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The peripheral light-harvesting complexes from purple sulfur bacteria have different 'ring' sizes

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Abstract The integral membrane light-harvesting (LH) proteins from purple photosynthetic bacteria form circular oligomers of an elementary unit that is composed of two very hydrophobic polypeptides, termed α and β. These apoprotein dimers are known to associate into closed circular arrays of 8, 9 and 16 α/β-mers. We report the existence of peripheral LH proteins purified from *Allochromatium vinosum* with two intermediate ring sizes and postulate that one is a 13 α/β-mer. This shows that LH proteins are able to form membrane rings of continuously increasing diameter from 68 to 115 Å. The presence of these new ring sizes warrants further study, as it will help to further validate the structure–function models of LH proteins currently found in the literature.
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1. Introduction

Photosynthetic organisms have developed efficient systems for harvesting and converting sunlight into biochemical energy. The only endergonic event in this process consists of the absorption of the photon, which is achieved by specialized protein–pigment complexes, termed light-harvesting (LH) proteins. Upon absorption, light-energy is converted in excitation energy, which is subsequently transferred to the reaction centre (RC), or photosystems, where it is transduced into chemical potential energy through redox reactions that ultimately lead to the synthesis of ATP [1]. In the past fifteen years, our knowledge of the structure and function of photosynthetic LH proteins has dramatically progressed, partly because the cofactors ((bacterio) chlorophyll and carotenoid molecules) bound to these proteins act as intrinsic molecular probes, allowing the use of a battery of non-invasive, spectroscopic techniques to monitor protein structure and stability.

The LH complexes from purple non-sulfur photosynthetic bacteria constitute a family of membrane proteins that are probably the best-characterized from both a structural and functional point of view [2–7]. The light-harvesting system in these organisms comprises a limited number of proteins, the structure of which has been elucidated using either X-ray crystallography or a combination of two-dimensional crystallography combined with electron microscopy [8–15]. The LH complexes are formed from the non-covalent association of two short, very hydrophobic, membrane-spanning, peptides termed α and β. Heterodimers of these peptides further associate into circular oligomers to form the final, ring-like structure. To date, two major classes of such annular structures, or 'rings', have been described. The first class consists of small closed rings composed of 8 or 9 polypeptide dimers and this seems to be the general basic structure of the peripheral LH (or LH2, light-harvesting complex 2 proteins) [2,6]. These complexes do not deliver excitation energy directly to the reaction centres, but to the second class of structures that are called the 'core' LH or LH1 (light-harvesting complex 1) antennae. These latter antenna complexes are made of much larger rings which comprise 16 polypeptide dimers when closed [9]. Related atomic force microscopy (AFM) has reported an annular structure with 10-fold symmetry in *Rhodospirillum* (*Rsp.*) *photometricum* membranes, however, no corresponding purified antenna has been forthcoming [16]. It has been proposed using stimulated emission spectroscopy that reconstituted LH1 complexes may also form smaller 10-mer 'rings' when a sub-population of Mg-Bchl (bacteriochlorophyll) molecules are replaced with Ni-Bchl during the reconstitution protocol [17]. The spectroscopic studies on these artificial antennae were based on the excitation trap approach and needs to be verified by detailed structural studies. AFM studies have highlighted the highly species-dependent architecture of the in vivo photosynthetic membrane and the structural role played by proteins such as PufX (e.g. [16,18–20]).

As a consequence of the ring-like quaternary structure of the LH, the cofactors bound to these proteins are organized in circular arrays. Each α/β-polypeptide dimer generally binds two Bchl molecules, which are located in the membrane phase [2], and, in LH2, an additional Bchl molecule is bound per α/β-dimer. The latter molecules form a ring of weakly interacting molecules, responsible of the absorption transition at ca.

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Abbreviations: Alc., *Allochromatium*; Bchl, bacteriochlorophyll; CD, circular diochroism; LH1, light-harvesting complex 1; LH2, light-harvesting complex 2; RC, photochemical reaction centre; *Rbl.*, *Rhodoblastus*; *Phs.*, *Phaeospirillum*; *Rsp.*, *Rhodospirillum*

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800 nm, while the former are in strongly excitonically-coupled, and responsible for the lowest energy transition of the LH complexes that ranges, depending on the complex studied, from ~850 (LH2) to ~890 nm (LH1), and ensures the excitation energy transfer to the reaction centres. In the LH2 from the purple sulfur bacterium *Allochromatium* (*Alc.*) *vinosum*, the 800 nm transition splits into two well-defined peaks at low temperature (Fig. 1a, red curve). The relative amplitude of these two transitions does not vary during the purification procedure.

The relationship between the overall symmetry of an LH protein and its electronic properties has been the subject of an intense controversy [21–27]. Given the strength of the intermolecular interactions which can be deduced both from the crystallographic structures and from spectroscopic measurements (ca. 300-cm^{-1}) from detergent-isolated complexes, the Bchl arrays should behave as a giant supermolecule, the electronic properties of which should result from the combination of the individual Bchl transitions. However, such a pure excitonic model leads to wrong predictions, such as that the LH proteins should not be fluorescent at low temperature [22]. However, if the structure of the protein exhibits fluctuations (or static deviations from a pure circular symmetry) around its canonic structure, deduced from X-ray crystallography, the different protein-bound Bchl will no longer share the exact same electronic properties. Modelling LH properties taking into account the disorder in their structure induces a partial localisation of the excited state onto a subset of Bchl molecules, and leads to much more accurate predictions of their electronic properties. Disorder had thus to be introduced into the interacting ensemble to correctly predict the electronic properties and biological function of LH proteins. There is however still strong disagreement about whether local disorder is large enough to wipe out electronic properties due to the symmetry of the Bchl arrays or not [22–27].

Access to a larger range of LH rings of progressively increasing sizes, where additional subunits such as PufX, or the recently described "W" polypeptide [14] are absent, would help in further testing the current theories mentioned above and in designing a framework for new hypotheses. Unfortunately, screening LH proteins from the many purple bacterial strains currently known is a difficult task, as up to now, only very few properties could be related to the overall structure of the ring. It is known, for instance, that the lower energy electronic transition of LH generally exhibits a larger inhomogeneous broadening in large rings. The dichroism of this transition is also sensitive to the overall structure of the ring: in small rings the CD (circular diochroism) signal is intense and conservative, while in rings or partial of rings, of larger diameter it is much weaker and non-conservative [28,29].

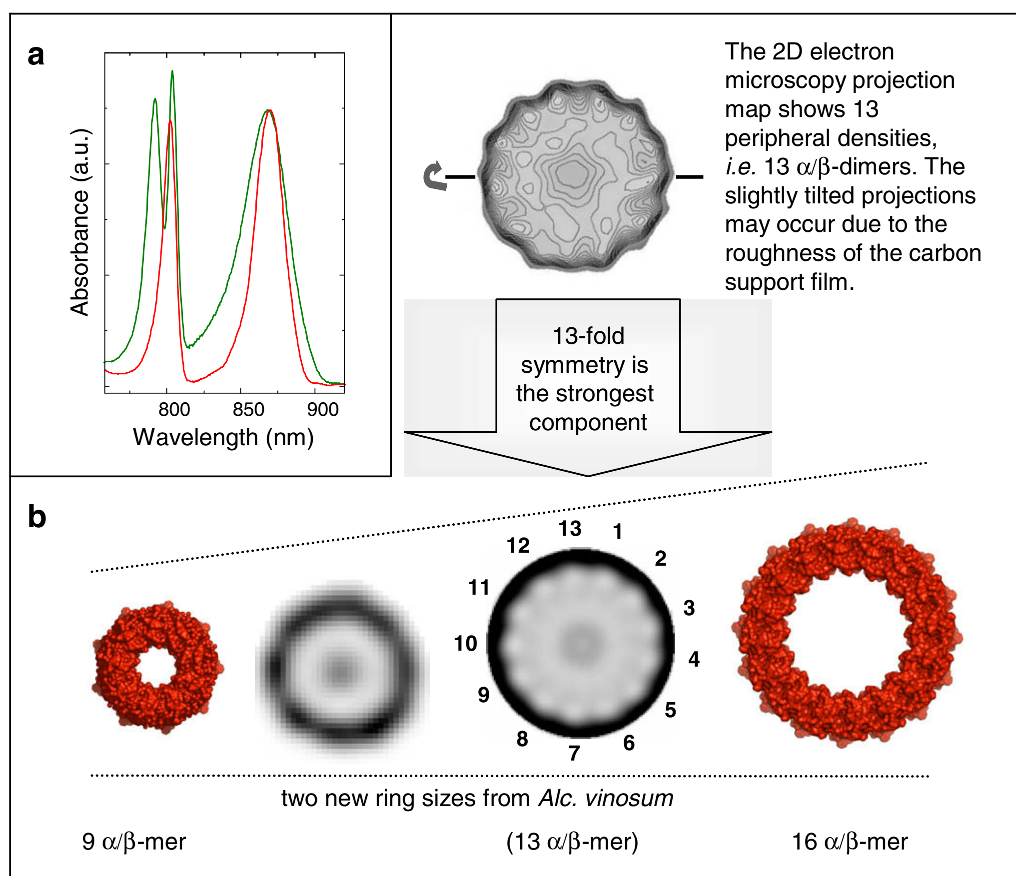

Fig. 1. Light-harvesting antenna proteins with different ring sizes. (a) 10 K absorption spectra of detergent-isolated LH2 complexes from a typical non-sulfur bacterium (*Rbl. acidophilus*, red curve) and the sulfur bacterium *Alc. vinosum* (green curve). (b) The variation of antenna ring sizes in purple photosynthetic bacteria. Each "subunit" is comprised of an inner α-apoprotein and an outer β-apoprotein and these α/β-dimers form the annular structures in the membrane. The new LH proteins from *Alc. vinosum* have intermediate ring sizes between 8/9-α/β-mers (LH2) and 16-α/β-mers (LH1).

In contrast to the LH2 complexes from non-sulfur bacteria, there is relatively little structural information on the LH antennae from purple sulfur bacteria although these were amongst the first complexes to be subjected to biochemical protocols [30]. This is mainly due to the greater biochemical complexity of these antenna complexes (see below), the lack of detailed genetic information and complete primary sequences of the LH peptides as well as difficulties in growing these anaerobes. Nevertheless, it has been known for over 20 years that LH2 complexes from purple sulfur bacteria, such as *Alc. vinosum*, when purified in the presence of mild detergents that the lower energy electronic transition exhibits a larger inhomogeneous broadening, a split 800 nm transition ([31,32] and Fig. 1a) and a weak, non-conservative, CD signal (e.g. [31]). These electronic properties from the *Chromatium* spp. LH2 complexes are reminiscent of LH1 proteins, i.e. of large polypeptide rings, suggesting a possible new quaternary structure for these integral membrane LH2 proteins. In this paper we have reexamined the detergent-purified LH2 complexes from *Alc. vinosum* and present evidence that, indeed, these proteins exhibit a different quaternary, and larger, structure from isolated LH2 complexes characterized so far.

2. Materials and methods

2.1. Sample preparation

Alc. vinosum DSM180*, Rsp. rubrum* S1 *and Rhodobacter sphaeroides* 2.4.1 were grown anaerobically at 28 °C in the presence of incandescent light (10 Wm^{-2}) in their respective liquid media [33,34] (DSMZGmbH, Braunschweig, Germany). Cells were harvested then resuspended in MES buffer (20 mM MES, 100 mM KCl, pH 6.8) and stored at −20 °C until required, except for the FFEM measurements where fresh cells were used. Based on an established protocol cells from *Alc. vinosum* were harvested and membranes prepared and the antennae purified in the presence of the mild non-ionic detergent *n*-dodecyl-β-D-maltoside ((DDM), Biomol GmbH, Hamburg, Germany) [8,35]. The antenna complexes were prepared by diluting the membranes to an OD of 25 cm^{-1} at the 800-nm peak with 20 mM Tris · Cl, pH 8.3, and then solubilized for 30 min with 2% (w/v) DDM. Following dilution of the detergent, a low-speed centrifugation step ensured removal of any unsolubilized debris (10 000 × *g*, 8 min, 4 °C). On the basis of the protocol in [36], the solubilized photosynthetic membranes were isolated using a linear (0.2–1.2 M) sucrose gradient centrifugation step. The pigmented band was collected, and the LH proteins were purified by anion-exchange chromatography (10–400 mM NaCl, Resource Q, GE Healthcare, Uppsala, Sweden) followed by separation by size exclusion (Superdex 200, GE Healthcare, Uppsala, Sweden) steps. The final buffer was 0.05% (w/v) DDM, 100 mM NaCl, 20 mM Tris · Cl, pH 8.3. Low temperature (10 K) absorption spectra of purified antennae (in 60% w/v sucrose, 20 mM Tris · Cl, pH 8.3) were collected in a SMC-TBT flow cryostat (Air Liquide, Sassenage, France), cooled with liquid helium, using a Varian Cary E5 spectrophotometer.

2.2. Single molecule particle sizes

For the electron microscopy and single molecule data analysis the purified LH complexes were negatively stained with 2% uranyl acetate on glow-discharged, carbon coated copper grids. Electron microscopy was performed on a CM120 electron microscope (Philips, Eindhoven, The Netherlands) operated at 120 kV. Images were recorded under low dose conditions (total dose ∼ 25e-/Å2) with a 4000 SP 4 K slow-scan camera (Gatan, Pleasanton, CA) at −340 nm defocus and at magnification 97 500 with a pixel size (after binning) of 3.07 Å at the specimen level. GRACE software for semi-automated specimen selection and data acquisition was employed [37]. In total, about 2400 images were recorded and 38 000 single particle projections were selected for analysis. A major part of projections was selected using Boxer, a graphical program from the EMAN software package [38]. Single particle analysis was performed with the Groningen Image Processing software package. Selected single particle projections were aligned by multi-reference and reference-free alignment procedures [37,39] then subjected to multivariate statistical analysis followed by hierarchical classification [39]. Resolution was measured using Fourier-ring correlation and the 3σ criterion [40].

2.3. Freeze-fracture electron microscopy

The bacteria were fixed for 20 min with 2% glutaraldehyde, rinsed, and cryoprotected in 30% (v/v) glycerol. Cell suspensions were deposited on freeze-fracture supports and frozen by immersion in liquid N_2-cooled Freon22. Specimens were fractured at −130 °C in a Balzers (Balzers, Switzerland) freeze-fracture device and were shadowed by depositing platinum at an angle of 45°, followed by carbon at 90°. Replicas were cleaned in bleach followed by distilled water wash and mounted on EM grids. Micrographs of bacteria membranes were taken at 28 000× magnification in a Philips EM400 electron microscope. Room-temperature electronic absorption spectra of membranes (imbedded in 82% (w/v) sucrose, 20 mM Tris · Cl, pH 8.0) were recorded with a Perkin–Elmer LS35 spectrometer. The relative ratio of LH2 and RC-LH1 complexes expressed in the *Rba. sphaeroides* membrane was calculated using previously determined in vivo extinction coefficients and pigment extraction using an acetone:methanol (7:2) mixture [41]. We estimated the range of particle sizes obtained from analysing the FFEM images that are associated with the LH2 complexes, the distribution of particle sizes ascribed to the RC-LH1 of *Alc. vinosum* was removed by subtracting the distribution of particle sizes (accounting for relative abundance in the in vivo membrane) that were obtained from the $LH2^{-}$ *Rsp. rubrum* sample. The negatives obtained by the EM400 microscope were digitized and the mean diameter of each intramembrane particle (transmembrane protein) was deduced from the surface area of its contour, at a display resolution of 2.654 pixels nm^{-1}. Between 379 and 424 particles were measured per sample. The distribution of particle sizes associated with the LH2 complexes from *Alc. vinosum* was estimated by subtracting the distribution of particle sizes attributed to a membrane packed with RC-LH1s (namely the data from *Rsp. rubrum*), weighted for the relative abundance of these complexes as determined by absorption spectroscopy.

3. Results and discussion

The LH2 complexes from *Alc. vinosum* purified in the presence of the mild non-ionic detergent *n*-dodecyl-β-D-maltoside (Biomol) were studied by electron microscopy followed by single particle analysis. A large data set of 38 000 projections was analyzed by single particle analysis, including statistical analysis and classification containing no bias towards predefined structures or symmetries. We found that the usual purification procedure lead to a mixture of two types of LH proteins, with ring-like structures of different apparent diameters of 9.55 ± 0.25 nm and 10.45 ± 0.45 nm, respectively. These values, however, do not represent true diameters because the antennae are in a detergent shell that is not (fully) penetrated by the negative stain-contrasting agent [42,43]. The smaller-sized projections (about 30% of the proteins) did not allow substantial gain of resolution upon averaging (Fig. 1b). It must be underlined here that these objects are at the extreme limit of single particle analysis as previously reported by Boekema and colleagues on estimating the correct ring symmetries of the smaller LH2 complexes from *Rba. sphaeroides* and *Phaeospirillum* (*Phs.*) *molischianum* [42,43]. In the case of the larger-sized projections (70% of the proteins) two independent approaches of processing the data, without imposing any predefined bias towards a particular symmetry, were employed and in each case ‘rings’ with 13-fold symmetry was by far the most favoured structural geometry. The first approach involved particle averaging of all the objects with no symmetry bias

imposed to produce a 20 Å resolution projection. The individual α- and β-apoproteins cannot be individually seen but the projection clearly shows the overall shape of a ring-like structure. There is a stain-filled open centre and multiple small stain-excluding densities at the periphery (Fig. 1b, upper EM projection). The structure appears to be slightly out of plane, presumably due to the roughness of the carbon support film, but the distances between these densities were only compatible with a 13-fold symmetrical particle. This rotational symmetry was therefore imposed on the final sum (Fig. 1b, lower EM projection). In the second approach we imposed increasing (9–15×) rotational symmetry on the best 17 class-sums of projections, each comprising about 1000 particles (see Fig. 2). Strong enhancement of peripheral densities was mainly observed when a 13-fold symmetry was imposed (8 classes, see red boxes). Peripheral densities were also somewhat enhanced with 12-fold (3 classes) and 11-fold symmetry (1 class). In general, other symmetries resulted in a decrease of the statistical significance of these densities.

We thus conclude that the LH2 system from *Alc. vinosum* comprises at least two types of ring-like proteins, one probably composed of 13 α/β-dimers, the other is smaller, but larger than other previously reported 8 or 9-mer LH2 complexes from species such as *Rhodoblastus acidophilus*, *Phs. molischianum* and *Rba. sphaeroides* that have been elucidated by X-ray, or 2D, crystallography [8,10,13].

When grown photosynthetically in the absence of oxygen the membrane-bound photosynthetic apparatus makes up to 55% of the total number of membrane proteins present in the microbe [44,45]. Thus, analysis of the distribution of particle sizes from freeze-fracture electron microscopy (FFEM) micrographs [45], in the 4–15 nm range, embedded within the native membrane will in general represent the structure of the photosynthetic apparatus [44,45]. The near-IR absorption spectra of the membranes investigated by FFEM in this work are shown in Fig. 3. *Rsp. rubrum* S1 (Fig. 3a) does not produce LH2 and thus lacks the characteristic 800/850 nm absorption profile of this membrane protein. The low intensity peak at about 802 nm is due to the presence of the photochemical reaction centre (RC) which is a constituent of the RC-LH1 complex. The absorption spectrum of *Rba. sphaeroides* (Fig. 3b), which contains both RC-LH1 and LH2 complexes, is dominated by LH2. The absorption of LH1 complexes contributes to these spectra only as a small shoulder at about 875 nm on the red-wing of the 850 nm peak of LH2. In contrast, the LH1 transition is not fully masked by the 800/850 nm Bchl transitions in *Alc. vinosum* (Fig. 3c) and is due to the fact that the transition of *Alc. vinosum* LH1 is slightly red-shifted (Fig. 3b). Although this may suggest that the amount of LH1 in the later membrane could be higher the abundance of the RC-LH1, compared to LH2, is similar in these two membranes (32% and 34% for *Rba. sphaeroides* and *Alc. vinosum*, respectively). Finally, to confirm that the NIR absorption spectra of the isolated pigment–protein complexes reflects the situation in the in vivo membrane the absorption spectrum of the membrane was recreated from the individually purified complexes and found to be alike (see Supplementary Information Figure 1).

There is an obvious difference between the distribution of particle sizes in membranes from *Rsp. rubrum* (Fig. 3d) and *Rba. sphaeroides* (Fig. 3e). Compared to *Rba. sphaeroides*, in *Rsp. rubrum* there is a greatly reduced relative intensity of particle sizes in the 7–8 nm region, which corresponds to the diameter of the LH2 complex. This is fully consistent with *Rsp. rubrum* being a LH2 deficient species. The RC-LH1 is the dominant multi-subunit membrane complex in the photosynthetic membrane from *Rsp. rubrum*, therefore, it is not surprising that the distribution of particle sizes is centred at approximately 11–12 nm, the well-established diameter for this complex [12,46]. The particle distribution observed in *Alc. vinosum* membranes (Fig. 3f) contains relatively more objects in the 9–10 nm range than in *Rba. sphaeroides* (Fig. 3e).

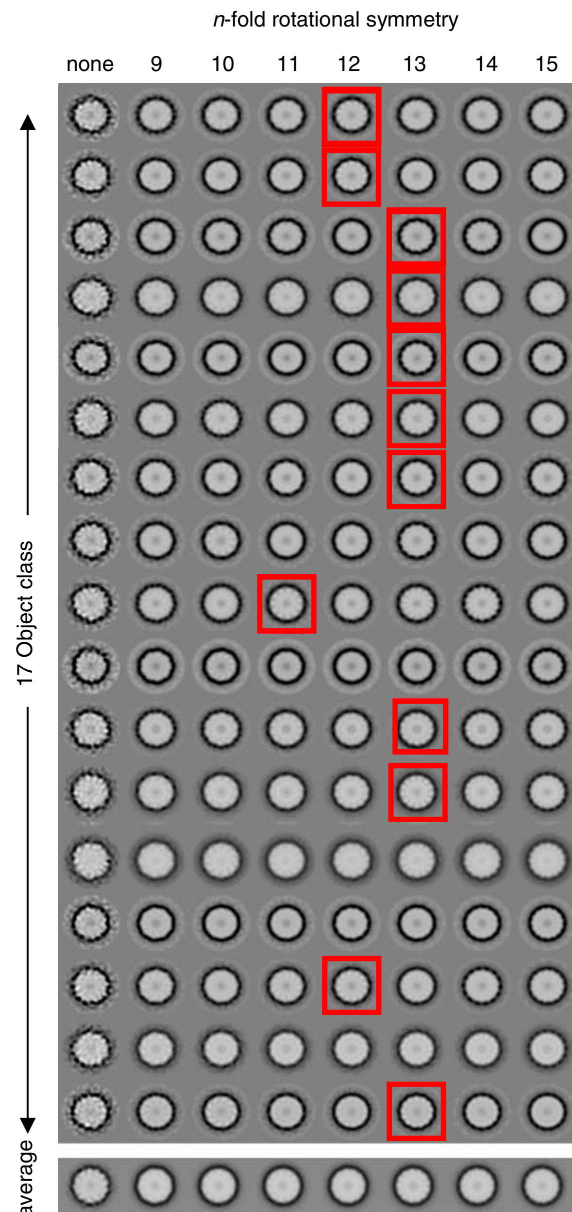

Fig. 2. Single particle electron microscopy of 25000 projections of the 'larger' LH complex from *Alc. vinosum*. To investigate the number of α/β-dimers of the LH 'rings', 9-fold to 15-fold rotational symmetry was imposed on each of the classes after completion of the analysis (horizontal rows). Strongest *n*-fold rotational symmetry is indicated by red boxes. Lower line: sum of the best 8 classes without (left) and with symmetry imposed.

The distribution of particle sizes in the membranes associated with the LH2-only fraction was then estimated by subtracting the distribution of particle sizes attributed to a

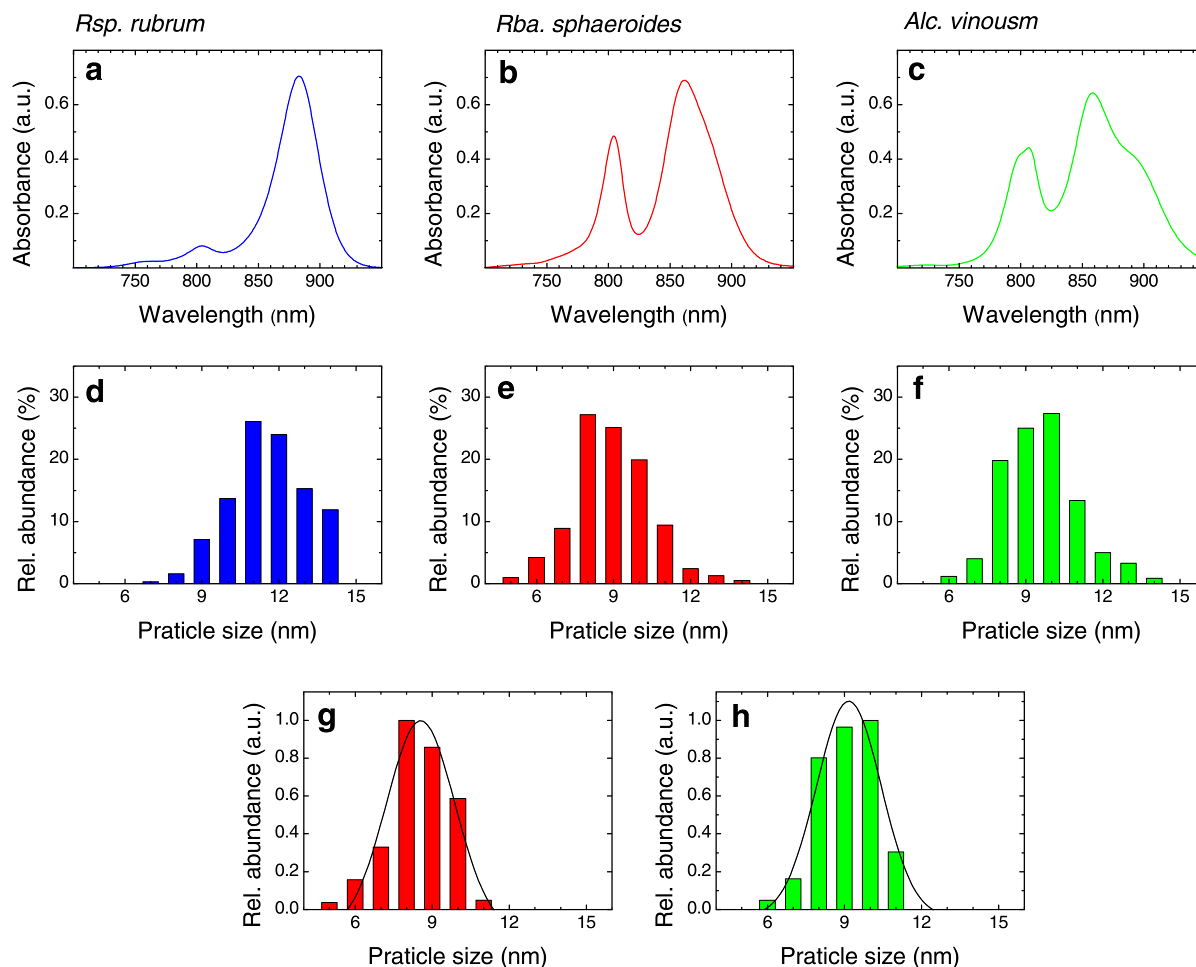

Fig. 3. The near-IR absorption spectra (a–c) and the distribution of particle sizes observed by FFEM (d–f) of membranes from *Rsp. rubrum* (blue) *Rps. sphaeroides* (red) and *Alc. vinosum* (green). The relative abundance of particle sizes associated with the LH2-fraction from *Rps. sphaeroides* (g) and *Alc. vinosum* (h) after subtracting the RC-LH1 component. Overlaid are Gaussian distributions and indicates that the dominant particle size is 8.5 ± 0.5 nm and 9.2 ± 0.5 nm in *Rba. sphaeroides* (g) and *Alc. vinosum* (h), respectively.

membrane packed with RC-LH1s (namely the data from *Rsp. rubrum*), weighted by the relative abundance of these complexes as determined by absorption spectroscopy and pigment analysis (see Section 2). From the distribution of particle sizes it is apparent that the LH2-fraction from *Alc. vinosum* (Fig. 3h) is somewhat broader, with more larger objects, than the corresponding population from *Rba. sphaeroides* (Fig. 3g) (this work and [42]). Finally, overlaid are Gaussian distributions and indicates that the dominant 'LH2' particle size is 8.5 ± 0.5 nm and 9.2 ± 0.5 nm in *Rba. sphaeroides* (g) and *Alc. vinosum* (h), respectively. While FFEM is not as elegant as studies on single molecules it has enabled us to verify that the distribution of particle sizes in *Alc. vinosum* native membranes is indeed significantly different to that observed in *Rba. sphaeroides* when grown under the same anoxygenic (and light) growth conditions.

The bacterium *Alc. vinosum* is known to express multiple types of α- and β-apoproteins but their primary sequences have only been partially determined (e.g. see [2]). Although the primary sequences of the LH2 from exhibit the same overall homology in their Bchl binding pockets with the non-sulfur photosynthetic species there is a near compete lack of information about the C- and N-terminal regions. This is actually why it has been the subject of so few studies in the recent past. In order to fully characterize these proteins, and to understand the parameters that govern their unusual quaternary structures, it is now necessary to develop new purification protocols that will allow separating the proposed different *Alc. vinosum* LH2 complexes that have been revealed by electron microscopy, to perform a full characterisation of their polypeptides. This may help in elucidating the molecular origins of the split 800 nm transition (Fig. 1a, green curve) and, in the long term, these once-purified new ring-like proteins will help in addressing the essential question of the influence of symmetry on LH electronic properties. However, in the present state of our investigations, we can already conclude that the members of the LH protein family contain membrane rings of continuously increasing diameter from 68 to 115 Å.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.09.050.

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