Two-dimensional Crystals Formed from Photosynthetic Reaction Centers

KENNETH R. MILLER and JULES S. JACOB
Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

ABSTRACT Photosynthetic reaction centers from the bacterium *Rhodopseudomonas viridis* were prepared after detergent solubilization of photosynthetic membranes. The purified reaction centers, in agreement with reports from other laboratories, contain four distinct polypeptides ranging in molecular weight from 28,000 to 41,000. When the detergent was gradually removed by dialysis under appropriate conditions, large two-dimensional sheets of reaction centers were formed, suitable for analysis by electron microscopy. The crystals were rectangular, and the dimensions of a single unit cell were 121 × 129 Å. Each unit cell contained four distinct subunits, each with approximate dimensions of 45 × 60 Å. The thickness of the sheet was 60 Å. Preliminary studies of the sheets with negative staining indicated that the sheets show a high degree of order: as many as six orders are visible in transforms of the images. Because of the fact that in *R. viridis* the native membrane from which these reaction centers were purified also displays a crystal-like structure, comparative studies between a membrane and one of its components, each analyzed by Fourier techniques, are now possible.

Rhodopseudomonas viridis is a purple, nonsulfur photosynthetic bacterium. The light reactions of photosynthesis take place within the cell on specialized photosynthetic membranes which exist as large flattened saccules. *R. viridis* is of special interest for structural studies owing to the regular arrangement of subunits within the membrane. These subunits are arranged in the form of a "membrane crystal" that allows images of the membrane to be analyzed by Fourier techniques for detailed structure determinations. In the case of *R. viridis*, both two-dimensional (7, 10, 11) and three-dimensional (8) analyses of membrane structure have been carried out.

Recently, Michel (6) has reported the first three-dimensional crystals of purified reaction centers obtained from *R. viridis*. The reaction centers used for these preparations constitute a subset of membrane proteins: there are six polypeptides in the native membrane (3, 4) and four in the purified reaction complex (3, 4, 6, 9). In this paper, we describe the preparation of two-dimensional crystals suitable for high resolution analysis by electron microscopy and electron diffraction.

MATERIALS AND METHODS

Cultures of *R. viridis* were grown photosynthetically in succinate medium and cells were harvested as described previously (3). In a typical isolation, cells were harvested from 2 liters of culture after a 2-wk growth period following inoculation. The cells were homogenized using a French pressure cell and the photosynthetic membranes were isolated to nearly 100% purity by electron microscopic criteria using sucrose density gradient centrifugation (3).

The purified membranes were washed free of sucrose by resuspension in 10 mM sodium phosphate buffer containing 1 mM tetrasodium EDTA, pH 7.2. The membranes were pelleted at 40,000 *g* for 30 min at 4°C, using a Sorvall SS-34 rotor, resuspended and pooled in a fresh volume of phosphate buffer and stored as a concentrated sock at −20°C until needed.

Isolation of Reaction Centers from Purified Photosynthetic Membranes: Typically, one-half of the frozen stock of membranes from an isolation would be thawed, pelleted at 40,000 *g* for 30 min at 4°C using the SS-34 rotor (Beckman Instruments, Inc., Palo Alto, CA) and the supernatant fluid discarded. The membranes were then resuspended in 5 ml of 50 mM Tris containing 0.1% LDAO[1] (*N,N*-dimethyldodecylamine-*N*-oxide) (vol/vol), 0.01% sodium azide (wt/vol), pH 7.25 (23°C). 1 ml of 30% LDAO (vol/vol) was added to the membrane suspension and solubilization was carried out at 4°C. After 20 min, the mixture was centrifuged at 40,000 *g* for 60 min at a 4°C using the SS-34 rotor to remove LDAO-insoluble material.

The supernatant fluid was then applied to an 80 ml (packed volume) column (1.5 × 50 cm) of hydroxylapatite using 10 mM potassium phosphate containing 0.1% LDAO (vol/vol), 0.01% sodium azide (wt/vol), pH 7.0, as the column buffer. All column isolation procedures were performed at 4°C. Light-harvesting bacteriochlorophyll-protein complexes were eluted by passing ~150 ml of the phosphate buffer through the column at a flow rate of 15 ml/h. Reaction center was eluted by passing 200 ml of a linear 0.01/0.3 M potassium phosphate (containing 0.1% LDAO, pH 7.0) gradient through the column. The reaction center fraction was collected at a concentration of ~0.15 M potassium phosphate. Typically, the ratio OD_{280}/OD_{830} was in the range 2.3 to 2.6. Reaction center fractions were either used immediately or could be stored at −20°C. for at least several months without noticeable changes in spectral or biochemical characteristics. Reaction center fractions were used without any further purification.

[1] *Abbreviations used in this paper:* LDAO, *N,N*-dimethyldodecylamine-*N*-oxide.

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Formation of Reaction Center Crystals: 2-ml aliquots of the reaction center fraction were dialyzed for at least 24 h against 0.3 l of 0.1 M sodium phosphate buffer containing 0.01% sodium azide, pH 5.3, at 23°C. The dialysis tubing used had a maximum pore size of 12,000–14,000 mol wt. The protein concentration of the dialysate was in the range of 1–2-mg reaction center protein/ml, as judged by the optical density of the reaction center fraction at 280 nm. This particular condition was optimal, although changes in ionic strength (0.01–0.3 M sodium phosphate) and pH (pH 5.0–6.6) of the dialysis fluid produced useful crystals. Probably most important was the influence of temperature on crystal formation. Initially, the recrystallization experiments were performed at 4°C with sporadic success. Changing the temperature of the dialysis to 23°C produced improved reaction center crystals. Similar results have been reported for crystals of cytochrome oxidase (2).

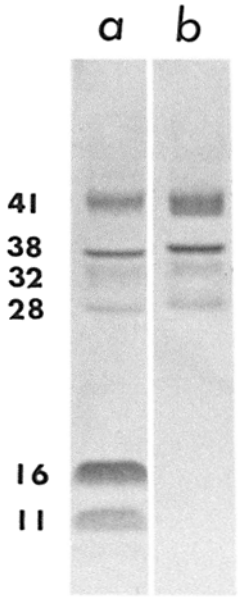


FIGURE 1 Electrophoresis of intact membranes (*a*) and reaction centers (*b*). Bands at the 41,000-, 38,000-, and 28,000-mol-wt markers are reaction center-associated. The bands at 16,000- and 11,000-mol-wt markers are light-harvesting components. The major biochemical difference between the native photosynthetic membrane and the purified reaction center is the absence of light-harvesting components from the latter.

At the end of the dialysis, the contents of the dialysis bags were centrifuged at 14,000 *g* for 1 min at 4°C using the Fisher microfuge. The pellets were resuspended in 0.5 ml of fresh 0.1 M sodium phosphate buffer containing 0.01% sodium azide, pH 5.3, and could be stored at 4°C for several weeks without obvious deterioration.

Electron Microscopic Techniques: Samples of the reaction center crystals were negatively stained with 2% aqueous uranyl acetate (wt/vol) and air-dried on carbon-substrate copper mesh grids and examined at 80 kV using a JEM 100B electron microscope.

Optical transforms of the electron microscopic images were recorded and digitized using a rotating drum scanning device. Fourier transforms of the digitized images were run on a VAX 11/780 minicomputer. Transform coordinates were selected to fit an ideal reciprocal lattice with p1 plane group symmetry. Images were displayed on a 256 gray level TV graphics screen and recorded photographically.

Gel Electrophoresis: Electrophoretic separations were performed on 12% acrylamide SDS slab gels using a modification of Laemmli's procedure (5) as described previously (3). Gels were stained with either Coomassie Blue R-250 or else the Gelcode color-silver stain procedure (Upjohn Diagnostics, Kalamazoo, MI).

LDAO was purchased from Continental Chemical Co. (Clifton, NJ) and was used as supplied. Acrylamide was purchased from Eastman Kodak (Rochester, NY) and purified using mixed-bed ion exchange resins to remove residual acrylic acid. Other reagents for SDS acrylamide gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA) and were of "electrophoresis quality." All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO), and were of the highest quality available from the supplier. Water was deionized and distilled.

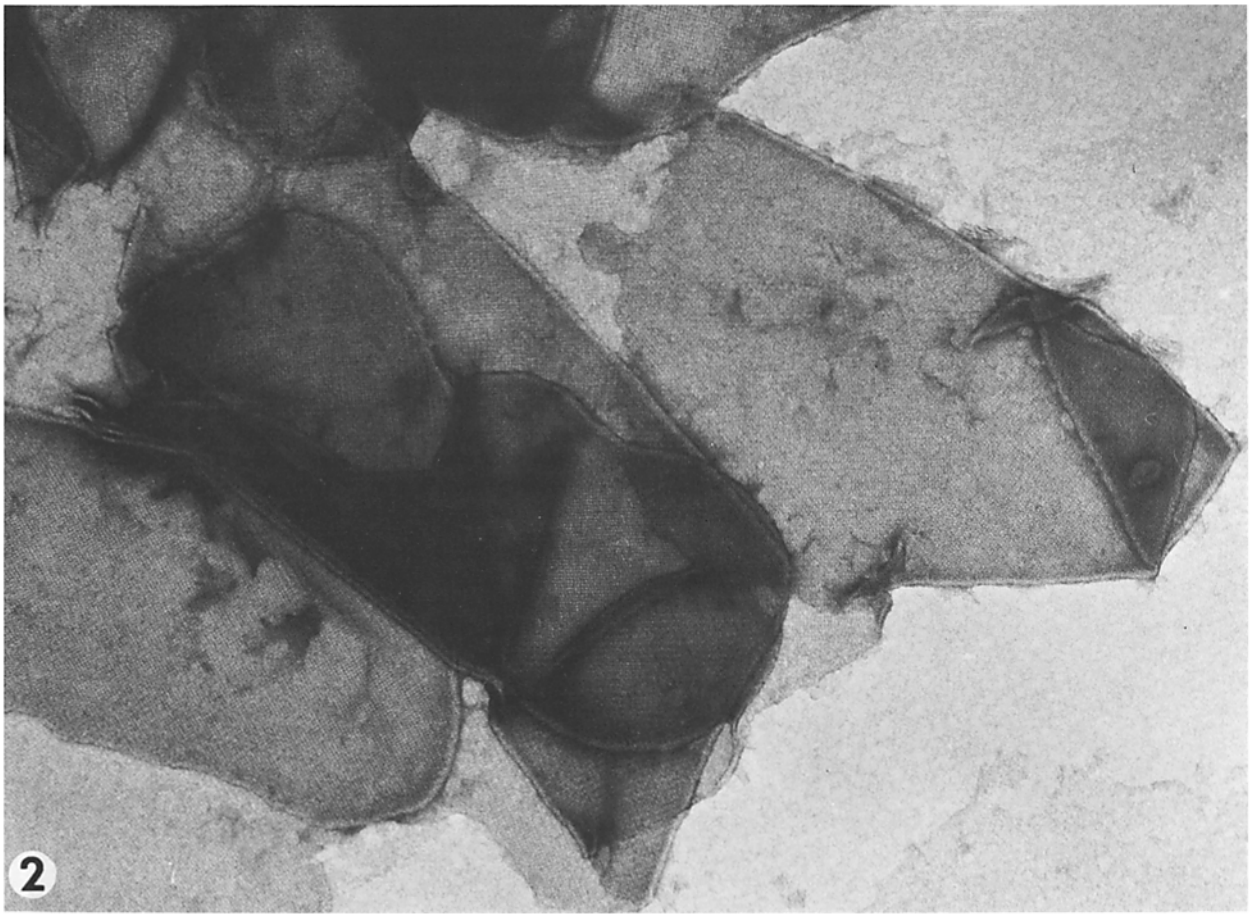
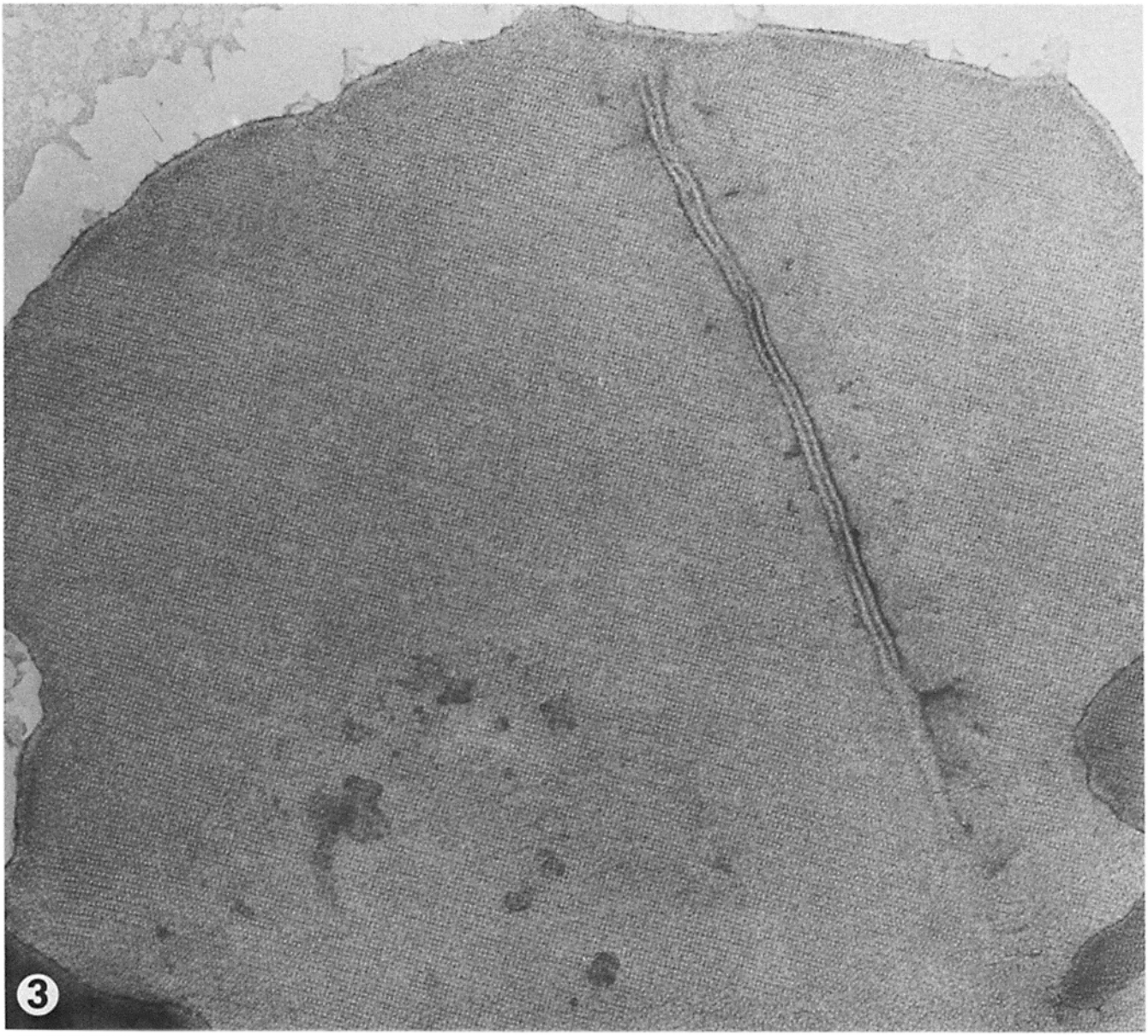
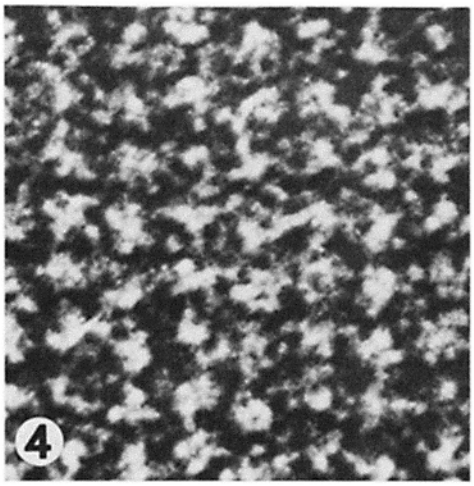
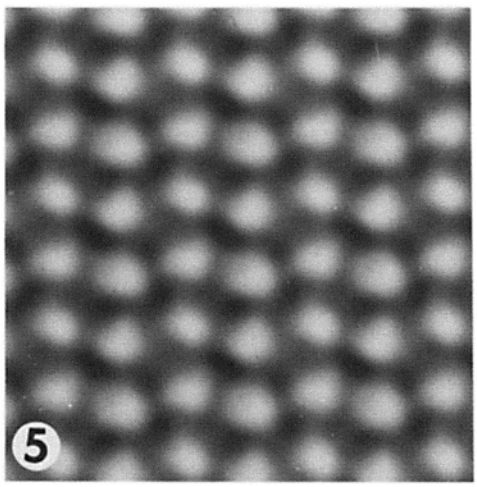
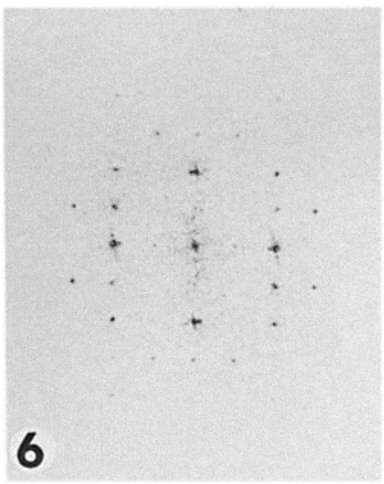


FIGURE 2 Two-dimensional crystalline sheets resulting from detergent dialysis of the LDAO-solubilized reaction center fraction. These sheets form at low pH and at near room temperatures, are present in large numbers and are highly ordered. × 70,000

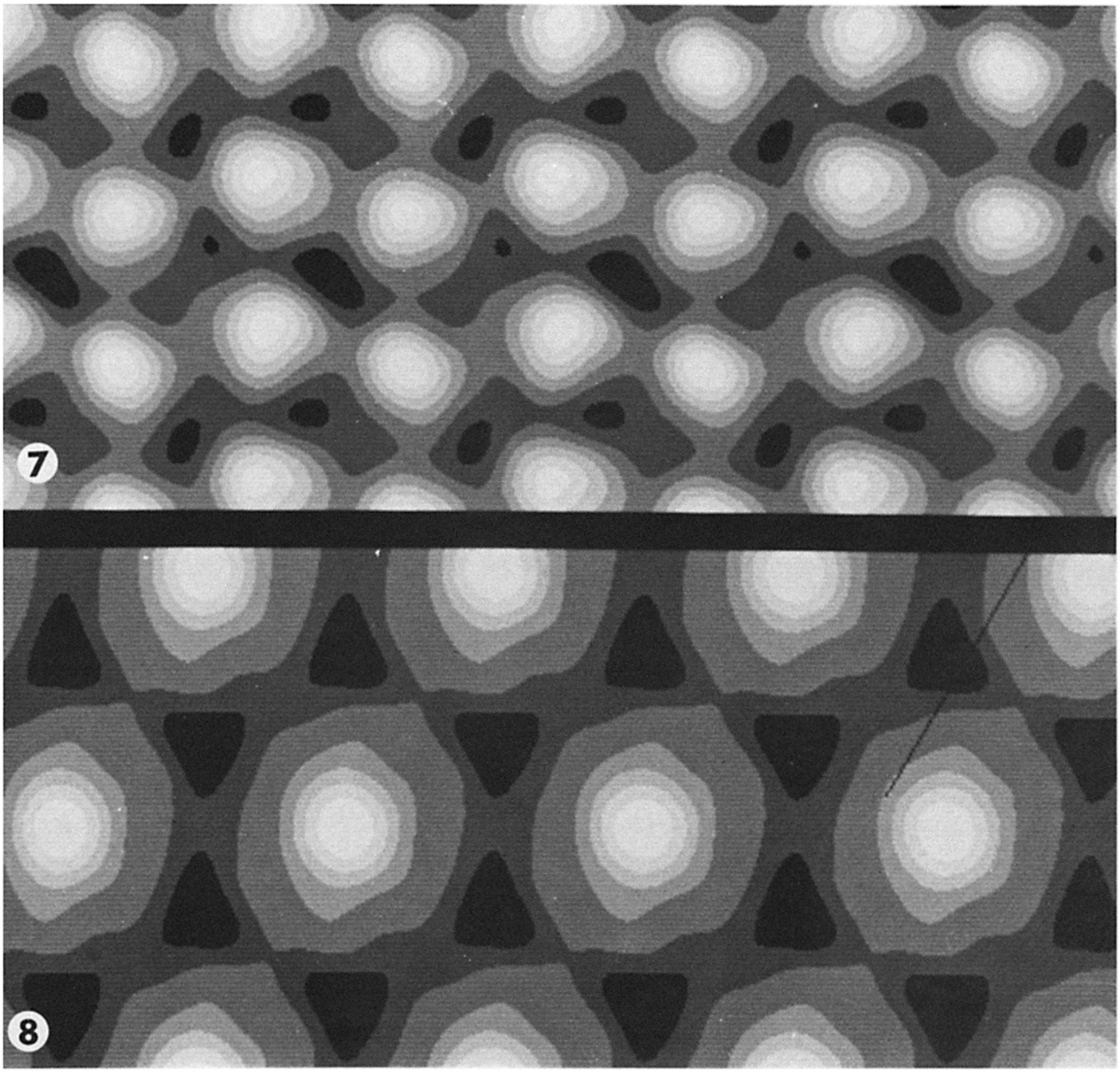








FIGURES 3–6 Fig. 3: Individual sheet showing a large, well-ordered area. A fold in the sheet is visible along the right side of the micrograph which permits the thickness of the membrane to be estimated. × 95,000. Fig. 4: Highly magnified detail of Fig. 3, illustrating subunit arrangement in the sheet of purified reaction centers. × 1,400,000. Fig. 5: Image of sheet similar to Fig. 4 but derived from Fourier filtering methods. The enhancement of detail by image processing affords a clearer view of the individual subunits. × 1,400,000. Fig. 6: Computed Fourier transform of electron micrograph of purified reaction center sheet. Unit cell spacings are 121 Å in the horizontal direction, and 129 Å in the vertical direction.



FIGURES 7 and 8 Fig. 7: Filtered image of a small portion of the reaction center sheet. The approximate center-to-center spacing between stain-excluding subunits (shown as light areas) is 65 Å. When compared with Fig. 8, the native membrane, a much closer packing of stain-excluding regions is observed. This image was prepared from a single micrograph and was not averaged for symmetry. Fig. 8: Detail of a filtered image of the native *R. viridis* membrane from which the reaction centers were isolated. The membrane subunits pack in a hexagonal lattice, and a center-to-center spacing of ~130 Å is observed. Note the increased spacing between stain-excluding regions. This image was prepared from a single micrograph, and was not averaged for symmetry.

RESULTS

We have characterized the reaction centers prepared as described in Materials and Methods according to two essential characteristics: absorption maxima and polypeptide composition, as determined by SDS PAGE. The ratio of absorbance at 830 nm to that at 280 nm provides a useful standard of comparison for the purity of our preparation to those reported elsewhere. For the preparations used here, that value ranged between 2.3 and 2.5 (OD_{830}/OD_{280}).

PAGE of *R. viridis* membranes and purified reaction centers is shown in Fig. 1. These gels illustrate the main biochemical difference between the native membrane and the purified reaction centers. Two low molecular weight polypeptide bands (at 11,000 and 16,000 mol wt) are absent from the reaction center preparations.

Detergent dialysis of purified reaction centers results in the formation of regular sheets as shown in Fig. 2. These sheets were formed under a wide variety of experimental conditions, although the optimum conditions for their formation included dialysis at low pH (5.3) and room temperature, as described in Materials and Methods.

The sheets show a high degree of order and close packing of individual subunits, as illustrated in Fig. 3. In contrast to the native *R. viridis* photosynthetic membrane, the subunits arranged within these sheets are ordered in a rectangular

lattice rather than a hexagonal one (7, 10, 11). Optical diffraction of images of these sheets in negative stain shows a rectangular pattern with unit cell dimensions of 121 × 129 Å (mean of 10 measurements) and the best patterns extend to six orders in the smaller dimension indicating an image resolution of ~20 Å. No special precautions were taken during microscopy to limit electron dose, and we expect that this value will be markedly improved in future studies.

Individual micrographs were digitized and used for Fourier analysis to enhance the regular features of the sheets. Figs. 4 and 5 show a comparison between comparable units of the membrane before and after Fourier image processing. The individual subunits within the lattice are clearly seen as electron-transparent, stain-excluding structures at intervals of ~65 Å in either direction.

Figs. 7 and 8 show, for purposes of comparison, a detailed view of the two-dimensional projected image of the reaction center crystal and, at the same scale, a similar processed image from the native *R. viridis* membrane from which the reaction centers were purified. Neither image has been averaged for symmetry, and each is the product of a single micrograph. The approximate dimensions of each subunit within the reaction center sheet is 50 × 60 Å. Other micrographs (Fig. 3) allow the thickness of the reaction center sheets to be estimated from edge-on views, and we have measured the thickness of the sheet in negative stain to be 60 Å.

DISCUSSION

The formation of two-dimensional sheets from purified reaction centers of *R. viridis* allows the direct analysis of a major membrane component with the native membrane from which it has been prepared. This is made possible because both the native membrane and the sheets containing reaction centers are organized in crystalline fashion. In a recent study of the three-dimensional architecture of the native membrane (8), one of us estimated the volume of the entire complexes within the native membrane to be 550,000 Å^3. The volume of each subunit within the two-dimensional sheets is approximately 180,000 Å^3. Studies from our lab and others (3, 4, 6, 11) indicate that the reaction center of *R. viridis* is composed of four polypeptides with apparent molecular weights of 28,000, 34,000, 38,000, and 41,000. The total molecular weight of a single reaction center, if all components were present at the same molar ratio, would be 141,000 mol wt. Assuming a value of 1.3 A^3 per molecular weight (1), this would predict a volume for the reaction center of 183,000 Å^3. These calculations allow us to conclude that the subunits within the sheets are indeed individual reaction centers.

Although a three-dimensional analysis of the reaction center sheets is in progress, it is not possible from the data available now to directly relate the subunits within the reaction center preparation to the native membrane. The native membrane is characterized, however, by a large structure which protrudes from both surfaces of the membrane (see also Fig. 7), and it is quite likely that this in fact is the position of the reaction center in the native membrane. A recent study in our laboratory (3), which showed that the reaction center polypeptides are preferentially accessible to proteolysis, also supports this view.

These results, especially when taken in light of Michel's recent success in preparing three-dimensional crystals of the *R. viridis* reaction center (6), indicate that *R. viridis* is an ideal system in which to analyze the structures of photosynthesis. The relationship between our two-dimensional crystals and Michel's three-dimensional crystals (6) is not clear. He reports a unit cell dimension of 223 × 223 × 114 Å, which would approximate the volume of eight unit cells (containing 32 subunits) from our two-dimensional crystals. In addition, the space group of the tetragonal three-dimensional crystals is reported to be $P4_1^2{}_1^2$ or $P4_3^2{}_1^2$ (6). In contrast, the highest symmetry which can be assigned to our two-dimensional crystals is P2 (there is a twofold rotational axis perpendicular to the plane of the crystalline sheet).

The advantages presented by the *R. viridis* system, including the ability to analyze both the native membrane and one of its purified components by Fourier techniques, are considerable. Work is currently underway in this laboratory to increase the available resolution of the images and to prepare a comparative description of both specimens in three dimensions.

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