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The structure of the photoreceptor unit of *Rhodopseudomonas viridis*

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The thylakoid membrane of *Rhodopseudomonas viridis* contains extensive, regular arrays of photoreceptor complexes arranged on a hexagonal lattice with a repeat distance of ~130 Å. Single membrane sheets were obtained by mild treatment of the thylakoid fraction with the detergent Triton X-100. Heavy metal shadowing and electron microscopy of isolated thylakoids indicated a strong asymmetry of the membrane, showing a smooth plasmic and a rough exoplasmic side. Fourier processing of rotary-shadowed specimens showed the different surface relief on both sides of the membrane. Structural units on both sides were roughly circular and showed 6-fold symmetry at a resolution close to 20 Å. The structural unit was characterised by a central core that seemed to extend through the membrane, protruding on the exoplasmic side. The core was surrounded by a ring showing 12 subunits on the plasmic side. Rotary-shadowed as well as negatively-stained membranes indicated a handedness of the structure. Treatment of thylakoid vesicles with higher detergent concentrations yielded a fraction of particles showing the same features as Fourier maps of the structural units. The isolated particles therefore appeared to represent structurally intact units of photosynthesis.

Key words: electron microscopy/image analysis/photoreceptor unit/pigment-protein complex/*Rhodopseudomonas viridis*

Introduction

Photosynthesis in plants and bacteria is effected by chlorophyll-protein complexes of the photosynthetic (thylakoid) membrane. Bacterial photosynthetic membranes contain only one photosystem. In comparison with the thylakoids of plants and algae, which have two different photosystems, their structural organisation is simple. They are therefore useful for studying the structure and function of proteins involved in the trapping and fixation of solar energy.

A small class of photosynthetic bacteria using a rare form of chlorophyll, bacteriochlorophyll b, share an unusual feature that makes them attractive to electron microscopists. The thylakoid membranes of these bacteria contain regular, often extensive arrays of photoreceptor units (Garcia *et al.*, 1968; Engelhardt *et al.*, 1983) which are highly suitable for structure analysis by image processing methods.

We have studied the structure of the thylakoid membrane of the most widely known species, *Rhodopseudomonas viridis*, a purple, non-sulphur-containing bacterium that was discovered ~20 years ago (Eimhjellen *et al.*, 1963; Drews and Giesbrecht, 1965, 1966). The photosynthetic apparatus of this bacterium is well characterised. Its reaction centre consists of four polypeptides (Thornber, 1971; Pucheu *et al.*, 1976; Michel, 1982; Jacob and Miller, 1983) of 24 000–38 000 apparent mol. wts. (Jay *et al.*, 1983a) and forms crystals suitable for X-ray structure analysis (Michel, 1982). In addition to the reaction centre polypeptides, the thylakoid membranes contain three smaller polypeptides of 6800, 6100 and 4000 (B1015-α,β,γ) mol. wt. which have been sequenced (Brunisholz *et al.*, in preparation). The homology of these polypeptides with those found in native isolated light-harvesting complexes of related species (Broglie *et al.*, 1980; Cogdell *et al.*, 1982, 1983; Picorel *et al.*, 1983; sequenced by Brunisholz *et al.*, in preparation and Theiler *et al.*, in preparation) implies that they are constituents of the light-harvesting complex (B1015).

Electron microscopy of thylakoid membranes from *R. viridis* (Miller, 1979; Wehrli and Kübler, 1980; Welte and Kreutz, 1982) and related species (Engelhardt *et al.*, 1983) has revealed roughly circular structural membrane units of ~130 Å diameter arranged on a two-dimensional hexagonal lattice. The units appear to consist of a core of 45 Å diameter surrounded by a ring roughly 20 Å wide in projection. It has been assumed that the core represents the reaction centre complex, and that the ring contains the light-harvesting complexes. The structural units can be isolated and therefore exist as separate entities (see preceding paper by Jay *et al.*). The first step in photosynthesis results in a charge separation across the thylakoid membrane. This requires a membrane that is asymmetric at the molecular level. Detailed analysis of negatively-stained and rotary-shadowed thylakoid membranes using electron microscopy and Fourier processing of micrographs shows that the asymmetry of the thylakoid membrane is clearly expressed in the structure of the unit.

Results

Cells of *R. viridis* contain stacks of flattened, appressed thylakoids which are easily isolated by mechanical disruption of the cells and fractionated by centrifugation. Heavy metal shadowing of specimens prepared for electron microscopy shows that the thylakoid fraction is a mixture of stacked and unstacked vesicles, and fragments of both. Two different aspects of the thylakoid membrane can be distinguished. The outer (plasmic) surface of closed, flattened vesicles appears rather smooth (Figure 1a). The hexagonal lattice of structural membrane units is nevertheless visible. Fragmented vesicles reveal the inner (exoplasmic) surface of the thylakoid membrane which appears rough (Figure 1b). The shadow cast at the edge of single membranes indicates a thickness of 60–70 Å. This does not include the particles which give the exoplasmic side its rough appearance. These protrude ~40 Å above the membrane surface. The total thickness of the structural units is thus ~110 Å. The maximum thickness of the structural unit, measured on thin sections of freeze-substituted cells (Müller *et al.*, 1980) is 80–90 Å (results not shown).

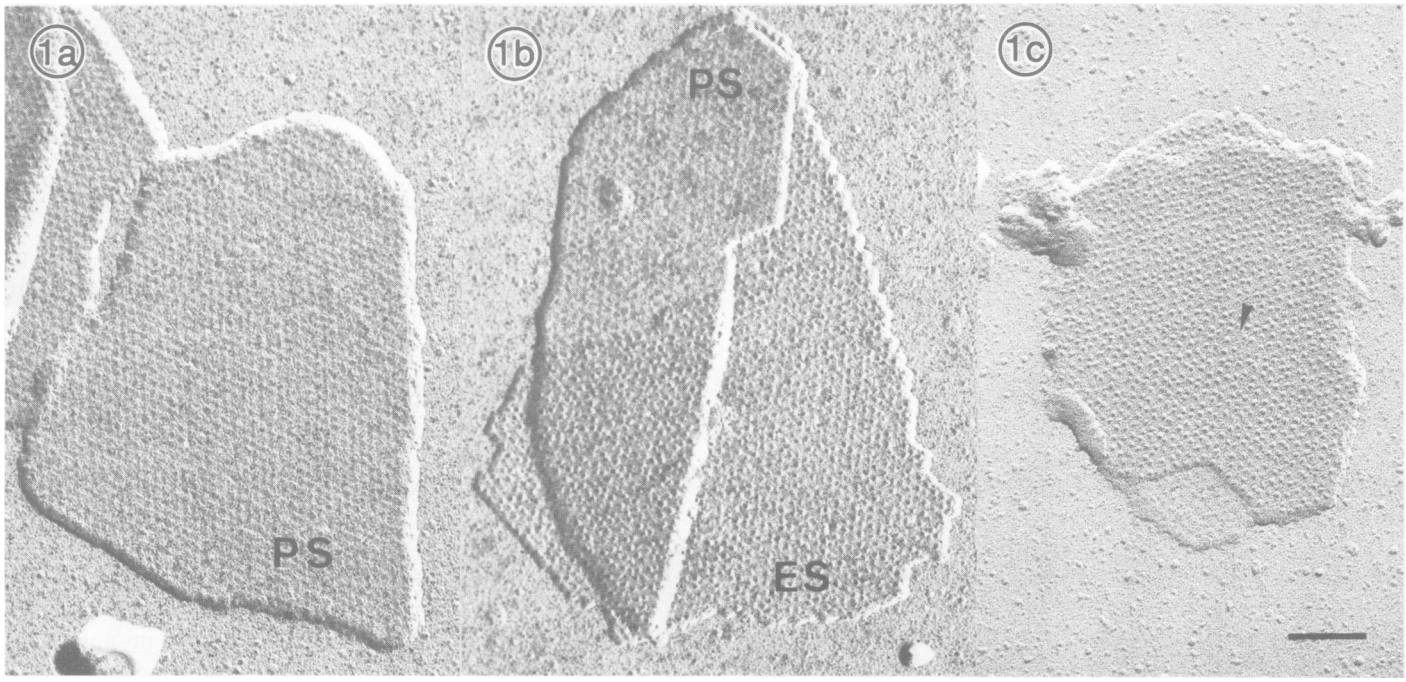


Fig. 1. Electron micrographs of Pt/C shadowed membranes of *R. viridis*. **(a)** A flattened vesicle with its plasmic surface (PS) facing upwards. **(b)** A fragmented vesicle which exhibits both the plasmic (PS) and the exoplasmic side (ES). **(c)** Double membrane with the exoplasmic surface facing upwards and the two plasmic surfaces are in contact. The lattices of both membranes are in register as is often observed. Occasionally, the protruding particles on the exoplasmic surface are absent, see arrow. Scale bar = 1000 Å.

A close examination of the shadowed rough membrane surface shows that the protruding particles are missing from some of the repeating units and thus seem to represent a part of the photosynthetic apparatus that can be cleaved off the membrane (Figure 1c). Fragments are frequently found in which two membranes are in contact with their plasmic surface, forming a symmetrical double layer. The hexagonal lattices of both layers are usually in register (Figure 1c). Sheets of single membranes are less common. The repeat distance of photoreceptor units on the hexagonal lattice was measured by optical diffraction of well-ordered areas on electron micrographs of either freeze-dried and heavy metal-shadowed or negatively-stained membrane preparations. Calibration with negatively-stained catalase crystals (Wrigley, 1968) gave a centre-to-centre distance of 130 Å in both cases.

Treatment of the thylakoid fraction with low concentrations of the non-ionic detergent, Triton X-100, and subsequent sucrose density gradient centrifugation gave a sharp band at 1.19 g/cm^3 density. Electron microscopy showed that this band contained mostly single membranes and some double membranes with both layers in register. Measurement of the lattice repeat distance gave a value of 125 Å for negatively-stained and 123 Å for freeze-dried membranes. SDS-polyacrylamide gel electrophoresis of the gradient fraction indicated that all seven polypeptides of the thylakoid membrane were present (results not shown).

To reveal the surface structure on the plasmic and on the exoplasmic side of the thylakoid membrane, specimens of Triton-treated membrane sheets were rotary shadowed with tantalum/tungsten. Shadowing clearly showed the structural differences between the smooth and rough side of detergent-treated membranes (Figure 2a and c). Contour maps of both sides (Figure 2b and d) were obtained by Fourier processing of electron micrographs, using diffraction spots above background level out to the (3,3) reflection which corresponds to a resolution of 21 Å. The phase residual calculated for P6 symmetry was 21° for the plasmic side and 21.6° for the exoplasmic side.

The structural unit on the plasmic side resembles a ring of 120 Å diameter with a central core measuring ~45 Å across which rises steeply to a plateau. The ring and the core are separated by six depressions 15–20 Å wide. Within the ring, 12 sites on which metal is deposited, each presumably corresponding to one subunit, are arranged in six pairs. Within a pair the distance between the centres of the subunits is 22 Å. The distance to the nearest neighbour in the adjacent pair is 30 Å. The distance between the centre of the core and the centre of a subunit is 48 Å. A handedness of the structural unit is evident in the relative position of the six depressions. The same features, including the handedness of the structure, were observed in symmetrised and unsymmetrised contour maps derived from images of other thylakoid membranes.

The rough exoplasmic surface of the membrane (Figure 2c) and its grey level map (Figure 2d) differ significantly from the structure seen on the plasmic surface (Figure 2b). The central core is the dominating feature while the ring is not evident. Instead, six small protrusions on which metal is deposited seem to rise from a plateau. The distance between these peripheral protrusions is 40 Å; the distance between the centre of a protrusion and the centre of the core is 42 Å. The diameter of the unit on this side is 100 Å, 20 Å less than on the plasmic side. Again, contour maps derived from other images of the exoplasmic surface showed the same feature before and after imposing 6-fold symmetry.

Single membrane sheets negatively contrasted with uranyl acetate show the characteristic pattern of hexagonally packed circular units (Figure 3a). Occasionally, the subunits in the ring can be distinguished. Detergent-treated membranes in negative stain showed a particularly high degree of order, giving diffraction spots out to the (6,0) reflection which cor-

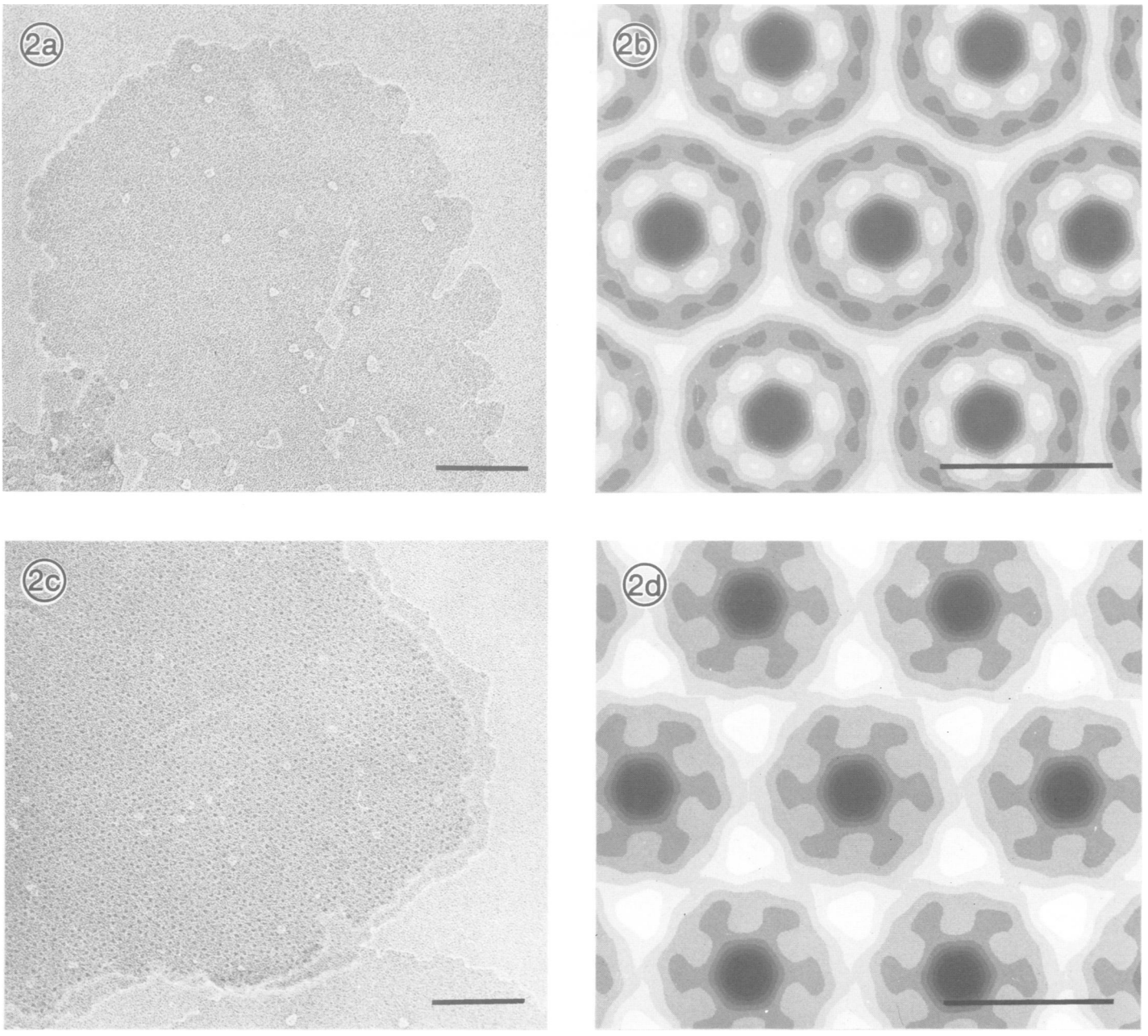
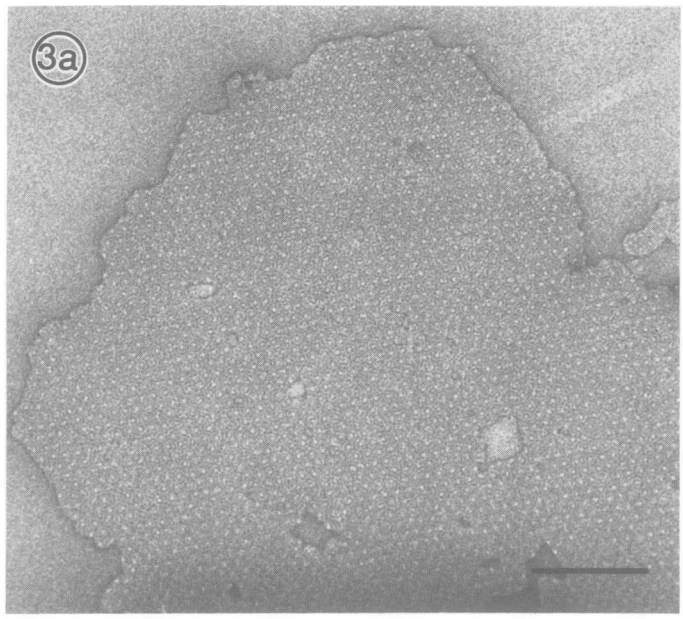


Fig. 2. Electron micrographs and Fourier processed images of Ta/W rotary-shadowed, Triton X-100-treated membranes. **(a)** A single membrane sheet with the plasmic surface upwards and its related grey level map **(b)**. **(c)** A double membrane with the exoplasmic surface uppermost and respective grey level map **(d)**. Scale bar = 1000 Å **(a,c)**, 100 Å **(b,d)**.

responds to a resolution of 18 Å (Figure 3c). Fourier processing of selected, well-ordered areas on micrographs yielded a projection map (Figure 3b) showing the characteristic central core and a ring similar to Figure 2b. The phase residual calculated for P6 symmetry of this negatively-stained membrane was 13.6°. The unsymmetrised projection map of Figure 3d gives a clear indication of 6-fold symmetry. Owing to the superposition of structural detail on both membrane surfaces the 12 subunits of the ring seen on the plasmic surface (Figure 2b) are not resolved in the projection map. However, the handedness of the photoreceptor unit is manifest in small, but significant, differences in amplitude of the (1,2), (2,1) and (1,3), (3,1) pairs of reflections, respectively. These differences are expressed in small deviations from mirror symmetry of the projection map (Figure 2b and d). Out of 53 projection maps of negatively-stained single membrane sheets, 35 could be assigned to one of two mirror symmetric projections, depending on whether the plasmic or exoplasmic side faced up in the electron microscope. It was thus possible to calculate an average map of the three best images judged by their phase residual and number of diffraction orders (Figure 3e). Comparison of the average map with Figure 2b suggests that in this projection the plasmic side of the membrane faces up.

Treatment of the thylakoid fraction with higher Triton X-100 concentrations leads to increasing solubilisation of the photosynthetic membrane. Electron microscopy of a negatively-stained specimen (Figure 4) shows a large number of ~130 Å particles. A detailed characterisation of these units is given in the accompanying paper by Jay *et al.* The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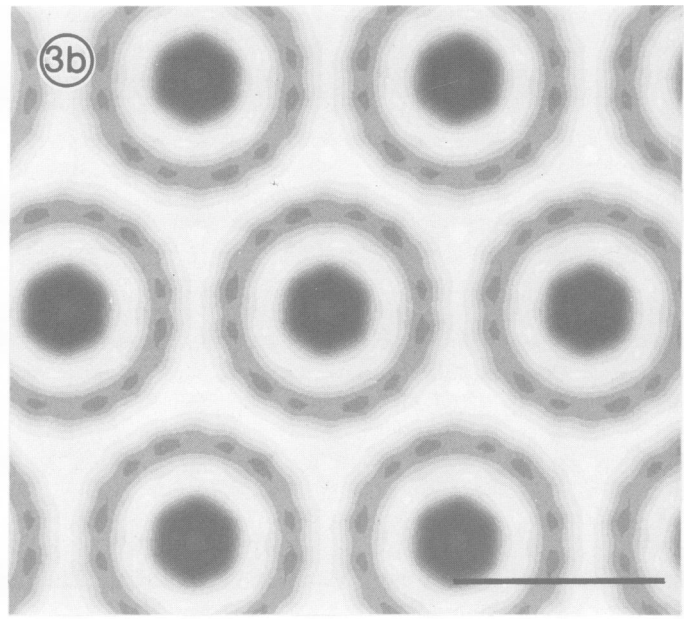
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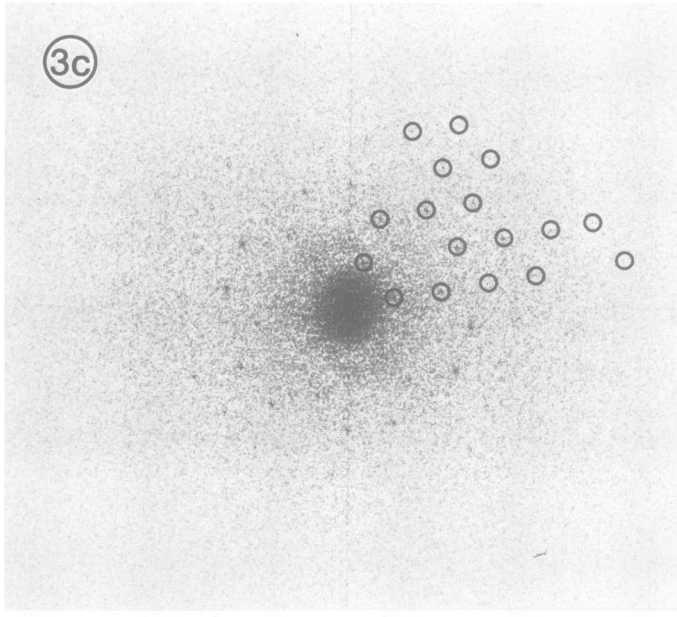
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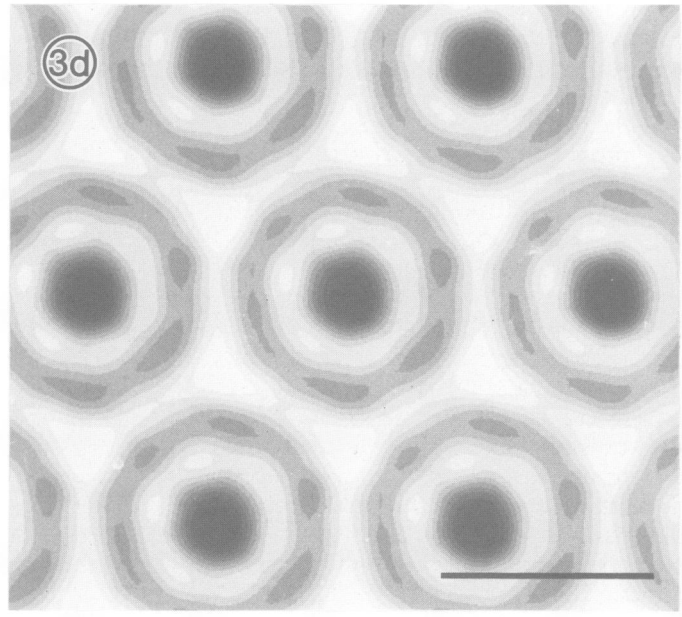
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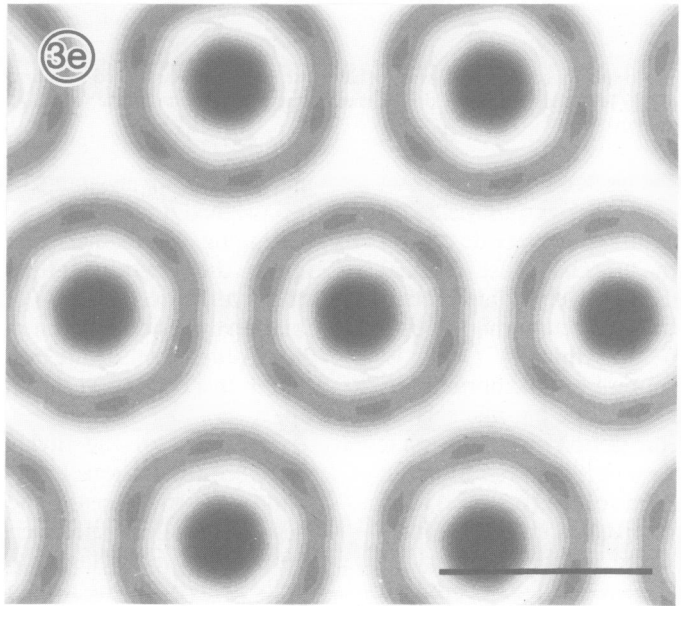
3e

structure of these particles, with a central core and ring in which up to 12 subunits can be counted (Figure 4), suggests that they are structurally intact photoreceptor units containing a full set of reaction centre and light-harvesting proteins.

Discussion

Photosynthesis requires an asymmetrical structure of the thylakoid membrane. Differences in molecular composition are clearly expressed in terms of surface structure. The vertical asymmetry of the photoreceptor unit manifests itself (i) in the central core which protrudes by ~40 Å in the exoplasmic side, (ii) in the occurrence of a ring around the core composed of 12 subunits on the plasmic side and (iii) in the diameter of the unit which is ~120 Å on the plasmic side and ~100 Å on the exoplasmic side. A schematic drawing (Figure 5) summarises these findings. To take account of the difference in diameter, the subunits of the ring are tilted by ~5° towards the central axis. The plasmic surface of the unit may have a functional role in membrane interaction. The fact that lattices of appressed membranes are usually in register suggests that this interaction is specific.

The handedness of the unit, apparent from Figures 3e and 2b, has only been observed in negatively-stained and rotary-shadowed single membranes. Fourier maps of negatively-stained double membranes with both layers in register do not show the handedness of the unit since the two layers add up to a mirror symmetric structure in projection.

Mol. wts. reported for isolated *R. viridis* reaction centre complexes range from 110 000 (Thornber, 1971) to 240 000 (Pucheu *et al.*, 1976) with a likely value between 160 000 (Thornber *et al.*, 1980) and 190 000 (Clayton *et al.*, 1978). A volume estimate based on the dimensions of the photoreceptor unit derived from our measurements suggests that the core can accommodate one or, at most, two complexes of this mol. wt. range. Two-dimensional crystals of *R. viridis* reaction centres (Miller and Jacob, 1983) indicate that the complex is similar in shape and size to the core of the photoreceptor unit, suggesting that the core does in fact represent the reaction centre. The ring appears to be composed of 12 subunits which in size are similar to light-harvesting complexes isolated from related, bacteriochlorophyll a-containing species. The light-harvesting complex of *R. sphaeroides* (Broglie *et al.*, 1980) and *Rhodospirillum rubrum* (Cogdell *et al.*, 1982; Picorel *et al.*, 1983) indicates that the monomer is composed of one α and β chain each and two bacteriochlorophyll molecules, giving a total apparent mol. wt. of 20 000–25 000. In addition, the *R. viridis* light-harvesting complex may also contain the small γ polypeptide. A total mol. wt. of 12 units in this mol. wt. range is compatible with the estimated volume of the ring. The details exposed on both surfaces of the membrane suggest that the 12 subunits are arranged in six V- or Y-shaped pairs, each pair exposing two sites on the plasmic and one (or two which are not resolved) on the exoplasmic side.

Antibody labelling and surface iodination of the membranes (Jay *et al.*, 1983a, 1983b; and in preparation) confirms the plasmatic location of all three light-harvesting polypeptides. At present there is a discrepancy between the labelling data and structural evidence regarding the exoplasmic location of these polypeptides since they are not accessible to antibodies or iodination. However, the amino acid sequences of the α, β and γ polypeptides contain hydrophobic stretches long enough to span the membrane (Brunisholz, in preparation).

The photoreceptor unit of *R. viridis* thus seems to be composed of one or, at most, two reaction centre complexes surrounded by 12 light-harvesting complexes arranged in six dimers. It follows that, at a molecular level, the reaction centre cannot have 6-fold symmetry. This is also suggested by Fourier maps of two-dimensional reaction centre crystals (Jacob and Miller, 1983) which show the reaction centre coordinated to four adjacent complexes. In the photoreceptor unit, intermolecular contacts are made between ring and core which do seem to have near 6-fold rotational symmetry. The Fourier maps of Figures 2 and 3 should therefore perhaps be regarded as averages over six very similar possible orientations of a structural unit without intrinsic rotational symmetry packed into a hexagonal lattice.

Distances between the centres of photoreceptor units in the thylakoid membrane of *R. viridis* between 100 Å (Miller, 1979) and 133 Å (Welte and Kreutz, 1982) have been reported. The majority of the values for native membranes range from 128 to 133 Å, irrespective of the method used for measuring them. Smaller values, ranging from 120 to 125 Å are found for Triton X-100-treated membranes.

This decrease of the interparticle distance which goes along with an increase of membrane density is due to a partial loss of lipids (Welte and Kreutz, 1982). Detergent treatment does not alter the absorption characteristics of the photoreceptor units significantly (data not shown) but does seem to improve the long-range order of the membrane, presumably due to closer packing of the photoreceptor units.

Of the four maps of the structures of bacterial photosynthetic membranes recently published, three differ significantly from our results. The projection maps of Welte and Kreutz (1982), Miller and Jacob (1983), and the three-dimensional map by Miller (1982) both show a more or less mirror-symmetric structure with large central core and six peripheral peaks that are more or less continuous with the centre but well separated from one another. It seems that both maps suffer from a lack of resolution beyond 33 Å. When we truncate our data at this limit, a very similar projection map results. The lack of resolution may be due to poor crystalline order of the membrane or to radiation damage. We have found that a dose of ~100 electrons per Å^2 is sufficient to cause loss of detail in the 20–30 Å resolution range, the most dramatic effect being the disappearance of the staining region that separates the core from the ring. The effect of radiation damage to a *R. viridis* thylakoid membrane is obvious in an image shown by Miller and Jacob (1983) which was recorded without taking precautions to minimise irradiation of the specimen. This is obviously a serious problem when recording all micrographs used in a tilted-view reconstruction from a single membrane. It is therefore perhaps not surprising that the three-dimensional map of Miller (1982) does not show the asymmetry of the membrane which is evident in shadowed as well as in negatively-stained specimens.

A projection map derived by correlation averaging of the negatively-stained thylakoid membrane of *Ectothiorhodo-*

Fig. 3. Electron micrographs and Fourier processed images of negatively-stained, Triton X-100-treated, single membrane sheets. **(a)** Single membrane sheet, **(b)** grey level map, with P6 symmetry, **(c)** computer-generated diffraction pattern, **(d)** grey level map without imposed symmetry, **(e)** average from the three best images. Scale bar = 1000 Å **(a)**, 100 Å **(b,d,e)**.

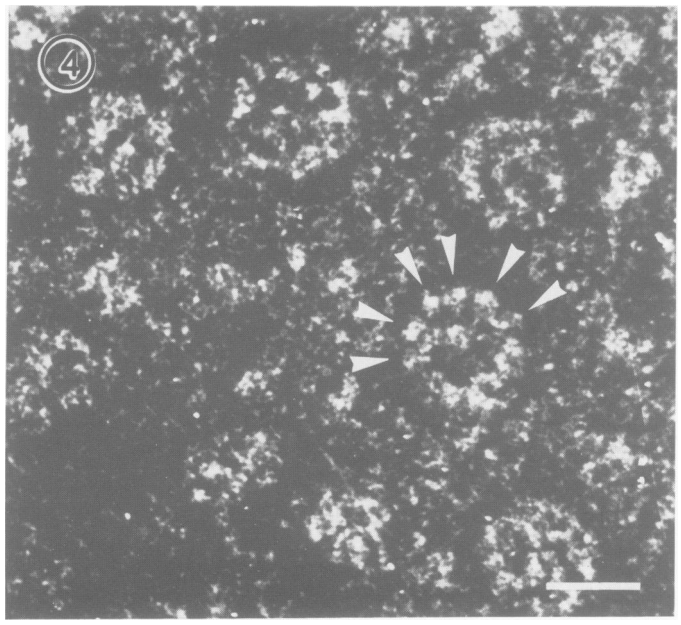

Fig. 4. Electron micrograph of isolated, negatively stained photoreceptor units. Scale bar = 100 Å.

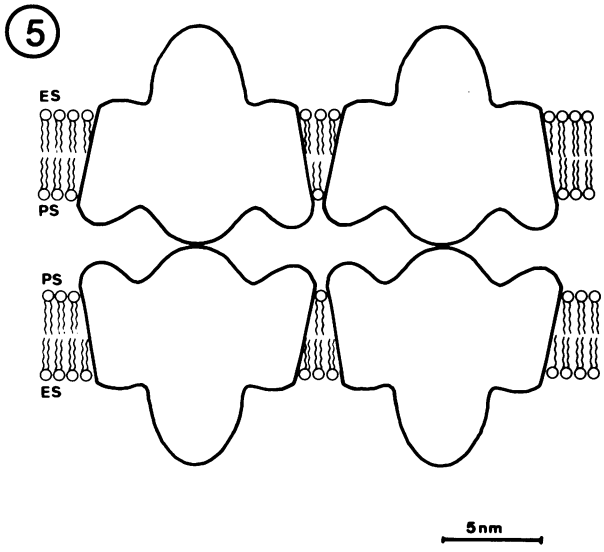

Fig. 5. This model drawing showing the suggested transverse arrangement of photoreceptor units in the membrane.

spira halochloris (Engelhardt *et al.*, 1983) closely resembles our projection map (Figure 3b). The absence of 6-fold symmetry in unsymmetrised averages may indicate, however, some rotational disorder of the units in the membrane. Nevertheless, the similarity to our projection map supports the conclusion of Engelhardt *et al.* (1983) that this structure is a feature common to all photosynthetic membranes containing bacteriochlorophyll b.

Structurally intact photoreceptor units can be isolated by detergent solubilisation of the thylakoid membrane. These units appear to contain a full set of light-harvesting and reaction centre complexes (see preceding paper by Jay *et al.*). The smallest unit of photosynthesis, the 'quantasome' originally postulated by Park and Biggins (1964) for thylakoids of green plants thus does seem to exist, at least in bacterial photosynthetic membranes.

Materials and methods

Growth conditions and isolation of photosynthetic membranes

A culture of *R. viridis* (strain ATCC 19567) was kindly provided by N.Pfennig, University of Konstanz, FRG. Cells were grown anaerobically in 500 ml flasks according to the method of Ormerod *et al.* (1961) modified by Hanselmann *et al.* (1979). The isolation of the intracellular membranes was carried out as described by Jay *et al.* (1983a).

Preparation of single membrane sheets

Isolated membrane sheets were incubated with Triton X-100 (Sigma Chemicals, St. Louis, MO) at a ratio of 0.3 mg protein/mg Triton X-100 for 20 min at 25°C. Immediately after solubilisation the solution was loaded onto a 10–60% sucrose density gradient and centrifuged (110 000 *g*, 15 h, 4°C). Unsolubilised thylakoid membranes banded at a density of 1.168 g/cm^3. Single membrane sheets formed a sharp band at 1.19 g/cm^3. A similar method is also described by Garcia *et al.* (1968).

Preparation of the structural membrane units

Isolation methods and the characterisation of these structural membrane units are shown in the accompanying paper by Jay *et al.*

Specimen preparation for electron microscopy

Membranes were adsorbed onto carbon-coated copper grids and contrasted with 2% uranyl acetate. Other specimens were frozen in super-cooled nitrogen, and freeze-dried in a Balzers BAF 300 freeze-etch unit (Balzers Union Inc., Balzers, Liechtenstein). Freeze-dried specimens were either rotary shadowed with tantalum/tungsten (Ta/W) or unidirectionally with platinum/carbon (Pt/C) at an elevation angle of 45°. The amount of heavy metal corresponded to a homogenous 5 Å thick layer which was backed with a 50 Å thick carbon layer (Wildhaber *et al.*, 1982).

Electron microscopy

Electron micrographs were taken at an acceleration voltage of 100 kV in a Jeol 100 C electron microscope equipped with a minimal dose attachment. Images were recorded on Agfa Scientia films (Agfa Gevaert, Leverkusen, FRG) with an estimated radiation dose of 10–15 e/Å^2 at ~0.5 μm underfocus. Negatively-stained membranes were located and positioned at low magnification and recorded at x 33 000. The magnification was calibrated using negatively-stained, glutaraldehyde-fixed catalase as a standard (Wrigley, 1968).

Image analysis

Well-ordered areas of electron micrographs selected by laser diffraction were densitometered on an Optronix P 1700 photoscan/photowrite unit (Optronix International Inc., Chelmsford, MA). Square arrays of 512 x 512 steps were digitised with a step size and aperture of 25 μm, corresponding to 7.6 Å at the specimen. Fourier transforms of the densitometered areas were calculated on a CDC Cyber 722 computer (Cyber Systems, Anaheim, CA) using a fast Fourier transform routine by O.Kübler (Institute für Kommunikationstechnik, ETH Zürich). Amplitudes and phases of reflections above background level were collected and averaged for P6 symmetry. Contour maps were calculated from symmetrised and unsymmetrised data obtained from individual images, and overlaid with a grey scale using programmes by O.Kübler.

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