is offset for clarity.

As observed in $LH2_{HL}$, there are many $LH2_{LL}$ complexes that fluoresce only in the 870 nm spectral region, undergoing only small spectral jumps (less than 10 nm). This would imply that a single conformational coordinate is active in these proteins, and is responsible for relatively small spectral shifts of individual Bchl pigments (*i.e.* no more than 300 cm^{-1}) [31, 34-36]. Since these 'rings' are able to form the strongest H-bonds to the Bchl-B850 molecules

then statistically they will only contain $PucAB_a$ and $PucAB_b$ (*i.e.* no $pucB_d$ or may have one or only very few $pucB_d$ 'blue'-subunits in the ring that will hardly affect the FLP position but may broaden its distribution), as their bulk absorption spectra closely resemble those in reference [41] as well as the fluorescence properties of the $LH2_{HL}$ sample. Hence, these high-light-like $PucAB_{ab}$ 'rings' represent a localized energy minimum in the bulk LL membrane, where relatively small spectral shifts of individual Bchl pigments occur. Since the overall exciton energy transfer goes from high- to low-energy one could imagine that in the *in vivo* membrane these 'rings' would be situated closer to the LH1 complexes than the other LH2 'rings' in the various published models of the bacterial photosynthetic unit (*e.g.* [56-58]), but there is no direct evidence for this.

We must conclude that even in low-light cultures, if sufficient PucA and $PucB_{ab}$ peptides are present then the cellular machinery will preferentially assemble high-light 'rings', indicating that the unknown assembly process of LH2 is non-random. One could also imagine that some of these HL 'rings' could statically contain dimers with different ratios of $PucB_a$ and $PucB_b$ [59], resulting in a gradual spectral shift in the fluorescence properties which is not observed as no sub-populations were observed in $LH2_{HL}$. This could simply be due to the fact that at physiological temperature the inhomogeneous broadening is larger than the variance of the individual Bchl site-energies in the different 'rings'. However, 'rings' containing dimers with different $PucB_a/PucB_b$ ratios, with the addition of $PucB_d$ peptides (targeted by chaperons and an unknown assembly process), could manifest itself in step-wise increments in the FLP position, assuming that the exciton manifold is still maintained over multiple PucAB dimers [52]. The shift of the site energies of at least 1/4 -1/3 of the BChl in the ring *via* the insertion of multiple $PucB_d$ peptides are necessary to influence the FLP position due to the interplay of two main factors: (1) lack of exciton couplings between the red Bchls which will move the FLP to the blue, and (2) more localized red exciton states which are characterized by bigger reorganization shifts, and therefore tend to move the FLP to the red. The result will depend on the number of shifted sites and the ratio of the three parameters: exciton coupling, disorder, and amplitude of the blue shift. This, in part, would be determined by the quaternary structure of each individual 'ring'. Clearly, if the cellular machinery employs an unknown chaperon-directed assembly process to create each quaternary structure, based on the relative quantity of available 'blue' subunits, then one would observe the same step-wise variation in FLP position in the $LH2_{IL}$ and $LH2_{LL}$ samples, but the ratio of the different sub-populations would be different – which is exactly what happens. The notion that LH2 dimers may contain mixed

PucAB combinations has been established by chemical crosslinking experiments [15]. Extending this logic further, the inclusion of additional $PucB_d$ in the 'rings' would perturb the overall B850 exciton manifold and result in complexes that are more spectrally dynamic (*e.g.* Figures 2B and 5). When the vast majority, if not all, of the PucBs present in the LH2 complex are non-hydrogen-bonding $PucB_d$ peptides it would result in the in the assembly of spectroscopically pure '816 nm-rings' (Figure 2B, blue dots) that are only able to undergo small (no more than *ca.* 10 nm) spectral shifts. The 816 nm-rings would thus represent another local minimum in the energy landscape of the photosynthetic unit in *Rps. palustris*.

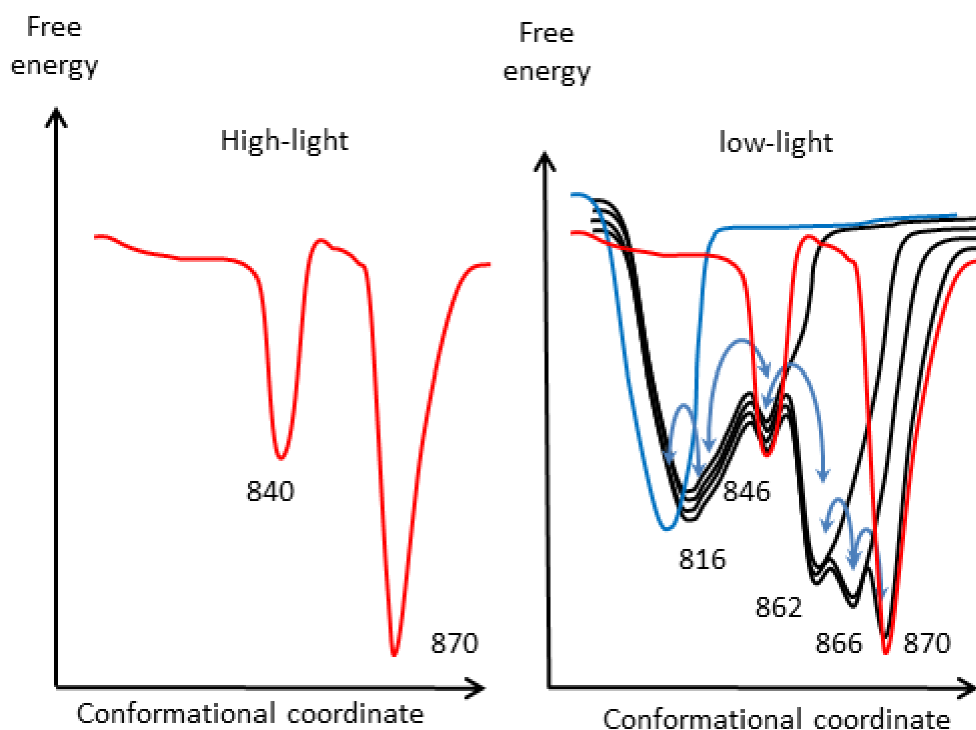

Figure 6. Schematic representation of the observed LH2 conformational changes in the protein energy landscape of the photosynthetic membrane of *Rps. palustris* under high-light (left) and low-light (right) culturing conditions. Left: Energy landscape associated with HL 'rings' that exhibit one conformational state at 870 nm (red trace), where occasional jumps occur to 840 nm. A large barrier height between these local minima points to infrequent transitions between these states. Right: under low-light culturing conditions additional ring types (black traces) are assembled, including the 816 nm-rings (blue trace) that are unable to jump to lower energy positions (blue arrows).

It is possible to construct an energy landscape diagram of the different LH2 proteins present in the photosynthetic unit (PSU) of the bacterium *Rps. palustris* under different culturing conditions – see figure 6. In $LH2_{HL}$, the energy landscape resembles LH2 proteins from other species that express one type of PucAB heterodimer [27, 28, 30, 31]. However, in $LH2_{LL}$ at

least 4 ‘ring’ sub-populations are present, as evidenced by the energy wells and the paths linking them (blue arrows). The precise significance of the presence of the 816 nm-rings is unclear but they must have a beneficial role under stressed conditions, otherwise evolution would have eliminated them. What is clear is that the addition of additional PucB apoprotein types in the B850 ‘rings’ is a very effective procedure to augment the available cross-section available for light-harvesting in this bacterium.

Conclusions

In agreement with previous studies on the fluorescence properties of individual LH2 complexes from high-light adapted cells which exhibit typical B800-850 absorption properties there is a single fluorescence cluster at *ca*. 870 nm [27, 28, 30, 31, 33]. However, the LH2 complexes isolated from the bacterium *Rps. palustris* grown under light-stressed conditions, which are known to express multiple types of PucAB apo-proteins [17, 39, 41], possess a more complex distribution of fluorescence properties. Indeed, as we pass from high-light to intermediate-light conditions the cluster at *ca*. 870 nm is actually composed of additional sub-populations with other clusters at *ca.* 840 and 816 nm. These sub-populations are more apparent as the light intensity is further reduced (*cf.* low-light). This trend of increasing heterogeneity is a result of LH2 ‘ring-type’ containing PucAB dimers containing different $PucB_a/PucB_b$ ratios, with the addition of $PucB_d$ peptide. Indeed the later cluster at *ca*. 816 nm is reminiscent of the B800-only LH2 proteins previously considered to lack the H-bonding network in the B850 binding-pocket including the $PucAB_d$ mutant from *Rps. palustris* [54, 55].

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