Evidence that Envelope and Thylakoid Membranes from Pea Chloroplasts Lack Glycoproteins

KENNETH KEEGSTRA AND KENNETH CLINE
Department of Botany, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

Envelope and thylakoid membranes from pea (Pisum sativum var. Laxton's Progress No. 9) chloroplasts were analyzed for the presence of glycoproteins using two different approaches. First, the sugar composition of delipidated membrane polypeptides was measured directly using gas chromatographic analysis. The virtual absence of sugars suggests that plastid membranes lack glycoproteins. Second, membrane polypeptides separated by sodium dodecyl sulfate gel electrophoresis were tested for reactivity toward three different lectins: Concanavalin A, Ricinus communis agglutinin, and wheat germ agglutinin. In each case, there was no reactivity between any of the lectins and the plastid polypeptides. Microsomal membranes from pea tissues were used as a positive control. Glycoproteins were readily detectable in microsomal membranes using either of the two techniques. From these results it was concluded that pea chloroplast membranes do not contain glycosylated polypeptides.

In both plant and animal cells, many membrane proteins are posttranslationally modified by the addition of carbohydrate moieties. The initial glycosylation steps occur in the ER (8, 21), with additional carbohydrate often being added in the golgi. The resulting membrane glycoproteins are found in all of the membranes of the endomembrane system, i.e. the ER, golgi, and plasma membrane (9). However, glycoproteins have also been reported to be present in other internal membrane systems. For example, mitochondrial membranes in both animal (9, 11) and plant cells (14) have been reported to contain glycoproteins. Evidence has also been presented to indicate that chloroplasts contain glycoproteins (1, 2, 15, 19, 20, 22), although some of this evidence has been disputed (18). In particular, Racusen and Poincelot (20) reported that chloroplast envelope membranes contain significant levels of protein-bound hexosamine, suggesting that these membranes contain glycoproteins. The presence of glycoproteins in mitochondria and chloroplasts has important implications for the biogenesis of these glycosylated polypeptides. Since the ER and golgi are the only documented sites of glycosylation (8, 21), this suggests that organelar glycoproteins are glycosylated in the ER and golgi, and are subsequently transported to the proper organelle.

Because the presence of glycoproteins in chloroplast membranes has potential significance for understanding their biogenesis, we have attempted to reconfirm the earlier report (20), and to identify and further characterize any glycoproteins which might exist in the envelope and thylakoid membranes of pea chloroplasts.

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2 Abbreviations: Con A, concanavalin A; WGA, wheat germ agglutinin; RCA, Ricinus communis agglutinin.
Preparation of Microsomal Membranes. For preparation of microsomal membranes, the filtered homogenate of pea seedlings (see above) was centrifuged at 20,000g_{max} for 10 min in a Sorvall GSA rotor. Microsomal membranes were then obtained from the supernatant by centrifugation at 90,000g_{max} for 1 h in a Beckman type 30 rotor. The membrane pellet was washed once by resuspension in 10 mM Tricine/NaOH (pH 7.5) containing 4 mM MgCl_{2} followed by centrifugation at 90,000g_{max} for 1 h.

Sugar Compositional Analysis of Membrane Components. Membrane samples were prepared for sugar compositional analysis by removal of the sucrose or sorbitol used in membrane preparation. One of two methods was used. In the first, resuspended membrane pellets were subjected to dialysis against distilled H_{2}O. The dialysis was carried out at 4°C for 48 h with several changes of water. In the second method the membranes were washed 3 times by pelleting and resuspension in 10 mM Tricine/NaOH (pH 7.5), 4 mM MgCl_{2}, and then washed 3 times in the same manner using distilled H_{2}O. Envelope and microsomal membranes were pelleted by centrifugation for 90,000g_{max} for 30 min in a Beckman Type 65 rotor. Thylakoid membranes were pelleted by centrifugation at 20,000g_{max} for 10 min in a Sorvall HB-4 rotor.

Lipids were separated from membrane proteins by organic solvent extraction. The suspension of dialyzed or washed membranes was made 80% in acetone by addition of volumes of acetone. The insoluble protein residue was recovered by centrifugation and washed 4 times with 80% acetone. The combined soluble fractions were analyzed as the lipid fraction while the insoluble material was analyzed as the protein fraction.

Sugar analyses were performed as described previously (4). Briefly, this involves release of the sugars by acid hydrolysis using 2 N trifluoroacetic acid for 2 h at 121°C. Free sugars were reduced with sodium borohydride and the resulting alditols were acetylated with acetic anhydride. The resulting alditol acetates were extracted into chloroform and analyzed by GLC using a Hewlett-Packard Model 5830A gas chromatograph fitted with glass columns (1.82 m x 2 mm) packed with 3% OV-275 on gas chrom Q.

Lectin Staining of SDS-Polyacrylamide Gels. Con A, RCA, and WGA (1-2 mg in 0.05 M Tris-HCl [pH 7.5]) containing 0.15 M NaCl and 0.1% NaN_{3}) were iodinated with 125I (1 mCi) in the presence of inhibitory saccharides by the chloramine T method described by Burridge (5) with the exception that iodination was carried out for 15 min. After iodination, the lectins were purified by gel filtration chromatography on BioGel P-10. Con A was further purified by affinity chromatography on Sephadex G-50. RCA was further purified by affinity chromatography on Sepharose 4B. The repurified iodinated lectins were dialyzed extensively against 0.05 M Tris-HCl (pH 7.5) containing 0.15 M NaCl and 0.1% NaN_{3}. Bovine hemoglobin was added to 125I-lectin preparations as a carrier protein at a concentration of 2.5 mg/ml. The specific activities of the iodinated lectins ranged from 1.5 to 10^{6} cpm/mg protein.

SDS-polyacylamide gel electrophoresis was carried out in 0.8-mm thick slab gels using the discontinuous buffer system of Laemmli (12). The stacking gel was 1 to 2 cm long and was polymerized from a 4% acrylamide solution. The separating gel (8 cm) was polymerized from a 10% acrylamide solution. Membrane samples were prepared for electrophoresis by dissolving pellets in sample buffer at room temperature. Stromal proteins were first precipitated with 80% acetone and then dissolved in sample buffer at room temperature. Electrophoresis was carried out at a constant current of 12 mamp for 4 to 5 h. After electrophoresis, gels destined for Con A or RCA overlay were washed with Coomassie blue R250 and destained (5). Gels for WGA overlay were fixed in methanol:water:acetic acid (5:5:1) for 2 h. Gels were then equilibrated in 0.05 M Tris-HCl (pH 7.5) containing 0.15 M NaCl + 0.1% (w/v) NaN_{3} as described (5). For demonstration of hapten reversibility of lectin binding, the respective inhibitory saccharides were included at 20 to 40 mg/ml in the equilibration buffer. Gels were subsequently incubated with lectins as described by Burridge (5). Briefly, the gels were overlaid with radioactive lectin (at a concentration of 10^{6} cpm/ml) with or without inhibitory saccharide (40 mg/ml) for 1.5 h at room temperature. The gels were then washed extensively with 0.05 M Tris/HCl (pH 7.5) containing 0.15 M NaCl and 0.1% NaN_{3} (with or without inhibitory saccharides). Gels overlaid with either Con A or RCA were dried and then exposed to X-ray film. Gels overlaid with WGA were stained with Coomassie and destained prior to drying and autoradiography.

RESULTS

Sugar Compositions of Chloroplast Membrane Components. Purified intact chloroplasts from young pea seedlings were used to prepare envelope and thylakoid membranes as described in Methods. Inasmuch as both of these membranes are known to contain large amounts of glycolipids (7), the membranes used for compositional studies were first fractionated into lipid and protein portions by organic solvent extraction. The acetone-soluble lipid fraction was analyzed separately from the insoluble protein fraction and the results are shown in Tables I and II, respectively.

The lipid fractions of chloroplast membranes contained galactose as the predominant sugar. This was expected since galactolipids were the predominant phospholipids.

Table I. Neutral and Amino Sugar Compositions of Membrane Lipid Extracts

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Envelope</th>
<th>Thylakoid</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mg protein</td>
<td>µg/mg protein</td>
<td>µg/mg protein</td>
</tr>
<tr>
<td>Fucose</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
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<tr>
<td>Ribose</td>
<td>13.2</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Arabinose</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Xylose</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Mannose</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
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<tr>
<td>Galactose</td>
<td>397</td>
<td>100.0</td>
<td>16.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>65.3</td>
<td>4.1</td>
<td>21.5</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
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</tbody>
</table>

Table II. Neutral and Amino Sugar Composition of Membrane Protein Samples

Results are expressed as the amount of sugar found in the acetone-insoluble residue/mg of protein present in the membrane sample before acetone extraction. Each value represents the average of duplicate or triplicate analyses of a single membrane preparation. Similar results were obtained with other membrane preparations.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Envelope</th>
<th>Thylakoid</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mg protein</td>
<td>µg/mg protein</td>
<td>µg/mg protein</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.07</td>
<td>0.12</td>
<td>2.3</td>
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<tr>
<td>Ribose</td>
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<td>82.3</td>
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<td>&lt;0.04</td>
<td>108.0</td>
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<tr>
<td>Xylose</td>
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<td>&lt;0.04</td>
<td>5.4</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.21</td>
<td>0.12</td>
<td>15.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>2.4</td>
<td>0.97</td>
<td>62.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>20.4</td>
<td>5.3</td>
<td>20.8</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>8.1</td>
</tr>
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</table>
ids comprise a large proportion of the polar lipids of chloroplast membranes (7). Some lipid-bound galactose is also present in the microsomal membranes, most likely resulting from plastid membrane contamination of the microsomal fraction. The lipid fractions of envelope and microsomal membranes also contain significant quantities of glucose. This glucose most probably originates from nonlipid sources. For example, residual sucrose remaining in the dialyzed or washed membranes would be soluble in the 80% acetone used for preparation of the lipid fractions.

The protein fractions from the plastid membranes contain only very low levels of carbohydrate. More importantly, the sugars commonly found on glycoproteins (10), i.e. fucose, xylose, arabinose, mannose, and N-acetylgalactosamine are either not detected or present in very low levels. For example, mannose is found at a maximum level of 0.2 μg/mg protein. If one assumes that the mol wt of an average membrane polypeptide is 50,000 daltons, this represents only one mannose residue on 1/20 of the polypeptides, or 5 mannose residues on 1% of the polypeptides.

Those sugars which are present at readily detectable levels most likely arise from molecules other than glycoproteins. For example, the ribose found in the insoluble fraction from all three membranes probably derives from RNA. The glucose levels of all three types of membranes were variable from one preparation to another. It seems likely that the glucose is a contaminant derived from sources other than membrane glycoproteins.

To ensure that these methods were capable of detecting sugar residues in membranes which contain glycoproteins, microsomal membranes were analyzed. The results in Table II demonstrate that the protein fraction of the microsomal membranes contains readily measurable levels of a number of sugars, including those commonly found on glycoproteins (10), i.e. fucose, arabinose, xylose, mannose, and N-acetylgalactosamine. These results indicate that plastid membranes contain few, if any, glycoproteins while microsomal membranes contain easily detectable levels. However, there are at least two limitations to the direct analytical approach. First, it remains possible that the plastid membranes contain only a few glycoproteins, each with low levels of carbohydrate. In this case, the total levels of carbohydrate might be below the level of detectability of the compositional studies.

A second limitation is that the organic solvent extraction used to remove the glycolipids may also remove some polypeptides. To test this possibility, SDS-polyacrylamide gel electrophoresis was used to analyze the polypeptide profiles of envelope and thylakoid membranes before and after organic solvent extraction (Fig. 1). It is clear from the results in Figure 1 (Lanes 1 and 3) that several polypeptides of the envelope are removed by the acetone treatment used in preparing envelopes for carbohydrate analysis. The most significant loss is the disappearance of the 29,000 dalton polypeptide which represents nearly 15% of the Coomassie-staining protein of the envelope. In addition, some other less prominent bands are lost. These same polypeptides were lost if the envelope membranes were heated in sample buffer before electrophoresis (Fig. 1, Lane 2). Inasmuch as these polypeptides are presumably also lost during the preparation of envelope polypeptides for carbohydrate analysis, it remains possible that these polypeptides may contain carbohydrate. Similarly, acetone treatment results in some loss of thylakoid polypeptides (Fig. 1, Lanes 4 and 5).

Analysis of Plastid Membranes Proteins for Lectin Reactivity. In an effort to overcome the limitations in the analytical approach discussed above, a sensitive procedure which allows the detection of very low levels of individual glycoproteins was utilized. In this procedure, membrane polypeptides are first separated by SDS-polyacrylamide gel electrophoresis. The acrylamide gels are then reacted with radioactive lectin. The lectin binds to glycosylated polypeptides containing those sugars recognized by the lectin. After removal of unbound lectins, the gel is dried, and glycoproteins are detected as radioactive bands by autoradiography.

For these analyses three lectins were chosen which are known to react with a broad spectrum of plant and animal glycoproteins. They are: Con A, which is specific for α-mannosyl and α-glucosyl residues (14); RCA, which is specific for galactosyl residues (14); and WGA, which is specific for N-acetylgalactosaminy and sialyl residues (14). Figures 2, 3, and 4 show the results of analyzing plastid membrane polypeptides using these three lectins. In each case, the specificity of lectin binding was confirmed by treating duplicate samples with lectin in the presence of the appropriate sugar hapten.

Con A does not show a reaction with the polypeptides from any of the plastid fractions, i.e. stroma, thylakoids, or envelopes (Fig. 2). Under these same conditions, Con A does react in a hapten reversible manner with a large number of polypeptides from the microsomal membrane fraction (Fig. 2). The sensitivity of this
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Fig. 2. Staining of SDS-polyacrylamide gels of chloroplast and microsomal proteins with Coomassie and 125I-Con A. Electrophoresis of protein samples (60 µg) was carried out in a 10% acrylamide gel as described under "Materials and Methods." The gel was stained, destained, and equilibrated as described. The left half of the gel was then overlaid with 125I-Con A, whereas the right half was overlaid with 125I-Con A in the presence of the hapten, α-methylglucoside (40 mg/ml). The gel was subsequently washed, dried, and subjected to autoradiography. The upper portion of the figure is the Coomassie-stained gel. The lower portion is the autoradiogram of the same gel. Lanes: E, pea chloroplast envelope membranes; T, thylakoid membranes; S, chloroplast stromal proteins; M, pea microsomal membranes; MW, mol wt standards (1 µg of each protein). The mol wt standards were the same as described in Figure 1 except that ovalbumin (45,000) was included as a positive control for 125I-Con A or 125I-WGA staining. Mol wt standards were marked with a dot of radioactive ink to aid alignment of the autoradiogram with the Coomassie-stained gel.

Fig. 3. Staining of SDS-polyacrylamide gels of chloroplast and microsomal proteins with Coomassie and 125I-RCA. Electrophoresis, staining, and overlaying of gels was carried out as described in Figure 2 except that the left half of the gel was overlaid with 125I-RCA, whereas the right half was overlaid with 125I-RCA in the presence of lactose (40 mg/ml). The upper portion of the figure is the Coomassie-stained gel, the lower portion is the autoradiogram of the same gel. Lanes: RS, rabbit serum, contains glycoproteins which react with RCA; all remaining lane designations are as described in Figure 2.

Technique is demonstrated by the reaction which Con A shows with the mol wt standards. As expected, Con A binding to the 1 µg ovalbumin is easily detectable. In addition, Con A binds in a hapten-reversible manner to two impurities which are not visible by Coomassie staining. Because this stain can generally detect 0.1 µg of a protein, it follows that Con A binding can detect less than 0.1 µg of some glycoproteins. It should be noted, however, that the detection limit will depend upon the binding constant of the lectin for the carbohydrate residues as well as the number of carbohydrate moieties on a glycoprotein, and therefore will vary from one glycoprotein to another.

RCA also does not react with any polypeptides from the plastid fractions (Fig. 3). However, it does show hapten-reversible binding to a component of thylakoid and envelope membranes which precedes the dye front. This component is most likely galactolipid, which would be expected to move near the dye front and also to bind RCA. RCA also binds to a component preceding the dye front in the microsomal membrane fraction, suggesting that this fraction contains some galactolipids. This agrees with the composition data (Table I) which demonstrated lipid-soluble galactose present in the microsomal fraction. However, RCA fails to react with any of the polypeptides from the microsomal membranes. As
Fig. 4. Staining of SDS polyacrylamide gels of chloroplast and microsomal proteins with Coomassie and 125I-WGA. Electrophoresis was carried out as with Figure 2. The gel was then fixed and equilibrated as described under "Materials and Methods." The left half of the gel was overlaid with 125I-WGA. The right half of the gel was overlaid with 125I-WGA in the presence of N-acetylgalactosamine (40 mg/ml). The gel was then washed, stained with Coomassie, destained, dried, and subjected to autoradiography as described under "Materials and Methods." The upper portion of the figure is the Coomassie-stained gel. The lower portion is the autoradiogram of the same gel. Lanes: RS, rabbit serum, contains glycoproteins which react with WGA. All remaining lane designations are as described in Figure 2.

a positive control for the technique, the polypeptides of rabbit serum were tested. As expected, several polypeptides from rabbit serum showed hapten-reversible binding of RCA.

WGA also fails to react with chloroplast proteins (Fig. 4). In our hands, a considerable amount of nonspecific binding of 125I-WGA resulted when Coomassie-stained gels were overlaid with WGA. This nonspecific reaction was eliminated when gels were first overlaid with radioactive lectin, then washed and subsequently stained. When these conditions were used, WGA did not bind either chloroplast or microsomal proteins. As expected, WGA showed hapten-reversible binding to a number of rabbit serum proteins as well as to ovalbumin.

DISCUSSION

The results presented here lead to the conclusion that chloroplast membranes do not contain glycoproteins. This conclusion is based on the compositional analysis which show the virtual absence of those sugars commonly found in glycoproteins (Table II). In addition, the lectin-binding studies demonstrate that lectins which bind to a variety of plant and animal glycoproteins do not bind to any of the chloroplast membrane proteins. However, it does remain possible that chloroplast membranes contain low levels of glycoproteins which are below the detectability limits of the compositional studies. If present, these glycoproteins must also have unusual carbohydrate moieties, inasmuch as they do not bind any of the lectins employed here.

Our conclusions do not agree with those of Racusen and Poincelot (20) and Racusen and Foote (19) who concluded from their studies that chloroplasts contain significant levels of glycoproteins, mainly in the envelope membranes. They used a specific color test to identify hexosamines released from chloroplast proteins by acid hydrolysis. Inasmuch as the level of hexosamine that they reported would have been easily detected by our methods, we have no concrete explanation for the differences between our results and theirs. However, color tests are often subject to false positive reactions and it is possible that components other than hexosamines gave the positive results that they obtained.

Because of the negative results obtained with the plastid membranes, it was important to demonstrate the sensitivity and reliability of the techniques utilized. This was accomplished by analyzing microsomal membranes which are reported to contain glycoproteins. Glycoproteins were easily detected in microsomal membranes by both the compositional studies and by the Con A staining procedure. The results obtained with microsomal membranes are qualitatively similar to the results of Mellor et al. (17) who found the same sugar constituents in the ER of castor bean endosperm. Some of the quantitative differences between their results and ours may reflect tissue or species differences or the fact that our microsomal membrane fraction contains a mixture of membranes and not just ER.

One unusual aspect of the sugar composition of microsomal polypeptides presented here and by Mellor et al. (17) is the high level of galactose and arabinose. While these sugars may be derived from glycoproteins, for example, the hydroxyproline-rich protein found in plant cells (13), it remains possible that these sugars are derived from polysaccharides. This latter interpretation is supported by two lines of evidence. First is the observation that the levels of galactose and arabinose are variable from one preparation of microsomal membranes to another (data not shown). Second, the lectin-binding studies demonstrate that RCA, which binds galactosyl residues, does not bind to any of the microsomal membrane polypeptides which migrate into the SDS gels. This suggests that these polypeptides do not contain galactosyl residues, although it remains possible that they contain only terminal galactosyl residues which are not available for lectin binding or that the galactose is contained on glycoproteins which do not enter the gels.

The differential lectin binding of the plastid and microsomal membranes allows a conclusion concerning cross contamination between these fractions. Because of the intense labeling of microsomal membranes with Con A, even a small contamination of the envelope or thylakoid membranes with microsomal membranes should be detectable. The lack of labeling of the plastid membranes indicates they do not contain a significant quantity of microsomal membranes. On the other hand, the RCA labeling of the dye front of the microsomal membrane fraction suggests that this fraction includes some plastid membranes. This agrees with visual observations of green color in the microsomal fraction indicating thylakoid contamination of the microsomes.

Our conclusion that chloroplast envelope and thylakoid mem-
branes do not contain glycoproteins has significance for understanding the biogenesis of their polypeptides. Previous reports suggested that these polypeptides contain carbohydrate. Inasmuch as the only known sites of glycosylation are the ER and Golgi, the implication is that these glycosylated proteins are synthesized in the ER. Our results do not rule out the possibility that the chloroplast membrane proteins pass through the ER during their biogenesis. However, they do eliminate the need to invoke a role for the ER in glycosylation of plastid polypeptides.

LITERATURE CITED


