STRUCTURE OF THE PHOTOSYNTHETIC APPARATUS IN PROTEIN-EMBEDDED CHLOROPLASTS

GARTH L. NICOLSON. From the Department of Biology, University of California, San Diego, La Jolla, California 92037

INTRODUCTION

The photosynthetic apparatus in higher plants has been found to be located in complex lipoprotein membrane systems in the chloroplasts called grana. Electron microscopy has provided invaluable information in determining the fine structure of grana (6–9, 11, 21, 28–30). However, due to the harsh methods employed in fixation (usually by osmium tetroxide or potassium permanganate) and dehydration in lipid solvents (usually alcohol or acetone), the preservation of native structure has been questioned (11, 12, 16, 20, 22). Utilization of the freeze-etch technique for electron microscopy (1, 3, 23–26) and low angle X-ray scattering (13, 14, 18, 19) have successfully avoided some potential artifacts, but the resolution of these techniques has not been sufficient to visualize the cross-sectional molecular structure of grana.

Recently, Farrant and McLean (5) developed procedures for embedding biological materials in bovine serum albumin (BSA), without the harsh treatment with heavy metal fixatives and lipid solvents. In the present study, isolated spinach chloroplasts were fixed in low concentrations of glutaraldehyde, formaldehyde, or diethylmalonimidate and were embedded in BSA. Chloroplasts in cross-linked BSA sections were examined by transmission electron microscopy.

METHODS

Spinach chloroplasts were prepared in Dr. Warren Butler's laboratory by blending 50 g of deribbed ice-cold leaves in 150 ml of modified pyrophosphate-

sucrose buffer (10), 580 milliosmols (mosmols), or phosphate-sucrose buffer (0.1 M sodium phosphate, pH 7.9, 580 mosmols) at 0°C for a total of 1 min at 20-sec intervals. The mixture was passed through two layers of bolting cloth and immediately centrifuged at 300 *g* for 1 min to remove cell debris. The chloroplast fraction was washed three times by centrifuging at 600 *g* for 7 min and resuspending the pellet in buffer. Washed chloroplasts were fixed with 1% glutaraldehyde or 1% formaldehyde (in the appropriate buffer) at 5°C for 1 hr, or with 0.2 M diethylmalonimidate (DEM) (4) in buffer (pH 8.5) for 3 hr at 5°C (DEM is less reactive than aldehyde fixatives [16]). The fixed samples were washed several times with buffer and suspended in 10–30% BSA following the method of Farrant and McLean (5). The chloroplast-BSA suspension was transferred to a small tube (diameter 1 cm) with a dialysis membrane on the bottom. The tube was placed in Aquacide II (Calbiochem, Los Angeles, Calif.), and the suspension was allowed to dry to a firm gel. The protein block was cut into approximately 1-mm cubes and further dried over silica gel. The cubes were cross-linked with 1–2% glutaraldehyde for 1–6 hr, washed extensively, and redried over silica gel. If formaldehyde is used to cross-link the BSA, it is usually added directly to the tube containing the dried protein gel at the bottom. After allowing the fixative to penetrate the block for a few hours at room temperature, the block is washed and dried and then cut into small cubes. In the drying steps, vacuum was avoided to prevent bubbles from forming in the embedding matrix.

The dry, cross-linked cubes were mounted on the tips of Epon blocks with sealing wax and cut on a Porter-Blum MT-1 microtome using a du Pont diamond knife (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Silver-to-gold sections cut onto water at the lowest microtome settings were picked up on grids covered with carbon-collodion or carbon-Formvar (Belden Mfg. Co., Chicago, Ill.) micromesh (0.1–1 μ diameter holes). The grids were floated on 2% potassium phosphotungstate (PTA), pH 6.8, or 2% uranyl acetate (UAc), pH 4.8, for a few seconds to a few minutes and the excess stain was slowly withdrawn with a filter paper, leaving a thin film of stain to dry down on the substrate. Thin-section staining with a ferritin-antibody (anti-BSA) conjugate followed the procedure of McLean and Singer (17).

Through-focus electron micrographs were taken on a Phillips Model EM-300 or EM-200 electron microscope at 60 kv accelerating voltage. Photographic contrast was reversed for one of the figures by enlarging the original electron micrograph plate onto Panatomic X (Eastman Kodak Co., Rochester, N.Y.) in the Zeiss Ultraphot II microscope.

RESULTS

The membranes of BSA-embedded spinach chloroplasts stained with PTA or UAc appear unstained in contrast to the other regions of the chloroplasts which bind stain intensely (Figs. 1 and 3). It is postulated that the hydrophobic internal nature of these membranes (2, 15, 28) prevents the binding of highly charged stains. Because the material is cross-linked under very mild conditions and is not dehydrated in lipid solvents, the membrane lipid is expected to be present in approximately normal amounts in the embedded material. Efforts to determine the degree of lipid extraction from photosynthetic bacteria during this same fixation and dehydration procedure have shown that no detectable amount of lipid is removed (G. L. Nicolson, unpublished results).

In some regions (Fig. 1) the chloroplast separated from the embedding matrix. This is probably due to the different swelling behaviors of the chloroplast proteins and the BSA. As McLean and Singer (17) found with red blood cells, the embedding protein does not penetrate into the intact plastid. This is demonstrated by incubating protein thin sections with an electron-opaque ferritin-conjugated anti-BSA. The ferritin-conjugated antibodies are localized completely outside the chloroplasts in the embedding matrix. The binding of ferritin-conjugated anti-BSA is specific, as the sections do not bind a nonhomologous ferritin-conjugated antibody, ferritin-anti (human) spectrin.

Staining the BSA sections with UAc or PTA (Figs. 1 and 3) using techniques similar to negative staining yields essentially the same results. Thus the binding of UAc or PTA may depend not on the negative or positive charge of the stain, but instead on the hydrophilic nature of the substrate. The staining contrast is not greatly affected by staining time or section thickness, but washing the grid with a few drops of water (or buffer) or using a stain of lower concentration reduces contrast drastically. It is possible that PTA and UAc may fill in some of the open areas of the substrate that were predominately water-filled before desiccation.

Occasionally a crystal-like array is found inside the intact, BSA-embedded chloroplasts (Fig. 2). The crystal-like structure has a repeating distance of about 100 A and can account for up to one-half of the chloroplast area exposed by sectioning. This

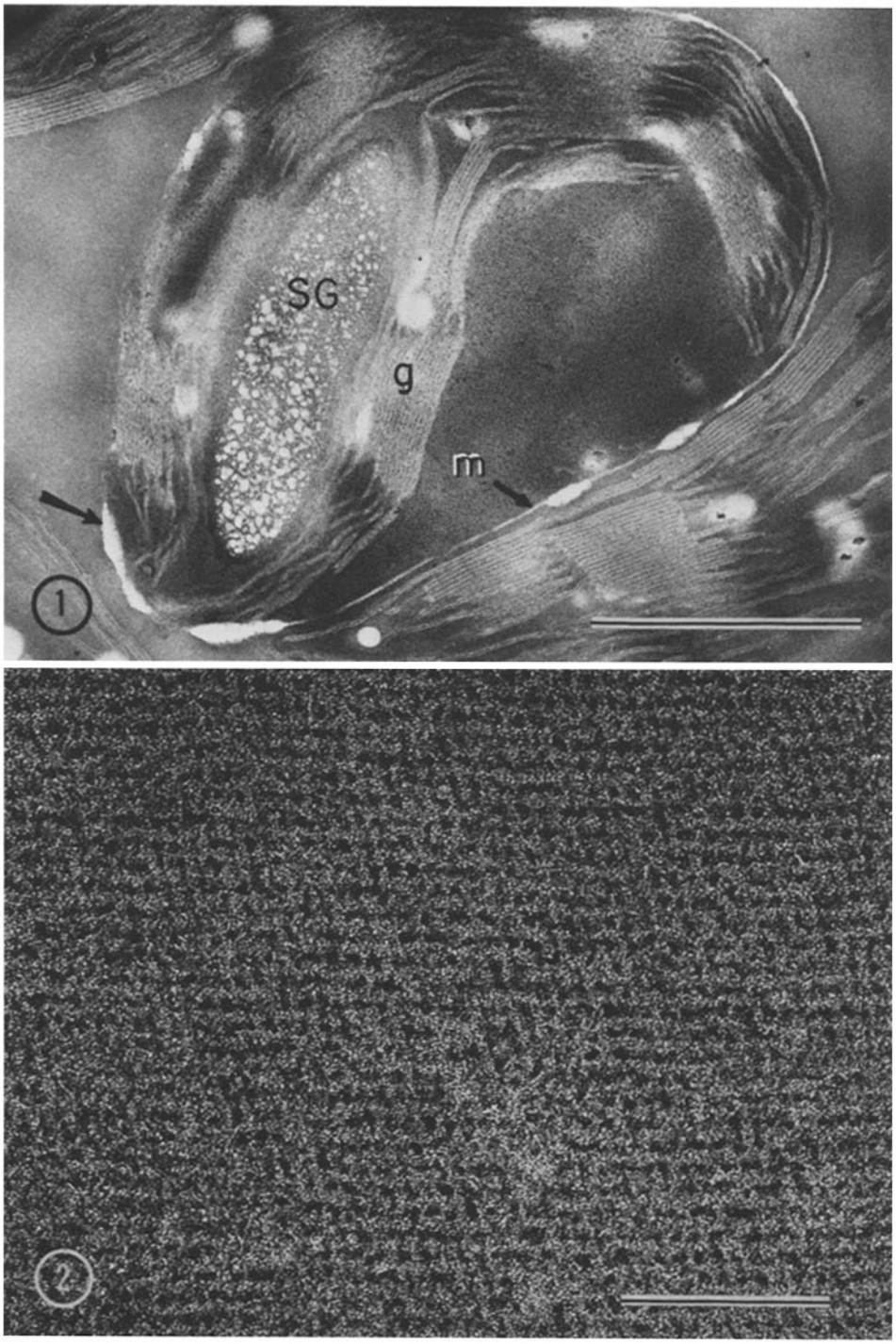

FIGURE 1 Glutaraldehyde-fixed spinach chloroplast embedded in bovine serum albumin. The outer membrane (*m*), grana membranes (*g*), and the starch granule (*SG*) appear in opposite contrast with respect to the rest of the chloroplast interior and the embedding matrix. The arrow shows where the chloroplast and embedding matrix have separated (see text). The section was stained with uranyl acetate. Bar equals 1 μm. × 39,000.

FIGURE 2 Crystal-like region of a protein-embedded chloroplast; section stained with phosphotungstate. Crystal periodicity is approximately 100 A. Bar equals 0.1 μm. × 284,000.

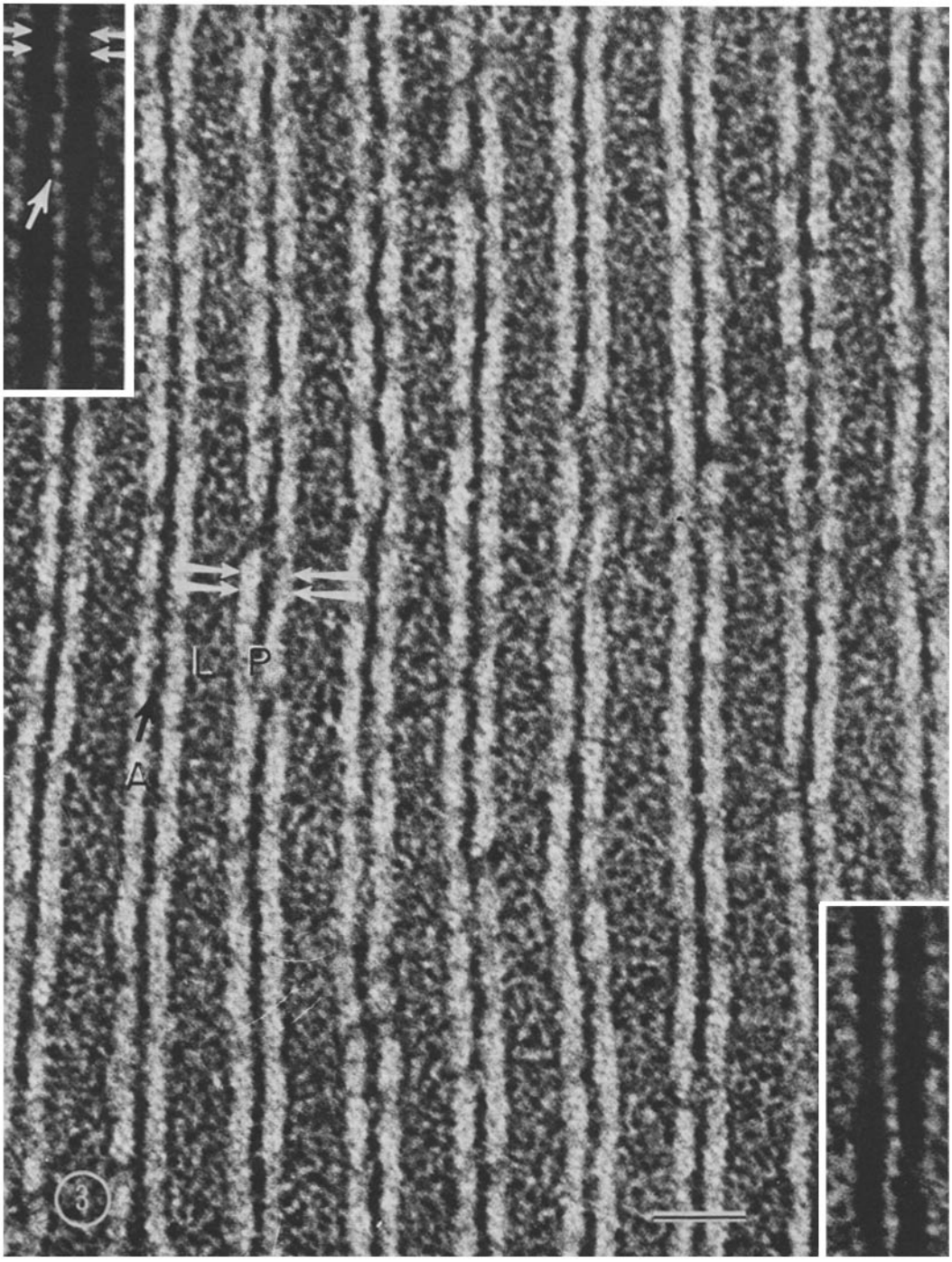

FIGURE 3 A region of a chloroplast granum in a protein-embedded chloroplast. Double arrows indicate the dimension of a partition (105–115 A) (*P*). The loculi (*L*) and the A spaces (*A*) stain intensely with phosphotungstate. *Inserts* have been photographically phase reversed and increased in contrast to show structures (arrow) in the A spaces. Bar equals 0.02 μm. × 656,000; *inserts*, × 1,100,000.

may be fraction I protein which is present in very high amounts in the chloroplast (27).

The chloroplast grana (Figs. 1 and 3) stain densely with PTA in two regions: (*a*) the intrathylakoid regions (loculi) which are enclosed by double-membrane structures called partitions, and (*b*) the thin interthylakoid regions ("A spaces") which separate the two nonstaining membranes of the partitions. The two nonstaining membrane regions of each partition (Fig. 3) are approximately 40–45 A thick and are separated by an ~20 A thick A space. Thus, the total partition thickness (40–45 A membrane: 20 A–A space: 40–45 A membrane) is about 105–115 A. Photographic reversal of the partition regions (inserts, Fig. 3) reveals structures in the A spaces that have a periodicity of approximately 30 A.

DISCUSSION

It has been postulated (7, 11, 29), on the basis of chloroplast grana swelling experiments, that the hydrophobic regions of the grana are in the double-membrane regions called partitions. The results here also show that the hydrophobic regions of the grana are in the partitions, not in the loculi (membrane-enclosed regions separating adjacent partitions) which stain intensely with heavy-metal negative stains. However, the A spaces (thin zones between the membranes in the partitions) also stain intensely with negative stains, which suggests that the A spaces are hydrophilic in nature. The postulate that the binding of heavy-metal negative stains is dependent on the hydrophilic nature of the substrate is supported by the following observations: (*a*) membranes which are known to be internally hydrophobic in nature (2, 15, 28) do not bind the negative stains in protein sections; (*b*) chloroplasts that have been fixed in glutaraldehyde and extracted with methanol-chloroform solutions before embedding in protein now appear to bind negative stains to their membranes (or at least the contrast between the membranes and other regions of the section is dramatically reduced); and (*c*) synthetically prepared lecithin-cholesterol (6:1 mole/mole) membranes (31) embedded in protein show the same staining characteristics as biological membranes (G. L. Nicolson, unpublished results).

Menke (18, 19) and Kreutz (13, 14) have proposed, on the basis of X-ray scattering data, models which place lipid and porphyrin on the opposite side from protein in the thylakoid membranes. Menke has presented a model (18, 19) that places lipid and porphyrin in the loculus regions. The hydrophobic regions of the grana do not appear to be in the loculi, but rather in the partition membranes which do not bind negative stains. Weier and Benson (28) interpret the data of Menke and Kreutz as evidence for the localization of light harvesting pigments and other lipids within the membranes of the partitions, near or partially in the A spaces. Weier and Benson do not assign a structural role to the A spaces, except as hydrophobic regions containing porphyrin heads and other lipid material. In the present study the A spaces appeared to contain hydrophilic structures that probably maintain the spacing between the membranes of the partitions at about 20 A.

On the basis of these and other studies (5, 17), protein embedding appears to be a useful method for studying the structure of membrane systems by electron microscopy. Studies are currently in progress which utilize postembedding, ferritin-antibody and ferritin-plant agglutinin (G. L. Nicolson and S. J. Singer. *Proc. Nat. Acad. Sci. U.S.A.* In press) staining on thin sections to determine the localization of specific macromolecules and oligosaccharides. The mildness of this embedding technique permits the preservation of protein antigenic structures and does not extract membrane lipids.

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