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FORMATION, STRUCTURE AND COMPOSITION OF A PLANAR HEXAGONAL LATTICE COMPOSED OF SPECIFIC PROTEIN-LIPID COMPLEXES IN THE THYLAKOID MEMBRANES OF *RHODOPSEUDOMONAS VIRIDIS*

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The hexagonal particle lattice in the thylakoids of the phototrophic bacterium *Rhodopseudomonas viridis* was investigated by use of electron microscopy, small-angle X-ray scattering, SDS-polyacrylamide gel electrophoresis and lipid analysis by thin-layer and gas chromatography. The thylakoids were treated with detergents and centrifuged to equilibrium in a linear 36–45% sucrose gradient to optimize the yield of the hexagonal particle lattice: up to a weight ratio of Triton X-100/protein of 4.0 or of cholate/protein of 7.7 several effects are observed: (1) The phospholipid/protein weight ratio changes from 0.338 for untreated membranes to 0.125 for the above given upper limit of detergent treatment. The main lipids of the membrane are not removed parallel to their sequence of abundancy in the untreated membrane, so that this sequence changes from phosphatidylethanolamine, phosphatidylcholine, cardiolipin to cardiolipin, phosphatidylcholine, phosphatidylethanolamine. (2) The density of the membranes increases from 1.157 g/cm^3 to 1.192 g/cm^3. (3) Small-angle X-ray diffraction and electron microscopy of freeze-fractured thylakoids indicate an increasing yield of ordered hexagonal particle arrangements with a decreasing interparticle distance from about 13.5 nm to 12.2 nm. With decreasing phospholipid content the membrane patches show polygon-like edges. The resolution of the resulting hexagonally ordered membranes both in small-angle X-ray diffraction and electron microscopy is about 3.0 nm. The distribution of the negative staining material, evaluated by numerical filtering, shows a central region of higher density surrounded by a ring of lower density with a radius of 4.0 nm. (4) The protein pattern as seen in SDS-polyacrylamide gel electrophoresis remains qualitatively invariant with increasing detergent concentration.

Introduction

Two-dimensional ordered protein arrangements in membranes have attracted much interest in recent years due to new electron-microscopic methods for extracting structural information from them [1].

The hexagonally ordered particles seen in the thylakoids of *Rhodopseudomonas viridis* [2,3] have recently been studied by Miller [4] and Wehrli [5,6] and by us [7]. Furthermore, the electron density profile of this membrane was evaluated by Hodapp and Kreutz [7].

A model proposed by Wehrli [6] and independently by us [7], states that the particles seen in the membrane consist of a reaction center complex surrounded by several light-harvesting complexes. Yet in these studies there was little experimental evidence to prove the model.

Further experiments on structure and composi-

tion of the hexagonal arrangements of particles therefore seemed to be necessary to confirm or reject the model.

Materials and Methods

Growth of the bacteria. Cultures of *R. viridis* were kindly provided by Dr. J. Weckesser of the Institut für Mikrobiologie, Freiburg. Cells were grown in a medium for Athiorhodaceae [8] in 1 litre bottles with a diameter of 10 cm. The intensity of light of ordinary bulbs was about 400 lux at the surface of the bottles and the temperature was 30–33°C within the culture. Before harvesting, the absorbance at 700 nm for 1 cm pathlength was measured in a Beckman Spectrophotometer Acta II. The absorbance of the harvested bacteria varied between about 1.0 and 1.5 during our experiments.

Isolation of the thylakoids. After washing three times with 50 mM Tris-HCl buffer (pH 8.0) the bacterial pellet was weighed and ground with aluminium oxide (90 active, 70–230 mesh, Merck) at a weight ratio of 0.7 g aluminium oxide/g bacterial pellet. After resuspension with Tris-HCl buffer, the cell homogenate was centrifuged at $2450 \times g$ for 30 min to remove cellular debris and aluminium oxide. The supernatant was centrifuged at $23\,500 \times g$ for 30 min and the pellet was resuspended.

Purification of the thylakoids and density gradient centrifugation. The latter material was layered on top of 31–44% (w/w) sucrose density gradients in 50 mM Tris-HCl and centrifuged to equilibrium at $25\,000 \times g$ in a swing-out rotor at 10°C for 12 h. The density of the thylakoids was measured by pipetting some droplets with a pasteur pipette out of the center of each pigmented band and measuring its density with a Zeiss refractometer at room temperature. For negative staining and SDS-polyacrylamide gel electrophoresis, the bands were collected and washed once with Tris-HCl buffer. Such material is called large fraction x% where x is the density of the band given in percentage sucrose (w/w) concentration.

Protein. Protein was determined according to Lowry et al. [9].

Lipid extraction. Lipid extraction was performed according to Awasthi et al. [10] with the third extraction medium altered to chloroform/methanol/25% ammonia = 66.5 : 23.5 : 4.

Lipid separation by two-dimensional TLC, and phosphorus determination. These were carried out according to Ronai and Wunderlich [11]. Amino lipids were identified by ninhydrin staining. Presence or absence of lipids was tested, using purchased phosphatidylcholine, phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine, cardiolipin. The purity of these was tested by thin-layer chromatography prior to co-chromatography with the unknown lipid mixture.

Analysis of the fatty acids. The analysis was done as described in Ref. 12.

SDS-polyacrylamide gel electrophoresis. The method of Laemmli [13] was used with a 11.5–16.5% polyacrylamide linear gradient gel. Apparent molecular weights were calculated from 11.5% polyacrylamide gels by calibration of a log M_r vs. migration distance plot with the following proteins: bovine serum albumin (68 000); ovalbumin (43 000); chymotrypsinogen (25 000); myoglobin (17 200); cytochrome *c* (12 500).

Negative staining and freeze etching of mica-adsorbed thylakoids. Isolated thylakoids were spread on carbon or Formvar coated grids and immediately stained with 1% phosphotungstic acid solution for 45–60 s at the appropriate pH of the thylakoid suspension. Alternatively, thylakoids were spread on freshly cleaved mica, a second mica piece was slightly pressed against the latter and the sandwich frozen quickly by dipping into liquid propane. The sandwich was fractured, using a device similar to that described by Nermut and Williams [14].

Electron microscopy. All micrographs were made with a Siemens Elmiskop 1 A operating at 80 kV. Magnification was calibrated by use of a grating replica.

X-ray diffraction. Pellets of thylakoids were resuspended in as much buffer as necessary in order to fill a mark capillary by capillary action. For X-ray diffraction of the latter material, a Rigaku Rotaflex RU 2000 V rotating anode X-ray source with a copper anode ($\gamma = 1.54$ Å), which was run at 45 kV/200 mA, was used. A point-collimated Kratky camera with a rectangular crossection of the primary beam of 100×200 μm at the position of the sample was attached to the X-ray source.

The intensity distribution was recorded with Kodak No Screen film.

Results

Thylakoid isolation.

Isopycnic sucrose density gradient centrifugation of the large fraction always yields one or both of two bands at densities corresponding to 32 ± 1% (w/w) sucrose = 1.136 g/cm^3 and 36 ± 1% = 1.157 g/cm^3. The estimated standard deviations are caused by the natural width of the bands. Small-angle X-ray diffraction of a concentrated suspension of 36% gives one ring at 1/11.5 nm^{-1}. It seems reasonable to interpret it as the first order of a hexagonal lattice with a interparticle distance of 13.5 nm. Micrographs of the large fraction 36% predominantly show the hexagonal lattice with a interparticle distance of 12.5 ± 1 nm, similar to the corresponding values in whole cells [15,16]. Micrographs of the negatively stained large fractions 32% thylakoids show predominantly a disordered particle arrangement; occasionally the hexagonal lattice is seen. The large fraction 32% and 36% consist of thylakoids with a diameter of 100–1000 nm. Fig. 1 shows mica-absorbed, freeze-etched large fraction 36% thylakoids.

These results seems to be independent of the technique used for breaking the cells, since we observed the two bands after grinding with aluminium oxide, ultrasonication, use of a Bühler homogenizer and the French Press. They also were insensitive to changes of the pH value of the buffer between pH 7 and 8, for temperatures between 17 and 25°C and for concentrations of the Tris-HCl buffer between 50 mM and 0.7 M. The phosphorus determination yielded 13.5 μg phosphorus/mg protein for the large fraction 36%. Assuming an average molecular weight for phospholipids of 775 [17], this is equivalent to a phospholipid-protein weight ratio of 0.338 for the large fraction 36%.

Large fraction thylakoids after detergent treatment and density gradient centrifugation

The large fraction 36% thylakoids at a protein concentration of 2 mg/ml were treated with different amounts of Triton X-100, corresponding to a Triton/protein weight ratio of 0, 1, 2, 3, 4 and 5 by slowly adding 10% Triton solution at 4°C and stirring for further 30 min. The samples were layered on top of six linear sucrose density gradients 36–44% (w/w) and centrifuged to equilibrium as described in Materials and Methods. The same type of experiment was carried out with sodium cholate as the detergent.

As shown in Fig. 2, the pigmented bands showed increasing density with incresing detergent/protein weight ratio. The bands became narrower, as their density approached to 42–44% (w/w) sucrose density.

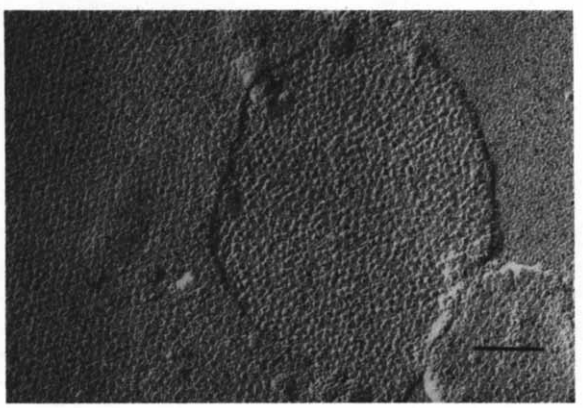

Fig. 1. Freeze-etched, mica-adsorbed large fraction 36% thylakoids. The lower membrane, adsorbed to the mica surface, shows the disordered arrangement of particles, the upper membrane a distorted hexagonal arrangement of particles. The bar represents 1 μm.

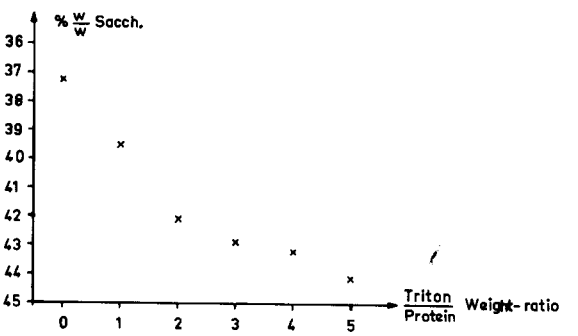

Fig. 2. Position of pigmented bands of Triton-treated thylakoids in a saccharose continuous density gradient 36–45% (w/w) in 50 mM Tris-HCl, pH 8 after Triton treatment at detergent/protein weight ratios of 1–5.

The membranes were then tested for removal of Triton by comparing their extinction at 276 nm and their density before and after stirring with bio-beads [18]. Only minor changes within the limits of measurements were observed, indicating that most of the Triton had been removed from the thylakoids by the density gradient centrifugation. The absorption spectra of the membranes between 350 and 1050 nm before and after Triton treatment were virtually identical.

Electron microscopic determination of the maximal yield of hexagonal particle arrangements by detergent treatment

Thylakoid smples, treated alternatively with increasing amounts of Triton X-100 or sodium cholate, show the same sequence of characteristics as observed by electron microscopy of negatively stained samples. Up to a characteristic weight ratio of 6 for Triton/protein at pH 7 (of 4 at pH 8) and 7.7 for cholate/protein at pH 7 or 8, the yield of hexagonal lattices is seen to increase to a level at which all thylakoids show the same hexagonal particle lattice with a lattice constant of 12.2 nm. This happens at a thylakoid density of 43% (w/w) sucrose density = 1.192 g/cm^3 both for Triton- and cholate-treated thylakoids. This material will therefore be called 'Triton fraction 43%' and 'cholate fraction 43%', respectively. Concurrently, the smooth appearance of the rim of the flattened thylakoid vesicles changes to a irregular polygon, whose edges follow the lattice lines. Figs. 3 and 4 show mica-absorbed and negatively stained samples of this material.

At higher detergent-to protein weight ratios, the membranes seem to break into smaller patches. Simultaneously they tend to aggregate and, finally,

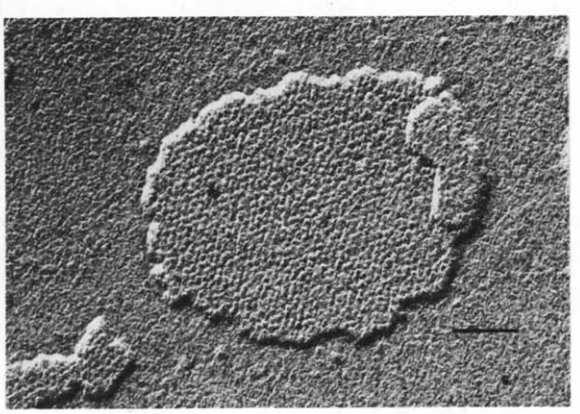

Fig. 3. Freeze-etched, mica-adsorbed Triton fraction 43% thylakoids. The membrane shows the hexagonal arrangement of particles with an interparticle distance of 12.3 nm. The rim of the thylakoids appears to be brittle and follows the lattice lines. The bar represents 1 μm.

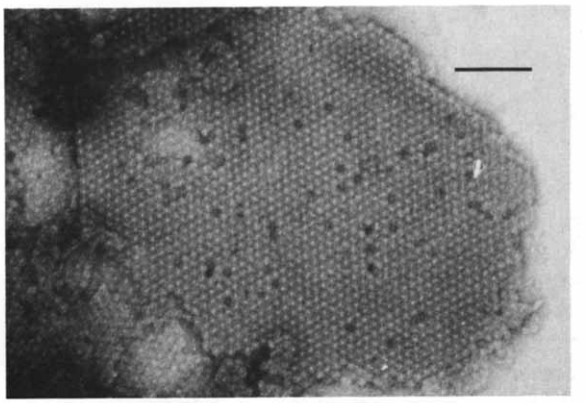

Fig. 4. Negatively stained thylakoids of the Triton fraction 43%. Even without image enhancement, a substructure of the particles is seen, similar to a ring around a central particle. The interparticle distance is 12.2 nm. The bar represents 1 μm.

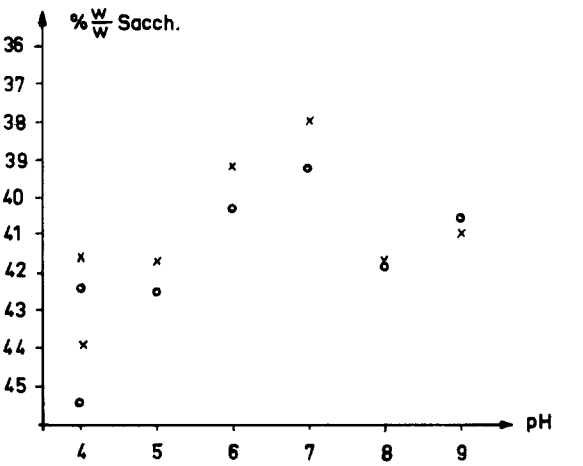

Fig. 5. pH dependence of the position of pigmented bands of Triton-treated thylakoids in continuous saccharose gradients 36–45% (w/v) after centrifugation to equilibrium. The pH values and the buffers used were: pH 4 and 5: 0.2 M acetate buffer; pH 6 and 7: 0.1 M phosphate buffer; pH 8 and 9: 0.05 M Tris-HCl buffer. ×, treatment at a Triton/protein weight ratio of 2; ○, treatment at a Triton/protein weight ratio of 3.

amorphous aggregates predominate. Preparation of a membrane sample with maximal yield of hexagonal lattices from thylakoids of different harvests can require somewhat different detergent/protein weight ratios; yet the density found for these samples always is equal to 43% (w/w) sucrose density. In addition, the density of the thylakoid band for a fixed Triton/protein weight ratio depends on the pH during detergent treatment and centrifugation. At a Triton/protein weight ratio of 2 and 3 a minimal density was observed at pH 7. The density at several other pH values is given in Fig. 5. The differences may be not only caused by the pH, but also by the different buffers used, which were 0.2 M acetate buffer for pH 4 and 5, 0.1 M phosphate buffer for pH 6 and 7 and 50 mM Tris-HCl buffer for pH 8 and 9. The thylakoids treated with the optimal detergent/protein weight ratio, showed much stronger aggregation at pH 7 than at pH 8, as seen in micrographs of negatively stained samples and by formation of 'flakes' in the density gradient and precipitation after washing.

At pH values lower than 7, aggregation was even stronger and density gradient centrifugation yielded several pigmented bands, even at low detergent/protein weight ratios. Nevertheless, all these bands showed qualitatively the same protein pattern in SDS-polyacrylamide gel electrophoresis. For all further work we therefore used 50 mM Tris-HCl buffer (pH 8) and a detergent/protein weight ratio of 4.0 for Triton and 7.7 for sodium cholate.

Phosphorus determination of Triton fraction 43%

This determination gave 5 μg phosphorus/mg protein, corresponding to a weight ratio of 0.125 of phospholipid/protein.

Lipid pattern of large fraction 36% and Triton fraction 43%

Two-dimensional silica-gel TLC of lipid extracts of untreated large fraction 36% thylakoids gave five major spots (spots 2, 3, 4, 5, 6) as seen by rhodamine fluorescence by illuminating at 360 nm. A number of minor spots, changing from one harvest to the other, were also seen.

Measurable amounts of phospholipids over 10 μg were found in spots 2, 3, 4 only. Spot 4 is also stained by ninhydrin and co-chromatographs with phosphatidylethanolamine. Spot 2 co-chromatographs with cardiolipin, spot 3 with phosphatidylcholine. Spot 5, which contains no appreciable amount of phosphorus, is stained by ninhydrin and moves close to phosphatidylethanolamine. These observations indicate that spot 5 might be an amide I or amide II ornithine lipid [19]. Thin-layer chromatography of Triton fraction 43% yields measurable amounts of phospholipid only for spots 2, 3 and 4, corresponding to cardiolipin, phosphatidylcholine and phosphatidylethanolamine. Spot 5 is very faint and does not show visible staining by ninhydrin. Neglecting the minor lipids, lipid/protein weight ratios for large fraction 36% and Triton fraction 43% membranes can be calculated as given in Table I.

Distribution of fatty acid chain lengths and degree of unsaturation

The extracts of the three major spots of large fraction 36% as well as of Triton fraction 43% membranes were subjected to a fatty acid analysis by gas chromatography. The results in Table II are given as molar percentages of the total amount of fatty acids found in each spot.

Protein pattern of large fraction 36% and Triton fraction 43% membranes

Fig. 6 shows traces of untreated large fraction 36% thylakoids as well as of Triton fraction 43%

TABLE I

LIPID/PROTEIN WEIGHT RATIOS IN LARGE FRACTION 36% (LF 36) AND TRITON FRACTION 43% (TF 43) THYLAKOIDS FOR THE THREE MOST PREDOMINANT PHOSPHOLIPIDS

For the calculation, the remaining minor lipid species were neglected. The data are the mean values and their standard deviations. The standard deviations (S) are less for material of a single harvest. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

	PE	PC	CL
LF 36	0.172 S 0.02	0.090 S 0.016	0.075 S 0.016
TF 43	0.033 S 0.009	0.036 S 0.006	0.057 S 0.003

TABLE II

MOLAR PRECENTAGES OF FATTY-ACIDS FOUND IN THE THREE MAJOR SPOTS OF THIN-LAYER CHROMATOGRAPHY OF LARGE FRACTION (LF) AND TRITON FRACTION (TF) 43% MEMBRANES

		12:0	14:0	16:0	16:1	18:0	18:1	20:0	20:1
LF 36%	CL	12	3	7	4	1	66		7
	PC			5	8		78		9
	PE			8	10		74		9
TF 43%	CL	16	4	8	4	2	60		6
	PC			5	7		80		8
	PE			11	13		76		

thylakoids, the latter being entirely hexagonally ordered as judged by electron microscopy. For cholate treatment identical results were obtained. As can be seen, there is no qualitative change in the protein pattern by Triton treatment. No attempt was made to compare the gel patterns quantitatively. The apparent molecular weights of the three prominent bands in the mid-region of the traces are 34, 27 and 22 kDa. No estimate was attempted for the two bands at the bottom of the trace, as molecular weight estimates are incorrect in this region. We also found that the 34 kDa band was pigmented with an absorption band at 409 nm characteristic for heme groups [34]. The band at 34 kDa behaved irreproducibly in our experiments. Sometimes it was smeared out in the high molecular weight direction, sometimes it was totally absent. Between the 27 and 22 kDa bands at about 26 kDa, a faint broad shoulder is usually seen which is resolved into two broad bands in some experiments. Furthermore, the 22 kDa band could be removed by heat-treatment of the SDS-solubilized thylakoids prior to application onto the gel.

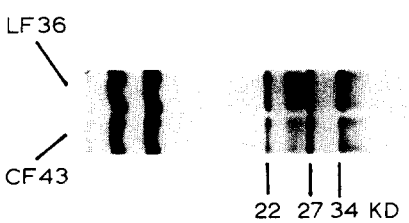

Fig. 6. SDS-polyacrylamide gel electrophoresis of the large fraction 36% (LF 36) and cholate fraction 43% (CF 43) thylakoids. The gel was made according to Laemmli [13] with a linear acrylamide gradient 11.5–16.5% (w/v). The thylakoids were solubilized at room temperature with a denaturing buffer containing 3% (w/v) SDS and 2% (w/v) mercaptoethanol. The apparent molecular weights were evaluated separately from a 11.5% acrylamide gel: the number represent molecular weights ($\times 10^{-3}$).

Micrographs of negatively stained Triton fraction 43% and cholate fraction 43% thylakoids and the reciprocal lattice peaks of their corresponding Fourier transforms

Fig. 4 shows a negatively stained Triton fraction 43% thylakoid. The interparticle distance obtained by evaluation of 70 different thylakoids from several different preparations is 12.2 ± 0.5 nm. The reciprocal lattice obtained by Fourier transformation of a digitized micrograph showed a C_6 symmetry axis and, with the exception of the outermost weak reflexion (3,1), two orthogonal mirror lines, giving 6_{mm} symmetry in good ap-

TABLE III

AMPLITUDES AND PHASES OF THE RECIPROCAL LATTICE POINTS OF TRITON FRACTION 43% AND CHOLATE FRACTION 43% THYLAKOIDS AS FOUND FROM FOURIER-TRANSFORMATION OF DIGITIZED ELECTRON-MICROGRAPHS AND FROM SMALL-ANGLE X-RAY INTENSITY MEASUREMENTS.

h	k	From negative staining		From small-angle X-ray
		A	ϕ	A
1	0	700	0	152
2	0	177	0	110
1	2	288	0	94
2	1	282	0	94
3	1	47	180	–
1	1	142	180	–

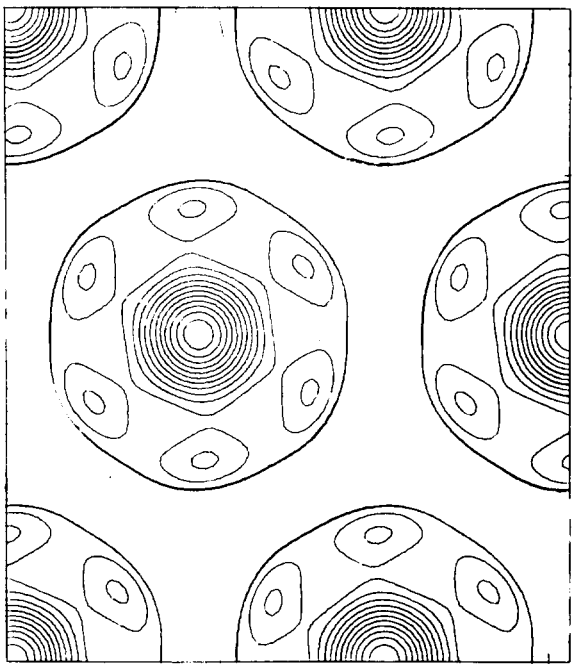

Fig. 7. Map of the averaged staining material distribution of negatively stained Triton fraction 43% thylakoids, calculated by Fourier filtering.

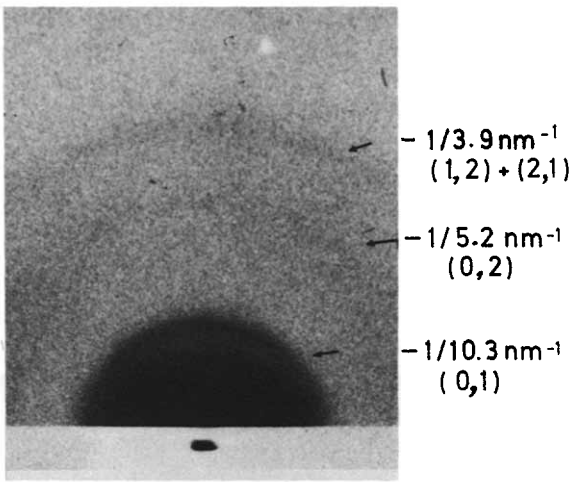

Fig. 8. Small angle X-ray scattering intensity of a sample of wet Triton fraction 43% thylakoids. The material was filled in a mark capillary of 1 mm diameter and the exposure time was 70 h. The distance between the sample and the X-ray film was 200 nm. Three Debye-Scherrer rings at 1/10.3, 1/5.2 and 1/3.9 nm^{-1} are seen, which fit into the reciprocal of a hexagonal lattice with an interparticle distance of 12 nm.

proximation. The two mirror lines of the two-dimensional map may well be a result of the superposition of two membranes formed by folding of a single membrane sheet.

The amplitudes and phases found are given in Table III together with their Miller indices. The corresponding Fourier-synthesis of the map of the staining material distribution is shown in Fig. 7. The map consists of a central and deepest minimum surrounded by a ring of approximate diameter of 8 nm.

Freeze-fractured Triton fraction 43% thylakoids

Sheets with hexagonal particle arrangements similar to the mica-adsorbed material of Fig. 3 were found with a interparticle distance of 12.2 ± 0.4 nm. The sheets usually consisted of two adjacent membranes.

Small-angle X-ray diffraction of large fraction 36% and Triton fraction 43%

Scattering intensities of large fraction 36% show no or at most one ring. If this ring is attributed to the (1,0) reflection of a planar hexagonal lattice, a interparticle distance of 13.3 nm results.

Scattering intensity patterns of Triton fraction 43% as shown in Fig. 8 consist of three rings at 1/10.3 nm^{-1}, 1/5.2 nm^{-1} and 1/3.9 nm^{-1}. They can be attributed to a plane hexagonal lattice with an interparticle distance of 12.0 ± 0.2 nm which agrees well with the corresponding value found in electron micrographs. The Miller indices of the corresponding small-angle X-ray diffraction reciprocal lattice are (0,1) for the first and (0,2) for the second ring. The third ring is a superposition of (1,2) and (2,1) reflexions. As it was our aim to evaluate the projection of the electron density onto the membrane plane from the small-angle X-ray diffraction rings, we had to assign amplitudes and phases to these reciprocal lattice points. In a first step, since there is no reason to assume that staining changes the symmetry of the unit cell within the low resolution we are dealing with, a 6_{mm} symmetry for the electron density projection was assumed. As a consequence the intensity of the third ring was assigned to equal parts to the (1,2) and (2,1) reflection and the same phase was given to both. In a second step we had to choose among eight different phase choices for the three

independent amplitudes. Among the corresponding eight electron-density maps, two agreed qualitatively with the electron microscopic result with respect to the following criteria: electron microscopic results as shown in Figs. 4 and 7, show a characteristic distribution of the staining material in the unit cell. Around a white core of about 1.6 nm diameter a black ring is seen, followed by a concentric white ring having a radius of about 4.0 nm. A further dark ring follows which separates neighbouring unit cells and has a diameter identical to the interparticle distance. If it is assumed that the staining material fills mainly the regions between membrane protruding particles on both surfaces of the membrane, the electron density projection is to be expected to be complementary to the negative staining map, so that low staining densities correspond to high electron densities and vice versa. Six maps can clearly be excluded by these criteria, the remaining two being very similar to the negative-staining map of Fig. 7. One of these even has the same set of phases as the latter.

Moiré patterns

The lattices of the two superimposed thylakoid membranes usually seen in negatively stained thylakoids frequently are not in register to each other. This can be concluded from Moiré patterns. It is possible to separate the two superimposed lattices on a micrograph by a Gilev-filter [20] between the micrograph and the camera at the proper distance to enhance the lattice periodicity. When rotating the filter through an axis parallel to the camera axis, two angular positions are observed, at which the usual hexagonal lattice is seen. At these positions only one lattice is enhanced by the filter, the other being suppressed.

Discussion

Effect of detergent treatment on composition and structure of the thylakoids

As reported, the large fraction 36% has a weight ratio of phospholipid/protein of 0.338. After treatment with Triton at a weight ratio of 4.0 or with cholate at 7.7, this ratio shifted to 0.125. Simultaneously the protein pattern remains qualitatively unchanged. At the same time, X-ray diffraction and micrographs of freeze-fractured thylakoids indicate an increased yield of hexagonal lattices in the plane of the thylakoids with an interparticle distance of 12.2 nm. Micrographs of negatively stained thylakoids of these preparations show a polygon-like rim of these membranes. At higher detergent concentrations, the ordered membrane patches break into smaller patches and finally only amorphous aggregates of particles are seen.

These events are similar to those reported in cytochrome *c* oxidase model membranes of decreasing phospholipid/protein ratio as reported by Jost et al. [17,21]. It is evident that the detergent treatment predominantly removes phospholipid from the thylakoids, but nevertheless there is still an amount of phospholipid present in the Triton fraction 43% and cholate fraction 43% membranes and the leveling off of the thylakoid density at higher detergent concentration possibly indicates closer contact of the remaining phospholipids with the proteins. This view is also supported by the fact that removal of further lipids from the ordered particle arrangements causes breakdown of their sheet-like association.

In the case of the cytochrome *c* oxidase model membrane it was shown that one phospholipid, cardiolipin, is removed from the enzyme only after others have been removed [10]. Furthermore, this enzyme has an absolute cardiolipin requirement for its activity [22,23], which also indicates a tight association of this phospholipid with the enzyme. A tight association of some lipids to bacterial photosynthetic proteins was also found by mobility measurements with spin-label and fluorescent polarization probes [24–27].

Such an association is called an annulus [21]. Though it is likely that lipid annuli defined by different parameters such as enzyme activity, mobility and ease of lipid removal are different, the concept nevertheless indicates a gradual change in physical and chemical properties of the lipids with distance from a membrane protein or protein complex. In this respect our observation of a difference in lipid composition between Triton-extracted lipids with the concomitant change from mainly disordered to hexagonally arranged particles and the remaining lipids of these latter membrane sheets also indicates a lipid annulus specific in its composition compared to the more distant lipids,

surrounding the photosynthetic protein complex. It may be significant that cardiolipin with its two negative charges represents the most abundant of the remaining lipids, which according to our observations is essential for the existence of membrane-like sheets. In fact Birrell et al. [24] found an increased association of negatively charged doxylstearic acid spin labels with photosynthetic proteins. Birrell et al. [24] conclude that the proteins bear excess positive charge on its hydrophobic surface. Such charges on proteins should be pH-dependent, and so should be the ease of lipid removal by detergents. This might be the explanation for the observed pH-dependence of the thylakoid density shown in Fig. 5.

It could be supposed that cardiolipin might interact preferentially with the proteins not by its head group, but by a specific distribution of fatty acids. In fact, a preferential association of certain fatty acid types was found in ATPase preparations of *Rhodosprillum rubrum* membranes [28]. In the thylakoids of *R. viridis* the fatty-acid distribution of the major lipid species of large fraction 36% and Triton fraction 43% membranes, given in Table II, was found to be very similar and thus the polar head groups seem to be responsible for the specific interactions.

The thylakoids of *R. viridis* are extraordinary with respect to their high cardiolipin content compared to some related photosynthetic bacteria. Table IV is taken from Oelze and Drews [29] with our data added.

TABLE IV

PHOSPHOLIPID COMPOSITION OF CHROMATOPHORES OF DIFFERENT PHOTOSYNTHETIC BACTERIA GIVEN AS WEIGHT PERCENTAGES OF TOTAL PHOSPHOLIPID

Data of the first three rows are from Ref. 29. For *R. viridis* our data for large fraction 36% thylakoids have been inserted. CL, cardiolipin; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine

	PC	PG	CL	PE
R. rubrum	6	29	8	57
R. sphaeroides	23	34		35
R. capsulata	11	41		48
R. viridis	27		22	51

The lattice constant observed in freeze-fractured whole cells [16] ($d = 12.8$ nm) is in good agreement with the one of the Triton fraction 43% membranes (12.2 nm). However, the lattice constant observed by small-angle X-ray diffraction of large fraction 36% ($d = 13.3$ nm) is significantly larger. We suppose, that during breakage of the cells lipid is added to the thylakoids possibly from a pool of the kind postulated by Cain et al. [30]. The protein pattern remains qualitatively invariant as compared before and after detergent treatment.

According to Thornber et al. [31], isolated reaction center particles of *R. viridis* consist of four protein bands of apparent molecular weights 38, 35, 28, 24 kDa and an estimated stoichiometry of 2 : 1 : 1 : 1. The 28 and 24 kDa bands are reported to disappear on heating and the 38 kDa band is found to be pigmented, with absorption characteristics of heme. In our experiments, heating removed the 22 kDa band only, while the 34 kDa band is pigmented with heme absorption characteristics. We therefore suppose that the 22 and 27 kDa bands we observed are identical to the 24 and 28 kDa proteins. Furthermore, we suppose that the 34 and 27 kDa bands seen by us are related to the 38 and 35 kDa reaction center proteins. The two remaining strong bands at the bottom of our gels most likely represent the antenna-pigment complex. Crystals of pure antenna-pigment protein complexes can exhibit lattices similar to those seen in our experiments [32,33].

The Moiré pattern clearly indicates that the hexagonal lattice can exist within a single membrane, without close association with a second one.

Discussion of structural results

X-ray diffraction patterns and micrographs of freeze-fractured Triton fraction 43% thylakoids indicate the existence of a hexagonal lattice with an interparticle distance of about 12.2 nm. X-ray diffraction shows only three Debye-Scherrer rings; optical diffraction of negatively stained micrographs of the same samples exhibits about the same resolution. Therefore, our initial goal to prepare highly ordered membrane crystals by detergent treatment was not achieved.

In a model proposed by Wehrli [6] and independently by us [7], the particles seen in the

thylakoids are assumed to consist of a central reaction center complex surrounded by peripheral antenna-pigment complexes. Wehrli [6] postulates some 12 particles and shows image reconstructions supporting this model. Though we believe in the general features of this model, we suggest care in interpretations of the filtered negative-staining map on the basis of this model. The main problem of our electron-density and negative-staining distribution maps is the low resolution of only some 3.0 nm, and X-ray diffraction indicates that this is an intrinsic property of the wet thylakoids and is not caused by the negative staining procedure. The low-resolution of an electron density projection could be caused by a matching of the fine spatial details in the projection procedure, but another and more probable explanation seems us to be inherent disorder. The main aim of further experimental work therefore must be to find and remove the source of disorder, to be able to evaluate a map of higher resolution.

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References

1 Unwin, P.N.T. and Henderson, R. (1975) J. Mol. Biol. 94, 425–440
2 Giesbrecht, P. and Drews, G. (1966) Arch. Mikrobiol. 54, 297–330
3 Garcia, A., Vernon, L.P., Ke, B. and Mollenhauer, H. (1968) Biochemistry 7, 326–332
4 Miller, K.R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 6415–6419
5 Wehrli, E., Kübler, O. and Koller, T. (1979) Experientia 35, 984
6 Wehrli, E. and Kübler, O. (1980) in Electron Microscopy at Molecular Dimensions (Baumeister, W. and Vogell, W., eds.), pp. 48–56, Springer-Verlag, Berlin
7 Hodapp, N. and Kreutz, W. (1980) Biophys. Struct. Mech. 7, 65–95
8 Drews, G. (1965) Bakteriol. Paras. Infektionskr. Hyg. Abtl. Orig. Suppl. 1, 170–178
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L.and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
10 Awasthi, Y.C., Chuang, T.F., Keenan, T.W. and Crane, F.L. (1971) Biochim. Biophys. Acta 226, 42
11 Ronai, A. and Wunderlich, F. (1975) J. Membrane Biol. 24, 381–399
12 Ferber, E., DePasquale, G.G. and Resch, K. (1975) Biochim. Biophys. Acta 398, 364–376
13 Laemmli, U.K. (1970) Nature 227, 680–685
14 Nermut, M.V. and Williams, L.D. (1977) J. Microsc. 110, 121–132
15 Stange, U., Hodapp, N., Welte, W., Kreutz, W. (1980) 7th European Congress on Electron Microscopy, The Hague, Vol. 2, 464–465
16 Welte, W., Hodapp, N., Aehnelt, C. and Kreutz, W. (1981) Biophys. Struct. Mech. 7, 209–212
17 Jost, P.C., Griffith, O.H., Capaldi, R.A. and Vanderkooi, G. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 480–484
18 Holloway, P.W. (1973) Anal. Biochem. 53, 304–308
19 Kenyon, C.N. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), Ch. 13, Plenum, New York
20 Gilev, V.P. (1979) Ultramicroscopy 4, 323–336
21 Jost, P.C., Capaldi, R.A. and Vanderkooi, G. (1973) J. Supramol. Struct. 1, 269–280
22 Fry, M. and Green, D.E. (1980) Biochem. Biophys. Res. Commun. 93, 1238–1246
23 Fry, M., Blondin, G.A. and Green, D.E. (1980) J. Biol. Chem. 255, 9967–9970
24 Birrell, G.B., Sistrom, W.R. and Griffith, O.H. (1978) Biochemistry 17, 3768
25 Fraley, R.T., Jameson, D.M. and Kaplan, S. (1978) Biochim. Biophys. Acta 51, 52–69
26 Fraley, R.T., Yen, G.S.L., Lueking, D. and Kaplan, S. (1979) J. Biol. Chem. 254, 1987–1991
27 Kaplan, S. (1981) Photochem. Photobiol. 34, 769–774
28 Oelze, T., Kaiser, I. and Schrader, M. (1980) FEMS Microbiol. Lett. 9, 145
29 Oelze, J. and Drews, G. (1981) Membranes of Phototrophic bacteria, in: Organization of Procaryotic Cell Membranes (Ghosh, B., ed.), CRC Press, Boca Raton, FL
30 Cain, B.D., Deal, C.D., Fraley, R.T. and Kaplan, S. (1981) J. Bacteriol. 145, 1154–1166
31 Thornber, J.P., Cogdell, R.J., Seftor, R.E.B. and Webster, G.D. (1980) Biochim. Biophys. Acta 593, 60–75
32 McDonnel, A. and Staehelin, A. (1980) J. Cell Biol. 84, 40–56
33 Mullet, J.E. and Arntzen, C.J. (1980) Biochim. Biophys. Acta 589, 100–117
34 Ross, E. and Schatz, G. (1976) J. Biol. Chem. 251, 1991