The Effects of Galactolipid Depletion on the Structure of a Photosynthetic Membrane

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Abstract. The galactolipids monogalactosyldiglyceride and digalactosyldiglyceride together comprise more than 77% of the photosynthetic membrane lipids of higher plant chloroplasts. We have isolated a lipase from the chloroplasts of runner beans (*Phaseolus vulgaris*) which is highly specific for these galactolipids. This galactolipase promotes the hydrolysis of monogalactosyldiglyceride and digalactosyldiglyceride, in the process liberating two free fatty acids into the membrane bilayer, leaving the residual galactosyl glyceride group to diffuse into the aqueous bulk phase.

Isolated spinach photosynthetic membranes were treated with this enzyme preparation and changes in membrane composition were studied with thin layer chromatography (for lipids), gel electrophoresis (proteins), and freeze–etching (membrane structure). After 30 min of lipolysis, nearly 100% of the galactolipids had been converted into membrane-associated fatty acids and water-soluble galactosyl glycerides. SDS PAGE showed that two proteins, one of which is possibly associated with the reaction center of photosystem II, were removed by the treatment.

Despite the minor nature of changes in membrane protein composition, freeze–fracture and freeze–etch studies showed that striking changes in membrane structure had taken place. The large freeze–fracture particle on the E fracture face had disappeared in stacked regions of the membrane system. In addition, a tetrameric particle visible at the inner surface of the membrane had apparently dissociated into individual monomeric particles. The fact that these two structures are so dramatically affected by the loss of galactolipids strongly suggests that these lipids play a crucial role in maintaining their structure. Both structures are believed to be different views of the same transmembrane unit: a membrane-spanning complex associated with photosystem II. Our results are consistent with two possible interpretations: the intramembrane particles may be lipidic in nature, and hence lipolysis causes their disappearance; or galactolipids are necessary for the organization of a complex photosystem II–associated structure which is composed of a number of different molecular species.

THE photosynthetic membranes of chloroplasts have been the subject of extensive study and have proven to be an ideal system for the correlation of membrane structure and function. The thylakoids, or inner membranous sacs, are the site for the light-driven reactions of photosynthesis. The membranes contain the two primary components of the photosynthetic apparatus, the reaction centers comprising photosystems I and II, as well as a battery of chlorophyll–protein complexes, carotenoids, and accessory pigments associated with the collection and transfer of energy to the reaction center molecules (1, 23, 39).

Most models for the organization of the reaction centers in the membrane have centered on the importance of the pigments and their apoproteins (1, 23, 39) in the energy–transfer process, while the function of lipids in the maintenance of the membrane architecture has been ascribed a largely passive or supportive role. To date, most studies of chloroplast membrane lipids have concerned themselves with either the nonspecific addition of lipids to thylakoids, using liposomes as vehicles for the transfer (25, 35), or else the use of general acyl hydrolases to degrade a wide spectrum of membrane lipids (11, 15, 27, 40). However, Anderson et al. (2) have described the isolation of a galactolipid-specific lipase from runner beans. Galactolipids comprise as much as 77% of the total thylakoid lipids (9), and may play a major role in membrane organization. The enzymatic characteristics of this galactolipase have been well documented, and its actions on the energy–transfer activities of chloroplasts have been thoroughly investigated (2, 33). We report here the effects of galactolipolysis on the morphology of thylakoid membranes, using the techniques of freeze–fracture/etch, and its effects on the protein complexes responsible for photosynthesis.

Materials and Methods

Isolation of Thylakoid Membranes

Approximately 50 g of market spinach was deveined, rinsed three times in distilled water, and homogenized for 15 s in 50 ml of ice-cold 150 mM NaCl, 50 mM Tris, 2 mM MgCl₂, pH 7.5, (isolation buffer) in a Waring blender

(Waring Products Div., Dynamics Corp. of America, New Hartford, CT), set at the maximum speed. All steps in the isolation procedure were performed at 4°C. The crude homogenate was passed through eight layers of gauze and the filtrate was centrifuged for 3 min at 200 g average in the SS-34 rotor of a Sorvall RC-5B centrifuge (DuPont Co., Wilmington, DE). The pellets were discarded and the supernatant fluids were centrifuged for 10 min at 5,200 g average in the same rotor. The pellets from this centrifugation were resuspended in 5 mM sodium phosphate buffer, pH 7.0, containing 5 mM tetrasodium EDTA. After 15 min, the membranes were again pelleted and resuspended in fresh unstacking buffer and left on ice for 60 min.

Isolation of Galactolipase

The galactolipase was prepared from aqueous extracts of acetone powders of homogenized runner bean leaf chloroplasts, according to the method of Anderson et al. (2). The crude enzyme extract was partially purified by precipitation with ammonium sulfate to 65% saturation and the precipitate was dialyzed against 50 mM sodium phosphate buffer, pH 7.0, and stored frozen at −20°C until needed. The specific activity of the galactolipase was ∼1.0 U/mg of protein (31), a higher value than that reported by Anderson et al. (2). We tested for protease activity in the galactolipase preparation by incubating the extract with a 1 mg/ml solution of BSA for 60 min at 37°C. No proteolysis of the BSA sample was detected. Subsequent gel electrophoresis of photosynthetic membranes after galactolipase treatment also failed to detect any proteolytic activity (see Fig. 2).

Galactolipase Treatment of Thylakoid Membranes

Unstacked thylakoid membranes were centrifuged for 10 min at 6,000 g average using the SS-34 rotor, the supernatant fluids were discarded, and the membrane pellets resuspended in 10 ml of ice-cold 50 mM sodium phosphate, pH 7.0 (incubation buffer). Equal aliquots were pipetted into 1.5-ml microfuge tubes and centrifuged at 14,000 g for 4 min in a Fisher Microfuge (Fisher Scientific, Medford, MA). The supernatant fluids were discarded and the pellets, containing typically 0.4–0.5 mg of total chlorophyll, were resuspended in 1.0 ml of either fresh incubation buffer or else the galactolipase preparation in incubation buffer. Membranes were incubated at either 30 or 37°C for intervals of 20 to 60 min with shaking. At the end of the incubation, the thylakoids were again centrifuged and the reaction quenched by resuspending the membranes in 1.5 ml of ice-cold incubation buffer. The galactolipase has very little activity at 0–4°C, and at temperatures exceeding 15°C, the enzyme has a Q10 of approximately 2 (2). The washing procedure was repeated twice in the course of 10 min. Replicate samples were used for high-performance thin-layer chromatography (HPTLC)[1], SDS PAGE, spectrophotometry, and freeze-fracture/etch analysis.

Lipid Analyses

Thylakoid membrane pellets were extracted for lipids with 2.5 ml of ice-cold, chloroform/methanol 2:1 (vol/vol) at 4°C for 20 min, according to the method of Folch et al. (7). The solvents contained butylated hydroxytoluene to inhibit oxidative degradation of lipids. The extracts were shaken with 0.5 ml of ice-cold, 0.8% KCl, centrifuged for 20 min at 2,000 g and the upper, aqueous phase and interfacial protein precipitate removed by aspiration. The lower, lipid-rich chloroform phase was further concentrated by evaporation of the solvents under a gentle stream of dry nitrogen gas at 4°C. Aliquots of the lipid extracts were applied to activated, silica gel 60 HPTLC plates (E. M. Laboratories, Darmstedt, FRG). The plates were developed in a filter paper–lined chamber using a solvent system of chloroform/methanol/acetic acid/water, 170:30:20:6 (vol/vol/vol/vol). After drying, the plates were sprayed with 5 N sulfuric acid and lipids were visualized by heating in a 60°C oven for 10 min. Individual lipid components were identified on the basis of relative mobility and characteristic staining behavior using group-specific reagents.

Densitometry of the lipid chromatography was carried out using contrast-reversed negatives of photographs of the HPTLC plates. Negatives were scanned with a 0.3 × 3 mm slit, at a speed of 2.9 cm/min, at 500 nm using an E-C model 910 densitometer (E-C Apparatus Corp., St. Petersburg, FL) attached to a Hewlett-Packard model 3390 reporting integrator (Hewlett-Packard Co., Avondale, PA).

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1. *Abbreviations used in this paper*: DGDG, digalactosyldiacylglyceride; EF, exoplasmic fracture face; ES, exoplasmic, or inner, surface; FFA, free fatty acid; HPTLC, high-performance thin-layer chromatography; MGDG, monogalactosyldiacylglyceride; PF, protoplasmic fracture face.

Chlorophyll Determinations

Chlorophyll concentrations were determined from 80% acetone (vol/vol) extracts and analyzed in a Cary model 219 spectrophotometer (Varian Associates, Inc., Palo Alto, CA) using the equations of Arnon (3).

SDS PAGE

Gel electrophoresis was performed on 15% acrylamide, 0.75-mm thickness slab gels, according to the method of Laemmli (18), using a mini-gel apparatus (Idea Scientific, Corvallis, OR). A 5% acrylamide stacking gel was used.

The sample solution contained 3% SDS, 3% mercaptoethanol, 50 mM Tris, pH 6.8, and 10% glycerol (vol/vol). The completed gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA).

Freeze–Etch and Freeze–Fracture

Samples for freeze-fracture were slowly infiltrated with glycerol to a final concentration of 20% (vol/vol) over the course of an hour at 4°C. Freeze-etch samples were resuspended in distilled water. Membranes were pelleted at 14,000 g for 4 min in the microfuge, resuspended in a minimal volume of fresh fluid and frozen as thickly concentrated samples by immersion in Freon 22 (DuPont Co., Wilmington, DE). For freeze-etching, the samples were fractured at −100°C, etched for 3 to 5 min at the same temperature, and then rotary shadowed with platinum and carbon. For freeze-fracture, the samples were fractured at −110°C and immediately shadowed from one side. A BAF 400 freeze-etch device (Balzers, Hudson, NH) was used for both fracture and etch operations. The replicas were cleaned in bleach, rinsed with distilled water, and examined on an EM 410 electron microscope equipped with a goniometer stage (Philips Electronics Instruments, Inc., Mahwah, NJ) at 100 kV.

Materials

Runner bean (Kentucky wonder) seeds were purchased from Sears, Roebuck and Co., germinated in vermiculite, and leaves were harvested after 3 wk. Primary and secondary leaves were used as the source for the galactolipase solution. All other chemicals were of reagent quality or better. Water was deionized and distilled.

Results

Hydrolysis of Membrane Galactolipids

Other workers have reported the successful isolation of galactolipase from the cytoplasm (10, 30) and chloroplasts (2) of runner bean leaf cells. For these studies we chose to use an enzyme preparation from runner bean chloroplasts, because it is well characterized (2, 33) and shows a higher activity (2). The enzyme hydrolyzes galactolipids. In the case of the major photosynthetic membrane lipids, monogalactosyldiacylglyceride (MGDG) and digalactosyldiacylglyceride (DGDG), runner bean galactolipase releases two fatty acids and a galactosyl glyceride from each galactolipid hydrolyzed (31). Our own measurements of lipid/chlorophyll ratios during these experiments indicated that galactolipase treatment does not change the actual lipid content of the membrane: lipid/chlorophyll ratios average 29:1 (wt/wt) both before and after enzyme treatment. Such measurements indicated that the fatty acid moieties which originally formed part of galactolipid molecules were still associated with the membrane after enzyme treatment.

The lipids of the photosynthetic membrane can be separated by the use of HPTLC as shown in Fig. 1. MGDG and DGDG, as well as a number of other membrane lipids are easily identifiable on HPTLC plates on the basis of relative mobility (Rf values) and staining with group-specific re-
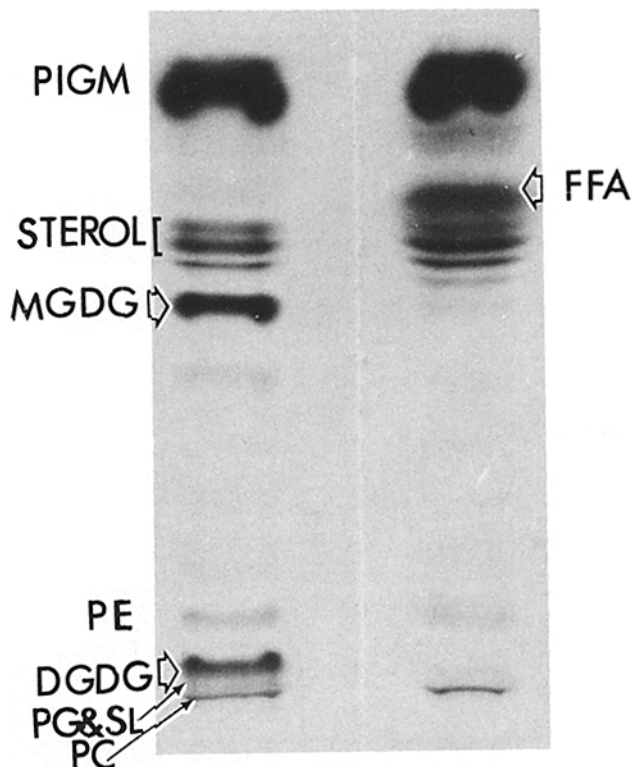
Figure 1. HPTLC of lipids from control (*left*) and runner bean galactolipase-treated (*right*) spinach thylakoid membranes. Membranes were incubated for 30 min with or without the enzyme preparation. The principal lipids of the thylakoids are identified: namely, pigments (*PIGM*), sterols, MGDG, phosphatidylethanolamine (*PE*), DGDG, phosphatidylcholine (*PC*), and sulfolipid (*SL*) (PG and SL co-chromatograph at the same rate in this particular solvent system). FFAs are not normal constituents of the membrane, but rather are by-products of galactolipid hydrolysis. The lipase hydrolyzes MGDG and DGDG rapidly, but also removes a small amount of phosphatidylethanolamine which becomes noticeable only in extended incubations. Lipids were made visible by spraying with 6 N sulfuric acid and heating at 60°C.

agents. HPTLC provides a rapid and sensitive measure of the lipid composition of our membranes.

When isolated photosynthetic membranes are incubated with purified runner bean galactolipase at 30° or 37°C, for a minimum of 20 min, there is an almost total loss of galactolipids from the HPTLC plate, as shown in Fig. 1. No other membrane lipids are affected significantly by this treatment. Some variation in the amount of galactolipid hydrolysis can be achieved by altering the concentration of the enzyme or the time and temperature of the incubation. The galactolipase-induced loss of MGDG and DGDG was associated with a dramatic increase in the free fatty acid (FFA) content of the membrane, as might be expected (Fig. 1). The complete hydrolysis of these two lipid species indicated that galactolipids in all regions of the membrane system were accessible to the enzyme.

Galactolipase Has Little Effect on Protein Composition

To judge whether galactolipase treatment affected membrane proteins we analyzed enzyme-treated samples with SDS PAGE (Fig. 2). The results of these experiments were rela-

tively straightforward: the overall polypeptide profile of the membranes was essentially unaltered by the enzyme treatment. The major membrane proteins, including those associated with the membrane-bound light-harvesting complex, were not affected by the enzyme treatment. Slight alterations were seen in the gel patterns at an apparent molecular weight of 32,000 and 48,000, however (arrows, Fig. 2), which were clearly associated with enzyme treatment. We are investigating the possibility that a small number of proteins in the membrane may be specifically associated with galactolipids, and hence may be subject to removal after galactolipid hydrolysis.

Galactolipase Affects Membrane Structure

Because the structural organization of the photosynthetic membrane has been well characterized by freeze–fracturing and freeze–etching (22, 23, 36, 38), we decided to apply these techniques to galactolipase-treated membranes. Fig. 3, *a* and *b*, illustrates the typical internal organization of the photosynthetic membrane as revealed by freeze–fracture techniques. The two fracture faces (exoplasmic fracture face [EF] and protoplasmic fracture face [PF] following the nomenclature of Branton et al. [6]) are easily distinguished from each other on the basis of intramembrane particles sizes, and their characteristic differentiation in stacked and unstacked regions of the membrane (for a review see reference 22).

Freeze–fracture demonstrates that a substantial change in the organization of galactolipase-treated membranes has
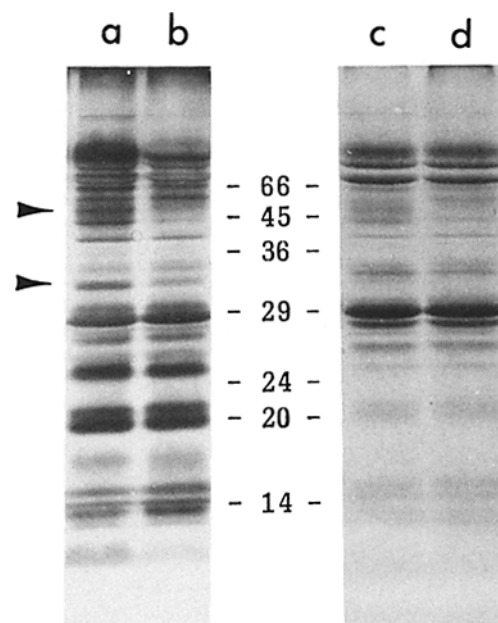

Figure 2. SDS PAGE of polypeptides from control (*a* and *c*) and lipase-treated (*b* and *d*) membranes after incubation for 30 min at 30°C. (Lanes *a* and *b* have been silver stained, and wells *c* and *d* have been stained with Coomassie Blue.) Under these conditions, nearly 100% of the MGDG and DGDG have been hydrolyzed. Only two polypeptides, with apparent molecular weights of 32,000 and 48,000, are lost from the membrane by this treatment (*arrows*). Galactolipase treatment causes only minor changes in membrane protein composition. The positions of molecular weight standards ($\times10^{-3}$) are indicated.
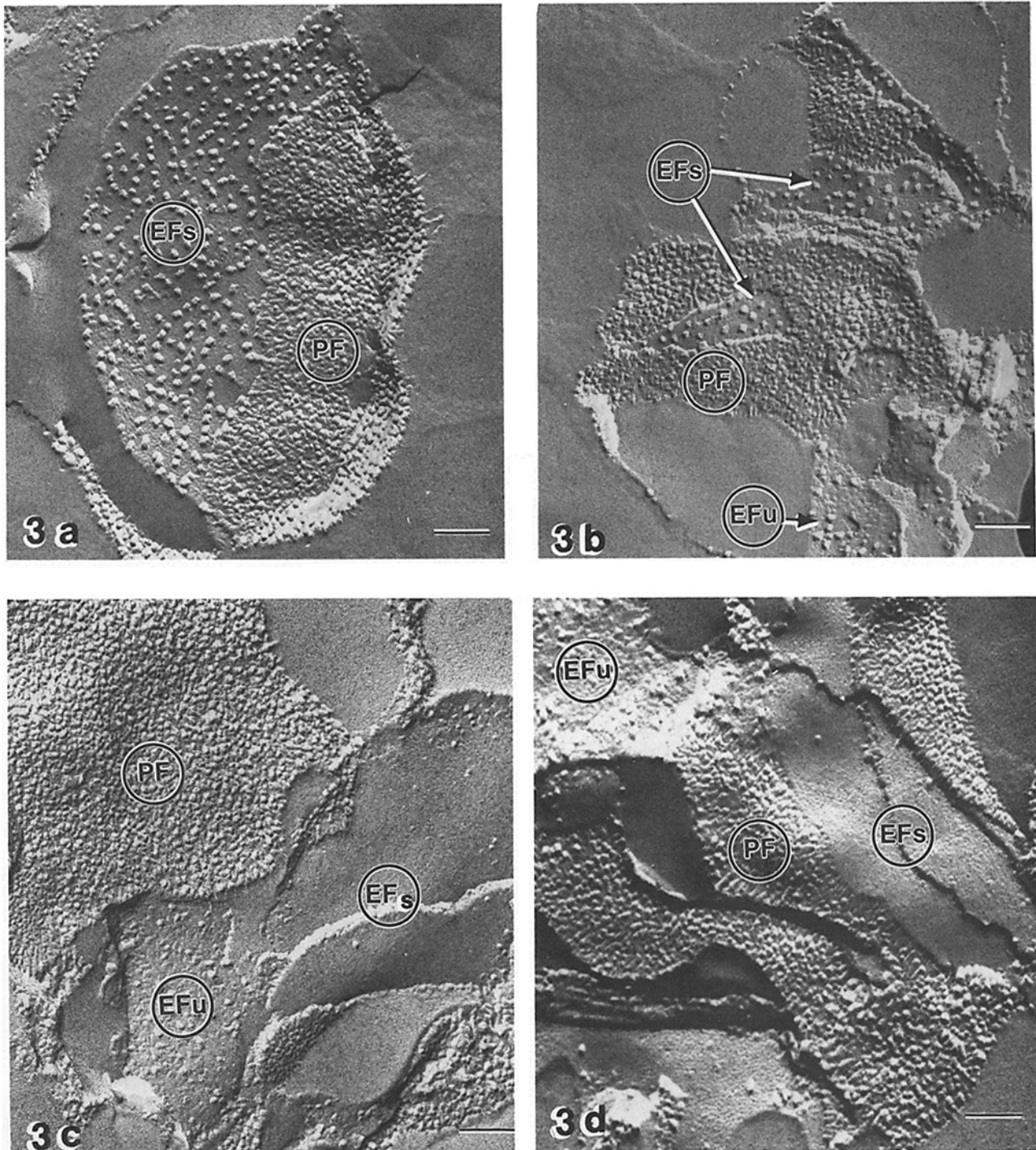
Figure 3. Freeze–fracture morphology of control (*a* and *b*) and galactolipase-treated photosynthetic membranes (*c* and *d*) after incubation for 30 min at 30°C. Again, the conditions result in nearly total abolition of membrane galactolipids. It is possible to identify regions of membrane stacking in both samples. The PF is easily distinguished by its high density of small particles. This fracture face is apparently unaffected by galactolipolysis. The EF in stacked membrane regions is recognizable by the appearance of distinctive, larger particles which average 140 Å in diameter. As shown in *c* and *d*, galactolipase treatment results in the disappearance of the EF particles, producing smooth fracture faces which are devoid of intramembrane particles. Despite this dramatic change in stacked membrane regions, particles in the unstacked regions (*EFu*) are only moderately affected, as evidenced by a modest reduction in both size and number of membrane particles. Bar, 0.1 µm.

taken place (Fig. 3, *c* and *d*). The large particles characteristic of the EFs, which measure ∼140 Å in diameter, are virtually absent from the enzyme-treated membranes. The other fracture faces, most notably the PFs, are changed very little by the enzyme treatment. Fig. 3 *d* emphasizes the point that

some intramembrane particles are still visible on the EFu (arrows), while the EF is nearly smooth. This loss of the EF particle, the largest intramembrane particle in the photosynthetic membrane, was observed in all of our enzyme-treated samples.
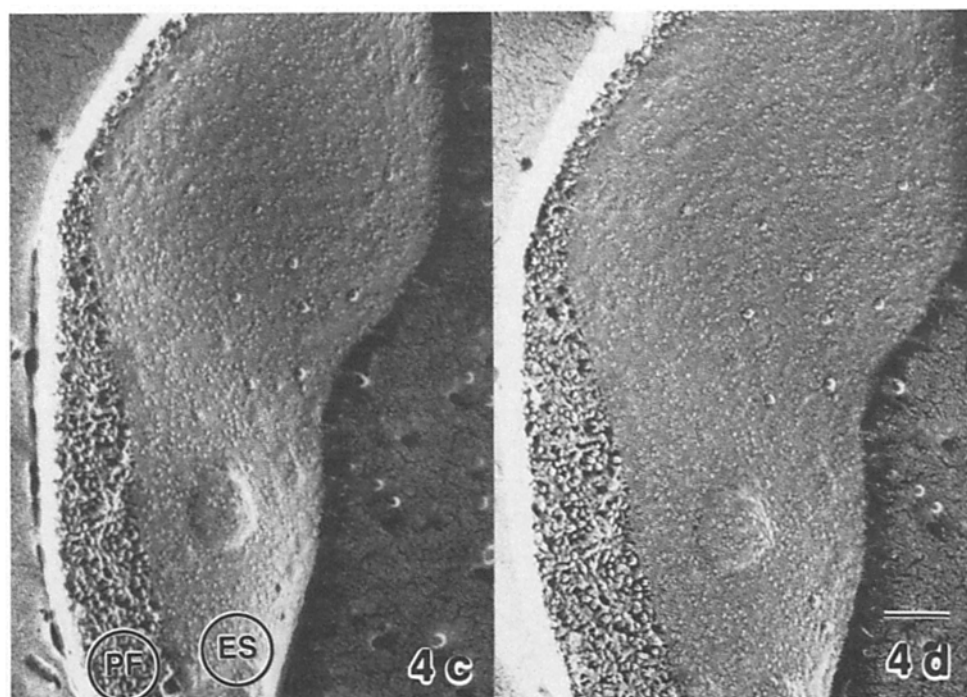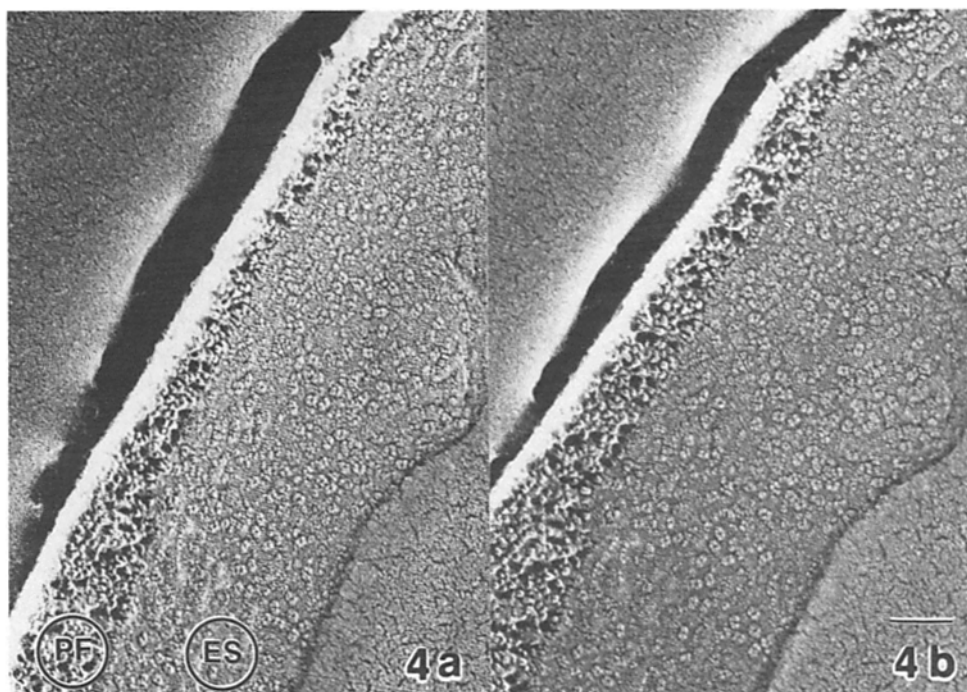
Figure 4. Stereo views of inner surfaces (*ES*) of control (*a* and *b*) and galactolipase-treated (*c* and *d*) membranes prepared by fracturing and deep etching. The ES surface may be positively identified using stereo microscopy and also by the juxtaposition of the inner surface with the PF during the fracturing process (as explained in the text). The characteristic tetrameric complexes, found only on stacked regions of ES, are missing in galactolipase-treated membranes. Smaller particles predominate instead. Membranes were incubated for 30 min at 30°C, with or without the enzyme preparation, and the resulting hydrolysis of galactolipids was nearly 100% in the experimental samples. Bar, 0.1 μm.

Earlier studies of the photosynthetic membrane have indicated a clear association of the EF particle with a characteristic particle on the inner surface of the membrane (21). Therefore, we prepared control and enzyme-treated membranes by freeze–fracturing and deep–etching. In this procedure, sublimination of ice from the fractured sample allows true membrane surfaces to be exposed and examined in the replica, often adjacent to fracture faces of the same membrane. Fig. 4 shows the effect of galactolipase treatment on the structure of the inner surface of the membrane. Control

membranes (Fig. 4, *a* and *b*) show a characteristic tetrameric particle protruding from the inner membrane surface.

Galactolipase-treated membranes show a distinctly different structure at the inner surface of the membrane, as shown in Fig. 4, *c* and *d*. While particles at the membrane surface are still visible, they are no longer organized as tetramers. Instead, the surfaces convey the strong impression that the tetramers have been dissociated by the enzyme treatment, and that individual, monomeric particles remain. It is possible, incidentally, to unequivocally identify these surfaces as
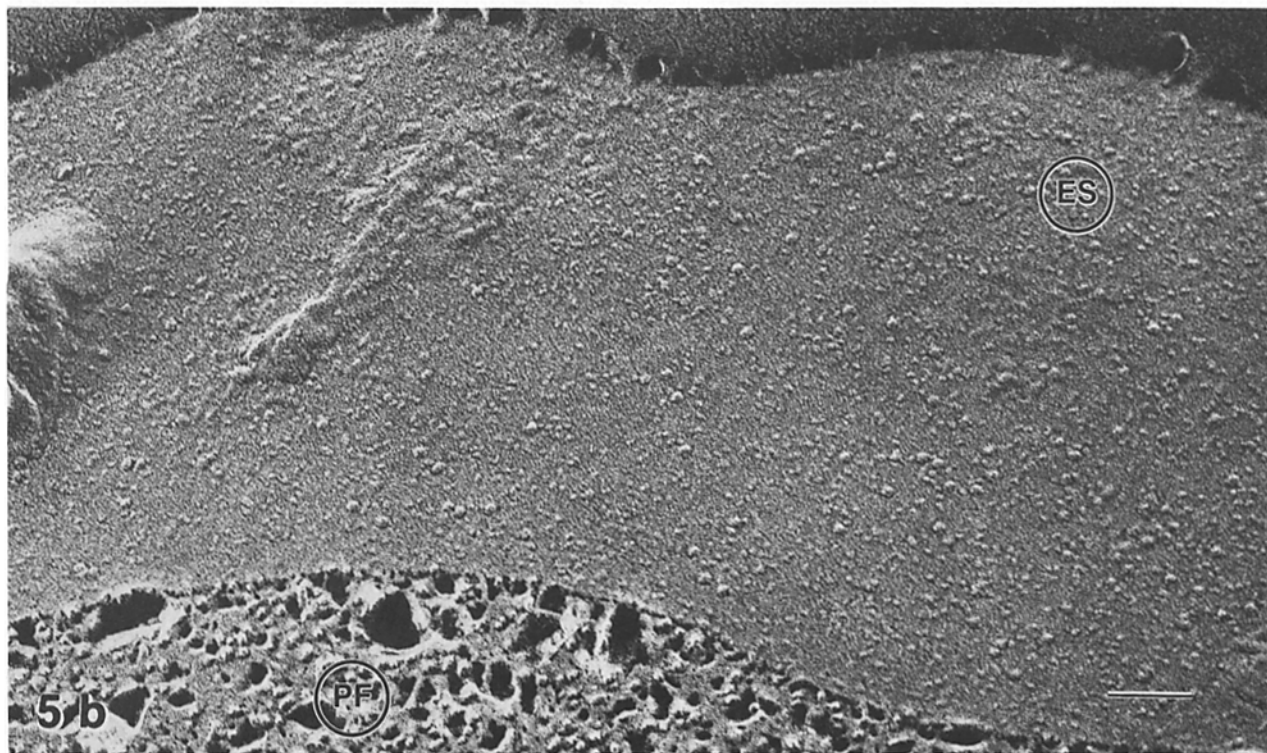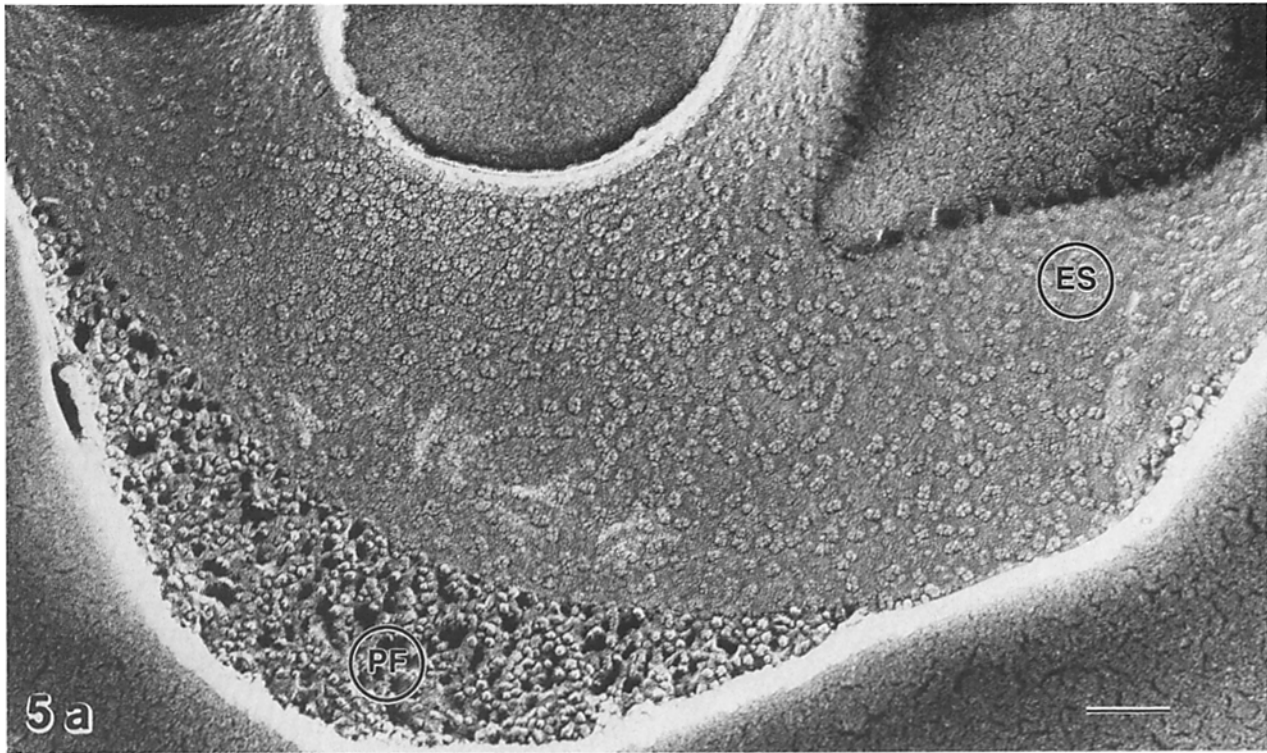
Figure 5. Slightly higher magnification view of photosynthetic membranes clearly shows the difference between the control (*a*) and enzyme-treated (*b*) membranes. The loss of the tetramers has been seen in every membrane observed in the lipase-treated samples. The effect is absolute: we have never observed the coexistence of domains of tetramers alongside the smaller, "new" particles, even under conditions of partial galactolipid hydrolysis. The experimental conditions were similar to those cited in Fig. 4. Bar, 0.1 μm.

the photosynthetic membrane inner surface. This can be done in images where a fracture face is directly adjacent to the etched surface of the same membrane (Fig. 4 *b* and 5 *b*). The PF, with its characteristic complement of small, tightly

packed particles, should be exposed wherever a membrane has been fractured just beneath the inner surface, and the figures presented here display that fracture face clearly.

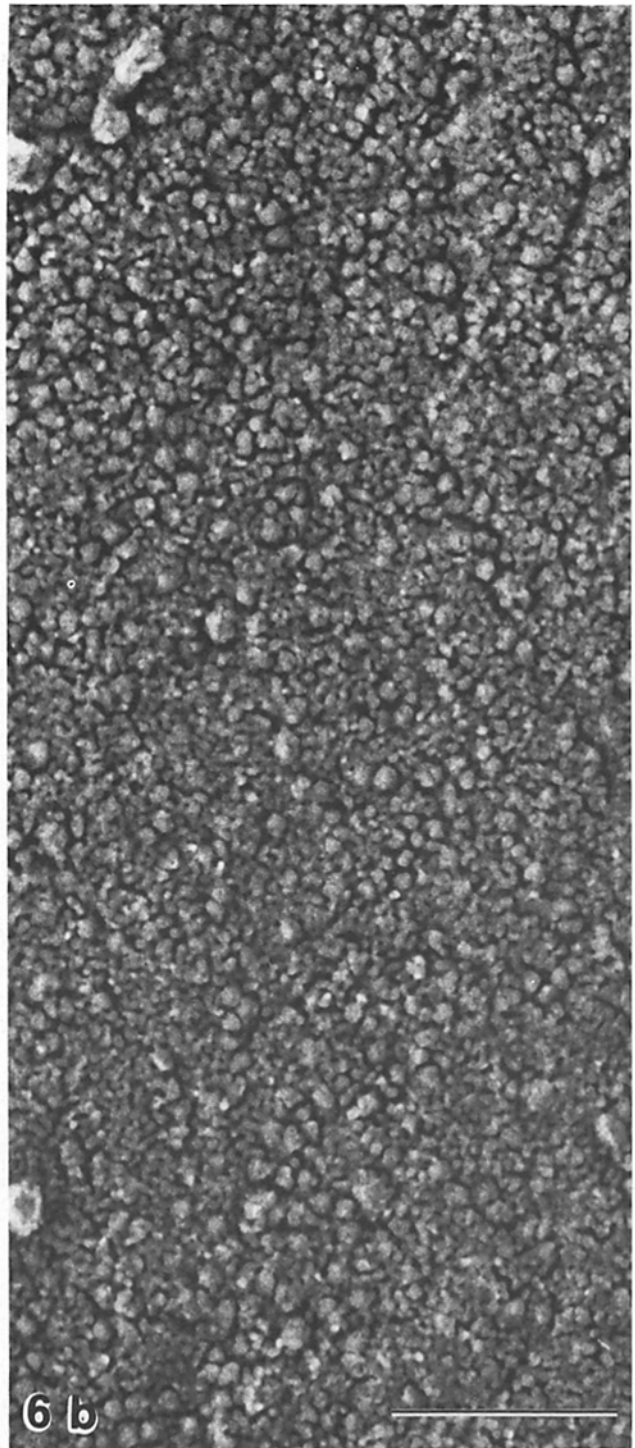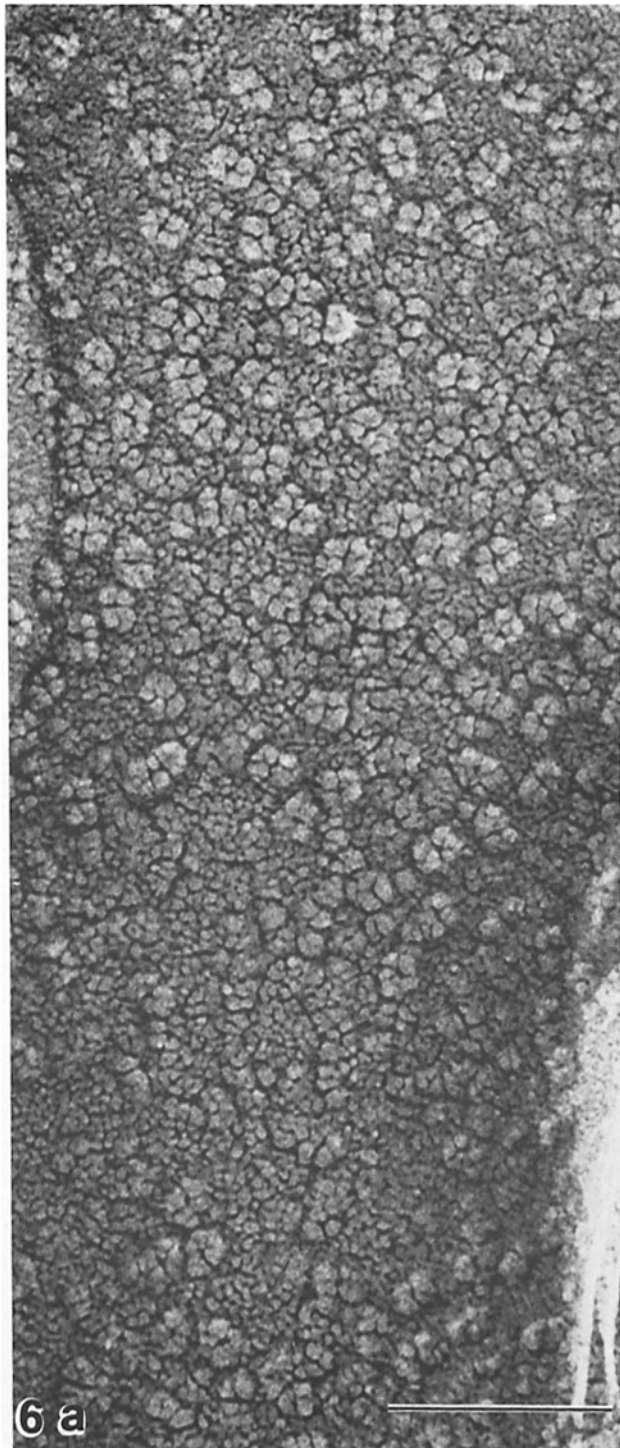Fig. 5, *a* and *b*, shows the effect of enzyme treatment at

Figure 6. Examination of the control (*a*) and lipase-treated thylakoids (*b*) at high magnification confirms the general impression that the "new" particles appearing on the ES of membranes after galactolipolysis are, in fact, the monomers of the membrane-spanning, EF/ES complex. The "monomers" appear approximately four times more frequently than the corresponding tetramers and measure about one-quarter the size of the larger particle (see Tables I and II). Experimental conditions were similar to those described in Fig. 4. Bar, 0.1 μm.

a higher magnification. Again, the individual exoplasmic, or inner, surface (ES) tetramers are clearly visible in the control sample, and seem to be dissociated in the enzyme-treated samples. Fig. 6, *a* and *b*, displays control and treated samples at high magnification to facilitate a side-by-side comparison. The loss of the tetramer is clearly seen in a comparison of the two images. Our measurements of particle sizes and

numbers of particles per unit surface area (Tables I and II) also support the impression conveyed by the micrograph. The particles visible on the enzyme-treated membrane surfaces are about half the diameter of particles on control membranes, and their numbers per unit membrane surface area are increased almost fourfold over the control tetramers.

Table I. Dimensions of Particles on the Inner Surface (ES)*

Tetramers (control membranes)	
Long axis	15.8 ± 0.3 (24)
Short axis	13.1 ± 0.3 (24)
Monomers (lipase-treated membranes)	
Diameter	6.4 ± 0.1 (77)

* Measurements are expressed as mean ± standard error. Number of measurements are given in parentheses.

Table II. Frequency of Particles on the Inner Surface (ES)*

	Particles/μm^2
Tetramers − control membranes	1,101 ± 22 (516)
Monomers − lipase-treated membranes	3,811 ± 426 (2,226)

* Measurements are given as mean ± standard error. Number of particles measured are given in parentheses.

Are Free Fatty Acids the Causes of These Changes?

Two principal lipid-related effects are produced by treating photosynthetic membranes with galactolipase: the specific loss of galactolipids, and the accumulation of FFAs produced by the lipolysis (see Fig. 1). The toxic effects of fatty acids on chloroplast membranes have been well documented (8, 13, 20, 33). Either of these effects might be related to the enzyme treatment. Therefore we devised an experiment to remove fatty acids from the membranes. Delipidated BSA has been widely used as a "scavenger molecule" for membrane-associated fatty acids. BSA has high affinity sites for fatty acid binding (5) and is the principal means by which FFAs are transported in mammalian blood plasma. HPTLC analysis (Fig. 7) demonstrated that supplemental incubation of enzyme-treated membranes with 3–6% delipidated BSA was effective in removing the FFAs produced during galactolipase treatment. Examination of the membranes after FFA removal, however, showed that this treatment did not reverse the effects of galactolipase treatment. The large EFs particle did not reappear and the inner surface tetramers did not reassociate (not shown). Therefore, it seems likely that galactolipid removal, rather than FFA production, is the major cause for the structural changes we have noted.

Discussion

The goal of the experiments reported here was to make a preliminary assessment of the role of galactolipids in maintaining the structure of the photosynthetic membrane. The use of a galactolipase to carry out such an investigation has several distinct advantages. First, the enzyme is specific for the major lipids of the membrane, MGDG and DGDG, and does not significantly hydrolyze any other lipid constituent. Second, the galactolipase is not a protease. Only two polypeptides, with apparent molecular weights of 32,000 and 48,000, are affected by the galactolipolysis, and even then only after extensive hydrolysis.

Krupa has also carried out a series of experiments in which the protein composition of galactolipase-treated thylakoid membranes was examined by gel electrophoresis (14, 15). His experiments showed a more extensive loss of membrane polypeptides than we have reported here, including reductions in the amounts of several chlorophyll–protein complexes associated with the membrane (14, 15). The
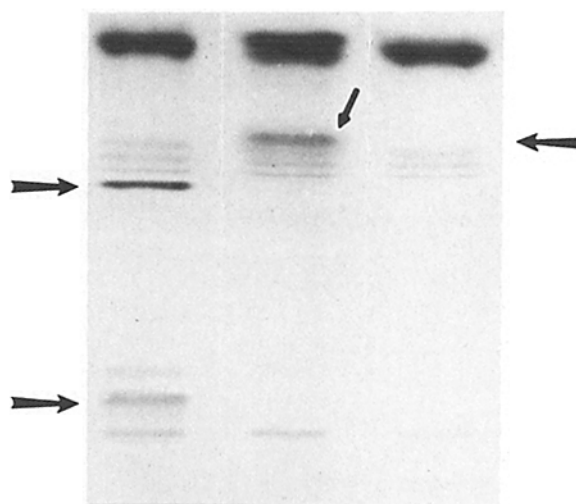

Figure 7. HPTLC of chloroplast lipids from membranes incubated for 30 min at 30°C: in a control buffer (left), with galactolipase (middle), and with galactolipase followed by an incubation with 3% delipidated BSA, for 30 min at 30°C (right). Arrows are used to indicate the location of MGDG and DGDG (left), accumulation of FFA (middle), and removal of FFA by BSA (right). The supplemental BSA treatment is effective in removing 100% of the FFA that accumulates in the membrane as a consequence of galactolipid hydrolysis. It was not possible to include BSA in the medium with the enzyme, since galactolipid hydrolysis was severely impaired (incubation of membranes with 3% BSA and lipase for up to 3 h at 37°C resulted in only 20% hydrolysis).

breakdown of the oligomeric form of the light-harvesting chlorophyll–protein complex into its monomers was another consequence of galactolipase treatment (15). Our results differ from these, in that we found no such changes even with nearly 100% degradation of the galactolipids. However, the earlier studies (14, 15) used runner bean chloroplasts, which are themselves the source of the enzyme, as experimental material. Therefore, it is possible that the addition of exogenous enzyme to the incubation medium might have activated the endogenous lipase associated with the chloroplast membrane and resulted in an amplification of the enzyme's actions. The experiments reported here do not necessarily contradict Krupa's results (14, 15), but rather present a unified approach in which alterations in membrane structure can be directly compared to biochemical studies of the same system.

Treatment of thylakoid membranes with galactolipase differs from the use of other perturbants of the lipid environment, such as detergents or liposomes, in that the galactolipase does not induce widespread changes in the architecture of the membrane. In our studies, major changes in membrane morphology have been limited to the inner surface of the thylakoid, and the membrane itself has clearly remained intact.

The earlier studies of Anderson et al. (2) and Shaw et al. (33) showed that galactolipase treatment caused substantial inhibition of light-driven electron flow in the photosynthetic membrane. Electron transport pathways which were significantly inhibited by the treatment included the flow of electrons from water to methylviologen, water to ferricyanide, and water to dichlorophenol indophenol. Experiments done by the same investigators (2, 33) also showed that these effects could be attributed to the accumulation of FFAs

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within the photosynthetic membrane, and therefore measurements of photosynthetic activity in galactolipase-treated membranes are not necessarily good indicators of the activities of individual membrane components.

Effects of Galactolipase on Membrane Structure

The major structural effect of galactolipase treatment was to dissociate the tetrameric particles normally found on the inner surface of the thylakoid sac. These particles vanish in galactolipase-treated membranes, and in their stead we find distinct individual particles on the inner surface of the thylakoid membrane. Superficially, this change suggests that the individual subunits of the tetrameric particles have been dissociated by the enzyme treatment. The sizes of these particles and their density per unit area are indeed consistent with dissociation of the tetramers.

Enzyme treatment also resulted in the loss of the large EF particles normally observed in stacked membrane regions in freeze–fracture. Studies on a number of systems (21–23) have associated the EF particle with the ES tetramer, and it seems likely that each represents a view of the same structure in the photosynthetic membrane: a large, membrane-spanning complex which is concentrated into the stacked regions of the thylakoid membrane system (23, 37, 38). Therefore, the organization of this structure, which has been directly associated with photosystem II activity in the membrane (22–24, 37, 38), is dependent on galactolipid.

This view is also supported by the observation mentioned earlier, that treatment of the photosynthetic membranes with galactolipase resulted in the removal of a polypeptide with an apparent molecular weight of 32,000. Other studies have identified polypeptides, having molecular weights of 32,000–34,000, with the photosystem II reaction center (12, 16, 17, 19). Therefore, it is possible that hydrolysis of galactolipids disrupted the organization of the EF/ES complex resulting in the release of a photosystem II–related polypeptide into the aqueous medium.

The interpretation of results from experiments using lipases as probes of membrane organization have been complicated by the concomitant release of FFA into the lipid bilayer during hydrolysis. Endogenous FFAs have been shown to inhibit electron transport in chloroplasts (2, 8, 13, 21, 33) and mitochondria (4). The inhibition of photosystem II activity by various nonspecific acyl hydrolases and galactolipases is well documented and is probably due in large part to accumulation of FFA in the membrane (2, 8, 13, 20, 33). In addition, the release of plastocyanin, an intermediate in the electron transport scheme, has been reported as a result of galactolipase treatment (14, 15).

Therefore, it was of interest to us to determine whether the structural effects which we have reported were a direct result of the removal of galactolipid, or whether they were caused by the accumulation of FFA formed during lipolysis. To address this question, we used delipidated BSA as a "sink" to absorb endogenous FFA. Supplemental incubation of galactolipase-treated membranes with BSA removes all of the liberated FFA (see Fig. 7), but does not reverse the dissociation of the EF and ES complexes (not shown). Therefore, removal of endogenous FFA from the membrane does not promote the reassembly of the disrupted complexes. Unfortunately, it was not possible to include BSA during the enzymatic treatment, because the enzyme activity was strongly

inhibited by BSA itself, as previously reported (20). The tetramers did not dissociate under these conditions (results not shown), but we did not consider these results conclusive, because lipolysis was not complete.

The Role of Galactolipids In Thylakoid Organization

In these experiments we have characterized the structural and biochemical changes associated with the hydrolysis of MGDG and DGDG, the major lipids of the photosynthetic membrane. Because a number of workers have clearly demonstrated that these lipids are capable of producing non-bilayer structures in model membrane systems, it is particularly important to consider whether or not these results suggest that non-bilayer structures play a role in the organization of the photosynthetic membrane.

Several groups have demonstrated that mixtures of several lipid types can be induced to form non-bilayer structures which produce distinct particles after freeze-fracturing of model membrane systems (32, 42). In most cases, these structures can be associated with the ability of certain lipids to form "inverted" micelles in which the hydrophilic head groups face inward (42). Several workers have suggested that inverted micelle structures might be important in the organization of the photosynthetic membrane (26, 29). Specifically, Murphy (26) has argued that the conical shape of MGDG favors its insertion in those regions of the thylakoid membrane which display a high surface curvature. Most notably, such regions would include the inner-facing surfaces of the membrane near the margins of thylakoid sacs. Kinetic analysis of galactolipase treatment of thylakoid membranes by Rawyler and Siegenthaler (28) suggests that there is indeed an asymmetry to the location of MGDG and DGDG in the thylakoid membrane.

There is no consensus, however, on the precise location of these two galactolipids in the thylakoid membrane. Experiments which have analyzed the rates at which each lipid type is hydrolyzed by galactolipase (28) have suggested that MGDG is distributed in a 65:35 ratio between the outer and inner leaflet of the membrane, while the outer/inner ratio for DGDG is 15:85. Earlier experiments, involving lipolysis of thylakoid membranes by Rhizopus arrhizus lipase (41), produced quite different results, leading other investigators to conclude that MGDG was largely located on the inner membrane leaflet, while DGDG was distributed evenly between the inner and outer leaflets.

The experiments presented here do not address the issue of galactolipid localization. They also do not test the possibility that the two major galactolipids display lateral heterogeneity between stacked and nonstacked regions of the thylakoid membrane system, as others have suggested (41). However, these experiments do show that hydrolysis of the major galactolipids produce profound structural changes within the photosynthetic membrane. We find it significant that major changes occur at the inner surface of the photosynthetic membrane, clearly demonstrating that galactolipase action is not necessarily confined to the exposed outer leaflet of the membrane, in agreement with conclusions reached earlier by Thomas et al. (40). The changes which we have noted have primarily affected a large, membrane-spanning structure associated, as noted earlier, with photosystem II activity. This structure is concentrated in the stacked regions of the thylakoid membrane under normal

conditions, and therefore it is worth noting that the most dramatic changes associated with galactolipid hydrolysis do indeed occur in stacked membrane regions.

The fact that a major class of freeze-fracture particles disappears upon galactolipase treatment might be taken to suggest that these particles are formed by inverted micelle structures organized within the plane of the thylakoid membrane. This is indeed a possibility, and Murphy (26) has suggested a distinct role for such structures in the organization of the membrane. However, lipidic particles produced under controlled conditions were reported by Verkleij (42) to range between 80 and 100 Å, and the EF particles are considerably larger (130 to 150 Å).

A more likely possibility is that these particles represent protein or lipoprotein assemblies which rely on galactolipid for their structural integrity, and rapidly disassemble when MGDG or DGDG are degraded by enzyme action. Siefermann-Harms et al. (34) have provided evidence that MGDG is required for effective transfer of light energy from the light-harvesting chlorophyll a/b protein complex to photosystems I and II. No other lipid can reconstitute this activity, indicating that the requirement for this galactolipid is absolute and specific.

Clearly the galactolipids do much more than play a supportive role in the organization of the membrane. The fact that MGDG and DGDG together comprise ~77% of the membrane lipids supports the findings that depletion of the galactolipids significantly alters the molecular nature of the thylakoid. Further studies with the specific addition of MGDG or DGDG to the thylakoid membrane, before and after delipidation, will provide insight into the biophysical properties at work in the organization of the photosynthetic apparatus.

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