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REGULAR PAPER

Variation in supramolecular organisation of the photosynthetic membrane of *Rhodobacter sphaeroides* induced by alteration of PufX

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Abstract In purple bacteria of the genus *Rhodobacter* (*Rba.*), an LH1 antenna complex surrounds the photochemical reaction centre (RC) with a PufX protein preventing the LH1 complex from completely encircling the RC. In membranes of *Rba. sphaeroides*, RC–LH1 complexes associate as dimers which in turn assemble into longer range ordered arrays. The present work uses linear dichroism (LD) and dark-minus-light difference LD (ΔLD) to probe the organisation of genetically altered RC–LH1 complexes in intact membranes. The data support previous proposals that *Rba. capsulatus,* and *Rba. sphaeroides* heterologously expressing the PufX protein from *Rba. capsulatus*, produce monomeric core complexes in membranes that lack long-range order. Similarly, *Rba. sphaeroides* with a point mutation in the Gly 51 residue of PufX, which is located on the membrane-periplasm interface, assembles mainly non-ordered RC–LH1 complexes that are most likely monomeric. All the *Rba. sphaeroides* membranes in their ΔLD spectra exhibited a spectral fingerprint of small degree of organisation implying the possibility of ordering influence of LH1, and leading to an important conclusion that PufX itself has no influence on ordering RC–LH1 complexes, as long-range order appears to be induced only through its role of configuring RC–LH1 complexes into dimers.

Keywords Photosynthesis · *Rhodobacter sphaeroides* · RC–LH1 complex · PufX · Linear dichroism · Supramolecular organisation

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Introduction

The purple photosynthetic bacterium *Rhodobacter* (*Rba.*) *sphaeroides* has a photosystem comprising a reaction centre (RC) and two types of light harvesting complex. The so-called LH1 light harvesting complex is made up from protein, bacteriochlorophyll (BChl) and carotenoid, and forms a hollow cylinder around the RC within the photosynthetic membrane. This RC–LH1 complex is found throughout the purple photosynthetic bacteria, although there are differences in the detailed architecture between different species, particularly with regard to whether the LH1 forms a closed cylinder around the RC or whether there is a gap (Scheuring et al. 2005; Scheuring 2006). In many species, one or more additional light harvesting complexes are also present at variable levels depending on growth conditions such as light intensity or the presence/absence of oxygen (Cogdell et al. 2006).

Several years ago, it was established that the *Rba. sphaeroides* RC–LH1 complex can assemble in either a monomeric form, where the RC is almost completely surrounded by an LH1 antenna that forms a C-shape when viewed perpendicular to the plane of the membrane, or a dimeric form in which two RCs are surrounded by an

S-shaped antenna and related by an axis of twofold symmetry (Jungas et al. 1999; Siebert et al. 2004; Bahatyrova et al. 2004). An additional polypeptide, termed PufX, is present at a stoichiometry of one per RC and seems to determine the presence of a break in the continuity of the LH1 antenna around each RC and facilitate the assembly of dimers (Francia et al. 1999; Comayras et al. 2005; Walz et al. 1998). Dimeric RC–LH1 complexes have been imaged in photosynthetic membranes from *Rba. sphaeroides* by atomic force microscopy, in 2-D crystals by electron microscopy (EM) and in detergent solution by single particle EM (Bahatyrova et al. 2004; Sturgis et al. 1988; Qian et al. 2005, 2008; Scheuring et al. 2004; Semchonok et al. 2012).

PufX proteins have been identified with certainty only in five species of *Rba.* (Tsukatani et al. 2004; Holden-Dye et al. 2008), although equivalent minor components of the RC–LH1 complex may also be present in other species of purple bacteria with incomplete rings of LH1 pigment protein surrounding the central RC, such as *Rhodopseudomonas palustris* (Roszak et al. 2003). PufX has a strong influence on whether the *Rba. sphaeroides* RC–LH1 complex is assembled in a dimeric form, deletion of the corresponding *pufX* gene producing monomeric RC–LH1 complexes in which the central RC is completely encircled by an enlarged LH1 antenna (Jungas et al. 1999; Siebert et al. 2004); this structural change seems to involve the assembly of two additional pairs of LH1 α and β polypeptides into the cylinder surrounding the RC, along with four BChls and two carotenoids (Cogdell et al. 1996; McGlynn et al. 1994; Siebert et al. 2004). However, in other species of *Rhodobacter*, such as *Rba. veldkampii*, AFM analysis of photosynthetic membranes has suggested that the RC–LH1 complex is assembled only in a monomeric form despite the presence of a PufX protein that prevents closure of the LH1 ring (Liu et al. 2011). Given this, there has been interest in trying to identify correlations between the available sequences for PufX and the presence or absence of dimeric RC–LH1 complexes, as this may give some insights into the structural role played by PufX in determining the architecture of the *Rba. sphaeroides* RC–LH1 protein (Gubellini et al. 2006; Busselez et al. 2007; Hsin et al. 2009a; Liu et al. 2011).

In recent work it was proposed, on the basis of an analysis of the RC–LH1 complexes that can be extracted from photosynthetic membranes, that the dimeric form of this complex is assembled in *Rba. azotoformans* but not in *Rba. capsulatus* (Crouch and Jones 2012). Furthermore, replacement of the *Rba. sphaeroides pufX* gene with the counterpart from *Rba. capsulatus* resulted in a chimeric RC–LH1 complex which was photosynthetically competent but did not assemble in the dimeric form (Crouch and Jones 2012; Klug and Cohen 1988). The resulting strain of *Rba. sphaeroides* was named RCLH1sXc-g to denote that it possessed *Rba. sphaeroides* RC and LH1 proteins, *Rba. capsulatus* PufX and green carotenoids (principally neurosporene); the rationale behind use of a "green strain" is explained in the "Results" section. The chimeric monomers that were assembled in this strain had a normal complement of LH1 BChls per RC, indicating that the foreign PufX was assembled into the structure of the monomer but did not facilitate the further assembly of dimers in the same fashion as the native PufX (Crouch and Jones 2012).

A possible criticism of this analysis of the RC–LH1 complexes present in *Rba. capsulatus* and the chimeric *Rba. sphaeroides* strain is that it requires solubilisation of proteins from photosynthetic membranes using 4 % dodecylmaltoside, prior to size fractionation on sucrose density gradients. As controls, it was established that the extraction protocol is capable of solubilising high yields of RC–LH1 dimer when these are present in the starting membrane (Crouch and Jones 2012). In addition, the relative amounts of monomer and dimer obtained by this procedure were highly reproducible and did not depend on the precise conditions of the extraction (detergent concentration varied by ±2 %, time for extraction or temperature) (Crouch and Jones 2012). However, it cannot be completely ruled out that dimeric RC–LH1 complexes do indeed assemble in wild-type *Rba. capsulatus* cells, or cells of the RCLH1sXc-g *Rba. sphaeroides* strain, but that they dissociate into monomers when DDM is added to membranes.

One way to probe the organisation of RC–LH1 complexes in intact photosynthetic membranes is through linear dichroism (LD) spectroscopy, measuring the difference in absorption of light polarised linearly in vertical and horizontal directions by a sample displaying macroscopic order. It has been shown that membranes with PufX-containing dimeric *Rba. sphaeroides* RC–LH1 complexes can be distinguished from membranes with only PufX-deficient monomeric RC–LH1 complexes, the former giving specific spectral signatures of uniform orientation of the RC in both LD and dark-minus-light difference LD (ΔLD) spectra of oriented membranes (Frese et al. 2000). Upon orienting membranes suspended in a gel through compression in two perpendicular directions, the precise geometry of the transition dipole moments of the RC cofactors can be detected by LD. Membranes containing RC–LH1 complexes in a dimeric configuration display a pronouncedly different LD spectrum compared to isolated RC–LH1 complexes. The rationale is that dimeric RC–LH1 complexes form ordered arrays within the membranes with the RC locked in a unique orientation within the complex. In contrast, in PufX-deficient monomeric RC–LH1 complexes, the RC can adopt multiple orientations with respect to the surrounding LH1 ring, producing a characteristically

different LD or ΔLD spectrum. These spectra can be characterised as completely unordered when displaying features similar to those of isolated complexes (Frese et al. 2000), or partially ordered when membranes consist of coexisting domains of RCs that are rotationally locked in one orientation and another domain where this ordering is absent, as observed in PufX-deficient membranes that also contain LH2 complexes (Frese et al. 2008). Most likely, some ordering can be facilitated due to the flexibility of the LH1 helices which under packing and curvature can adapt to the asymmetric shape of the RC.

In the present work, we have investigated these effects in detail by comparing the supramolecular organisation in two bacterial strains that naturally contain LH2 complexes and RC–LH1 in a dimeric configuration. We have applied LD and ΔLD spectroscopy to oriented membranes of wild-type *Rba. capsulatus* and *Rba. sphaeroides* strain which contains PufX of *Rba. capsulatus* (RCLH1sXc-g), looking for the spectral features that would indicate the presence or absence of ordered RC–LH1 complexes. In addition, we have looked at the LD and ΔLD spectra of a strain of *Rba. sphaeroides* with a single point mutation in PufX that appears to prevent assembly of RC–LH1 dimers.

We find no evidence for the presence of long-range ordered arrays in intact membranes from any of these strains. From the ΔLD spectra of *Rba. sphaeroides* membranes with altered PufX, however, it transpires that these preparations are not completely unordered, corroborating our earlier observation that the presence or absence of order in the bacterial membrane depends not only on the presence of PufX (Frese et al. 2008). We conclude therefore that minute alterations of PufX can have severe ramifications for the supramolecular organisation within these membranes; not only can they prevent dimerization of RC–LH1 complexes and thus long-range ordering, but they can also alter the more partial effect of packing- and curvature-induced ordering.

Materials and methods

Strains of wild-type *Rba. sphaeroides* and *Rba. capsulatus* were NCIB8253 and Kb-1, respectively. *Rba. sphaeroides* was grown in M22+ medium at 34 °C (Hunter and Turner 1988) and *Rba. capsulatus* in RCV-PY medium at 30 °C (Beatty and Gest 1981), under either dark/semiaerobic or photosynthetic conditions as described in detail previously (Crouch and Jones 2012). The plasmids used to express the *Rba. capsulatus pufX* together with the *Rba. sphaeroides pufBALM* were as described recently, and were inserted into *Rba. sphaeroides* deletion strains DPF2/G (Hunter et al. 1991) and DD13/G (Jones et al. 1992) through conjugative crossing (Hunter and Turner 1988). The control strains used were DPF2/G and DD13/G complemented with plasmid pRKEH10 containing the *Rba. sphaeroides pufBALMX* operon or plasmid pRKEH10X^{-} which is a derivative of pRKEH10 lacking *pufX*.

The Gly to Leu mutation at position 51 of the *Rba. sphaeroides* PufX was generated using the QuikChange method (Stratagene) using plasmid pUCXB-3 as the template (Holden-Dye 2007). Nucleotide changes were confined to the target codon and were confirmed by DNA sequencing (Cogenics Inc.). Altered *pufX* genes were shuttled into the broad-host range vector pRKEH10. This was inserted into the *Rba. sphaeroides* strain DPF2/G or DD13/G through conjugative crossing (Hunter and Turner 1988).

Growth of the engineered strains of *Rba. sphaeroides* was carried out as described above for wild-type NCIB8253 except the medium was supplemented with neomycin, streptomycin and tetracycline where appropriate, as described previously (Crouch et al. 2010).

Intracytoplasmic membranes were extracted from cells using a French pressure cell (Jones et al. 1994). LH2 and RC–LH1 complexes were solubilised from these membranes using 4 % *n*-dodecyl-β-D-maltoside (DDM) (Siebert et al. 2004), as described in detail recently (Crouch et al. 2010). Solubilised pigment proteins were fractionated by ultracentrifugation on sucrose density gradients composed of five discrete steps of 20, 21.25, 22.5, 23.75 and 25 % (w/v) sucrose in 20 mM HEPES (pH 8)/0.04 % DDM in transparent ultracentrifuge tubes. Solubilised membrane proteins (150 µl of sample with an absorbance of 25 at 850 nm) were loaded on to each gradient, and these were centrifuged in a Sorvall TH-641 swing-out bucket rotor at 180,000×*g* for 20 h at 4 °C. After photography, sucrose gradients were fractionated for analysis by absorbance spectroscopy.

Isolated intracytoplasmic membranes were suspended in 20 mM Tris pH 8.0, 62 % (v/v) glycerol, 11.6 % acrylamide, 0.4 % *N*,*N*′-methylynebisacrylamide, 0.02 % ammonium persulfate, 0.02 % *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) which was left to polymerise. The gel was subsequently squeezed in two perpendicular directions while allowed to expand along *z*-axis, transferred to acrylic cuvette and cooled to 77 K in an Oxford cryostat. All spectra were recorded at 77 K on a home-built spectrometer (described in Frese et al. 2000) allowing for direct recording of either LD or ΔLD or ΔOD.

Results

Organisation of RC–LH1 complexes in *Rba. capsulatus*

First, we recorded 77 K LD and ΔLD spectra of *Rba. capsulatus* membranes and compared these to the well-

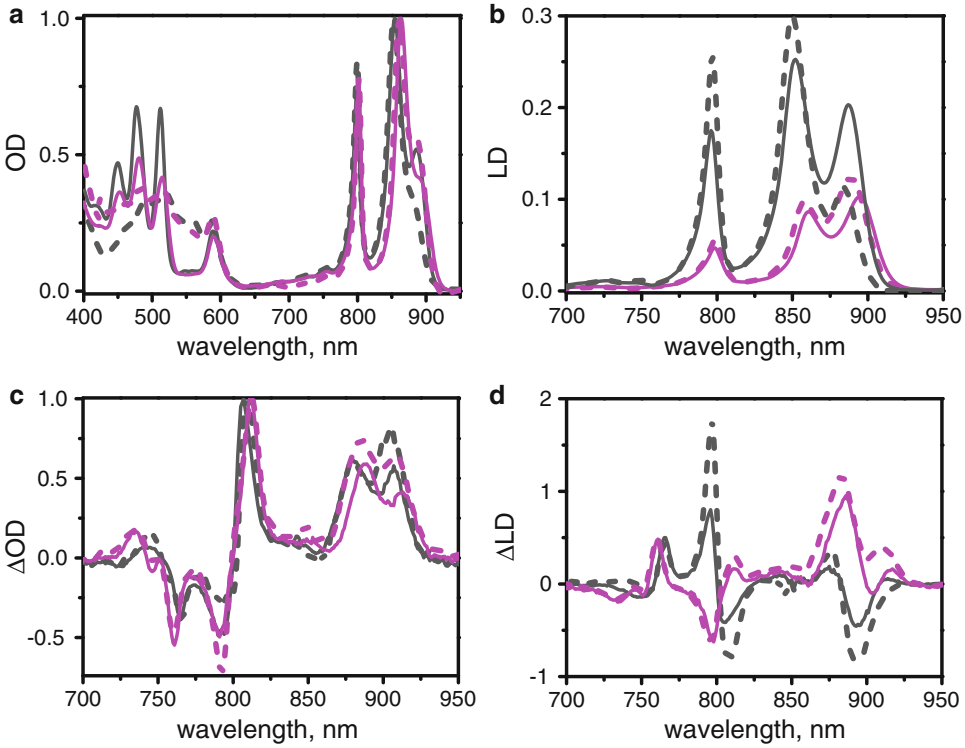

Fig. 1 77 K absorption (**a**), LD (**b**), ΔOD (**c**) and ΔLD (**d**) spectra of membranes from *Rba. sphaeroides* wild-type grown photosynthetically (*dark grey solid line*) and semi-aerobically (*dark grey dashed line*), and wild-type *Rba. capsulatus* grown photosynthetically (*magenta solid line*) and semi-aerobically (*magenta dashed line*). Absorption and ΔOD spectra are normalised at their respective maxima. ΔLD and LD spectra are normalised to the same OD of LH1

characterised membranes of *Rba. sphaeroides*. Both species are known to have very similar membrane architectures, producing near-spherical chromatophores with a diameter of a few tens of nm, but in *sphaeroides* RC–LH1 assembles itself primarily into dimers, whereas *capsulatus* is thought to form mainly monomers. Both wild-type strains were grown under either photosynthetic or dark/semiaerobic conditions (Table 1). For convenience, these two growth conditions are referred to as 'light' and 'dark', respectively, in the following. The 77 K absorbance spectra (Fig. 1a) comprised contributions from carotenoid between 400 and 550 nm, the BChl Q_x absorbance band at 590 nm, the Q_y bands of the LH2 BChls at ~800 and ~860 nm, and the Q_y band of the LH1 BChls at ~890 nm. The maxima of the Q_y absorption bands for *Rba. capsulatus* were red-shifted by a few nm with respect to their *Rba. sphaeroides* counterparts, most noticeably for LH2 where the lowest energy band was at 854/853 nm in the *Rba. sphaeroides* spectra and 861/864 nm in the *Rba. capsulatus* spectra (the spectra were normalised to the maximum of this band). The different growth conditions also produced variations in the relative amounts of LH2 and LH1 with, in particular, a relatively high amount of LH2 in membranes from *Rba. sphaeroides* grown in the dark.

LD spectra are shown in Fig. 1b. All observed bands have positive LD, which means that the absorption of the Q_y transitions of the B880 (LH1), B850 (LH2) and B800 (LH2) is stronger with parallel polarised light than with perpendicular polarised light, which means that all these transitions are in line with the orientation direction of the membranes. The RC chromophores are not observed in these spectra because they are hidden under the antenna bands. It is expected that in unordered membranes P displays a strongly positive LD, B weak positive LD and BPhe a negative LD. There are minor differences in the relative LD signals of LH1 and LH2 in both species, suggesting some differences in the way the membranes were oriented. When corrected for the intensity of absorbance, the overall intensity of the LD spectra for light- and dark-grown *Rba. capsulatus* and dark-grown *Rba. sphaeroides* was approximately half of that of light-grown *Rba. sphaeroides*. This suggests that the latter membranes had twice the macroscopic orientation as the former.

The absorbance difference spectra (Fig. 1c) probed spectral shifts due to the formation of the final $P^+Q_B^-$ charge separated state in the RC (where P is the primary electron donor BChl dimer and Q_B is the secondary acceptor quinone of the RC). The spectra (displayed as dark-minus-light or PQ_B minus $P^+Q_B^-$) were similar for all four sets of membranes, with a blue-shift of the absorbance band of the two RC bacteriopheophytins (BPhes) at 760 nm and a red-shift of the absorbance band of the two RC monomeric BChls at 800 nm. There were small noticeable shifts in the P-associated band between 860 and 930 nm that were difficult to quantify since this band was split. In addition, the BPheo band-shift exhibited additional fine structure at ~745 nm in both spectra for *Rba. capsulatus* membranes.

Table 1 Abbreviations and growth conditions of membranes used in this study

Sample	Growth conditions	RC	LH1	PufX	LH2	carotenoid
Rba. sphaeroides WT	Light/photosynthetic	Native	Native	Native	Native	Green
Rba. sphaeroides WT	Dark/semiaerobic	Native	Native	Native	Native	Red
Rba. capsulatus WT	Light/photosynthetic	Native	Native	Native	Native	Green
Rba. capsulatus WT	Dark/semiaerobic	Native	Native	Native	Native	Red
RCLH1sXs-g2	Dark/semiaerobic	Native	Native	Native	Native	Green
RCLH1sXs-g	Dark/semiaerobic	Native	Native	Native	None	Green
RCLH1sXd-g2	Dark/semiaerobic	Native	Native	None	Native	Green
RCLH1sXd-g	Dark/semiaerobic	Native	Native	None	None	Green
GX51L-g2	Dark/semiaerobic	Native	Native	Native with point mutation at Gly 51 swapped to Leu 51	Native	Green
GX51L-g	Dark/semiaerobic	Native	Native	Native with point mutation at Gly 51 swapped to Leu 51	None	Green
RCLH1sXc-g2	Dark/semiaerobic	Native	Native	From *Rba. capsulatus*	Native	Green
RCLH1sXc-g	Dark/semiaerobic	Native	Native	From *Rba. capsulatus*	None	Green

The ΔLD spectra (Fig. 1d) probed the polarisation dependency of these light-induced spectral shifts, and in previous work on *Rba. sphaeroides*, the details of this spectrum have been shown to be highly dependent on the supramolecular organisation of complexes within the membrane (Frese et al. 2000, 2004). The ΔLD spectra for *Rba. sphaeroides* membranes were characterised by strong negative LD of the 890 nm band arising from the P dimer and a band-shift centred at ~800 nm that corresponds to a negative LD for the RC accessory BChls. In marked contrast, in the ΔLD spectra for *Rba. capsulatus* membranes, the LD signs of both the P band signal and the B band-shift are positive, as was observed in isolated RC complexes (Rafferty and Clayton 1978; Abdourakhmanov et al. 1979; Tiede et al. 1985; Breton 1985). The conclusion drawn from these differences was that in the intact *Rba. capsulatus* membranes, the RCs did not display the order that is typical of their arrangement in *Rba. sphaeroides* membranes, a conclusion consistent with the lack of dimeric RC–LH1 complexes predicted from fractionation of detergent-solubilised *Rba. capsulatus* complexes in previous work (Crouch and Jones 2012).

Effect of PufX on the organisation of RC–LH1 complexes

In addition to concluding that the *Rba. capsulatus* RC–LH1 complex is not likely to assemble in the dimeric form in the photosynthetic membrane, a previous publication also proposed that RC–LH1 dimers do not assemble in *Rba. sphaeroides* if the native PufX is replaced by the *Rba. capsulatus* protein (Crouch and Jones 2012; Fulcher et al. 1998). This proposal was based on analysis of the complexes extracted from two strains of *Rba. sphaeroides* expressing this chimeric RC–LH1 complex, strain RCLH1sXc-g2 which also expresses LH2 and strain RCLH1sXc-g which lacks LH2. Both of these strains also express green carotenoids (principally neurosporene) rather than the native red/brown carotenoids spheroidenone or spheroidene, as the yield of dimeric RC–LH1 complexes that can be isolated from such 'green' strains is much higher than the equivalent 'red/brown' strain, particularly following growth under dark/semiaerobic conditions. The complexes obtained from strains RCLH1sXc-g2 and RCLH1sXc-g were compared with equivalent strains either expressing native RC–LH1 complexes (strains RCLH1sXs-g2 and RCLH1sXs-g) or PufX-deficient RC–LH1 complexes (strains RCLH1sXd-g2 and RCLH1sXd-g). Dimeric RC–LH1 complexes could be isolated from membranes of strain RCLH1sXs-g, but only monomeric RC–LH1 complexes could be isolated from strains RCLH1sXc-g or RCLH1sXd-g, an equivalent result being obtained with the LH2-containing strains. Unlike the PufX-deficient monomeric RC–RC–LH1 complexes isolated from control strain RCLH1sXd-g, the monomers obtained from the chimeric RCLH1sXc-g strain had an absorbance spectrum that implied that the *Rba. capsulatus* PufX protein was assembled into the complex (see below). In the present report, LD and ΔLD spectroscopy was applied to intact membranes from the same six strains grown under dark conditions.

In addition to this, LD and ΔLD spectroscopy were applied to membranes from a strain of *Rba. sphaeroides* with a single point mutation in the native *pufX* gene that results in replacement of residue glycine 51 with a larger leucine side chain. This mutation was made as part of a programme of mutagenesis of the *Rba. sphaeroides* PufX, including multiple changes of this conserved glycine 51 residue, that will be reported in detail elsewhere (Crouch

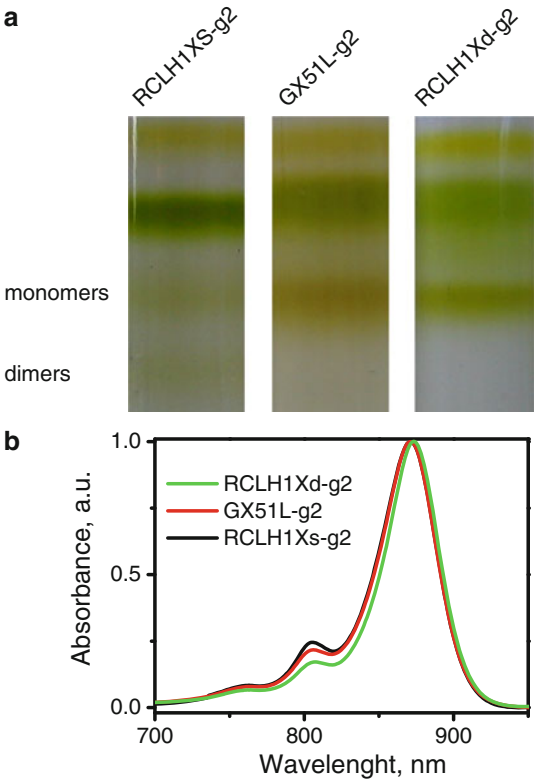

Fig. 2 Sucrose gradient fractionation of complexes extracted from photosynthetic membranes. **a** Coloured bands are attributable to (*top* to *bottom*) free carotenoid, LH2, RC–LH1 monomers and RC–LH1 dimers. **b** Absorbance spectra of RC–LH1 monomers, normalised to the same LH1 absorbance at 875 nm

and Jones, manuscript in preparation). The salient feature was that when RC–LH1 complexes containing this GX51L mutation were extracted from photosynthetic membranes and size fractionated on sucrose density gradients, the dimeric form of the RC–LH1 complex was not obtained.

Figure 2a shows sucrose density gradients used to fractionate LH2 and RC–LH1 proteins from strain GX51L-g2 and the PufX-containing and PufX-deleted control strains RCLH1sXs-g2 and RCLH1sXd-g2. Both monomeric and dimeric RC–LH1 complexes were obtained from the strain assembling native RC–LH1 complexes, but only monomers were obtained from strain GX51L-g2 or the PufX-deficient control. Equivalent results were obtained with strains lacking LH2 (data not shown). Absorbance spectra of RC–LH1 monomers removed from these sucrose gradients (Fig. 2a) are shown in Fig. 2b. The spectrum of monomers isolated from strain RCLH1sXd-g2 exhibited the elevated amount of LH1 absorbance at 875 nm relative to RC absorbance at 805 nm characteristic of the enlarged and completed circle of LH1 BChl surrounding each RC that is a consequence of the absence of PufX. In contrast, the spectrum recorded for monomers from strain GX51L-g2 did not show this increase in LH1 absorbance, indicating that the altered PufX protein was still an integral component of the RC–LH1 complex. Moreover, there was no blue-shift of LH1 absorption in the GX51L mutant, observed in RCLH1sXd and associated with the stronger excitonic interaction of the complete ring of excitonically coupled pigment. Additionally, the antibody assays analysis indicated the presence of PufX in the RC–LH1 complexes of the mutants (Ratcliffe and Hunter, personal communication). Thus the phenotype of strains containing this GX51L mutation in the *Rba. sphaeroides* PufX was identical to that of the strains described above in which the *Rba. sphaeroides* PufX was replaced by the *Rba. capsulatus* protein. Accordingly, LD and ΔLD spectroscopy was also applied to intact membranes from strains GX51L-g and GX51L-g2 to probe the organisation of RC–LH1 complexes in intact membranes.

LD and ΔLD spectroscopy of membranes lacking LH2

Low temperature absorbance spectra of membranes from dark-grown strains RCLH1sXs-g, RCLH1sXd-g, RCLH1sXc-g and GX51L-g are compared in Fig. 3a, normalised to the same absorbance at the maximum of the LH1 band around 884 nm. No major spectral differences were apparent, absorbance maxima varying by no more than a 2–3 nm across the four spectra. These LH2-deficient membranes exhibited three LD bands at around 754, 803 and 887 nm (Fig. 3b) originating from the RC BPhes, RC accessory BChls and LH1 BChls, respectively. Again, band maxima did not vary by more than 2–3 nm. The 887 nm band had positive amplitude in all preparations confirming that the orientation of LH1 was similar in all cases, and the small BPhe LD band at 755 nm had a similar negative amplitude across all membranes.

The sign and amplitude of the LD band at 800 nm corresponding to the RC monomeric BChls has been associated with the degree of order exhibited by RCs in oriented membranes. Consistent with published findings on similar strains of *Rba. sphaeroides* (Frese et al. 2000), this signal had a negative sign in the LD spectrum of membranes from the RCLH1sXs-g control strain with native RC–LH1 complexes, but had a positive sign in the LD spectrum of membranes from the RCLH1sXd-g strain with PufX-deficient RC–LH1 complexes (Fig. 3c). Despite the presence of a PufX, this signal also had a positive sign in LD spectra of membranes from the RCLH1sXc-g and GX51L-g strains (Fig. 3c). The LD for these latter three strains was not positive over the entire band; however, a negative contribution being visible at ~813 nm (Fig. 3c) originating from the upper exciton Q_y band of the P BChls that has been found to be always negative irrespective of existence or lack of order (due to being nearly orthogonal

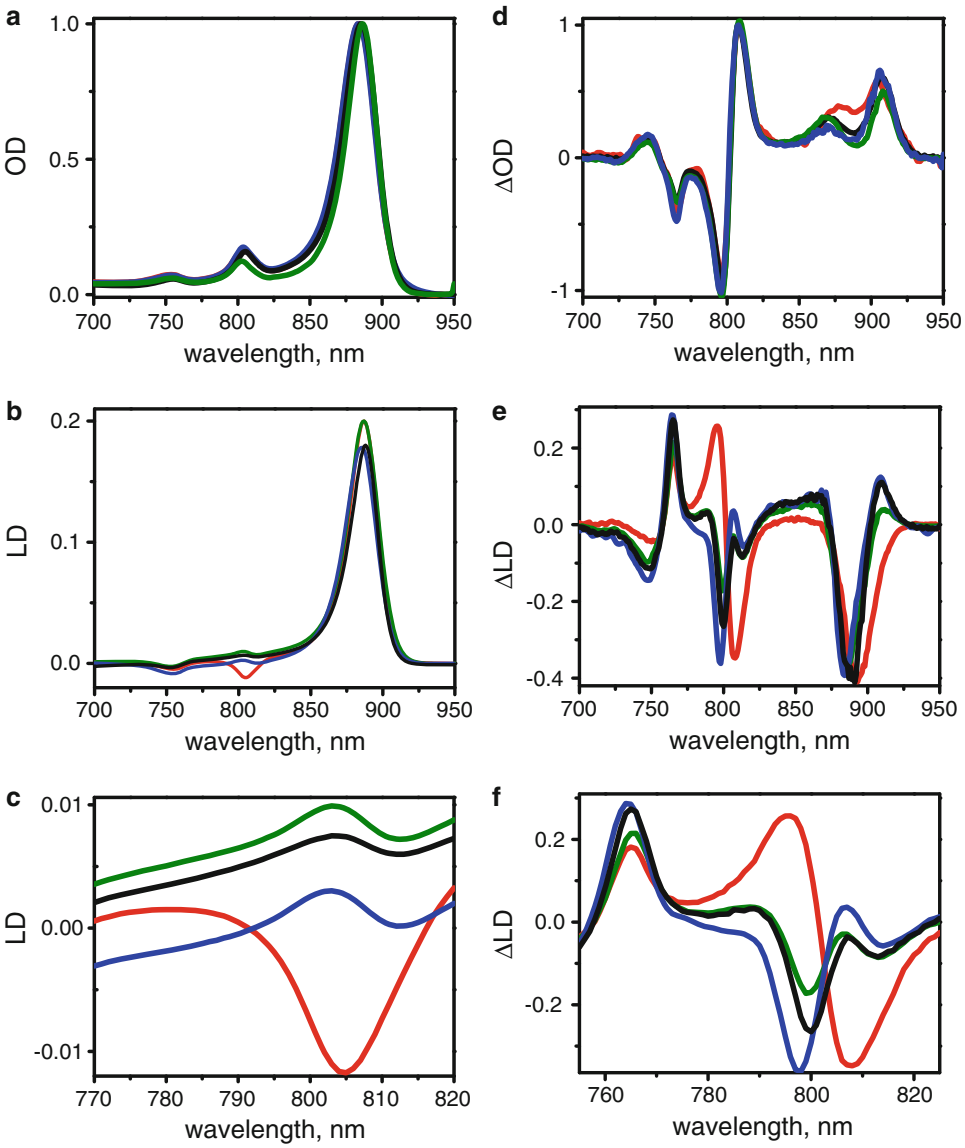

Fig. 3 77 K absorption (**a**) and LD (**b**, **c**), ΔOD (**d**) and ΔLD (**e**, **f**) spectra of membranes from *Rba. sphaeroides* LH2-deficient mutants: RCLH1Xs-g (*red*), RCLH1sXd-g (*green*), GX51L-g (*black*), RCLH1sXc-g (*blue*). LD and ΔLD spectra are normalised to the same OD of LH1. **c**, **f** Magnifications of the accessory BChl region of LD and ΔLD spectra, respectively

to the lower exciton band of special pair Q_y, and therefore perpendicular to the membrane) (Arnett et al. 1999; Frese et al. 2000). For these latter three strains, the absolute magnitude of the LD for the 800 nm band was lower than would be expected for a signal originating from a fully disordered sample, being smaller than the magnitude of the negative band in the LD spectrum of the RCLH1sXs-g control strain, indicative of ordered RCs.

The membranes from the LH2-deficient strains had almost identical absorbance difference spectra (Fig. 3d). The ΔLD spectrum of membranes from the RCLH1sXs-g control strain (Fig. 3e) reproduced the pattern of negative and positive bands characteristic for a membrane showing order in the arrangement of RC–LH1 complexes (Frese et al. 2000). It displayed a strongly negative signal at ~890 nm arising from the Q_y band of the RC P BChls and a split band centred at ~803 nm that was negative on the red side and positive on the blue side, in accordance with a reversed absorbance difference signal. In marked contrast in the ΔLD spectra of membranes from the remaining strains, the sign of this differential feature was reversed, with a negative band at 798–800 nm and a positive band at ~808 nm (Fig. 3f). The magnitude of this latter positive band was greater for the GX51L-g and RCLH1sXc-g mutants than for the PufX-deficient control. The small negative signal at ~815 nm in the spectra of all three mutants was probably a negative contribution of the upper exciton band of the RC P BChls—this was of course not visible in the spectrum of membranes from the control strain with a native PufX due to the reversed band-shift signal in this region. Despite the positive LD signal at ~800 nm, the 890 nm band of the ΔLD spectra of the mutant strains had negative sign with positive contributions at both red and blue side of that band possibly due to

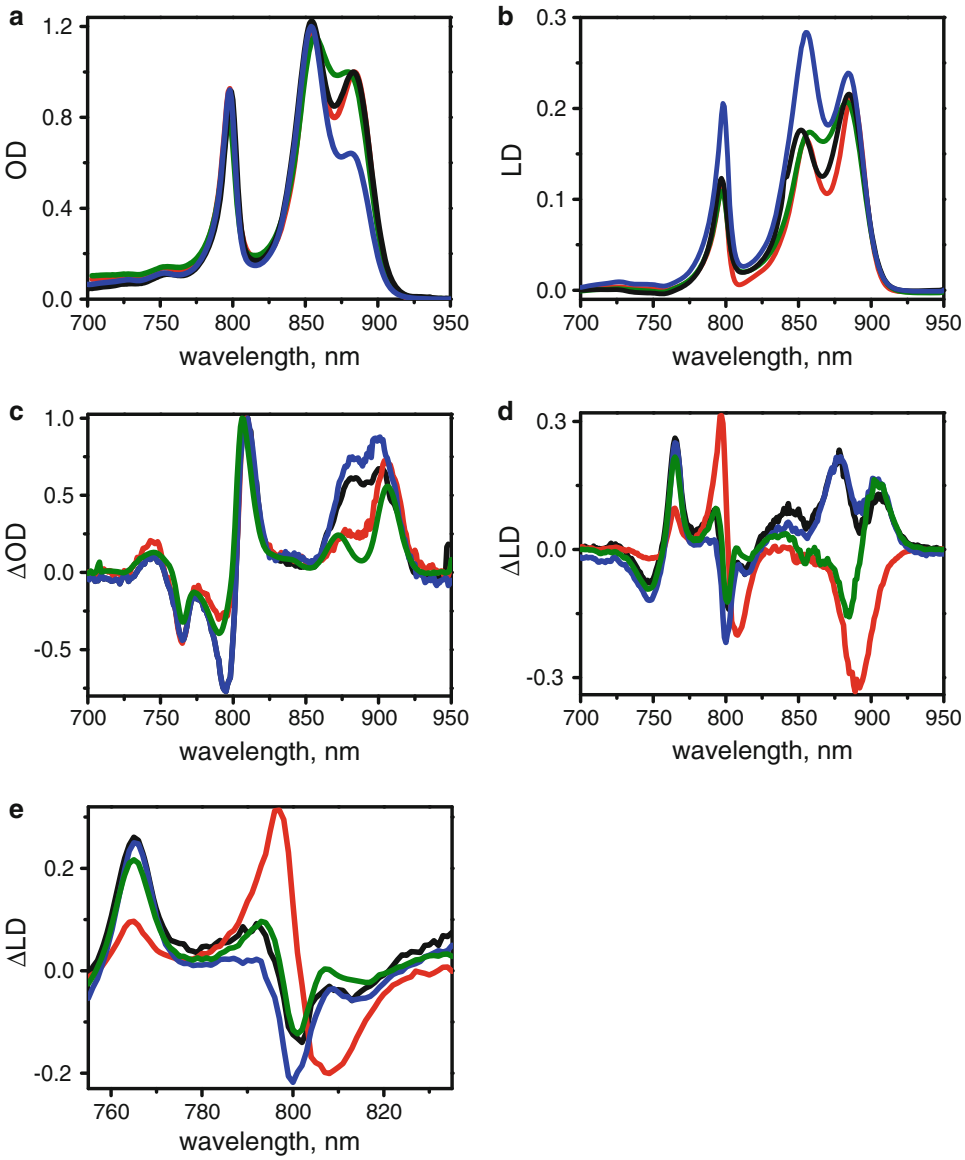

Fig. 4 77 K absorption (**a**), LD (**b**), ΔOD (**c**) and ΔLD (**d**, **e**) spectra of membranes from *Rba. sphaeroides* LH2-containing mutants: RCLH1Xs-g2 (*red*), RCLH1sXd-g (*green*), GX51L-g2 (*black*) and RCLH1sXc-g (*blue*). **b**, **d**, **e** Normalised to the same OD of LH1; **e** magnification of the accessory BChl region of **d**

additional band-shift signals from the LH antennae in these membranes.

To summarise, overall the LD and ΔLD spectra of membranes from the GX51L-g and RCLH1sXc-g mutants were similar to those for the PufX-deficient control and distinct from the spectra of the control strain with a native PufX, indicating a largely unordered arrangement of RCs within the oriented membranes despite the presence of a PufX protein.

LD and ΔLD spectroscopy of membranes containing LH2

It has been shown that clusters of LH2 complexes have membrane curvature-inducing properties (Chandler et al. 2009) and their presence can induce partial order in the arrangement of RC–LH1 complexes even in membranes lacking extensive intrinsic molecular ordering (Frese et al. 2004). To examine to what extent this effect was visible, polarised spectroscopy was performed on intact membranes from an equivalent set of mutants containing LH2.

In general, the spectroscopic analysis of these membranes reinforced the conclusions arrived at with their LH2-deficient counterparts. Absorbance and LD spectra at 77 K are shown in Fig. 4a, b, respectively. In the latter, the RC LD band at 800 nm was obscured by a strongly positive LD band arising from the 800 nm-absorbing BChls of LH2, and so information on the degree of order displayed by RCs in these membranes was derived from ΔOD and ΔLD spectra. As expected, the dark-minus-light absorbance difference spectra (Fig. 4c) were similar to one another, and to the corresponding difference spectra for

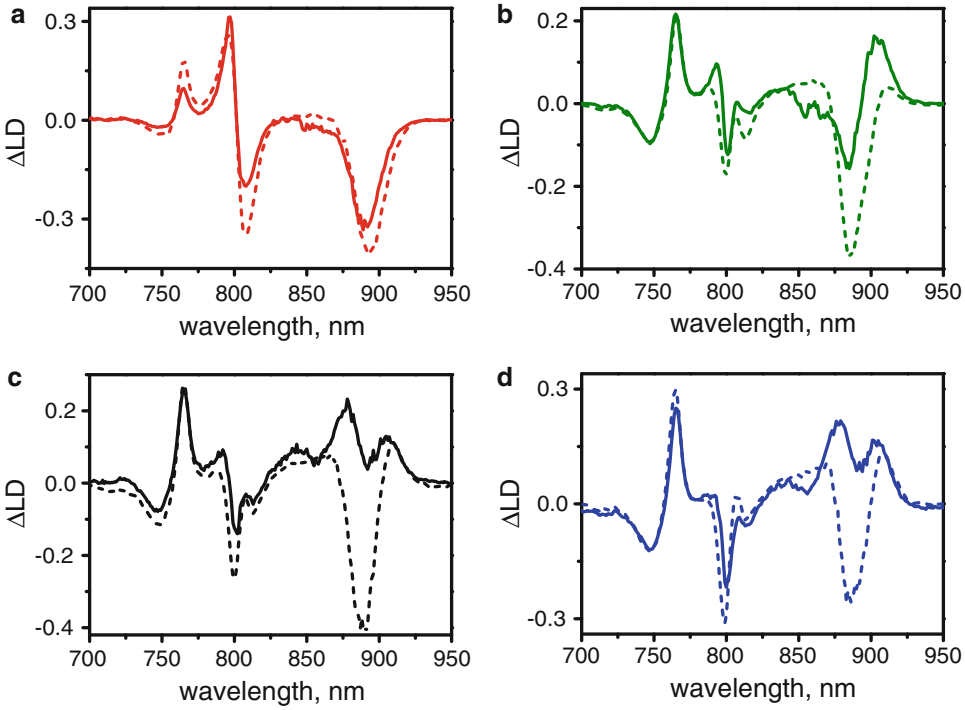

Fig. 5 Effect of LH2 on 77 K ΔLD spectra of membranes of LH2-deficient (*dashed line*) and LH2-containing (*solid line*) *Rba. sphaeroides* strains: **a** RCLH1Xs-g, **b** RCLH1sXd-g, **c** GX51L-g, **d** RCLH1sXc-g

LH2-deficient membranes (Fig. 3d), with a light-induced blueshift of the absorbance band of the RC BPhes at 760 nm, red-shift of the absorbance band of the RC monomeric BChls at 802 nm and a split absorbance increase in the region of the RC P dimer around 890 nm.

The ΔLD spectrum of membranes from strain RCLH1sXs-g2, with a native PufX, exhibited the features diagnostic of an ordered membrane with a pronounced negative LD signal at 890 nm (Fig. 4d) and a strong spectral blueshift in the region around 802 nm (shown expanded in Fig. 4e). Consistent with findings with LH2-deficient membranes, this band-shift was reversed in the ΔLD spectra of the remaining three strains, with a trough around 800 nm and a weak positive lobe at around 808 nm. This pattern was consistent with the general conclusion that the order in the arrangement of RCs in the membranes of the strain containing a native PufX was largely lost in membranes from the remaining strains with either a modified PufX or lacking a PufX. Small differences between the ΔLD spectra for these latter strains probably reflected different degrees of residual order in these membranes, as discussed below.

Direct comparisons between ΔLD spectra of LH2-deficient and LH2-containing membranes are presented in Fig. 5. All membranes exhibited signs of an ordering influence of LH2, visible as a change in the magnitude of the band-shift of the RC accessory BChls. As observed previously (Frese et al. 2004, 2008), changes in membrane shape due to the presence of the curvature-inducing LH2 protein is signalled by a change in the magnitudes of the

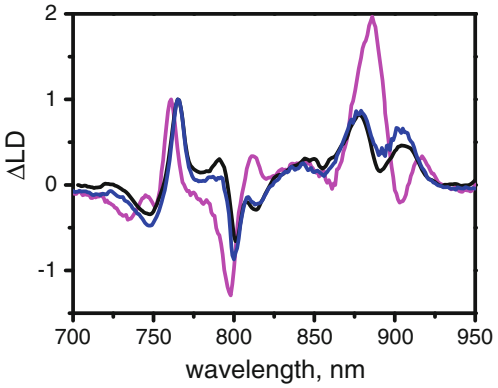

Fig. 6 Comparison of 77 K ΔLD spectra of *Rba. sphaeroides* LH2-containing mutants: GX51L-g2 (*black*) and RCLH1sXc-g2 (*blue*) and 77 K ΔLD spectra of *Rba. capsulatus* WT (*magenta*)

BPhe and BChl band-shifts (Fig. 5a). This organisational influence was most pronounced in the RCLH1sXd-g2 membranes (Fig. 5b), where there was a clear contribution of negative accessory BChl LD to the spectra. A similar change was evident in the membranes of GX51L-g2 (Fig. 5c). The RCLH1sXc-g2 mutant (Fig. 5d) was least affected by the presence of LH2, still exhibiting a strong finger-print of disorder in the form of positive LD of accessory BChl, despite having a higher ratio of LH2 to LH1 complexes than the remaining strains.

It is also interesting to note that when comparing the ΔLD spectra of membranes containing the *Rba. capsulatus* PufX with the GX51L-g2 mutant and the wild-type *Rba. capsulatus* (Fig. 6), the membranes with the single point

GX51L mutation had the smallest degree of disorder of those three, confirming the different organisational features between those membranes.

Discussion

Use of LD and ΔLD spectroscopy to probe organisation of photosynthetic membranes

The aim of the present study was to use LD and dark-minus-light ΔLD spectroscopy to probe the degree of organisation of RC–LH1 complexes within intact photosynthetic membranes, to throw further light on the observation that dimeric RC–LH1 complexes could not be isolated from wild-type *Rba. capsulatus*, from *Rba. sphaeroides* expressing the *Rba. capsulatus* PufX, or from *Rba. sphaeroides* expressing the native PufX with a single amino acid change of glycine 51 to leucine. In previous work, it has been shown that polarised light spectroscopy on oriented samples of membrane produces distinctive spectral features arising from the RC BChls that can be correlated with the order that arises from packing of dimeric RC–LH1 complexes into larger regular arrays (Frese et al. 2000, 2004, 2008). This macroscopic organisation is seen most spectacularly in membranes from LH2-deficient strains of *Rba. sphaeroides*, where RC–LH1 dimers pack into extensive, highly ordered arrays, the curved shape of the RC–LH1 dimer in the plane of the membrane leading to the formation of extensive tubular membranes that can run the entire length of a bacterial cell (Westerhuis et al. 2002; Chandler et al. 2008; Qian et al. 2008), or even connect cells (Kiley et al. 1988). In LH2-containing strains, the presence of the peripheral antenna complexes limits the extent of aggregation of RC–LH1 dimers, but nevertheless ordered arrays of several dimeric RC–LH1 complexes form and, in an oriented sample, give rise to characteristic spectral features in LD and, in particular, ΔLD spectra.

Previous studies on oriented *Rba. sphaeroides* membranes containing native RC–LH1 complexes have revealed that the Q_y transition of the P BChls of the RC is oriented almost perpendicular to the long axis of the membrane (Frese et al. 2000, 2004, 2008). This preferential orientation is characterised by a negative LD band arising from the RC accessory BChls at around 800 nm, a differential band in this region (blue positive/red negative) in the ΔLD spectrum, and a strong negative ΔLD band for the RC P BChls at around 890 nm (Frese et al. 2000, 2004). Key to the interpretation of these LD and ΔLD spectra as being signatures of long-range order in these oriented membranes was a comparison with the LD and ΔLD spectra of membranes from PufX-deficient variants of *Rba. sphaeroides*. For a PufX-deficient strain also lacking LH2 the sign of the negative LD band and differential ΔLD band around 800 nm was reversed, and the negative ΔLD band at 890 nm was replaced by two positive bands at around 870 and 910 nm. This was explained by the LH1 antenna forming a complete circle of pigment protein around the RC in the absence of PufX, leading to a loss of RC–LH1 dimers and a random orientation of RCs within the surrounding LH1 ring. These initial observations with LH2-deficient membranes were subsequently extended to oriented LH2-containing membranes from the wild-type *Rba. sphaeroides* (Frese et al. 2004), the ΔLD spectrum showing the differential blue positive/red negative RC signature around 800 nm and strong negative signature at 890 nm both indicative of order in the orientation of the RC bacteriochlorins relative to the axis of orientation. Wild-type *Rba. sphaeroides* produces the classical, spherical chromatophore membrane vesicles, and the fact that a polarised light spectrum indicating a high degree of RC order was obtained with these suggested that these membrane had undergone alignment during the two dimensional squeezing process used to produce alignment in the sample. It was reasoned that arrays of several RC–LH1 dimers cause elongation of these spherical membrane vesicles in one dimension, producing a more elliptical structure that then underwent alignment during the squeezing process.

Membrane organisation in wild-type *Rba. sphaeroides* and *capsulatus*

In the present case, although oriented samples of membranes isolated from cells of wild-type *Rba. sphaeroides* and wild-type *Rba. capsulatus* gave broadly similar absorbance and absorbance difference spectra, their LD and ΔLD spectra were sufficiently distinctive to permit the general conclusion that the order in the arrangement of RCs characteristic of native *Rba. sphaeroides* membranes was not present in the *Rba. capsulatus* membranes. There could be three possible reasons for this. First, if the gross morphology of the membranes were different in the two species then it could be that the *Rba. capsulatus* membranes were not oriented by the squeezing process. This seems unlikely, as *Rba. capsulatus* and *Rba. sphaeroides* are known to have very similar membrane architectures, producing near-spherical chromatophores with a diameter of a few tens of nm. A second possibility is that *Rba. capsulatus* assembles dimeric RC–LH1 complexes that do not associate into arrays, and therefore do not contribute to asymmetry of the chromatophore vesicle. The presence of short ordered arrays in *Rba. sphaeroides* induces an orientation of the almost spherical chromatophores by the gel squeezing technique required for the LD measurements and the absence of such arrays in *Rba. capsulatus* prevents such

preferential orientation and so producing polarised light spectra lacking signals that can be attributed to order. Finally, it is possible that, as proposed recently (Crouch and Jones 2012), dimeric RC–LH1 complexes do not assemble in *Rba. capsulatus*, with the result that again membrane vesicles do either not orient, or do orient but the component RC–LH1 monomers are not ordered with respect to the direction of the polarised light beams.

Both wild-type strains of *Rba.* were grown under both photosynthetic and semiaerobic/dark conditions. For membranes of *Rba. capsulatus* from both light- and dark-grown cells, the signs of ΔLD spectrum generally were reversed with respect to their *Rba. sphaeroides* counterparts implying that the LD signals of the accessory and special pair BChls were positive and thus the RCs were randomly oriented in these membranes. This is consistent with the prediction made from an analysis of RC–LH1 complexes isolated from *Rba. capsulatus*, that the RC–LH1 complex does not assemble in the dimeric form.

There was no difference in the magnitude of the BChl and BPheo band-shifts, and the similar value of overall LD signals implied that the membranes from light- and dark-grown *Rba. capsulatus* were similarly curved. In contrast, when corrected for the intensity of absorbance, the overall intensity of the LD spectra for membranes from dark-grown *Rba. sphaeroides* was approximately half of that of membranes from light-grown *Rba. sphaeroides*. This, due to the fact that the overall intensity of observed LD depends on the amount of orientation, suggests that the latter membranes had twice the macroscopic orientation as the former, implying a stronger deviation from a purely spherical architecture. This interpretation was further supported by the apparent disparity in the magnitudes of BPheo ΔLD band-shifts in dark- and light-grown *Rba. sphaeroides* membranes. Such occurrence, observed before between photosynthetically-grown *Rba. sphaeroides* containing and lacking LH2, originates in increased anisotropy due to curvature in the former membranes (Frese et al. 2000, 2004). One explaining factor could be relative amounts of RC–LH1 and LH2, but there is little or no difference reported in size of chromatophores from *Rba. sphaeroides* strains grown photosynthetically under low or high-light conditions, which affects the relative amounts of RC–LH1 and LH2 (Sturgis and Niedermann 1996; Adams and Hunter 2012). It is thus unlikely that any shape difference between chromatophores of light- or dark-grown *Rba. sphaeroides* inferred from the present data is solely due to the LH2 content. On the other hand, there is a distinctive difference between diameters of chromatophores from cells grown under light and dark conditions (Golecki et al. 1989) so there must be other factors that affect the global curvature of the membranes. An interesting point which may be relevant is that if RC–LH1 complexes are isolated from light-grown wild-type *Rba. sphaeroides* cells and fractionated on sucrose density gradients then almost all are in the dimeric form, whereas when this procedure is repeated using dark-grown cells almost all are in the monomeric form (Crouch and Jones 2012). This suggests differences in the detailed organisation of *Rba. sphaeroides* RC–LH1 complexes in cells grown under different conditions, a subject that is currently under investigation. Finally, the difference in the magnitude of ΔLD signal of the special pair between light- and dark-grown membranes of *Rba. sphaeroides* points to more complex implications. First, the dark-grown strain contains enormous amount of LH2 relative to LH1 and to be able to detect any signal of RC a high optical density of the sample is needed. In this particular case, we therefore employ the ΔLD spectra only non-quantitatively as a marker of the presence or absence of order in the membrane. Admittedly, the photo-oxidated-P-induced band-shifts of BPheo and accessory BChl are a complicated effect. They depend not only on the lower exciton Q_y transition but also on a an upper one as well as on membrane shape.

Effects of manipulation of PufX on membrane organisation

The second purpose of the present study was to investigate the organisation of RC–LH1 complexes in intact membranes of strains harbouring alterations to the PufX protein. The two alterations studied, a single point mutation in the *Rba. sphaeroides* PufX and replacement of the *Rba. sphaeroides* PufX by its *Rba. capsulatus* counterpart, showed the same phenotype, only monomeric RC–LH1 complexes being extractable from photosynthetic membranes under conditions where high levels of dimeric RC–LH1 complexes can be isolated from an otherwise equivalent strain with the native PufX (Fig. 2 and Crouch and Jones 2012). However, the absorbance spectra of those monomeric RC–LH1 complexes were similar to that of monomers from the control strain with a native PufX, and did not show the elevated absorbance of LH1 relative to the RC that is characteristic of RC–LH1 monomers lacking a PufX. Furthermore, the maximum of LH1 absorption band in those strains was blue-shifted with respect to the membranes lacking PufX, which is characteristic for a closed circle of excitonically coupled BChls (Hu et al. 1997, 2002). The conclusion was therefore, both with and without the presence of LH2, that these strains harbouring changes to PufX assemble monomeric RC–LH1 complexes with a normal composition and a gap in LH1.

Low temperature absorbance, LD, absorbance difference and ΔLD spectroscopy were first applied to membranes from strains lacking LH2. The interpretation of these spectra was straightforward, in that they indicated an

organisation very different from that exhibited by native *Rba. sphaeroides* RC–LH1 complexes but similar to that displayed by PufX-deficient RC–LH1 complexes. In particular, LD spectra contained a positive band at ~800 nm indicative of a lack of order in the orientation of the RC within the ring of LH1 (Frese et al. 2000), and in the ΔLD spectra, there was an inversion of the differential signal at ~800 nm from the sign obtained in the spectrum of membranes from the control strain with a native PufX. In the case of strains possessing LH2, it was not possible to assess the degree of order from the 800 nm region of the LD spectrum due to a strong positive band arising from the LH2 800 nm BChls; however, the same pattern of positive and negative bands around 800 nm was seen in the ΔLD spectra. These findings were consistent with the absence of ordered dimeric RC–LH1 complexes in membranes of strains expressing either the GX51L variant of the *Rba. sphaeroides* PufX or the *Rba. capsulatus* PufX.

Ordering effect of LH complexes on chromatophores

Given the above, it is notable that there were small differences in the magnitude of the accessory BChl band-shift in the ΔLD spectra (as seen in Figs. 3f, 4e) implying that partial order might exist in the membranes with an altered or deleted PufX. In the ΔLD signal of both LH2-deficient and LH2-containing membranes, the accessory BChl band-shift of the mutant with the *Rba. capsulatus* PufX was reversed in sign with respect to the control strain with a native PufX, a characteristic of an unordered membrane. The magnitudes of the band-shift in spectra of the PufX-deficient and GX51L mutants were between those two extremes, indicating the presence of some contribution from a negative LD for the accessory BChl. The mixed-sign contribution to LD was also visible in the ΔLD spectra of the LH2-less membranes at ~890 nm. This in turn means that there are ordered and disordered regions of membrane present simultaneously in the preparations. The reason for this could, in principle, be that some RC–LH1 complexes in membranes with an altered or deleted PufX are in fact in dimeric form that induces order in those membranes. This however seems unlikely, given that the ΔLD signal of dark-grown control strain *Rba. sphaeroides* membranes, that are known to produce only small proportion of RC–LH1 dimers on extraction as mentioned earlier (Crouch and Jones 2012), carries strong spectral fingerprint of organisation. Moreover, there were no dimeric complexes found in sucrose gradient and, we expect, if there were ordered dimeric core complexes present in the chromatophores in question, spectral features indicating order in their ΔLD spectra would be much more pronounced. Instead, it seems likely that, at least in the LH2-less membranes, this ordering effect comes from the LH1, which is elliptical and thus can induce a degree of ordered packing of monomeric RC–LH1 complexes. The presence of hexagonally clustered monomeric core complexes in PufX-deficient mutants of *Rba. sphaeroides* is well known (Siebert et al. 2004; Adams et al. 2011) and it is possible that the dense packing can induce some degree of order in this situation.

The ordering effect of LH2 complexes on membranes of *Rba. sphaeroides* has been observed before in PufX-deleted strains (Frese et al. 2008), where the packing of LH2 has induced partial order with about half of the chromatophores containing organised domains of oriented RCs resulting in a ΔLD spectrum that was a linear combination of PufX-containing and PufX-devoid LH2-less ΔLD spectra. Here, we cannot quantify the amount of order in partially ordered membranes by simple arithmetical combination of the completely ordered and completely unordered membranes. However, were we to use this combination to make a rough estimate of amount of order in the samples, we would arrive at the conclusion that in the mutant with a *Rba. capsulatus* PufX, the LH2-deficient membranes are at least in 90 % disordered, and the LH2-containing membranes are at least in 80 % disordered, whereas the LH2-deficient and LH2-containing membranes with the GX51L PufX are 80 and 70 % disordered, respectively.

The presence of a varying degree of disorder in membranes from the mutant strains has direct ramifications with respect to the influence of the PufX protein on the supramolecular organisation of bacterial membrane. It seems that the earlier assumption of a direct involvement of PufX in membrane ordering is somewhat overreaching. It is a fact that in membranes that contain only RC–LH1 complexes, PufX has been shown to determine both dimerization and long-range order. If however PufX indeed had, by itself, an ordering effect we would see it here in LH2 containing strains expressed as a much stronger rise of order in membranes with a point-mutated PufX then in membranes with deleted PufX, when comparing to strains without LH2. The intriguing fact that this is not a case points to a conclusion that the only influence the PufX exerts on ordering of the membrane is via facilitation of dimerization of core complexes which, having one long symmetry axis and being bent along the dimer interface (Qian et al. 2008), succumb to an ordering process governed by entropy (Eldridge et al. 1993; Frese et al. 2008; Hsin et al. 2009b).

Conclusions

The results presented in this study reinforce the findings of previous work that RC–LH1 complexes are unlikely to

assemble as dimers in *Rba. capsulatus* in vivo, their chromatophores having the spectral signature of unordered membranes.

Likewise, LD and ΔLD spectra in combination with sucrose gradients data indicate that neither the strains of *Rba. sphaeroides* containing the *Rba. capsulatus* PufX, nor the strains with a GX51L mutation in the native PufX, assemble ordered dimers.

Finally, we have observed LD signals that are combinations of positive and negative LD, reflecting the simultaneous existence of ordered and disordered regions of membrane in the strains with an alteration in PufX. That such effect is visible in LH2-less membranes, together with the fact that the amount of this order varies between the PufX-altered strains, may imply that the creation of ordered RC–LH1 domains depends not only on the presence of RC–LH1 dimers but also on a structure of LH1 itself. We therefore conclude that PufX does not play a direct role in ordering of the photosynthetic membrane but rather plays an indirect one, through the configuration of RC–LH1 dimers and that the presence of altered or non-native PufX can influence packing- and curvature-induced ordering.

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