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Two-dimensional crystals of LH2 light-harvesting complexes from *Ectothiorhodospira sp.* and *Rhodobacter capsulatus* investigated by electron microscopy

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Abstract

Two-dimensional crystals of LH2 (B800-850) light-harvesting complexes from *Ectothiorhodospira sp.* and *Rhodobacter capsulatus* were obtained by reconstitution of purified protein into phospholipid vesicles and characterized by electron microscopy. The size of the crystals was up to several micrometers. The crystals of LH2 from *Ectothiorhodospira sp.* were with a square unit cell ($p42_12$ plane group symmetry) and a repeat of 9.9 nm. By image processing of negatively stained crystals a projection map was obtained at 1.8 nm. It shows complexes with an outer diameter of about 6.5 nm and with an octagonal appearance. Crystals of *Rb. capsulatus* have a hexagonal unit cell with a repeat of 8.1 nm and a resolution of 2.7 nm. A ring-like complex with an outer diameter of about 7 nm and an inner stain-filled indentation with a diameter of 2 nm was obtained. This structure is similar to the one of LH2 from *Rhodopseudomonas acidophila*, determined at atomic resolution (McDermott et al. (1995) Nature 374, 517-521), which is a multimer of 9 α- and β-subunits. Taken together, the data indicate that isolated LH2 from *Rb. capsulatus* is a nonamer, whereas LH2 from *Ectothiorhodospira sp.* is an octamer.

Keywords: Light-harvesting complex; Photosynthesis; Two-dimensional crystallization; Purple bacterium; Electron microscopy; (*Ectothiorhodospira sp.*); (*Rb. capsulatus*)

1. Introduction

The light-harvesting complexes of the chromatophore membrane of photosynthetic bacteria are responsible for the collection and transfer of light energy to the photosynthetic reaction center [1]. Three different types of light-harvesting antenna complex, LH1, LH2 and LH3, have been described and isolated from various species of purple bacteria. All three antenna complexes consist of two types of subunit, α and β, each comprising about 50 amino acids. Together they form a heterodimeric unit (or protomer) to which bacteriochlorophyll (BChl) molecules are associated [2,3]. The units can further arrange into larger complexes.

Investigations on the structure of the antenna complexes include X-ray diffraction studies and electron microscopy (EM). LH1 or B875 is the most inner antenna complex and is directly associated with the reaction center. Isolated LH1 from *Rhodobacter (Rb.) sphaeroides* forms ring-like structures which easily collapse and split into two single particles each with a tentative $(\alpha\beta)6$ structure [4]. Crystals of LH1 rings from *Rhodopseudomonas (Rps.) marina* were also considered to contain 12 $\alpha\beta$ units [5]. In association with the reaction center, complexes with 12 $\alpha\beta$ units have been found for *Ectothiorhodospira halochloris* and *Rhodospirillum (Rs.) molischianum* [6,7], but more recently

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also the existence of rings without a reaction center but with 16 $\alpha\beta$ units was shown for *Rs. rubrum* [8].

LH2 or B800-850 forms a more peripheral antenna complex, which donates its excitation energy to LH1. Isolated LH2 from several bacteria has been crystallized [9]. Recently, the structure of the LH2 complex from *Rps. acidophila* was solved at atomic resolution [10]. It consists of a ring of 9 $\alpha\beta$ heterodimers. The nine α subunits are packed in an inner ring to form a hollow cylinder of radius 1.8 nm. The 9 β-subunits are arranged radially with respect to the α subunits to form an outer ring with an outer radius of 6.8 nm. A ring of 18 B850 BChl molecules are sandwiched between the α- and β-subunits and a further 9 B800 BChl molecules are positioned between the outer β-subunits [10]. From other LH2 structures only low-resolution information is present. On the other hand, a high-resolution structure exists for the major antenna complex of green plants, LHCII [11], which is very distinct from prokaryotic LH2. LH2 from *Rb. sphaeroides*, with an estimated outer diameter of 5.1 nm, is thought to be a hexameric structure with 6 $\alpha\beta$ units [4]. Mass determination by analytical ultracentrifugation indicated that the

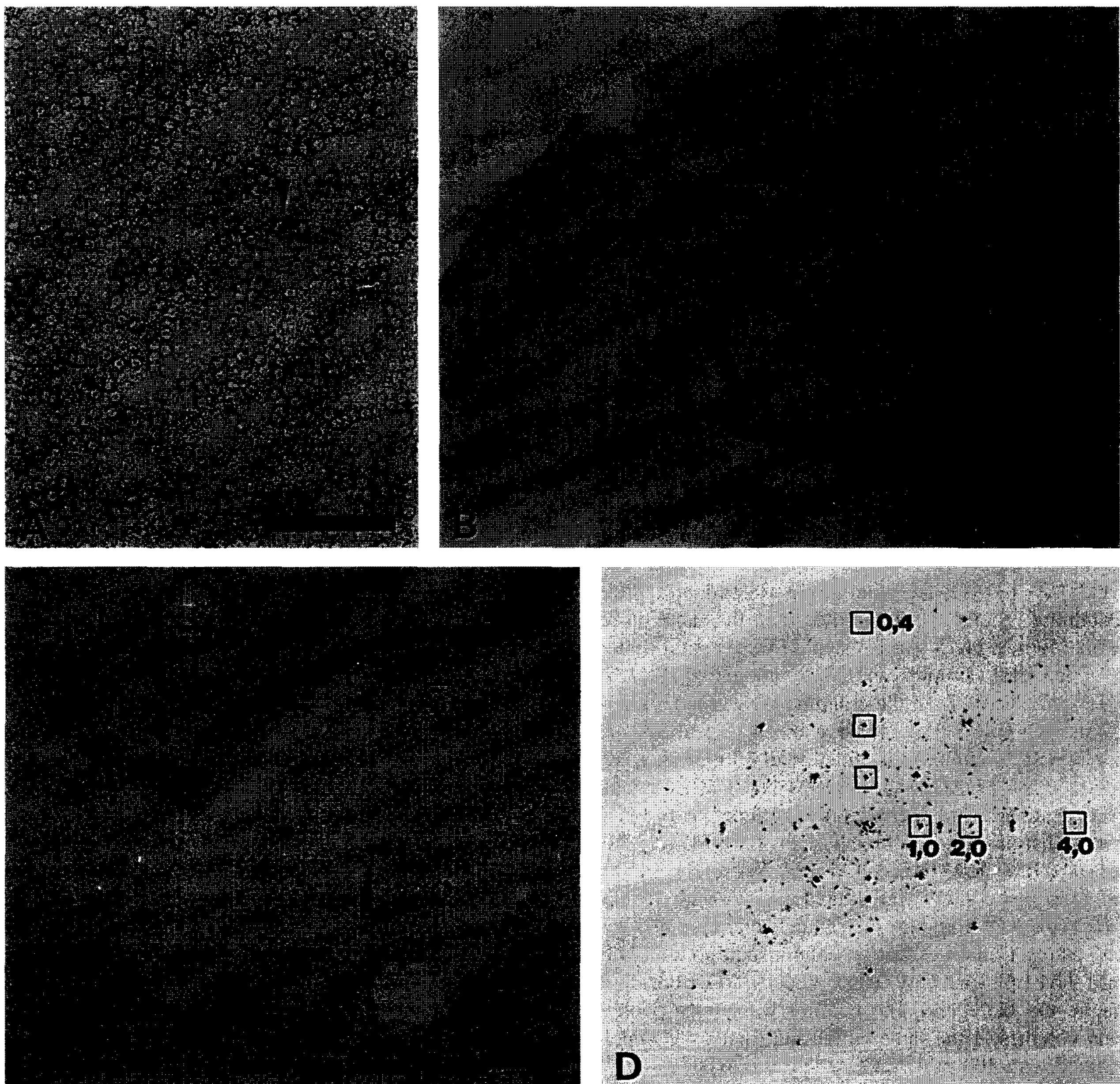

Fig. 1. Electron microscopy of *Ectothiorhodospira sp.* (A) single particles, negatively stained with 2% uranyl acetate. The arrow points to a larger particle consisting likely of a reaction center surrounded with LH1, this impurity consisted about 0.3% of the material; (B) Overview of a crystalline 2-D sheet; (C) single-layer part of the crystal of B at higher magnification; (D) computer-generated Fourier transform of a single-layer crystal. The scale bars in (A)–(C) represent 100 nm. Some peaks in (D) have been marked with a square.

LH2 complex from *Rs. molischianum* should be an octamer [12]. Thus, it seems that, depending on the source and possibly on the isolation procedure, LH2 can exist in various association states.

In the LH2 structure solved by X-ray diffraction exact 9-fold symmetry was observed, which means that all α- and β-subunits are at identical and equivalent positions [10]. Whether this is generally true for all types of LH2 needs to be established. To answer such questions, EM and crystallographic averaging was applied to two-dimensional (2-D) crystals which were obtained from the purple photosynthetic bacteria *Ectothiorhodospira sp.* and *Rb. capsulatus*.

2. Materials and methods

2.1. Isolation of the antenna complexes

The LH2 antenna complex from *Ectothiorhodospira sp.* was isolated as described in [13] with some modifications. A chromatophore suspension ($A_{857} = 5$) was mixed with an equal volume of 2% (v/v) lauryl dimethylamine *N*-oxide (LDAO) in 50 mM phosphate (pH 7.5) at 4°C in the dark. After 1 h incubation the suspension was centrifuged at $110000 \times g$ for 90 min. The pellet was resuspended in 50 mM phosphate (pH 7.5) to a final $A_{857} = 50$, and incubated in the presence of 1% LDAO at 4°C in the dark. After 1 h incubation the suspension was centrifuged at $110000 \times g$ for 90 min. The supernatant was dialyzed for 12 h against 10 mM Tris-HCl (pH 8.0) and loaded onto a Fractogel TSK-DEAE 650S anion-exchange column equilibrated with buffer A containing 10 mM Tris-HCl (pH 8.0) and 0.1% LDAO. The column was washed with buffer A containing 75 mM NaCl and then with the same buffer but with 150 mM NaCl. At this step all the reaction center, most of the LH1 complex and some amount of the LH2 complex are eluted. Then the column was washed with 10 mM Tris-HCl (pH 8.0) to remove the detergent and the remaining LH2 complex was eluted with 10 mM Tris-HCl (pH 8.0), 0.2% LDAO, and 400 mM NaCl. This sample was concentrated and loaded onto a Superose-6 FPLC column (Pharmacia) equilibrated with 10 mM Tris-HCl (pH 8.0), 0.05% LDAO and 400 mM NaCl. The sample eluted from this column was diluted 5-times with 10 mM Tris-HCl (pH 8.0) to reduce the salt concentration and was loaded onto an anion-exchange column equilibrated with 10 mM Tris-HCl (pH 8.0), 0.05% dodecyl β-maltoside to exchange the detergent. The column was rinsed with 3 column volumes of the same buffer and finally the sample was eluted with 400 mM NaCl.

LH2 from *Rb. capsulatus* was isolated as described in Welte et al. [14]. Protein concentrations were calculated from BChl absorption; a value of $A_{1cm\ 800nm} = 120$ corresponds to 10 mg/ml [14].

2.2. Crystallization

For 2-D crystallization, LH2 was reconstituted with soybean L-α-phosphatidylcholine (Sigma) or β-linoleoyl-γ-palmitoyl-L-α-phosphatidylcholine (Sigma). From aliquots of these lipids dissolved in chloroform, chloroform was removed by drying with argon gas. A buffer containing 10 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$ and 0.03% dodecyl β-maltoside was added to a final lipid concentration of 2 mg/ml. The lipid solution was placed on a Vortex stirrer for 10 s and used without further sonification or other treatment. Crystallization experiments were carried out by equilibrium dialysis in glass capillaries with an internal diameter of 1 mm.

2.3. Electron microscopy and image analysis

Images were recorded with a Jeol JEM 1200-EX electron microscope using 80 kV at 40000–60000 × magnification. Images were digitized with a Kodak Eikonix Model 1412 CCD camera. Image processing of 2-D crystals was carried out with IMAGIC software [15] complemented with programs for crystallographic averaging provided by V. Mallouh (Strasbourg).

3. Results

3.1. LH2 from Ectothiorhodospira sp

To check the quality of the material, LH2 samples were prepared for EM as single particles. Negatively stained specimens (Fig. 1A) show that it was almost free from impurities. Only a few larger particles were present (one marked by arrow), which are likely to be reaction centers surrounded by a ring of the LH1 antenna subunits because they had similar size and features as reaction center-LH1 complexes from *Rs. molischianum* [7].

2-D crystals were obtained by reconstitution of LH2 into phosphatidylcholine (PC) vesicles by means of equilibrium dialysis in glass capillaries. Large crystals could be grown at a protein concentration of 0.1 mg/ml and a protein-to-lipid ratio (mg/mg) of 1:3–4. The best results were obtained when detergent was removed by dialysis at 22°C. Detergent removal with Bio-Beads [16] turned out to result in crystals of poorer quality. The type of PC used was not of influence on the crystal quality. Crystals appeared after 1–2 days and crystalline sheets or vesicles measured up to several micrometers in diameter (Fig. 1B). They were of a mosaic type in which the largest homogeneous domains had a diameter of about 300 nm.

Three images of crystals were selected by optical diffraction and processed. Their Fourier transforms showed that LH2 crystals had a square unit cell with an average

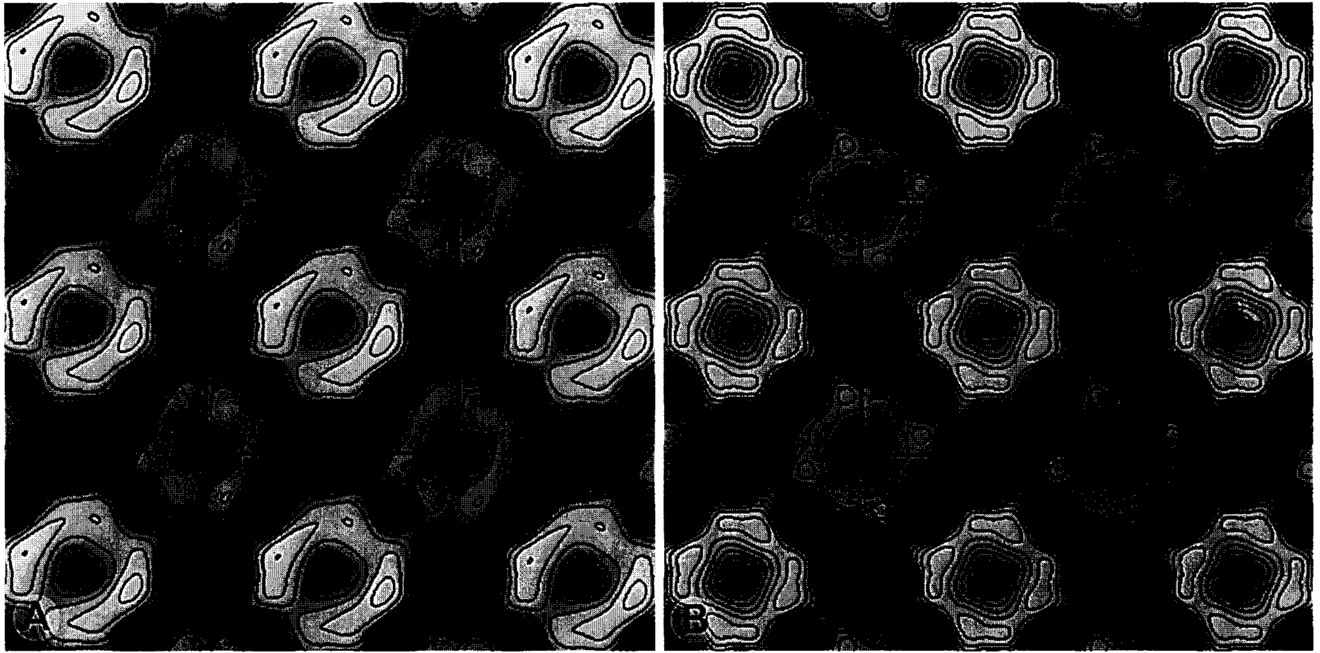

Fig. 2. Projection density map at 1.8 nm resolution of *Ectothiorhodospira sp.* LH2, (A) without applying symmetry and (B) with p4 symmetry imposed. The square unit cell of 9.9 × 9.9 nm (indicated by solid lines) contains two square-like LH2 complexes, which are oriented upside-up and upside-down in the membrane. The crystals have in fact the higher $p42_12$ symmetry, but this symmetry was not imposed on the unit cell to show the uneven stain distribution around the differently oriented complexes. Also shown are the 4-fold axes normal to the membrane plane (solid squares).

unit cell dimension of 9.9 nm and diffracted up to 1.8 nm (Fig. 1D). The phase and amplitude values indicated that the crystal has at least the symmetry of the plane group p4 (Table 1). The structure factors of Table 1 were used to construct 2-D projection maps without (Fig. 2A) and with (Fig. 2B) p4 symmetry imposed. The maps show square-like particles, with an outer diameter of about 6.5 nm and with a central stain-filled indentation, about 1.7 nm in diameter. Two particles with similar size and shape but with a different stain distribution are found within the unit cell. As single complexes of LH2 observed in the presence of detergents have a diameter of about 9 nm (Fig. 1A), we conclude that each unit cell contains two square-shaped LH2 complexes. The strong difference in stain distribution is likely to be due to the presence of LH2 complexes in two opposite orientations (up and down) in the crystal. The symmetry of the crystal is therefore likely to be $p42_12$. The presence of (0,1) and (1,0) reflections, which should be absent in $p42_12$ symmetry, is likely to result from an uneven staining of the two sides of the crystal. Preliminary results by cryo-EM of unstained crystals indicate indeed the absence of such reflections (data not shown).

3.2. *LH2 from Rb. capsulatus*

Ordered domains of LH2 from *Rb. capsulatus* were grown at 22°C with essentially the same method as described for LH2 from *Ectothiorhodospira sp.* The final protein concentration was 2 mg/ml and the protein-to-lipid ratio was 1–1.4 to 1. Large vesicles were obtained (Fig. 3A). Diffraction patterns show that the crystals have a hexagonal lattice, with unit cell dimensions of 8.1 × 8.1 nm and $\gamma = 60°$ (Fig. 3B). The ordering of the crystals is, however, of lesser quality than for LH2 from *Ectothiorhodospira sp.*, and the resolution was restricted to 2.7 nm. A Fourier synthesis shows the LH2 structure to be ring-like with an outer diameter of about 6–7 nm and with a central stain filled indentation with a diameter of 2 nm (Fig. 3C). Due to the limited resolution, the symmetry type of the crystals is difficult to determine. Thus, the unit cell in Fig. 3C is drawn with the most simple type of symmetry, which is p1.

4. Discussion

The molecular features of LH2 from *Rb. capsulatus*, as seen in the 2–D projection at low (2.7 nm) resolution (Fig. 3C), are compatible with those of LH2 from *Rps. acidophila*, which were determined at atomic (0.25 nm) resolution [10]. About the same outer diameter (6.8 nm) and diameter of the inner hole (1.8 nm) were found. Furthermore, the hexagonal lattice suggests that LH2 from *Rb. capsulatus* also has a nonameric assembly of $\alpha\beta$ units, but the apparent 9 subunits are not resolved in the projection map (Fig. 3C). Since individual units have a mass of only 15 kDa, it can be estimated that about 1 nm resolution should be obtained to resolve them by EM. This has not

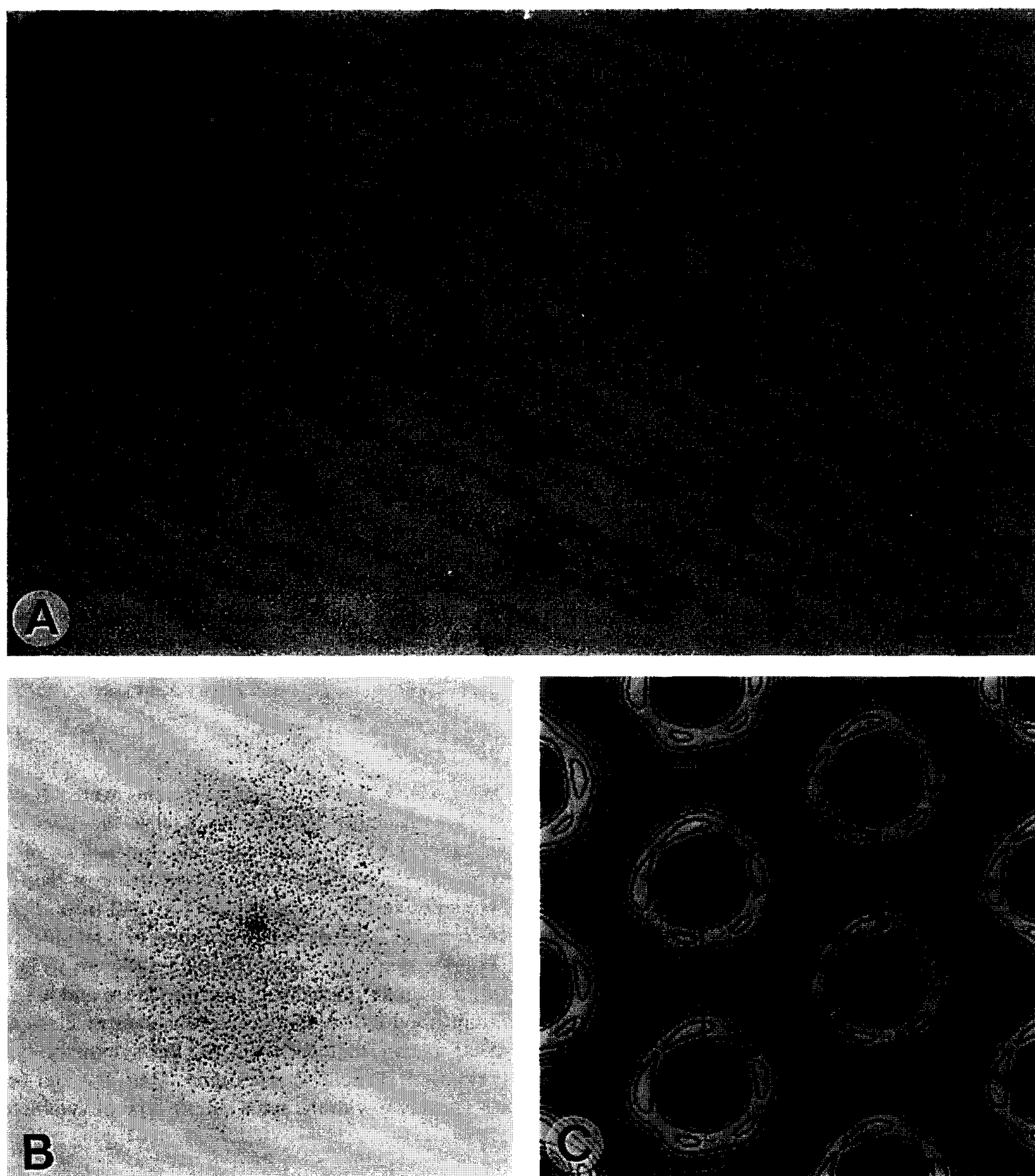

Fig. 3. (A) Electron micrograph of ordered domains of LH2 from *Rb. capsulatus*, obtained by reconstitution in phosphatidylcholine vesicles. Vesicles were negatively stained with uranyl acetate; (B) Fourier transform of a representative part of the image of (A); (C) 2-D projection map at 2.7 nm resolution. The unit cell (8.1 × 8.1 nm) is indicated by solid lines. The crystal symmetry could not be determined at the present resolution. The scale bar in (A) marks 100 nm.

been achieved, but even at low resolution (1.8 nm), it is obvious that LH2 from *Ectothiorhodospira sp.* has a different structure, because image processing indicates square-like complexes with an octagonal appearance, packed in a crystal with $P42_12$ symmetry (Fig. 2). Moreover, the repeating center-to-center distance of LH2 complexes in the *Ectothiorhodospira sp.* crystals is 7 nm, which is 1.1 nm smaller than the repeat in the *Rb. capsulatus* crystals. Such a difference is significant, because the crystal lattice repeats can be determined with a precision of a few tenths of a nanometer, even if the resolution of molecular features is limited to only 2–3 nm. From these data we conclude that *Ectothiorhodospira sp.* LH2 has an $(\alpha\beta)8$ configuration. This extends mass determination experiments by ultracentrifugation on LH2 from *Rs. molischianum*, the first LH2 that was proposed to have

Table 1
List of structure factors for LH2 2-D crystals from *Ectothiorhodospira sp.*

h	k	Ampl. obs.	Ph. obs.	Ampl. p4	Ph. p4
0	1	591	170	592	180
1	0	592	177	592	180
1	1	1594	0	1448	0
1	−1	1302	0	1448	0
0	2	589	−172	561	180
2	0	532	178	561	180
1	2	419	−1		
2	1	375	−4	359	0
−1	2	343	−1	359	0
2	2	1380	177	1247	180
2	−2	1113	179	1247	180
1	3	227	−177		
3	1	195	150		
0	4	279	180	306	180
4	0	333	178	306	180
1	4	209	172		
2	4	392	5	288	0
4	−2	185	−2	288	0
4	2	340	5	267	0
−2	4	194	11	267	0
5	3	159	147	131	180
−3	5	104	157	131	180
5	−3	99	171		
6	3	97	−174		

Columns 3 and 4: all factors with a phase differing by no more than 40° from either 0° or 180° after origin refinement for p2 symmetry. Columns 5 and 6: factors with p4 symmetry enforced. Only the pairs for which the (h,k) and $(-k,h)$ terms were simultaneously present were taken into account. (The average phase residual after this refinement was 8°, taken into account all 24 terms and 5° for p4 symmetry, taken into account 9 sets of p4-related terms. The phase origin is located at a potential 4-fold symmetry axis). (Ampl. obs. = amplitude observed; Ph. obs. = phase observed (in degrees); Ampl. p4 = amplitude with p4 symmetry enforced; Ph. p4 = phase with p4 symmetry enforced). The factors in the columns 3 and 4 were used to calculate a non-symmetrized map in Fig. 2A. The factors in the columns 5 and 6 were used to calculate the p4 symmetry map in Fig. 2B.

an $(\alpha\beta)8$ configuration [12]. The similarities of the aggregation state of LH2 from these two bacteria were also confirmed by site selection fluorescence polarization experiments (Ortiz de Zarate, Somsen, Picorel, Van Grondelle, unpublished results). Furthermore, the LH2 from *Ectothiorhodospira sp.* has a CD spectrum that is more similar to *Rps. molischianum* than to *Rb. capsulatus* (Ortiz de Zarate, Somsen, Picorel, Van Grondelle, unpublished results).

In LH2 of *Rps. acidophila* all $\alpha\beta$ units are packed in a similar way into a ring-like configuration [10]. If such a type of association is a general principle, one could expect for LH2 from *Ectothiorhodospira sp.* a structure with 8-fold symmetry in which all $\alpha\beta$ units are in equivalent position. This could very well be the case, although the 2-D projection is too low in resolution to show individual units.

In general, it can now be concluded that isolated LH2 of purple bacteria can have a different number of $\alpha\beta$ units. Our present work gives examples of an octamer and nonamer. The octamer of LH2 from *Ectothiorhodospira sp.* extends evidence for an octameric LH2 structure from *Rhodospirillum molischianum* [12]. Single particle averaging indicated that LH2 from *Rb. sphaeroides* is a hexamer [4]. When in the near future more LH2 structures will be solved at high- or low-resolution, it would be interesting to compare their amino acid sequences with special emphasis on determined contact areas between $\alpha\beta$ units, or modelled areas based on the *Rps. acidophila* structure [10]. Possibly, within bacteria species giving rise to the same type of LH2 multimer (octamer vs. nonamer) specific amino acids are important in determining the tendency of aggregation.

The general conclusion that in species of purple bacteria the LH2 complex can have a different number of $\alpha\beta$ units could also be valid for LH1 complexes surrounding the reaction center. In the latter case, however, the fixed shape and size of the reaction center in the membrane likely constraints the number of possibilities. Although a number of 16 $\alpha\beta$ units has been directly determined by EM [8], most investigations come to a number of 12 units [4–6,17]. In this light it is also relevant to note that the ratio of BChl to the reaction center can be accurately determined. For six species of bacteria that only contain a core antenna a constant number of 25 ± 3 antenna BChls is present per reaction center [18]. This number supports the current model in which the core antenna generally consists of 12 $\alpha\beta$ units surrounding the reaction centers. But if LH1 becomes separated from the reaction center it may be possible that artificial ring-like structures with lower [4] or higher numbers of units appear [8]. This may also be very well the case for LH2, and the association state of $\alpha\beta$ units in vivo should be a further point of interest, for both LH1 and LH2.

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