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

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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 nonaromatic ‘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.

1. 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 excitation 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 nonaromatic. Nine Bchl molecules are located in the space between PucA and PucB, and form a weakly coupled ring that is responsible for the absorption at 800 nm (B800). A second ring of 18 strongly coupled Bchls 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 20 nm absorption shift is due to altered pigment site energies via the replacement of residues Trp44 and Tyr45 that H-bond to the Bchl–B850 ring [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) 800 nm-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 caredfully 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.

2. Materials and methods

2.1. Protein purification

Rps. palustris, strain 2.6.1, was grown phototrophically 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 0.01 Wm⁻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 zwitterionic 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 LH2HL, LHIL and LH2LL, respectively. The purified antennae were stored in 100 mM NaCl, 0.05% (w/v) LDAO, 20 mM Tris.HCl, pH 8.5. The same (deoxygenated) buffer was used for the fluorescence measurements.

2.2. Spectroscopy

2.2.1. Spectrometer

Fluorescence images and spectra were acquired using 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 Q₀ 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 100 ×, 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), 46,065). 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 directed 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.

2.2.2. 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 × 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 Fig. S1).

2.2.3. 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 Fig. S2) The expression for the skewed Gaussian function is

\[ F(\lambda) = \Delta + A \exp\left\{ -\ln(2)/b^2 \ln[1 + 2b(\lambda - \lambda_{\text{m}})/\Delta\lambda]\right\} \]

where \( \Delta \) is the offset, \( A \) the amplitude, \( \lambda_{\text{m}} \) 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].
3. Results and discussion

Shown in Fig. 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 LH2HL, LH2IL and LH2LL, respectively. The absorption properties of the LH2 antennae are similar in the Bchl\textsubscript{800} and Bchl\textsubscript{850} bacteriochlorophyll molecules. As we go from HL to LL the Bchl\textsubscript{850} molecules show a blue-shift in the Bchl-Q\textsubscript{y} band (green bars). There is a subset of LH2LL 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).

The FLP positions, intensity and fwhm for the LH2HL, LH2IL and LH2LL proteins are plotted in Fig. 3. As in Fig. 2B, where a selection of LH2 molecules were randomly chosen, the full LH2 LL data set exhibits a large distribution of FLP positions (Fig. 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 (Fig. 3A). The spread of LH2HL data points in the sub 860 nm region is primarily due to the presence of some small spectral jumps (< 10 nm) 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 (< 1%), larger spectral jumps peaking at ca. 840 nm occur. Compared to the LH2HL proteins, the fwhm of the FLPs in the three LH2LL 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 LH2LL sample (Fig. 3B) and with the others it is evident that it is intermediate between the high-light and low-light data sets.

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 Fig. 4. The LH2HL, which contains Puc\textsubscript{ABa} and Puc\textsubscript{ABb} 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, LH2IL (Fig. 4C), which is known to also contains Puc\textsubscript{Bd} approtein [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 5 nm, 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 centred at ca. 816 nm (fwhm = 5.3 nm) and 814.6 nm (fwhm = 4.1 nm). Applying the initial Gaussian distributions obtained for the LH2HL and LH2IL samples, LH2IL 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\textsubscript{max}) obtained for each individual LH2 complex. Again, multiple LH2\textsubscript{LL} sub-populations are present in the 870 nm region (Fig. 5). At least 3 ‘ring types’ are present as the FLP\textsubscript{max} is again separated by increments of ca. 5 nm (870.6,
Fig. 2. Variation of the fluorescence emission spectra of LH2. (A) Averaged fluorescence spectra from three LH2LL (blue, green and red traces) complexes compared with a typical LH2HL (black trace) protein. (B) Variance of the fluorescence peak position in 15 LH2LL (coloured dots) and 15 LH2HL (black dots) proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Distribution of the FLP position for each analyzed fluorescence spectrum of the LH2HL (left column), LH2IL (middle column) and LH2LL (right column) data-sets plotted against FLP peak fwhm (A, B, C) and intensity (D, E, F).
as it is well known that it contained an 800 nm-only LH2 fraction after biochemical purification. Nonetheless, it is evident from this work that there is a sub-population now centred at 846.8 nm, is broad (11.3 nm) and the ca. 816 nm cluster slightly diﬀers from the blue (at most ca. 10 nm) from ca. 816 nm. This implies that these ‘816 nm-rings’ contain enough pucBd 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 LH2L ‘816 nm-rings’ are artefacts as it is well known that is eﬀectively impossible to separate LH2 (or B800–B850) from LH3 (or B800–830) from the same species (e.g. *Rhodobacter 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 reinstitute the culture in order to obtain a spectrosopically 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 sub-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 pucAa and pucBa genes [54,55] and it expresses a LH2L 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].

As observed in LH2L, there are many LH2L complexes that ﬂuoresce only in the 870 nm spectral region, undergoing only small spectral jumps (< 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 > 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 PucBa and PucBb (i.e. no pucBd or may have one or only very few pucBd ‘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 LH2L sample. Hence, these high-light-like PucABba ‘rings’ represent a localised energy minimum in the bulk LL membrane, where relatively small spectral shifts of individual Bchl pigments occur. Since the overall excitation 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 LH ‘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 PucBbp 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 localised 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\(_{hl}\) 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. Figs. 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’ (Fig. 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 \(Rps.\ palustris\).

It is possible to construct an energy landscape diagram of the different LH2 proteins present in the photosynthetic unit (PSU) of the bacterium \(Rps.\ palustris\), found, in the online version. The Transparency document associated with this article can be found, in the online version.

### 4. 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]. However, the LH2 complexes isolated from the bacterium \(Rps. palustris\) grown under light-stressed conditions, which are known to express multiple types of PucAB apoproteins [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\(_A\) mutant from \(Rps. palustris\) [54,55].


