Flow Cytometry of Spinach Chloroplasts

Determination of Intactness and Lectin-Binding Properties of the Envelope and the Thylakoid Membranes

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ABSTRACT

Intact spinach (Spinacia oleracea) chloroplasts, thylakoid membranes, and inside-out or right-side-out thylakoid vesicles have been characterized by flow cytometry with respect to forward angle light scatter, right angle light scatter, and chlorophyll fluorescence. Analysis of intact chloroplasts with respect to forward light scatter and the chlorophyll fluorescence parameter revealed the presence of truly “intact” and “disrupted” chloroplasts. The forward light scatter parameter, normally considered to reflect object size, was instead found to reflect the particle density. One essential advantage of flow cytometry is that additional parameters such as Ricinus communis agglutinin (linked to fluorescein isothiocyanate) fluorescence can be determined through logical conditions placed on bit-maps, amounting to an analytical purification procedure. In the present case, chloroplast subpopulations with fully preserved envelopes, thylakoid membrane, and inside-out or right-side-out thylakoid membranes vesicles can be distinguished. Flow cytometry is also a useful tool to address the question of availability of glycosyl moieties on the membrane surfaces if one keeps in mind that organelle-to-organelle interactions could be partially mediated through a recognition process. A high specific binding of R. communis agglutinin and peanut lectin to the chloroplast envelope was detected. This showed that galactose residues were exposed and accessible to specific lectins on the chloroplast surface. No exposed glucose, fucose, or mannose residues could be detected by the appropriate lectins. Ricin binding to the intact chloroplasts caused a strong aggregation. Disruption of these aggregates by resuspension or during passage in the flow cytometer induced partial breakage of the chloroplasts. Only minor binding of R. communis agglutinin and peanut lectin to the purified thylakoid membranes was detected; the binding was found to be low for both inside-out and right-side-out vesicles of the thylakoid membranes.

Among the tools available to characterize cellular compartments by morphological and functional criteria, flow cytometry occupies an important place. Advantages of this technique are the possibility of fast identification, quantification, and resolution of subpopulations in heterogeneous cell samples of small size on the basis of a multiparametric analysis of several optical signals.

Although the present generation of flow cytometers (cell sorters) is sufficiently flexible to handle diverse material such as living cells, prooplasts, chloroplasts, and mitochondria (5, 6, 9), little work has been done on isolated plant organelles and their subfractions. Chl fluorescence and light-scattering properties of plant cells (4, 5, 14, 16), algae (24, 25) and, more generally, phytoplankton (11, 23) can be easily detected by this technique. With respect to photosynthesis and/or respiration, few reports are available (3, 28, 35). Green plant material has a natural fluorophore, Chl. Because Chl fluorescence in flow cytometry is independent of the functioning of the photosynthetic apparatus (35), it is a good measure of the pigment content of the particles.

Both animal and plant cell surfaces have exposed glycosyl moieties bound to either proteins or lipids (17). Internal membrane systems such as ER, Golgi apparatus, glyoxysomes, and mitochondria have been found to contain surface-exposed sugar residues (19, 28). However, conflicting results have been reported for chloroplasts (7, 8, 15, 22, 32, 33). Glycosylated lipids have been suggested to play a role in membrane stability, shape, recognition, and ion binding (12), whereas glycosylated proteins have been proposed to be involved mainly in conformational stability, protease resistance, and charge and water-binding capacity (26).

One of the goals of this work was to use flow cytometry to investigate whether the galacto residues are accessible on the chloroplast envelope membrane surface to enable an interaction with other molecules or other cell membranes. The multiparametric capabilities of flow cytometry were used to distinguish intact and broken chloroplasts, i.e. envelope and thylakoid membrane surfaces. Various fluorescein-conjugated lectins were used to reveal the presence and availability of specific glycosyl residues on the chloroplast envelope. Light, fluorescence, and electron microscopy were performed to confirm the flow cytometric analysis. Because no glycoproteins were detected, the differences in lectin binding are discussed in terms of different lipid-to-protein ratios between the envelope and the thylakoid membrane.

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Figure 1. Cytograms of an intact chloroplast preparation analyzed for Chl fluorescence (IRFL) and FALS. Chloroplast intactness, 87% (determined by the ferricyanide test). A, Biparametric representation with three classes of frequency (Fr.) indicated by the contours >250 particles (center, black), 50 to 250 particles (medium gray), and 10 to 49 (light gray). Also shown are the two working statistical bit-maps MAP1, encircling intact chloroplasts, and MAP2, delimiting thylakoids. The corresponding histograms are shown as projections for IRFL (B) and for FALS (C). Their modal fluorescence intensity and modal light scatter values are indicated, as are the percentages of the whole population that they represent. A gate delimited the two subpopulations and the percentage of particles is given for the main subpopulation. D, Three-dimensional cytogram of IRFL (at 30° viewed angle) as a function of FALS for intact chloroplasts showing the distinct separation of the two populations. Frequency is plotted in the pseudo third dimension.

MATERIALS AND METHODS
Preparation of the Chloroplasts

Spinach (Spinacia oleracea) was obtained from the local market. Intact chloroplasts were prepared according to the method of Nakatani and Barber (21). These chloroplasts were further purified by isopycnic centrifugation (5000g for 20 min) after deposit on a preformed 50% (v/v) Percoll (Pharmacia) gradient (10,000g for 100 min) containing 300 mM mannitol, 30 mM Mops (pH 7.8), and 2 mM EDTA, which had been run at 10,000g for 100 min (20).

The purified intact chloroplasts (class I) were gently resuspended in a medium containing 300 mM mannitol, 30 mM Mops (pH 7.8), 10 mM NaCl, and 10 mM MgCl₂ (suspension medium). The intact Percoll-purified chloroplasts used in this paper showed, depending on spinach quality, an intactness of 92 ± 6% (measured by ferricyanide-dependent O₂ evolution). The CO₂-dependent O₂ evolution was normally about 100 μmol O₂ mg⁻¹ Chl h⁻¹ and chloroplasts had an average mass ratio of protein:Chl 17:1 (w/w), in agree-

Figure 2. Three-dimensional cytograms of preparations of highly intact chloroplasts (A), osmotically lysed chloroplasts (B), and purified thylakoid preparation (C) analyzed for Chl fluorescence (IRFL) and FALS. The third dimension is a pseudo dimension representing the frequency. The frequency in A is double that chosen for B and C to provide better visualization of the third dimension. The PMT setting was 1294 V in all cases.
ment with Mourioux and Douce (20).

Thylakoid membranes (class II) were prepared by osmotic disruption of the purified chloroplasts by resuspending them in 5 mM MgCl₂ and centrifuging 5 min at 5000g. The resulting pellet was washed three times with suspension medium to obtain purified thylakoid membranes.

Inside-out and right-side-out thylakoid vesicles were obtained by mechanical disintegration in a Yeda press of stacked thylakoid membranes, followed by partitioning in an aqueous dextran/polyethylene glycol two-phase system, as previously described by Akerlund and Andersson (1).

O₂ evolution was measured with a Clark-type electrode at 20°C in suspension medium. The intactness was determined by ferricyanide-dependent O₂ evolution, in the presence of 15 mM NH₄Cl to ensure uncoupling before and after an osmotic shock (18).

**Lectins**

The chloroplast and thylakoid preparations in the suspension medium were incubated for 30 min at 25°C with lectins (120 µg/mL) at a Chl:lectin mass ratio of 2:1. The treatment was made in the presence or in the absence of solutions of the corresponding sugars (60 mM) to competitively inhibit the specific membrane binding. Lectins used were RCA₁₂₀³ (from *Ricinus communis*) and PNA (from *Arachis hypogaea*), both specific for β-D-galactose; *Solanum tuberosum* agglutinin (from

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**Table 1. Flow Cytometric Analysis of Intact Chloroplasts and Purified Thylakoids**

In this experiment, the PMT setting was 1294 V, and a gate was set on the FALS channel. The number of particles analyzed was 10⁵.

<table>
<thead>
<tr>
<th>Preparations</th>
<th>FALS Frequency ≤ channel 11 %</th>
<th>FALS Frequency ≥ channel 12 %</th>
<th>RALS Mean AU</th>
<th>RALS Mean AU</th>
<th>IRFL Median channel AU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact chloroplasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major population</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(channel 12 to 63)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Minor population</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(channel 1 to 11)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Purified thylakoids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(channel 1 to 11)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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**Figure 3.** Histograms of intact chloroplast preparation (A, D, G) and osmotically lysed chloroplast preparation (B, E, H) analyzed for Chl fluorescence (IRFL), FALS, and 90° light scatter (RALS). The C, F, and I cytograms compare the intact (hatched histograms) and osmotically lysed chloroplasts. The y axis represents frequency. Settings are the same as in Figure 2. The arrow (in F) represents the fraction of particles that was discarded during purification of the osmotically lysed chloroplasts.
S. tuberosum), specific for glucose residues; Tetragonolobus agglutinin (from Tetragonolobus purpureas), specific for fucose residues; and ConA (from Canavalia ensiformis), specific for mannose residues. All lectins were FITC-labeled and were purchased from Sigma.

Flow Cytometric Analysis

The samples were analyzed with an EPICS V flow cytometer (Coulter Electronics) equipped with an argon laser (Spectra-physics 2025-05). A standard 76-μm nozzle was used. A confocal optic system was used to improve the light-scatter resolution. The sheath was water at 4°C. For fluorescein, emission filters were 515-nm LP interference, 515-nm LP absorbance, 525-nm BP absorbance, and 560-nm SP interference for PMT1 (IGFL). For Chl fluorescence, emission filters were 515-nm LP absorbance and 685-nm BP for PMT2 (IRFL). The excitation was 488 nm at 400 mW. The count rate was 1500 to 2000 objects s⁻¹. The resulting histograms correspond to 10⁵ particles analyzed, and each histogram is representative of two to five independent preparations.

When considering inside-out and right-side-out subthylakoid membrane vesicles, a quartz flow chamber was used in combination with the confocal optic system to improve

Figure 4. Biparametric representations of FALS versus LIFR, with the corresponding histograms for (A) right-side-out vesicles and (B) inside-out vesicles. For each type of vesicle, a three-dimensional cytogram, its projection, and the corresponding histograms are presented. Two classes of frequency (Fr.) are indicated by the contours, which delimit coordinates having >250 particles (center) and 50 to 250 particles (surrounding). The fraction of events in each quadrant is indicated.

Figure 5. Histogram of RCA₁₂₀-FITC binding to spinach chloroplast envelope analyzed by use of MAP1. A, Biparametric representation showing IGFL (log value of the “green” fluorescence of FITC, on 256 channels) versus chloroplast frequency. The samples are: control chloroplasts; chloroplasts incubated with 100 μg mL⁻¹ BSA-FITC; chloroplasts incubated with 100 μg mL⁻¹ RCA₁₂₀-FITC; and chloroplasts incubated with 100 μg mL⁻¹ RCA₁₂₀-FITC in the presence of β-D-galactose. The median channel values (of FITC fluorescence) are given. B, The histogram of RCA₁₂₀-FITC has been deconvoluted into its constituent parts by an algorithm (“immunoanalysis” of MDAIDS, Coulter) that models nonreactive populations (sequentially on autofluorescence and nonspecific fluorescence in this case). The reference was chloroplasts treated with RCA₁₂₀-FITC in presence of β-D-galactose. The following statistics are indicated for the positives (solid arrows) and the reference (open arrows): median, lower decile (LD), and upper decile (UD).
resolution in light-scatter properties.

Statistically delimited bit-maps allow us, in a complex preparation, to ascribe light scatter (FALS) or Chl fluorescence (IRFL) to subpopulations representing in the present case 100% intact chloroplasts or thylakoids, devoid of debris or aggregates. The working bit-maps are described in Figure 1A, where MAP1 represents the intact chloroplast population and MAP2 represents a thylakoid population. The essential advantage is that additional parameters such as FITC fluorescence can then be accumulated through a logical condition placed on the bit-maps, amounting to an analytical purification procedure.

For FITC fluorescence measurements expression, we used the pulse integral on a three-order log scale (IGFL), for which an increment of 27 channels (on 256 channels) represents a doubling. IGFL was calculated from the values of the modal fluorescence channel $\times$ (logarithmic scale) converted to linear scale with $y = 10 \exp (1.1149 \times x)$; data were normalized to the modal fluorescent channel as a reference within each experimental series. The variation around the modal value was estimated through the same procedure but taking as reference the channel value that limited the first decile of particles (10% lower fluorescent decile) and the channel corresponding to the upper decile (10% higher fluorescent decile). In the case of a linear scale, as for the FALS or the RALS, the simple arithmetic mean channel was used. Epics V histogram files were processed through Macintosh graphic (Cricket graph software) as described by Cameron (10). Three-dimensional representations were drawn through Boeing graph software run on a 386 IBM-PC computer.

Cytology

Intact chloroplasts and purified thylakoids were observed by differential interference contrast and epifluorescence microscopy. The epifluorescence filterblocks used were: B1, excitation 450 to 495 nm, emission LP 520 nm (Chl and FITC); B4, excitation 475 to 495, emission 520 to 560 (FITC); and G1, excitation 546/10, emission LP 590 (Chl). For black and white photos, Kodak T-MAX 100 was used, and for color photos, Kodak Ektachrome 1600. The pretreatment of the samples was the same as for flow cytometry.

For EM, the samples were incubated with RCA120 as de-

![Figure 6. Titration of the RCA120-FITC binding to spinach chloroplast envelope (A) and thylakoid membrane (B) through flow cytometric analysis. • and D, RCA120-FITC; O and D, RCA120-FITC in presence of β-D-galactose.](image)

Table II. Statistics Extracted from the Histograms Presented in Figure 5 for FITC-RCA120 Binding to Spinach Chloroplasts

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Negatives Class</th>
<th>Positive Class</th>
<th>∆ in Channels</th>
<th>Factor of Fluorescence Increase*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Percent</td>
<td>F median</td>
<td>Percent</td>
<td>F median</td>
</tr>
<tr>
<td>Control (autofluorescence)</td>
<td>100</td>
<td>23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ricin/Control</td>
<td>5</td>
<td>23</td>
<td>95</td>
<td>91</td>
</tr>
<tr>
<td>Ricin/β-D-gal + Ricin</td>
<td>32</td>
<td>44</td>
<td>68</td>
<td>102</td>
</tr>
</tbody>
</table>

* Converting log to linear with $y = 10 \exp (1.1149 \times x)$. 

For EM, the samples were incubated with RCA120 as described above (except that sucrose was used instead of mannitol in the sample buffer). The incubation was stopped by 10-fold dilution and glutaraldehyde was added to the samples to a final concentration of 2%. After 2 h of incubation in the dark, the samples were spun down and washed two times with sample buffer, postfixed in 1% osmium tetroxide.
in sample buffer for 2 h at 4°C, and washed with distilled water. Samples were then dehydrated in increasing ethanol concentrations and in propylene oxide, and embedded in araldite resin for 60 h at 48°C. Ultrathin sections of cells were obtained with a LKB Ultratome III ultramicrotome, stained in 9% uranyl acetate in methanol, poststained in lead citrate (Reynolds), and observed in a Hitachi HU 12 A electron microscope (75 kV).

**RESULTS**

Flow Cytometry of Intact Chloroplasts and Thylakoid Membranes

When a preparation of intact spinach chloroplasts (80% according to ferricyanide test) was analyzed by flow cytometry with respect to Chl fluorescence and FALS, two subpopulations were found (Fig. 1D), the major subpopulation representing 76% of the whole chloroplast population and the minor one representing 20%.

However, if a highly intact preparation (Fig. 2A) of spinach chloroplasts (Percoll purified, 92 ± 5% intact according to ferricyanide test) was analyzed, 96.5% had a FALS value of 36.5 AU, whereas a minor subpopulation, 3.5% of the population, showed a lower FALS value of 8.8 AU (Fig. 2A, Table I).

If intact chloroplasts were resuspended in distilled water or centrifuged harder, treatments normally considered to disrupt the chloroplasts, the major subpopulation of the intact chloroplasts disappeared, whereas the number of particles found in the initial minor population increased (Fig. 2B). A small subpopulation of lower red fluorescence and low-light scatter is detectable and could be debris with a low Chl content.

When analyzing purified thylakoids, there was only one discrete population (Fig. 2C), in which, in this case, 98% of the population exhibited a FALS value of 8.0 AU, not significantly different from the value determined for the minor population of the intact chloroplasts (Table I).

Whereas chloroplasts and thylakoids were clearly distinct in FALS (Fig. 2 and Fig. 3, A–C), their Chl fluorescence was the same (Fig. 3, D–F, Table I). This is understandable because Chl fluorescence is considered to be closely associated with intrinsic protein complexes in the thylakoid membranes and is not likely to be affected by removal of the non-Chl-containing envelope. In addition the RALS value for “intact” chloroplasts, “disrupted” chloroplasts, and purified thylakoids did not show any significant differences (Fig. 3, G–I, Table I) and therefore was not a useful parameter with which to distinguish the three classes.

**Flow Cytometry of Subthylakoid Vesicles**

Inside-out thylakoid membrane vesicles (originating from the grana region) and right-side-out thylakoid membrane vesicles (originating mainly from the stroma region) were analyzed by flow cytometry. To analyze these much smaller particles, a quartz flow chamber and a different setting of the flow parameters had to be used. Therefore, no direct comparison of data should be made with the chloroplasts or purified thylakoid membranes.

With respect to Chl fluorescence, 12.0 AU for right-side-out vesicles (Fig. 4B, IRFL) and 11.7 AU for inside-out vesicles (Fig. 4A, IRFL), no significant differences were detected. The inside-out vesicles were less dispersed on IRFL than the right-

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Inside-Out Vesicles</th>
<th>Right-Side-Out Vesicles</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FALS (Mean)</td>
<td>IRFL (Median)</td>
</tr>
<tr>
<td>Control</td>
<td>5.7</td>
<td>8</td>
</tr>
<tr>
<td>RCA120</td>
<td>6.6</td>
<td>9</td>
</tr>
<tr>
<td>β-D-Galactose + RCA120</td>
<td>5.8</td>
<td>8</td>
</tr>
</tbody>
</table>

**Figure 7.** Analysis of time dependence of chloroplast intactness, with and without RCA120 incubation, measured by ferricyanide-dependent O2 evolution on oxygen electrode. Control chloroplasts (●) and chloroplasts incubated with RCA120 (Ricin120) (O) as described in "Materials and Methods." In the last two points, the samples were centrifuged and resuspended after 60 min incubation as done prior to the flow cytometry.
Figure 8. Micrographs of intact chloroplast preparation and purified thylakoid membranes. Normarski interference contrast; at 1250X magnification the field covers 80 × 120 μm. A, Intact chloroplasts. B, Purified thylakoids. Notice similar size and shape but different internal aspect of the two population. The results of RCA_{120}, incubation of intact chloroplasts (C) and purified thylakoids (D) are also shown. The bars correspond to 20 μm.

Side-out vesicles. However, FALS revealed some differences. The inside-out vesicles exhibited a somewhat lower FALS of 2.4 AU (Fig. 4B) than did right-side-out vesicles, with FALS of 4.0 AU (Fig. 4A). The right-side-out vesicles have an asymmetric distribution, whereas the inside-out vesicles were divided into two subpopulations, characterized as FALS 2.4 AU and FALS 11.7 AU. A similar heterogeneity with a least two subpopulations with different Chl content has been reported upon sonication of inside-out vesicles (2). However, further experiments are needed to establish this.

Use of the Bit-Maps

By the use of bit-maps positioned on a two-dimensional histogram of a routinely prepared chloroplast preparation, with a pseudo-third dimension representing particle frequency (Fig. 1A), areas of interest can be encircled and analyzed separately. A major population (76%) was encircled and denoted MAP1 ("truly intact" chloroplasts) and a minor population (20%) was denoted MAP2 ("disrupted" chloroplasts, i.e. thylakoid membranes). Outside the two delimited areas, MAP1 and MAP2, few particles were found. Particles of low FALS and low IRFL (debris) represented less than 1% in "intact" chloroplast preparations and less than 1% in purified thylakoids.

These results allowed the use of the forward light scatter as a selecting parameter (Fig. 3) for further investigation of surface properties of either intact chloroplasts or purified thylakoid membranes. In this way, bit-maps allow the analysis of a third factor (like fluorescent lectin binding) on a discretely defined subpopulation.

For the purified thylakoids, the bit-maps were not really necessary, except as a control or to discard some occasional debris of low FALS and low IRFL. The "disrupted" chloroplasts and the purified thylakoids matched well.

Lectin Binding

Lectins are glycoproteins of nonimmune origin that agglutinate cells and/or precipitate complex carbohydrates. The agglutination activity of these highly specific carbohydrate-binding molecules is usually inhibited by a simple polysaccharide. To address the question of availability of glycosyl moities on the membrane surfaces, an intact chloroplast preparation was incubated with FITC-labeled RCA_{120}, a β-D-galactose-specific lectin. The FITC fluorescence was then analyzed in the two gating windows MAP1 and MAP2, i.e. "truly intact" or "disrupted" (thylakoid) chloroplasts. However, the binding was most pronounced in MAP1. Purified thylakoids were analyzed in the same way, and the binding was found to be less than in intact chloroplasts (not shown).
In both types of preparations, the binding was specifically inhibited by β-D-galactose, providing further evidence for specific binding.

The lectin binding was intense (median channel 96) and highly specific and the fixation of the RCA₁₂₀ was strongly inhibited by β-D-galactose (median channel 45) (Fig. 5A). To estimate the amount of FITC fluorescence that was due to any possible nonspecific binding, BSA-FITC was tested under the same conditions. The binding was found to be slightly lower (median channel 34) than that of RCA₁₂₀ in the presence of β-D-galactose and also slightly higher than the control, which indicates some minor nonspecific absorption of BSA-FITC on the chloroplast envelope. The statistics program ("Immunoanalysis," Coulter) gave 68.2% of the chloroplasts as positive, i.e. specifically stained by the RCA₁₂₀-FITC (Fig. 5B). A conversion of the data from Figure 5B (logarithmic to linear) is given in Table II, and one can estimate that the positively stained chloroplasts (compare with the β-D-galactose-treated chloroplasts) are 3.4 times more fluorescent for the lower decile and 8.2 times more fluorescent for the upper decile.

A titration with increasing RCA₁₂₀ concentration was performed with or without the competing β-D-galactose. Full saturation of the RCA₁₂₀ binding to the chloroplast envelope was reached at a concentration of 100 μg mL⁻¹ RCA₁₂₀ (Fig. 6A), whereas the saturation was 50 μg mL⁻¹ for the purified thylakoids (Fig. 6B). The inhibition of the RCA₁₂₀ binding by β-D-galactose was 85% complete for the chloroplast envelope and 50% or less for the thylakoid membranes.

This low binding to the thylakoid membrane was further
analyzed by the use of the right-side-out and inside-out thylakoid vesicles. The RCA$_{120}$ was found to bind to both types of vesicles, with a somewhat higher affinity for the right-side-out membranes (Table III).

To test if other sugar residues were exposed, four other FITC-labeled lectins were analyzed for binding to the chloroplast membranes. PNA (also β-D-galactose specific) also bound to the membranes, but the following did not: Solanum Tuberosum Agglutinin (specific for glucose residues), Tetra Globulus Purpurea (specific for fucose residues), and ConA (specific for mannose residues) (data not shown).

**Effect of the Lectin Binding**

While analyzing the RCA$_{120}$ binding to chloroplasts, a decrease in intactness was observed. The data obtained from the titration of RCA$_{120}$ were reanalyzed, this time with respect to intactness.

Increasing RCA$_{120}$ concentration decreased intactness, suggesting that RCA$_{120}$ binding to the envelope induced a breakage of the chloroplasts. Did RCA$_{120}$ binding induce a direct lysis of the chloroplasts or is the breakage indirect following destabilization and aggregation of the envelope membrane? To address these questions, an intact chloroplast preparation was incubated with RCA$_{120}$ at saturation level. After various intervals, the intactness was analyzed on the oxygen electrode by the ferricyanide-dependent oxygen evolution. No breakage of the chloroplasts during 60 min of incubation was detected, either with or without RCA$_{120}$ present (Fig. 7).

However, if the samples were centrifuged at 2000g for 60 s and gently resuspended in the same medium (same treatment as performed for flow cytometry), a breakage (80%) was observed for the RCA$_{120}$-treated chloroplasts, whereas the control chloroplasts remained intact after this treatment (Fig. 7). This clearly showed that the observed breakage of the chloroplasts upon RCA$_{120}$ incubation reflected aggregation level and disruption of the aggregates more than a direct lysis induced by RCA$_{120}$.

**Cytology**

To verify the results obtained by flow cytometric analysis, microscopy was performed. Nomarski interference contrast micrographs did not reveal any drastic differences between intact chloroplasts and thylakoid membranes (Fig. 8). The particles in both preparations had approximately the same size but the intact chloroplasts (Fig. 8A) had a smoother, distinct appearance than the purified thylakoids (Fig. 8B). When intact chloroplasts were incubated with RCA$_{120}$, a rapid and extensive aggregation was readily seen under the microscope (Fig. 8C). The purified thylakoids showed some aggregation, but it was not as extensive as that shown by intact chloroplasts (Fig. 8D). The aggregation was inhibited by the addition of β-D-galactose (not shown).

To localize the RCA$_{120}$ binding, fluorescence microscopy was used. For the intact chloroplasts, the FITC fluorescence was clearly found to decorate the envelope (Fig. 9, C and D). The binding to the purified thylakoid membranes was much lower and not distinctly localized, more like random spots on the particles (not shown). Also, in these experiments, it was found that the FITC fluorescence was strongly reduced by addition of β-D-galactose.

Finally, EM of the RCA$_{120}$-treated material was performed (Fig. 10). The control chloroplasts showed "normal" configuration (not shown). As expected, chloroplasts incubated with RCA$_{120}$ showed a high degree of aggregation, and occasionally the envelopes appeared to be fused (Fig. 10B). With β-D-galactose present, the RCA$_{120}$-induced aggregation was inhibited and the samples looked like the control material. The thylakoid membranes did not aggregate to the same degree as the intact chloroplasts, which was in accordance with the lower level of RCA$_{120}$ binding observed by flow cytometry.
DISCUSSION

Flow cytometry of intact chloroplast preparations revealed the presence of two populations. The characteristics of the major population came to reassemble those of the minor type upon lysis of the chloroplasts. Separately purified thylakoid membranes were found at this latter position (using the same flow settings). These results show that the major population of the intact chloroplasts were the "truly intact" chloroplasts and the minor population represented "disrupted" chloroplasts, i.e. thylakoid membranes. The somewhat lower percentage of intactness detected by flow cytometry compared with the ferricyanide test is probably due to a subsequent breakage of the fragile envelope by frictional motion through the flow injection system. These results and interpretations are in accordance with preliminary results reported by Petit et al. (29) on lettuce chloroplasts.

It is customary to consider that the FALS is proportional to particle size and most generally to the square of the radius of the particles (31). Despite the comparable size of intact chloroplasts and thylakoids (Fig. 8, A and B), the FALS signal distinguishes two populations. By contrast, RALS, thought to reflect the internal granularity of the particles, i.e. be dependent of the refractive index (31), reveals only one population. This was surprising because the intact chloroplasts contain the protein-rich stroma, which is washed away in the purified thylakoids. In contrast with the most common observations with whole cells, the FALS seems to reflect the density (internal structure) of the particles rather than their size in subcellular particles. A similar phenomenon was encountered in the case of both animal and plant mitochondria (27, 30).

Although the size of right-side-out and inside-out thylakoid membrane vesicles (<1 μm) was rather similar, the FALS analysis of the particles was improved by the use of a flow quartz cuvette. And in these conditions, the vesicles could be analyzed by flow cytometry (Fig. 4), which is of interest for either gated analysis or sorting.

Lectin (RCA120 or PNA) binding was mainly to the chloroplast envelope; however, a minor (side-unspecific) binding to the thylakoid membrane was observed. Because the purified thylakoids were washed three times, this is unlikely to be due to contaminating envelope fragments. To our knowledge, no glycoprotein has been found in chloroplasts. We also failed to detect any glycosylated chloroplast membrane proteins by lectin affinoblotting or direct sugar staining of gels or samples. Therefore, it is concluded that the binding of the RCA120 was to the galactolipids of the membranes.

Although the lipid composition of the envelope and thylakoid membranes has been reported to be essentially the same with an even lower galactolipid content in the chloroplast envelope (13), we observed a much higher binding to the chloroplast envelope than to the thylakoid membranes. Also, Billecocq et al. (8) found that antibodies against the galactosyldiglycerides exhibited a higher binding to the surface of the envelope than to the thylakoid membranes.

The amount of proteins associated with thylakoid membranes is much higher than that associated with the envelope. The protein-to-lipid ratio has been estimated to be 1/1.2 for the envelope and 1/0.5 for the thylakoids (13). The differences in RCA120 binding reported in this paper probably reflect differences in accessibility to the galactose residues due to steric hindrance. In the analysis of RCA120 interaction with the thylakoids, a higher binding was detected in the right-side-out vesicles than in the inside-out vesicles (Table III). These results are consistent with the transbilayer organization of the thylakoid galactolipids reported by Sundby and Larsson (34), namely 60% of the galactolipids in the outer bilayer and 40% in the inner. The binding of the RCA120 to intact chloroplasts does not by itself induce a breakage or induce leaky envelope membranes (Fig. 7). The increased breakage detected in flow cytometry is a result of cluster disruption, probably upon nozzle exit where pressure relaxes to atmospheric value. This gives an indication of the strength of the lectin-sugar interaction. However, some direct destabilization of the envelope due to RCA120 binding might also occur.

RCA120 (Fig. 6) and PNA exhibited the same binding and inhibition pattern. PNA confirms the presence of galactose residues. None of the other lectins tested were found to bind, which means that the chloroplast envelope and the thylakoid membranes do not expose any glucose, fucose, or mannose residues on their surfaces. The hypothesis that the differences in the accessibility to galactosyl residues between envelope and thylakoid membranes determines a fine control of certain reactions remains to be proven.

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LITERATURE CITED

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