Role of Galactolipids in Spinach Chloroplast Lamellar Membranes

II. EFFECTS OF GALACTOLIPID DEPLETION ON PHOSPHORYLATION AND ELECTRON FLOW.

Received for publication November 26, 1975 and in revised form February 2, 1976

ARTHUR B. SHAW, MARK M. ANDERSON, and RICHARD E. MCCARTY

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

ABSTRACT

A galactolipid lipase from primary bean (Phaseolus vulgaris) leaves has been used to partially deplete spinach chloroplast inner membranes of their galactolipids. Chloroplasts treated with the lipase in the absence of bovine serum albumin lost 91% of their monogalactosyl diglyceride, 83% of their digalactosyl diglyceride, all of their phosphatidyl choline, but none of their sulfatide. Electron microscopy of thin sections revealed that the treated chloroplasts were greatly enlarged and lacked membrane stacking. Linoleic acid had similar effects on the structure of the chloroplasts. Chlorophyll, carotenoids, and coupling factor I remained bound to the treated membranes.

To minimize the inhibition of phosphorylation and electron flow by fatty acids released by the lipase, bovine serum albumin (15–24 mg/ml) was added to the lipase incubation mixtures. Bovine serum albumin inhibited the extent, but not the initial rate, of fatty acid release by the lipase. Electron microscopy of chloroplasts treated with the lipase in the presence of bovine serum albumin showed that membrane stacking was partially maintained. Chloroplasts treated with lipase under these conditions retained about 30% of their monogalactosyl diglyceride, 50% of their digalactosyl diglyceride and phosphatidyl choline. The sulfatide and phosphatidyl glycerol contents were unchanged. Electron flow through photosystems I and II with artificial electron donors and acceptors was not affected by lipase treatment in the presence of bovine serum albumin. In contrast, oxygen evolution and phosphorylation were partially inhibited. These reactions are also very sensitive to fatty acids and it is possible that the inhibition is the result of interaction of fatty acids with the membrane prior to their binding to bovine serum albumin.

In view of the irreversible inactivation of electron flow and phosphorylation by fatty acids, it is difficult to assess the role of galactolipids in these processes when a specific lipase is used to deplete the membrane.

Galactosyl diglycerides are present in high concentrations in the photosynthetic membranes of higher plants and algae,
and electron donors and acceptors as given in figure and table legends. When phosphorylation was assayed, 3 mm ADP and 3 mm potassium phosphate buffer (pH 8) which also contained about 10⁶ cpm of carrier-free ³²P were also added. Unless otherwise stated, illuminations were performed with saturating white light at room temperature. Esterified ³²P was determined by the method of Avron (5). Methyl viologen reduction was followed by determining O₂ consumption with a Clark O₂ electrode, whereas ferricyanide reduction and DPIP⁵ reduction were estimated spectrophotometrically.

Lipids were extracted from chloroplasts or subchloroplast particles (6), and samples containing 30 to 150 µg of Chl were spotted on Merck Silica Gel G analytical thin layer plates (Brinkmann Instruments Co.). The lipids were chromatographed in two dimensions using CHCl₃/CH₃OH/H₂O (65:25:4, v/v) in the first direction and CHCl₃/CH₃OH/CH₃COOH/acetone/H₂O (100:20:40:5, v/v), in the second. After removal of the solvent, lipid spots were visualized by brief exposure of the plate to I₂ vapor. Excellent separations of individual lipids were obtained. Zones of the gel which contained the lipids were scraped from the plate and transferred to test tubes. Phospholipids were eluted from the silica gel by adding 8 ml of water, followed by exposure of the suspension to sonic oscillation in a sonic cleaner bath for about 5 sec. Three ml of a 1:2 mixture by volume of CHCl₃/CH₃OH were added, and the contents were mixed and centrifuged. The CHCl₃ layer was evaporated to dryness and phosphorous determined by the Ames-Dubin procedure (1). Sulfolipid (sulfoquinovosyldiglyceride) was determined according to Kean (16).

Since silica gel did not interfere with alkaline hydrolysis of galactolipids, the silica gel containing the galactolipids was treated in screw cap test tubes at 100 C for 2 hr with 1 ml of 4.25 N KOH and 1 ml of 95% ethanol. After cooling, 1 ml of H₂O, followed by 4 ml of petroleum ether (30–60 C boiling range) was added. After mixing, the petroleum ether phase was discarded and the extraction was repeated with the petroleum ether phase again being discarded. The mixture was then acidified by addition of 0.4 ml of 11.5 N HCl. Seven ml of petroleum ether was added, and the contents of the tube were vigorously mixed on a Vortex mixer. Following centrifugation the petroleum ether phase was saved and the extraction was repeated. The combined petroleum ether phases were washed three times with 3 ml of H₂O to remove HCl. Aliquots of the petroleum ether phases were evaporated to dryness, and the fatty acid content was estimated (2).

**RESULTS**

**Quantitative Lipid Analysis in Lipase-treated Chloroplasts and Subchloroplast Particles.** Chloroplasts, treated with lipase in the absence of BSA, lost most of their galactolipids and phosphatidyl choline. Phosphatidyl glycerol and sulfolipid were not markedly hydrolyzed (Table I). These results confirm the suggestion (3) that the lipase prefers uncharged or zwitterionic lipids as substrates. BSA (15 mg/ml) inhibited the extent of fatty acid release and preserved some of the galactolipids and phosphatidyl choline. Approximately 70% of the monogalactosyl diglyceride and 50% of the digalactosyl diglyceride and phosphatidyl choline were hydrolyzed by the lipase in the presence of BSA. The lipid content of the control chloroplasts was similar to that reported by others (15). A loss of a small amount of monogalactosyl diglyceride after 60 min of incubation in the absence of added lipase was also observed. This release was previously noted (11). A similar depletion of galactolipids and phosphatidyl choline by the lipase in subchloroplast particles was observed (Table II). In no case were components with mobilities expected for the monoacylated derivatives of galactolipids or phospholipids detected after TLC.

**Fig. 1. Effect of BSA on fatty acid release in chloroplasts by the lipase.** Chloroplasts (125 µg Chl) in 20 mm potassium phosphate buffer (pH 7) were incubated at 21 C in 0.25 ml with 1.25 µg of lipase in the presence and absence of 7.5 mg BSA for the indicated times.

**Table I. Estimation of Acyl Lipids in Lipase-treated and Control Chloroplasts**

<table>
<thead>
<tr>
<th>Lipid Component</th>
<th>Control</th>
<th>Control plus BSA</th>
<th>Lipase-treated</th>
<th>Lipase-treated plus BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG</td>
<td>1.10</td>
<td>1.00</td>
<td>0.10</td>
<td>0.30</td>
</tr>
<tr>
<td>DG</td>
<td>0.60</td>
<td>0.60</td>
<td>0.10</td>
<td>0.30</td>
</tr>
<tr>
<td>PG</td>
<td>0.13</td>
<td>0.10</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>SL</td>
<td>0.13</td>
<td>0.13</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>PC</td>
<td>0.08</td>
<td>0.07</td>
<td>0</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**Table II. Extent of Galactolipid and Phosphatidyl Choline Depletion in Subchloroplast Particles by Lipase Treatment**

Subchloroplast particles were treated with lipase in the presence of 15 mg/ml BSA under conditions similar to those given in Table I. Lipids were extracted from the total incubation mixture and quantitated as described under "Materials and Methods."

**Abbreviations:** DPIP: 2,6-dichlorophenolindophenol; PMS: N-methylphenazonium methosulfate; TMPD: N,N',N'-tetramethylphenylenediamine.

Copyright © 1976 American Society of Plant Biologists. All rights reserved.
presence of BSA still contained stacked membranes although there was some disruption of stacking and some swelling (Fig. 2A). In contrast, chloroplasts incubated with the lipase in the absence of BSA were greatly enlarged and contained no stacked membranes (Fig. 2B). The unstacking of the membranes by lipase treatment in the absence of BSA was probably the result of the liberation of fatty acid. Linolenic acid (3.8 μmoles/mg Chl), an amount similar to that formed by the lipase, also caused marked swelling and disruption of membrane stacking (Fig. 2C).

BSA also partially inhibited the extent of fatty acid release from most preparations of subchloroplast particles. The exposure of chloroplasts to sonic oscillation under the conditions used to prepare subchloroplast particles did not fully disrupt membrane stacking even though it fragmented the lamellar system. Electron micrographs of thin sections of subchloroplast particles showed many regions of stacked membranes which were disrupted by lipase treatment in the absence of BSA.

Even though major amounts of the galactolipids in the membrane were hydrolyzed by the lipase, the binding of at least three components of the membrane, coupling factor I, Chl, and carotenoids, was not grossly affected. No significant amount of these components was rendered soluble even after lipase treatment in the absence of BSA.

Effects of Lipase Treatment of Chloroplasts and Subchloroplasts Particles on Electron Transport and Phosphorylation. Up to 4 μg of fatty acid may be released from chloroplasts or subchloroplast particles equivalent to 1 mg of Chl (Fig. 1) when the incubation of either chloroplasts or subchloroplast particles was carried out in the absence of BSA. However, nearly all photochemical activity was lost. This result was expected since fatty acids inhibit electron flow (9, 7, 20, 23) and uncouple phosphorylation (20, 23). For example, 2 to 3 μmoles of linolenate/mg Chl nearly fully inhibit photosystem II-dependent electron flow and phosphorylation is even more sensitive. To assess the effects of lipid depletion on photochemical activity, conditions had to be found to minimize the inhibitory action of fatty acids. BSA (15–24 mg/ml) was routinely added to the incubation mixtures to bind fatty acids and low amounts of lipase were used with prolonged exposure times. Under these conditions of lipase treatment, subchloroplast particles retained much of their photochemical activity even though from 2.4 to 2.8 μmoles of fatty acid were liberated/mg Chl. The results of a number of different experiments are summarized in Table III. Although electron transport reactions which involve O2 evolution are partially sensitive to the lipase treatment, the photosystem I-dependent oxidation of dianimodurene by methyl viologen, assayed in the presence of DCMU, was totally insensitive. Phosphorylation supported by PMS-dependent cyclic electron flow through photosystem I was the most sensitive reaction studied. It is clear that the major inhibitory site of inhibition of electron flow is the O2 evolving apparatus. The extent of inhibition by lipase treatment was the same with water as the electron donor when oxidants which accept electrons from different positions in the electron transport chain were used. For example, the inhibition of the reduction of methyl viologen, which accepts electrons from photosystem I, was the same as that observed in the reduction of oxidized dianimodurene in the presence of dibromothymoquinone. Oxidized dianimodurene accepts electrons from a component close to photosystem II (10, 25) under these conditions. Similar results were obtained with chloroplasts.

The light intensity dependence of DPIP reduction and of dianimodurene oxidation by photosystem I was not affected by lipase treatment. Since the slight inhibition of electron flow by lipase treatment was independent of high intensity, it appears that lipase treatment does not markedly alter the quantum requirement for electron flow.

Although tris-treated chloroplasts cannot use H2O as the electron donor, artificial electron donators such as diphenylcarbazide restore electron flow through photosystem II (28). Whereas the reduction of DPIP with H2O as the electron donor was partially sensitive to the lipase treatment in the presence of BSA in control chloroplasts, DPIP reduction with diphenylcarbazide was insensitive in tris-treated chloroplasts to the lipase when BSA was also present (Table IV).

Noncyclic phosphorylation coupled to electron flow from H2O to ferricyanide in subchloroplast particles was inhibited more than the rate of electron flow by lipase treatment resulting in a decrease in the P/O ratio. Cyclic phosphorylation with PMS showed a similar sensitivity to lipase treatment. Light-dependent H+ uptake in chloroplasts (13), supported by PMS-catalyzed electron flow, was nearly as sensitive as phosphorylation to lipase treatment.

Attempts to Separate Effects of Fatty Acids from Those of Lipid Depletion. The fact that phosphorylation and O2 evolution exhibit the greatest sensitivity to lipase treatment is disturbing since these processes are also very sensitive to inhibition by fatty acids. Even though high concentrations of BSA were used to sequester fatty acids, it was possible that the fatty acids released at the membrane could interact with the inhibitory sites on the membrane before they were bound by BSA. BSA, even at only 9 mg/ml, can remove all of the fatty acid liberated by the lipase from subchloroplast particles. Subchloroplast particles were incubated with the lipase and were diluted into either H2O or 9 mg/ml of BSA. After centrifugation, fatty acids in the pellets and supernatant fluids were determined. Whereas nearly all of the fatty acid (2.79 μmoles/mg Chl) remained bound to the subchloroplast particles in the absence of BSA, no fatty acid was detected in the pellet after exposure to BSA and 2.62 μmoles/mg Chl was found in the supernatant. Since 5 nmoles of fatty acid could have been detected in the aliquot (0.1 mg Chl) of subchloroplast particles, the upper limit of the fatty acid content of lipase-treated subchloroplast particles after exposure to BSA is only 0.05 μmole/mg Chl. This amount of linolenate has no effect on phosphorylation or electron flow. Yet, these particles were photochemically inactive showing that fatty acids can irreversibly inhibit electron flow. Similar results were obtained with chloroplasts.

Linolenate is the major fatty acid in galactolipids (15). To attempt to separate effects of fatty acids from those of galactolipid depletion, a comparison of the inhibition of electron flow from H2O to oxidized dianimodurene by linolenate and by lipase treatment was made. In this experiment, either lipase or linolenic acid was added to incubation mixtures without BSA. BSA was added to some of the samples at timed intervals. Fifteen sec later the reaction mixture for electron flow was added, and O2 evolution was assayed. When BSA was added prior to the lipase, only 35% inhibition was observed after 30 min. If BSA was omitted, lipase treatment resulted in complete inhibition within only 2 min. BSA added after lipase treatment for 2 min restored 71% of the electron flow. If the incubation time with the lipase was increased to 5 min, BSA restored only 38% of the activity. In contrast, the inhibition by added linolenic acid (100 nmoles/100 μg Chl) was reversible for a longer time. Incubation of chloroplasts with linolenic acid for 2 min with no addition of BSA caused a 60% inhibition of electron flow. Addition of BSA after 2 min incubation with linolenic acid reversed almost all of the inhibition. Even after 5 min of incubation with linolenic acid, the inhibition of electron flow after addition of BSA was only 23%. It must be stressed that the amount of linolenic acid added was greater than the amount of fatty acid liberated by the lipase (88 nmoles/100 μg Chl determined in a separate experiment). Thus, inhibition by added fatty acid is more readily reversed by BSA than the inhibition by endogenous fatty acids liberated by lipase. Fatty acids liberated at the thylakoid membrane may exert their inhibitory effects before the BSA is able to bind them.
Fig. 2. Structure of lipase-treated chloroplasts. Chloroplasts (0.5 mg Chl) were treated in the usual manner with 5 µg lipase/ml in the presence and absence of 30 mg/ml BSA for 45 min at 21°C. Another sample was incubated 45 min at 21°C with 0.5 mg Chl in the absence of lipase and BSA. After centrifugation in 10,000g for 10 min, portions of the pellets were fixed, embedded, and sectioned (24). The sections were poststained with lead citrate and were examined with an AEI electron microscope. A: Lipase-treated plus 30 mg/ml BSA; B: lipase-treated in the absence of BSA; C: linolenic acid-treated.
Table III. Electron Flow Activities of Lipase-treated Subchloroplast Particles

Subchloroplast particles (0.4 mg Chl/ml) were incubated at 20 C for 60 to 120 min in a reaction mixture which contained 15 to 25 mg/ml BSA, 28 mM Tricine-NaOH (pH 8), 4 mM NaCl, 0.16 mM sucrose, and 8 to 20 μg/ml of galactolipid lipase. Control incubation mixtures were identical except that they contained no lipase. The results shown were obtained in a number of experiments with different lipase and subchloroplast particle preparations in which the extent of fatty acid liberation by the lipase varied from 2.4 to 2.9 μmol/mg Chl. Methyl viologen was present at 0.1 mM; ferricyanide at 1 mM; DPIP at 0.1 mM; diamidodurene, at 0.5 to 1.0 mM, and PMS, at 0.05 mM. Sodium azide (1 mM) was added to reaction mixtures with methyl viologen to inhibit catalase. Ascorbate (2 mM) and DCMU (5 μM) were added to reaction mixtures for the assay of electron flow from diamidodurene to methyl viologen. An excess of ferricyanide (2.2 mM) was added to oxidize diamidodurene (0.5 mM) and 0.5 μM dibromomethoxyquinone was also added in the assay of electron flow from water to diamidodurene.

Table IV. Effect of Lipase Incubation of Tris-treated Chloroplasts on DPIP Reduction with Diphencylcarbazide as Electron Donor

Chloroplasts (4.26 mg Chl/ml) were diluted with an equal volume of 1.8 mM unneutralized tris. After 5 min at 0 C, an equal volume of 0.4 mM sucrose, 0.02 mM Tricine-NaOH (pH 8), and 0.01 mM NaCl was added, and the suspensions were centrifuged at 10,000g for 10 min. The pellets were resuspended in the buffered sucrose solution. Aliquots of the tris-treated chloroplasts were incubated in the presence and absence of lipase and with and without BSA (15 mg/ml) for 60 min at 20 C in the usual manner. The diphenylcarbazide concentration was 0.5 mM and the DPIP concentration was 0.1 mM. NH₄Cl (2 mM) was also present in the assay mixtures for electron flow.

![Figure 3](https://www.plantphysiol.org/)  
**Figure 3.** Sucrose density gradient centrifugation of lipase-treated and control subchloroplast particles. Freshly prepared subchloroplast particles were incubated with and without lipase in the presence of 15 mg/ml of BSA for 90 min at 20 C as described in Table I. After incubation, the particles were collected by centrifugation at 104,000g for 30 min. The pellets were resuspended in 0.4 mM sucrose which also contained 0.02 mM Tricine-NaOH (pH 8) and 0.01 mM NaCl. Sucrose gradients (1.2–2.2 M) were prepared by layering 6-ml aliquots of 2 M, 1.6 M, 1.3 M, 1.4 M, 1.3 M, and 1.2 M sucrose solutions in a cellulose nitrate centrifuge tube. Each sucrose solution contained 5 mM Tricine-NaOH (pH 8) and 5 mg/ml BSA. After the gradients had been aged for about 5 hr at 4 C, control and lipase-treated subchloroplasts particles equivalent to 4 mg Chl were layered on top of the gradients. The tubes were centrifuged at 25,000 rpm in a Spinco Model SW 27.1 rotor at 4 C for 10 hr. After deceleration of the rotor without use of the brake, the bottoms of the tubes were punctured and 1-ml fractions collected. PMS-dependent cyclic phosphorylation with 50 μM PMS and Chl content were then assayed.

From 21 to 43% at the end of 60 min even though the same amount of fatty acid was released.

The hydrolysis of lipids by the lipase in either chloroplasts or subchloroplast particles in the presence of BSA is not complete. Heterogeneity with respect to the extent of lipid depletion in a population of lipase-treated subchloroplast particles might be expected. For example, much of the phosphorylation activity after lipase treatment could reside in subchloroplast particles which might be insensitive to the action of the lipase. To test for this, lipase-treated and control subchloroplast particles were layered on sucrose density gradients, and the tubes were centrifuged to equilibrium. Lipase-treated subchloroplast particles were more dense than the controls (Fig. 3). The increased density of the lipase-treated subchloroplast particles is consistent with the loss of lipid. In view of the close correspondence between the distribution of PMS-dependent cyclic phosphorylation and of Chl in the gradient, there appears to be little heterogeneity in the lipase-treated subchloroplast particles. Furthermore, it is remarkable that phosphorylation in subchloroplast particles survives sucrose density gradient centrifugation with little loss in activity.

**DISCUSSION**

Fifty to 60% of the acyl lipids of chloroplasts membranes may be removed without affecting the rate of electron flow through...
photon systems I or II. To accomplish this, the incubation must be carried out in the presence of high concentrations of BSA. Nearly total inhibition of all photochemical activities by treatment with the lipase was achieved when the incubation was carried out in the absence of BSA. A major part of this inhibition must be the result of fatty acid liberation, since 4 μmoles or more of fatty acid/mg Chl were liberated by the lipase in the absence of BSA. Oxygen evolution (9, 17, 20) and phosphorylation (20, 23) are abolished when 4 μmoles of linolenate were added/mg Chl. Moreover, exogenous linolenate is not as effective an inhibitor as linolenic acid released at the membrane by the lipase. Since nearly total depletion of galactolipids and phosphatidyl choline in chloroplast membranes may be achieved without causing release of either Chl, carotenoids or coupling factor I, it seems unlikely that these lipids stabilize the binding of these components to the membrane.

The inhibition of O₂ evolution and phosphorylation by lipase treatment in the presence of BSA could also be caused by interaction of the membranes with fatty acids. Two experiments reported here supported this hypothesis. First, even though BSA binds all of the fatty acid liberated by the lipase, chloroplast membranes also bind fatty acids. If fatty acids remain in contact with the membranes for more than a few minutes, BSA cannot reverse the inhibition of activity. Second, phosphorylation is less sensitive to lipase treatment when the rate of fatty acid release is reduced by adding small aliquots of the lipase over an extended time period rather than by adding the same amount of lipase in one addition. Presumably, a slower rate of fatty acid release would afford the BSA a better opportunity to compete with the membranes for the liberated fatty acids. It is also possible that small amounts of monoacylated galactolipids or phosphatidyl choline are generated in the membrane on hydrolysis by the lipase. These substances could also be potent inhibitors of phosphorylation and O₂ evolution (12).

The photosystem I-dependent oxidation of TMPD in chloroplasts that had been lyophilized was partially sensitive to lipase treatment (18). About 56% of the monogalactosyl diglyceride and 15% of the digalactosyl diglyceride were removed by the lipase. Since no BSA was present during the lipase treatment, much of the inhibition was probably caused by fatty acids. The lipase-treated chloroplasts were then lyophilized and galactosyl diglyceride partially restored TMPD oxidation to the lipase-treated, lyophilized chloroplasts. Galactolipids also partially restored electron flow in lyophilized, heptane-extracted chloroplasts, even though little galactolipid is removed by the heptane extraction. Since triacylglycerols (7) and α-tocopherol can also partially restore activity to the heptane-extracted chloroplasts, it is clear that the lipid requirement is quite nonspecific.

An obligatory role for galactolipids in photosynthetic phosphorylation seems unlikely. Jaynes and Vernon (14) reconstituted photosynthetic phosphorylation by incorporation of a photosystem I-enriched subchloroplast fragment, prepared by the action of Triton X-100 on chloroplasts, into phospholipid vesicles. Although major amounts of galactolipids may be removed by the lipase, it is not possible to exclude the possibility that at least some galactolipid is required for O₂ evolution and electron flow. However, galactolipids may be of primary importance in the assembly of the photosynthetic apparatus.

**Acknowledgements** - Electron microscopy was carried out with the expert assistance and guidance of J. Telford. V. Gong’s technical assistance with a few of the experiments is appreciated.

**LITERATURE CITED**