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Separation and characterization of inner and outer envelope membranes of pea chloroplasts

(freeze-thaw hypertonic lysis/flotation centrifugation/galactolipids/galactosyl transferase/protease-treated chloroplasts)

KENNETH CLINE, JAEN ANDREWS, BRENT MERSEY, ELDON H. NEWCOMB, AND KENNETH KEEGSTRA

Department of Botany, University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT **A procedure for purifying the chloroplast envelope subfractionates it into two membrane fractions of comparable quantities. This procedure differs from previous ones in that the chloroplasts are ruptured by freezing and thawing in hypertonic medium rather than by osmotic shock. The two membrane fractions have qualitatively similar polar lipid compositions but differ in their content of individual lipids, specifically monogalactosyldiacylglycerol and phosphatidylcholine. The two fractions also differ in their constituent polypeptides and in their appearance when examined by electron microscopy. The light (density = 1.08 g/ml) and heavy (density = 1.13 g/ml) membrane fractions have been tentatively identified as the outer and inner envelope membranes, respectively.**

The chloroplasts of higher plants are enclosed by a pair of closely spaced membranes, the envelope, consisting of an outer membrane in contact with the cytoplasm of the cell and an inner membrane bounding the matrix or stroma of the organelle. The chloroplast envelope mediates the complex interactions between the chloroplast and the cell cytoplasm. For example, both reactants and products of photosynthesis must be transported across the envelope (1). In addition, those chloroplast proteins that are synthesized on cytoplasmic ribosomes must cross the envelope to reach their correct location (2). The envelope is also the site of various biosynthetic reactions, including those responsible for the formation of the galactolipids, major components of both envelope and thylakoid membranes (1).

The two membranes of the envelope have major differences in both structure and function, as shown by studies of isolated intact chloroplasts. It has been shown by freeze-fracture electron microscopy that the density of intramembranous particles is much lower in the outer membrane than in the inner, suggesting that the protein content of the outer membrane is lower (3). Also, experimental evidence indicates that the outer membrane is nonspecifically permeable to low molecular weight compounds although the inner is impermeable to such compounds and contains several translocator systems for the transport of metabolites (4).

In previously published procedures for the purification of envelopes from intact chloroplasts, the plastids are first broken by hypotonic lysis and the envelopes are then isolated by centrifugation (1). Unfortunately, during isolation, the inner and outer membranes presumably become an inseparable mixture. This makes it impossible to use these preparations to investigate the reported differences between the two membranes or to determine which one contains which biosynthetic enzymes.

We report here a new procedure for preparing the chloroplast envelope that subfractionates the envelope into two distinct fractions tentatively identified as the inner and outer membranes.

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MATERIALS AND METHODS

Percoll, uridine-5′-diphosphogalactose, and trypsin inhibitor (Type I-P from beef pancreas) were from Sigma. Trypsin and chymotrypsin were from Boehringer Mannheim and uridine diphospho[^{3}H]galactose was from Amersham.

Purification of Intact Chloroplasts. Chloroplasts were purified from homogenates of 2- to 3-week-old seedlings of pea (*Pisum sativum* var Laxton's Progress 9) by differential centrifugation followed by centrifugation through gradients of Percoll as follows: Pea shoots (200 g) were homogenized in 800 ml of cold homogenization buffer (50 mM Hepes, pH 7.5/0.33 M sorbitol/0.1% bovine serum albumin) and filtered through eight layers of cheesecloth containing cotton between the top two layers. Chloroplasts were sedimented for 2 min at 2500 × *g* max in a Sorvall HS-4 rotor with hand braking. The pellet was suspended in 20 ml of homogenization buffer and layered onto two 30-ml Percoll gradients that had been preformed by centrifuging suspensions of Percoll (50% in homogenization buffer) at 43,000 × *g* max for 30 min in a Sorvall SS-34 rotor. The overlayered gradients were centrifuged for 30 min at 1000 × *g* max in a Sorvall HB-4 rotor. Intact chloroplasts, which form a band near the bottom of the gradient at a density of ≈1.12 g/ml, were recovered by pelleting at 2000 × *g* max for 7 min and then washed twice with 50 mM Hepes, pH 7.5/0.33 M sorbitol. The resulting chloroplasts are generally 85–95% intact, as determined by the ferricyanide reduction method (5). They have been purified from the filtered supernatant 100-fold relative to mitochondria as estimated by chlorophyll (6)/cytochrome *c* oxidase (EC 1.9.3.1) (7) ratios, 100-fold relative to peroxisomes as estimated by chlorophyll/catalase (EC 1.11.1.6) (8) ratios, and 4-fold relative to endoplasmic reticulum as estimated by chlorophyll/NADPH cytochrome reductase (EC 1.6.2.4) (9) ratios.

Purification and Fractionation of Chloroplast Envelope Membranes. Purified intact chloroplasts (25–35 mg of chlorophyll) were suspended in 15 ml of 0.6 M sucrose and incubated at 0°C for 10 min. The chloroplasts were then ruptured by a freeze-thaw cycle in which the suspension was placed in a −20°C freezer for 1 hr and then at room temperature until thawed. The suspension of broken chloroplasts was adjusted to 1.3 M sucrose and overlayered with 9 ml of 1.2 M sucrose and 6 ml of 0.3 M sucrose. Envelope membranes were isolated by flotation centrifugation at 113,000 × *g* max for ≈14 hr at 4°C in a Beckman SW27 rotor. During this centrifugation, the envelope membranes rose to the 0.3 M/1.2 M sucrose interface. The envelope membranes were diluted to 0.45 M sucrose and

Abbreviation: Tricine, *N*-Tris(hydroxymethyl)methylglycine.

then fractionated by sedimentation through a 29-ml linear 0.6 M to 1.2 M sucrose gradient at 113,000 × *g* max for 14 hr at 4°C in an SW27 rotor. All solutions used for preparing envelope membranes by this procedure were buffered with 10 mM *N*-Tris(hydroxymethyl)methylglycine (Tricine), pH 7.5/2 mM EDTA.

Chloroplast envelope membranes were also prepared by the method of Douce and Joyard (1), except that, after lysis, the chloroplast suspension was adjusted to 0.3 M sucrose and the envelopes were collected by sedimentation (90 min, 113,000 × *g* max, SW27 rotor) to a 0.6 M/0.98 M sucrose interface.

Envelope membranes prepared by either method were recovered by dilution with buffer followed by pelleting at 90,000 × *g* max for 1 hr.

Protease Treatment of Intact Chloroplasts. Purified intact chloroplasts (8 mg of chlorophyll) were suspended in 50 mM Hepes, pH 7.5/0.33 M sorbitol at 1.5 mg of chlorophyll per ml. Reactions were initiated by adding aliquots of a mixture of trypsin and chymotrypsin (10 mg of each per ml in 1 mM HCl) and terminated after 30 min at 25°C by addition of pancreatic trypsin inhibitor (1.5 μg of inhibitor per μg of total protease). Chloroplasts were pelleted (1000 × *g* max, 6 min), resuspended in 5 ml homogenization buffer, and repurified on Percoll gradients as described. The treated repurified chloroplasts were ruptured by incubation for 2 min in 10 mM Tricine, pH 7.5/2 mM EDTA. The suspension of broken chloroplasts was adjusted to 1.3 M sucrose, and envelopes were isolated by the flotation centrifugation procedure described above.

Assays. Protein was estimated by the modified Lowry procedure described by Markwell *et al.* (10) using bovine serum albumin as standard. Galactosyl transferase (UDPgalactose–1,2-diacylglycerol galactosyltransferase, EC 2.4.1.46) was measured according to Douce and Joyard (11), except that reaction mixtures were buffered with 10 mM Tricine, pH 7.5/4 mM $MgCl_2$. Samples were assayed for varying times (0–15 min). Initial reaction velocities were determined from plots of galactolipid product vs. time.

Identification and Quantitation of Membrane Polar Lipids. Membrane lipids were extracted into chloroform and separated by two-dimensional thin-layer chromatography as described by Douce and Joyard (11). Lipid spots were visualized by reversible staining with iodine vapor. Phospholipids and galactolipids were identified by reactivity with specific stains (12, 13), by R_F values, and by comparison with standards. For quantitation, the silica that contained each lipid was scraped from the plate and assayed directly for phosphorus (14) or carbohydrate (15).

Electron Microscopy. Samples of envelope membranes (5–10 μg of protein), suspended in the buffered sucrose solutions of the gradients, were adjusted to 4 mM $MgCl_2$/1% glutaraldehyde and incubated on ice for 1 hr. The fixed membranes were diluted with 0.025 M phosphate buffer, pH 7.5/4 mM $MgCl_2$ to a sucrose concentration of 0.3 M and pelleted in a refrigerated Eppendorf microfuge for 1 hr. The pellets were postfixed for 5 min in 2% (wt/vol) OsO_4 in 0.025 M phosphate buffer, pH 7.5/4 mM $MgCl_2$. Pellets were washed three times with the same buffer and further postfixed in buffered 2% OsO_4 for 1 hr at room temperature. After dehydration in acetone, the samples were embedded in Spurr's resin (16). Thin sections were poststained with uranyl acetate and lead citrate.

RESULTS

The procedure for purifying two membrane fractions from the chloroplast envelope consists of two steps. In the first step, intact chloroplasts are ruptured and the envelopes are separated from other chloroplast components by flotation centrifugation on a discontinuous density gradient. The second step involves fractionating the resulting envelope membranes by isopycnic density-gradient centrifugation.

Step 1: Preparation of the Chloroplast Envelope by Freeze-Thaw Lysis and Flotation Centrifugation. Purified intact chloroplasts suspended in a hypertonic sucrose solution are ruptured by a cycle of freezing and thawing. This regime usually results in breakage of 50–70% of the chloroplasts, as estimated by phase-contrast microscopy. The envelope membranes are then isolated from the broken chloroplasts by flotation centrifugation on a discontinuous sucrose gradient (see *Materials and Methods*). During this centrifugation, the envelope membranes

Table 1. Polar lipid composition of various envelope fractions

Lipid	Hypotonic lysis/ sedimented envelopes	Freeze-thaw/ floated envelopes	Pool 1 heavy membranes	Pool 4 light membranes
$GalAc_2Gro$	12.5 ± 1.8	18.3 ± 1.1	44.5 ± 2.0	6.4 ± 1.9
Gal_2Ac_2Gro	38.6 ± 1.4	34.1 ± 0.7	31.3 ± 0.9	32.7 ± 1.3
Gal_3Ac_2Gro	5.1 ± 0.3	1.9 ± 0.2	1.3 ± 0.5	1.0 ± 0.1
Gal_4Ac_2Gro	1.4 ± 0.2	0.9 ± 0.08	0.4 ± 0.2	0.6 ± 0.1
$SQAc_2Gro$	5.8 ± 1.1	2.4 ± 0.3	2.2 ± 0.5	3.3 ± 0.7
Total glycolipids	63.4 ± 4.8	57.6 ± 2.4	79.7 ± 4.1	44.0 ± 4.1
PtdEtn	0.9 ± 0.1	1.0 ± 0.08	0.9 ± 0.1	1.5 ± 0.1
PtdGro	7.2 ± 0.3	5.3 ± 0.2	6.7 ± 0.6	5.5 ± 0.5
PtdCho	21.7 ± 0.6	32.6 ± 0.6	10.1 ± 0.7	43.9 ± 1.8
PtdIns	4.1 ± 0.2	2.9 ± 0.2	2.2 ± 0.3	5.1 ± 0.7
Unknown phospholipid	2.7 ± 0.2	0.4 ± 0.05	0.5 ± 0.1	0.1 ± 0.06
Total phospholipids	36.6 ± 1.4	42.2 ± 1.1	20.4 ± 1.8	56.1 ± 3.2

Individual polar lipids were separated and the quantity of each was estimated as indicated in *Materials and Methods*. Values represent mean ± SEM of the mole percent of the total recovered polar lipids. Numbers of replicate determinations were as follows: hypotonic lysis/sedimented envelopes, eight replicates from three preparations; freeze-thaw/floated envelopes, nine replicates from three preparations; pool 1 membranes, nine replicates from three preparations; pool 4 membranes, six replicates from two preparations. Gal_2Ac_2Gro, monogalactosyldiacylglycerol; Gal_2Ac_2Gro, digalactosyldiacyglycerol; Gal_4Ac_2Gro, trigalactosyldiacylglycerol; Gal_4Ac_2Gro, tetragalactosyldiacylglycerol; $SQAc_2Gro$, sulfoquinovosyldiacylglycerol; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylglycerol; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol.

rise to a 0.3 M/1.2 M. sucrose interface and appear as bright yellow clumped material. Unbroken chloroplasts, thylakoid membranes, and stromal protein remain in the lower portion of the gradient. This procedure generally yields 40–70 μg of envelope protein per mg of chlorophyll of intact chloroplasts. Envelopes obtained in this manner are referred to as "freeze-thaw/floated membranes."

Characterization of Envelope Membranes Prepared by Freeze-Thaw/Flotation. The envelope membranes obtained in this first step have properties similar to those reported for envelopes from other higher plant chloroplasts (1). However, to establish that freeze-thaw/floated envelopes are essentially the same as those prepared by previously reported methods, we have compared them with pea chloroplast envelopes prepared by the method of Douce and Joyard (1). In their method, intact chloroplasts are ruptured by hypotonic lysis and the envelope membranes are isolated by sedimentation on a discontinuous sucrose gradient. In our hands, this procedure yields 20–30 μg of envelope protein per mg of chlorophyll of intact pea chloroplasts. Envelopes so prepared are referred to as "hypotonic lysis/sedimented membranes."

Envelopes obtained by the two procedures have similar lipid compositions; they are rich in galactolipids and phosphatidylcholine and poor in phosphatidylethanolamine (Table 1). They also have nearly identical polypeptide profiles when examined by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1, lanes E_f and E_s). A comparison with thylakoid membrane polypeptides (Fig. 1, lane T) shows the marked difference between polypeptide profiles of envelopes and thylakoids.

The envelopes that we have isolated by both procedures possess galactosyltransferase, a marker for the chloroplast envelope (1). The freeze-thaw/floated membranes have specific activities (25–40 nmol of galactose·min^{-1}·mg $protein^{-1}$) similar to those of hypotonic lysis/sedimentated membranes (37–44 nmol·min^{-1}·mg $protein^{-1}$).

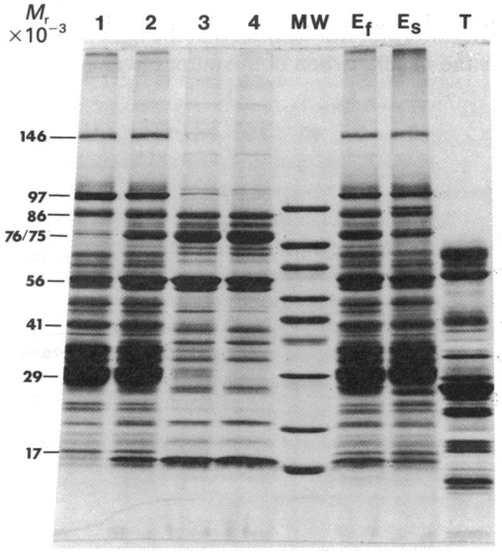

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoretogram of various chloroplast envelope fractions. Electrophoresis was carried out in 0.8-mm-thick gels according to Laemmli (17). The separating gel was a 7.5–15% linear acrylamide gradient accompanied by a 5–17.5% linear sucrose gradient. Membrane pellets were dissolved in sample buffer at room temperature. Lanes: 1–4, envelope pools 1–4 from density gradient as shown in Fig. 3; E_f, freeze-thaw/floated envelopes; E_s, hypotonic lysis/sedimented envelopes; T, thylakoid membranes from pea chloroplasts obtained from the flotation gradients described in *Materials and Methods*; MW, molecular weight markers [phosphorylase b (94,000), bovine serum albumin (68,000), catalase (57,000), fumarase (49,000), aldolase (40,000), malate dehydrogenase (34,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,500), and hemoglobin (14,800)]. M_r of envelope polypeptides were determined by using a standard curve of R_F vs. log M_r for the marker proteins.

One significant difference between the envelopes isolated by the two procedures can be seen when the membranes are examined by electron microscopy (Fig. 2). The envelopes obtained by hypotonic lysis/sedimentation contain a relatively high proportion of vesicles that are bounded by a closely spaced double membrane (arrows, Fig. 2*A*). Such double membrane vesicles are thought to contain both inner and outer envelope membranes (18). On the other hand, freeze-thaw/floated membranes contain a much lower proportion of such closely spaced double membrane vesicles (Fig. 2*B*).

These results show that the membranes obtained by freeze-thaw/flotation have properties similar to those of membranes obtained by previously reported methods of chloroplast envelope purification. The single difference—i.e., the lower proportion of closely spaced double membrane vesicles in the freeze-thaw/floated membranes—suggests that, in this preparation, inner and outer membranes are not confined to the same vesicles.

Step 2: Subfractionation of Envelope Membranes Prepared by Freeze-Thaw/Flotation. Fractionation of the freeze-thaw/floated membranes is achieved by sedimentation through a linear sucrose density gradient. This results in the resolution of two distinct bands of membranes: a whitish band near the top of the gradient and a yellowish band near the bottom.

This gradient, on fractionation, yields the optical density and buoyant density profiles shown in Fig. 3. The upper band (or "light membrane") has a buoyant density of 1.08 g/ml, and the lower band (or "heavy membrane") has a buoyant density of 1.13 g/ml. The small peak near the bottom of the gradient (fractions 1–3, Fig. 3) contains some green material (presumably thylakoids) that was present in the freeze-thaw/floated membranes.

Characterization of the Fractionated Envelope Membranes. We have recovered the membranes in the two bands by combining fractions from regions of the sucrose gradient (Fig. 3) and then collecting the membranes by centrifugation. The membrane pellets of all of the pools are yellow; however, the pellets from the heavy membrane appear more intensely colored than those of the light membrane. Generally, green cannot be detected in any pellets.

Examination by electron microscopy indicates that all of the pools contain vesicles bounded by single membranes. However, pool 2, the trailing edge of the heavy membrane peak, also contains a significant proportion of vesicles bounded by closely spaced double membranes (arrows, Fig. 2*D*). A noticeable difference between the heavy and the light membranes is that the vesicles of the former tend to be spherical (circular profiles in thin section, Fig. 2*C*) whereas the vesicles of the latter tend to have more distorted shapes (Fig. 2*F*).

The heavy and light membranes also differ in lipid composition (Table 1). The heavy membrane (pool 1) has a very high galactolipid content (≈80%). Monogalactosyldiacylglycerol is the predominant galactolipid, followed by digalactosyldiacylglycerol. In contrast, the light membrane (pool 4) has a much lower galactolipid content (≈44%), of which digalactosyldiacylglycerol is predominant. The light membrane also contains a large amount of phosphatidylcholine (44%).

A striking difference between the light and heavy membranes is seen when their polypeptide compositions are examined (Fig. 1). The polypeptides in the light membrane (pool 4) are a distinct subclass of those in the freeze-thaw/floated membranes from which they were derived (Fig. 1, lane 4). On the other hand, the heavy membrane (pool 1) exhibits a fairly complex polypeptide profile (Fig. 1, lane 1) in which the M_r 29,000 polypeptide is predominant. It is important to note that there is very

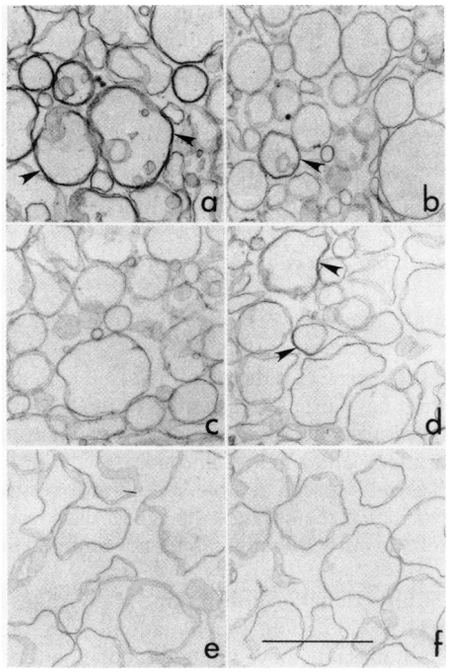

FIG. 2. Electron micrographs of envelope fractions. The micrographs are representative of central regions of the centrifuged pellets. (*a*) Hypotonic lysis/sedimented envelopes. (*b*) Freeze-thaw/floated envelopes. (*c*) Pool 1, as shown in Fig. 3. (*d*) Pool 2. (*e*) Pool 3. (*f*) Pool 4. Arrows indicate vesicles bounded by two closely spaced membranes. All figures are at the same magnification. Bar = 1 μm.

little overlap of polypeptide patterns between the heavy and light membranes—i.e., polypeptides present in the light membrane are generally absent in the heavy and vice versa (Fig. 1, lanes 1 and 4).

Identification of the Membrane Fractions. The fractionation of the chloroplast envelope into two distinct membranous components of comparable quantities suggests that these components may be the inner and outer envelope membranes. To determine which component is the outer and which the inner envelope membrane, marker polypeptides were sought. We have used the fact that large molecules cannot pass through the outer envelope membrane (4). Thus, exogenous proteases, when added to intact chloroplasts, should be able to hydrolyze only exposed outer envelope membrane proteins. Envelope polypeptides that are degraded by treatment of intact chloroplasts with proteases can thus be tentatively identified as outer membrane proteins.

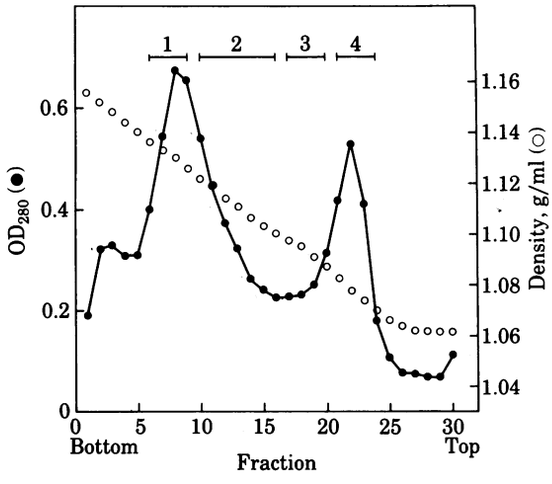

FIG. 3. Fractionation of chloroplast envelope membranes by density gradient centrifugation. Freeze-thaw/floated envelope membranes isolated from 30 mg of chlorophyll of intact chloroplasts were sedimented through a 0.6–1.2 M sucrose gradient as described in *Materials and Methods*. Gradient fractions are 1.25 ml. For analysis of the membranes, pools 1–4 were made as shown.

Such an analysis was conducted by treating intact chloroplasts with various amounts of trypsin/chymotrypsin (see *Materials and Methods*). Treatment with trypsin/chymotrypsin (up to 75 μg of each per ml) resulted in breakage of <30% of the chloroplasts compared with 15% breakage of control (no protease) chloroplasts. When the amount of protease was increased to 150 μg of each per ml, the breakage increased to 40%.

Envelope membranes obtained from repurified protease-treated chloroplasts (see *Materials and Methods*) were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 4). The protease treatment resulted in the disappearance of only a limited number of envelope polypeptides. Most of the major polypeptides, including the M_r 29,000 polypeptide, were unaffected. However, ≈10 polypeptides disappeared or were greatly decreased in quantity by this treatment (arrows, Fig. 4). Six of these polypeptides are characteristic of the light membrane, representing ≈30% of the total number of its polypeptides. Three of the protease-sensitive polypeptides are characteristic of the heavy membrane, representing ≈10% of its polypeptides. One protease-sensitive polypeptide is present in both membrane fractions. This pattern of polypeptide disappearance does not result simply because certain polypeptides are better substrates for the protease. When isolated envelope membranes are treated with trypsin/chymotrypsin, nonspecific destruction of envelope polypeptides occurs (not shown).

These results show that, when intact chloroplasts are treated with proteases, the polypeptides of the light envelope membrane are much more susceptible to hydrolysis than those of the heavy. We interpret this to mean that the light membrane band contains the major portion of the outer envelope membrane.

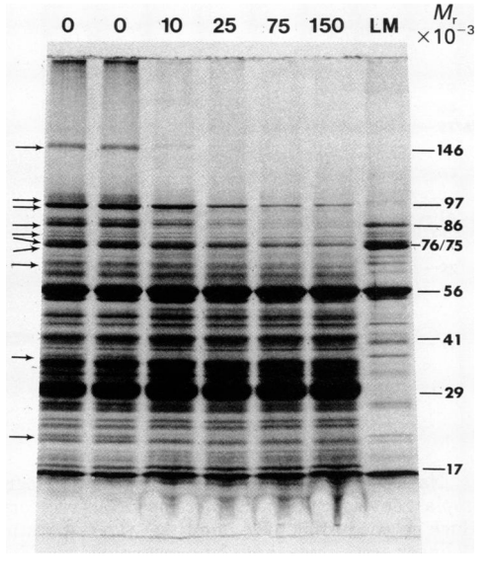

FIG. 4. Analysis of envelopes from protease-treated chloroplasts by $NaDodSO_4$/polyacrylamide gel electrophoresis. Intact chloroplasts were treated with various amounts of trypsin/chymotrypsin and repurified, and the envelopes were isolated (see *Materials and Methods*. Electrophoresis was performed as described in the legend to Fig. 1. Arrows on the left indicate polypeptides that disappear as a result of protease treatment. Lanes: 0, Independent controls (no protease); 10, 25, 75, and 150, chloroplasts treated with trypsin/chymotrypsin at 10, 25, 75, and 150 μg of each per ml, respectively; LM, light membrane preparation, included for comparison.

DISCUSSION

The procedure reported here for the purification of chloroplast envelope resolves two membrane fractions, which we call the light and heavy membrane. Evidence that both of these fractions originate from the chloroplast envelope is 3-fold. First, highly purified chloroplasts were used to prepare the membranes, making it improbable that they originate from other organelles. Second, the freeze-thaw/floated membranes, from which the two fractions were derived, have properties nearly identical to those of envelope membranes prepared by a procedure previously shown to yield high purity envelopes (1). Finally, the lipid compositions of both the light and the heavy membranes are characteristic of the chloroplast envelope (a high proportion of galactolipids and low levels of phosphatidylethanolamine) and very different from those of other cellular membranes (19).

Among the features of the purification scheme presented here, the most important for separating the two envelope membranes is the manner in which the chloroplasts are ruptured—i.e., freezing and thawing under hypertonic conditions. Incubation of chloroplasts in hypertonic solutions of low molecular weight solutes causes the inner envelope membrane to pull away from the outer (4). Thus, by rupturing the chloroplasts in hypertonic media, the envelope is broken when the inner and outer membranes are physically separated from one another.

Indeed, certain characteristics of the light and heavy membranes are precisely what one would expect if these two are the outer and inner envelope membranes. The light and heavy membranes are present in comparable quantities, as would be expected because, in the intact chloroplast, the outer and inner envelope membranes appear to be present in nearly equivalent quantities. Our procedure generally yields heavy and light membranes in a ratio of about 2:1 as judged by OD_{280}. In fact, the 2:1 ratio is probably an overestimate of the amount of heavy membrane present, as the trailing edge of the heavy membrane peak appears to be significantly contaminated with light membrane, as judged by polypeptide composition (Fig. 1, lane 2) and by the presence of double membrane vesicles in pool 2 (Fig. 2*D*). On the other hand, there appears to be little contamination of the light membrane peak by heavy membrane (Fig. 1, lanes 3 and 4). It was also expected that the inner and outer membranes would differ in their constituent proteins and lipids because they differ both structurally and functionally. As can be seen in Table 1 and Fig. 1, the light and heavy membranes have significantly different lipid compositions and polypeptide profiles.

Several criteria suggest that the light membrane is the outer envelope and the heavy membrane the inner. First, the light membrane has a lower buoyant density than the heavy (Fig. 3). It has previously been shown that the outer envelope membrane has a much lower density of intramembranous particles than the inner (3). As intramembranous particles are probably proteins, the outer membrane should have a lower protein content and consequently a lower buoyant density than the inner. Second, the light membrane is completely devoid of the M_r 29,000 polypeptide, the predominant one in the heavy membrane (Fig. 1, lanes 1 and 4). Evidence has been presented that, in spinach chloroplasts, the M_r 29,000 polypeptide is responsible for phosphate translocation (20), a function located in the inner membrane (4). The final criterion is that, when intact chloroplasts are treated with trypsin/chymotrypsin, many light membrane polypeptides are degraded (Fig. 4). This is interpreted to mean that these polypeptides are present on the cytoplasmic surface of the outer membrane and consequently that the light membrane is the outer envelope membrane.

However, several polypeptides of the heavy membrane are also destroyed by the protease treatment. This can be interpreted in several ways. For example, it may be that some of the protease gains access to the inner membrane. Breaks in the outer membrane can be detected in electron micrographs of intact chloroplasts. Another possibility is that, during fractionation, specialized regions of the outer membrane copurify with the inner. Electron microscopy of chloroplasts in hypertonic medium has shown zones of adhesion between the two envelope membranes (unpublished observations). It is possible that, during lysis and fractionation, small regions of outer membrane remain attached to the inner via these adhesion zones. Such a situation occurs in mitochondria, in which adhesion zones appear to keep portions of the mitochondrial outer membrane attached to the inner during fractionation (21).

Our data support the hypothesis that the light and heavy membranes are the outer and inner chloroplast envelope membranes, respectively. However, more definitive studies must be carried out before a positive identification can be made.

The separation of the component membranes of the chloroplast envelope now permits envelope functions to be studied in more detail by analyzing purified inner or outer membranes. By using this approach, we have obtained preliminary evidence that the galactosyltransferase enzymes are located in the outer envelope membrane.

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