Reconstitution of Light-harvesting Complexes and Photosystem II Cores into Galactolipid and Phospholipid Liposomes

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ABSTRACT Chlorophyll a/b light-harvesting complexes (chl a/b LHC) and photosystem II (PSII) cores were isolated from an octyl glucoside-containing sucrose gradient after solubilization of barley thylakoid membranes with Triton X-100 and octyl glucoside. No cation precipitation step was necessary to collect the chl a/b LHC. PAGE under mildly denaturing and fully denaturing conditions showed that the chl a/b LHC fraction contained chlorophyll-protein complexes CP27, CP29, and CP64. The PSII core material contained CP43 and CP47, and little contamination by other nonpigmented polypeptides. Freeze-fracture electron microscopy of the chl a/b LHC after reconstitution into digalactosyldiglyceride (DG) or phosphatidylcholine (PC) vesicles showed that the protein particles (~7.5 ± 1.6 nm) were ~99 and 90% randomly dispersed, respectively, in the liposomes. Addition of Mg ++ produced particle aggregation and membrane adhesion in chl a/b LHC-DG liposomes in a manner analogous to that described for LHC-PC liposomes. Reconstitution of PSII cores into DG vesicles also produced proteoliposomes with randomly dispersed particles (~7.5 ± 1.6 nm). In contrast, PSII-PC mixtures formed convoluted networks of tubular membranes that exhibited very few fracture faces. Most of the protein particles (~7.0 ± 1.5 nm) were seen trapped between, rather than embedded in, the membranes. The interaction between the zwitterionic head group of the phosphatidyl choline and the negatively charged PSII core may be responsible for the unusual membrane structures observed.

Since the early 1970's, it has become increasingly clear that chlorophyll is not part of the lipid bilayer in photosynthetic membranes, but rather that it is localized in chlorophyll-protein complexes embedded in the thylakoids (1). The behavior of some intact chlorophyll-protein complexes has been examined after their reconstitution into liposomes, with the intention of providing a model for some of their membrane-related functions (2–7). In this context, the chlorophyll a/b light-harvesting complex (chl a/b LHC) of photosystem II (PSII) has received the most attention because (a) large quantities are prepared easily by using the cation-precipitation method of Burke et al. (8), and (b) cation-mediated adhesion between chl a/b LHC-containing liposomes has been shown to mimic grana stacking (2–5, 9). However, the insertion of the chl a/b LHC particles into the bilayers from either side of the membrane and the tendency of the complexes to form extensive semicrystalline aggregates even before the addition of Mg ++ have proven impossible to control or eliminate. Semicrystalline aggregates of particles have been seen in all reconstituted chl a/b LHC preparations reported to date, even those in which the commonly used phosphatidylcholine vesicles (2, 3, 5) have been replaced with vesicles formed from a mixture of thylakoid lipids (4). These results suggest that aggregation could be a response of the chl a/b LHC to the isolation procedure, rather than to the nature of the lipids used for reconstitution.
To distinguish between these two possibilities, we investigated the role of sample preparation in the reconstitution of chlorophyll-containing protein complexes of chloroplast membranes by using a nonionic detergent solubilization method coupled with sucrose gradient centrifugation to isolate chl a/b LHC and PSII core complexes (10). This approach avoids any cation-precipitation step. The effect of the lipid component on the efficiency of reconstitution and on the organization of the reconstituted particles was tested by using liposomes formed from either charged phospholipids, which compose only ~13% of the thylakoid membrane in vivo, or uncharged galactolipids which contribute ~77% (11).

MATERIALS AND METHODS

Preparation of Photosystem II and LHC: A PSI- enriched pellet was prepared by a method (12) modified from that of Berthold et al. (13). This material was solubilized with octyl glucoside and fractionated on a sucrose gradient to produce PSII cores and chl a/b LHC (10).

Leaves (100 grams) from light-grown, 12-d-old barley (North American Plant Breeders, Berthoud, CO) were homogenized in (~500 ml) grinding medium (20 mM HEPES, pH 7.5, 0.4 M NaCl, 2 mM MgCl₂, 2 mg BSA/ml) in a Waring blender (20 s, high speed) and filtered through eight layers of cheesecloth. The filtrate was centrifuged for 10 min at 5,900 g at 4°C. The pellet was resuspended in washing buffer (20 mM HEPES, pH 7.5, 0.15 M NaCl, 4 mM MgCl₂) and re centrifuged at 5,900 g for 10 min. The washed pellet was resuspended in 50 mM HEPES, pH 7.6, 0.4 M sucrose, 10 mM NaCl, 10 mM MgCl₂ and accelerated to 480 g to remove large debris. The remaining supernatant was centrifuged at 5,900 g and the resultant pellet was taken up in solubilization buffer (50 mM 2-[N-morpholino]ethane sulfonic acid, pH 6.0, 15 mM NaCl, 5 mM MgCl₂, 1 mM ascorbate, 2 mg BSA/ml) to give a solution containing 5 mg chlorophyll/ml. Triton stock solution (25% [vol/vol] Triton X-100 in solubilization buffer without ascorbate or BSA) was added to give 25 mg Triton/mg chlorophyll. The solution was stirred in the dark at 4°C for 30 min and then centrifuged at 41,300 g for 30 min at 4°C.

The pellet from this centrifugation contained "PSII particles" (13), which were dried under nitrogen to a thin film. Tris-maleate buffer was added to give a detergent/chlorophyll = 10:1.

The octyl glucoside-solubilized material was layered over a discontinuous sucrose gradient (2.0 ml 25%, 2.0 ml 20%, 3.0 ml 17%, and 2.0 ml 10% (wt/wt) sucrose in Tris-maleate buffer that contained 30 mM octyl glucoside and ~1 mM EDTA. 1–2 mg of chlorophyll/ml was applied to each gradient and overlaid with Tris-maleate buffer. The gradients were centrifuged for 16 h at 30,200 rpm in a Beckman SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 4°C. The upper (chl a/b LHC) and lower (PSII core) green bands were collected, assayed for chlorophyll (14) and PSII activity (15), and evaluated for purity by PAGE under mildly denaturing (16) and fully denaturing conditions (17) in the presence of 4 M urea (10 and 12.5% acrylamide, respectively).

Absorption spectra of the various fractions were determined with a Perkin-Elmer model 330 spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, CT). Low temperature fluorescence was measured in liquid nitrogen with a Perkin-Elmer MPF-43 fluorescence spectrophotometer. Excitation was at 430 nm. Preparation of Liposomes: Crude soybean lecithin and purified spinach digalactosylglyceride (DG) (18) were taken up in chloroform and dried under nitrogen to a thin film. Triton-maleate buffer was added to give a lipid concentration of 10 mg/ml. Liposomes were formed by sonication at room temperature for 20 min in a bath sonicator.

Reconstitution of chl a/b LHC and PSII Core into Liposomes: Aliquots of the chl a/b LHC and PSII core gradient fractions were combined with phosphatidylcholine (PC) or DG liposomes (lipid/chlorophyll = 2:1) according to the method of Staehelin et al. (19). All samples were dialyzed overnight at room temperature against 3 x 2 liters of Triton-maleate buffer. The samples were observed by phase-contrast microscopy (x 400) before and after three cycles of freezing (in liquid nitrogen) and thawing. Each sample was glycinated to 35% (vol/vol) and concentrated by centrifugation (45,000 rpm, 30 Ti rotor, 30–60 min, 20°C). The pellets were frozen on copper bars by hand-dipping in liquid nitrogen-cooled Freon 12.

RESULTS

Isolation of chl a/b LHC and PSII Core Complexes

Solubilization and centrifugation in octyl glucoside of a Triton-extracted PSII pellet routinely produced an upper (chl a/b LHC) and a lower (PSII) green band, each of which was occasionally resolved into two bands. Absorption spectra of these two green bands are shown in Fig. 1. The spectrum of the lower green band resembles those reported for other PSII

Electron Microscopy: Freeze-fracture replicas were made at ~115°C in a Balzers 360M instrument equipped with electron guns (Balzers, Hudson, NH). Replicas were cleaned overnight in commercial bleach, chloroform/methanol (1:1), and distilled water. The magnification of the electron microscope was regularly checked with a calibration grid. Particles from two separate reconstitution experiments were measured from photomicrographs enlarged to x 200,000.

TABLE I

| Sample     | PSII activity* | Activity remaining |
|------------|----------------|--------------------|
| OGS applied to gradient, chl a/b = 1.76 | 62.5 ± 6.2* | (56–157)  |
| UGB 1, chl a/b = 1.52 | 10.2 | (6.1–14.9) |
| UGB 2, chl a/b = 1.63 | 11.2 | (6.1–14.9) |
| LGB, no detectable chl b | 208.4 ± 4.7 | (130.0–213.1) |
| LGB, 2 h, RT, dark | 173.3 | 81 |
| LGB + DG liposomes 2-h dialysis, RT, dark | 103.8 | 49 |
| Dialyzed, frozen, thawed | 89.4 | 42 |
| LGB + PC liposomes 2-h dialysis, RT, dark | 100.4 | 47 |
| Dialyzed, frozen, thawed | 86.5 | 41 |

* Micromoles of dichlorophenolindolphenol consumed per milligram of chlorophyll per hour.

Values given are for a single experiment. The ranges in activity observed for five preparations are given in parentheses.
preparations (20, 21) and shows only trace amounts of chlorophyll b (approximate maximum wavelength, 469 nm) contamination of this fraction. The upper green band, in contrast, is rich in chlorophyll b, indicating the presence of chl a/b LHC (20). Low temperature fluorescence emission maxima for the PSII and chl a/b LHC fractions were at 684-686 and 680-681 nm, respectively. Electron transfer rates (Table I) were typically between 130 and 215 μmol dichlorophenolindolphenol consumed/mg chlorophyll per h for the lower band(s) and between 10 and 15 μmol/mg chlorophyll per h for the upper band(s).

Electrophoresis of the gradient fractions under mildly denaturing conditions showed that the upper, less dense material was enriched in CP27 (CPII) and its oligomer CP64 (CPII*) (Fig. 2a), the chlorophyll–protein complexes associated with the chl a/b LHC, and CP29, a pigment–protein complex whose function is as yet undetermined. The lower band(s) contained CP43 and CP47 (Fig. 2a), the two chlorophyll–protein complexes presumed to be part of the PSII reaction center complex (10, 21). Analysis of the gradient fractions by SDS PAGE (Fig. 2b) showed little contamination of the PSII fraction with LHC and vice versa. Occasionally, Coomassie Blue staining revealed a slight contamination of the PSII fraction with the chl a/b LHC band CP27. Bands that migrated in the region of the α and β subunits of CF1 were sometimes present in the PSII fraction. The PSII preparation was depleted in a 34,000-mol-wt band which stained with Coomassie Blue but not with silver, and in a 32,000-mol-wt band which stained well with silver, but not with Coomassie Blue (not shown), in contrast to samples prepared by digitonin (22) or Triton plus dodecyl-β-D-maltoside (23) methods. On the basis of the staining patterns, these bands were tentatively identified as the Kuwabara and Murata protein (24) and the 32,000-mol-wt herbicide-binding protein.

Reconstitution of the chl a/b LHC

After dialysis of the freshly mixed lipid–protein samples at room temperature, no structures were visible by phase-contrast microscopy (x400), nor was any of the pigmented material sedimentable at 134,000 g for 60 min (50 Ti rotor). After freezing and thawing, liposomes (2-5 μm) were visible in both the chl a/b LHC-DG and the chl a/b LHC-PC samples (Fig. 3, a and b). The former were slightly smaller than the latter.

Freeze-fracture replicas of the reconstituted chl a/b LHC samples showed that the complexes produced particles, ~7.5 ± 1.6 nm diam, in liposomes of either DG or PC (Fig. 4).
Most of the particles visible in both the DG (Fig. 4a) and PC (Fig. 4b) liposomes of this study were randomly dispersed (~99 and 90%, respectively), in contrast to previous reports where 50% or more of the particles in chl a/b LHC-PC vesicles were aggregated. Addition of 5 mM Mg⁺⁺ induced both particle aggregation and membrane adhesion in chl a/b LHC-DG liposomes (Fig. 5) as has been reported for chl a/b LHC-PC vesicles (2). Since LHC particles are seen on both concave and convex fracture faces of all liposomes, we conclude that the complexes are randomly inserted into the membranes (Figs. 4a and 5).

Reconstitution of PSII Core Complexes

The extent of incorporation of the PSII core complexes into liposomal membranes varied with the lipid used for reconstitution. Light microscopy showed that the PSII-DG sample (Fig. 3c) consisted of large, mostly round liposomes. In contrast, the PSII-PC sample exhibited large, rod-shaped struc-

![Figure 3](image_url)

**Figure 3** Phase-contrast light micrographs of the chl a/b LHC (a and b) and PSII cores (c and d) reconstituted into DG (a and c) or PC (b and d) liposomes. The chlorophyll concentrations of the two PSII samples are comparable. The PSII-DG sample (c) contains many small vesicles that are not resolved by phase-contrast microscopy that were visible in freeze-fracture replicas (see Fig. 6a). × 740.

![Figure 4](image_url)

**Figure 4** Freeze-fracture electron micrographs of the chl a/b LHC reconstituted into DG (a) and PC (b) liposomes. Less than 1% of the particles seen in the DG samples were aggregated. In the LHC-PC sample, ~10% of the particles were aggregated as shown in b. (a) × 99,000; (b) × 97,000.

![Figure 5](image_url)

**Figure 5** Freeze-fracture electron micrograph of the chl a/b LHC reconstituted into DG liposomes after the addition of 5 mM MgCl₂. Extensive particle aggregation and membrane adhesion was observed in the presence of Mg⁺⁺. × 50,000.
FIGURE 6 Electron micrographs of freeze-fractured PSII cores reconstituted in DG (a) or PC (b and c). The PSII-DG sample (a) shows spherical vesicles with randomly dispersed particles. The PSII-PC sample (b) contained very few fracture faces with only an occasional particle. Most of the particles appeared between the membrane fracture faces (some examples marked with arrows, b). A lower magnification view of the PSII-PC sample (c) shows the lipid-protein aggregate of convoluted, tubular membranes with trapped particles (some examples indicated by arrows). (a and b) x 90,000; (c) x 41,000.

For PSII core complexes, the uncharged DG produces liposomes of normal spherical morphology and freeze-fracture faces with many particles, while the charged PC is very inefficient in incorporating complexes and cannot reform into spherical liposomes in the presence of PSII complex cores. The convoluted morphology of the PSII-PC membranes was quite unexpected. Possible mechanisms responsible for the structures seen in this sample involve (a) charge repulsions between protruding regions of adjacent negatively charged (27) PSII complexes; (b) charge interactions between the protein and the zwitterionic head groups of the PC; and (c) packing geometry of the isolated complexes (e.g., conical shape). If charge repulsion between exposed negative groups on adjacent PSII complexes or spatial crowding of conical PSII particles alone were responsible for the convoluted morphology, then a high monovalent salt concentration or a large excess of lipid should allow the vesicles to round up. Addition of 270 mM NaCl and 20 times more lipid to the PSII-PC samples did not change the morphology at the light microscope level. Finally, the roughly spherical shape of the PSII-DG vesicles indicates that protein–protein interactions alone cannot be responsible for the unusual morphology of the PSII-PC combination. Thus we believe that the charge interaction between the PC head group and the PSII cores is the underlying cause for the convoluted membrane system. In native thylakoids, PSII is primarily localized in the stacked, grana membranes (28). Not only does PC compose a very small portion (3%, reference 11) of the native thylakoid membrane lipid but from recent experimental studies (29, 30) and theoretical calculations (31), it also appears to be enriched in unstacked, stroma regions and depleted in stacked, grana regions of the thylakoids. Thus, the PSII core complexes in vivo are maintained in a very low PC environment. We find it rather astonishing that high salt did not affect the organization of the PSII-PC combination. We can only speculate at this time that any electrostatic interactions between PSII proteins and the zwitterionic PC are not readily perturbed by relatively high concentrations of monovalent ions.
Similarly, the chl a/b LHC-DG combination appears to better reproduce the in vivo assemblage of these proteins in that the reconstituted material lacks the semicrystalline particle arrays present when the chl a/b LHC is incorporated into PC liposomes. For the chl a/b LHC reconstitution, the method of complex isolation appears to be very important. Specifically, the avoidance of a cation-presentation step to collect the chl a/b LHC allows the chl a/b LHC to be reconstituted into PC liposomes with many fewer aggregated particles.

These observations emphasize the contribution of lipids to the overall structure of chloroplast membranes and suggest specific roles for lipids in the functional integration of protein complexes into lipid bilayers and their nonrandom distribution between grana and stroma membrane regions (32). Further support for the importance of the specific lipids comes from the relative effects of various thylakoid lipids on PSII functions. Addition of isolated thylakoid lipids to oxygen-evolving, Triton-treated membrane fragments showed that PC, but not PSII, supported the integrated energy distribution between the chlorophyll a/b light-harvesting complex isolated from chloroplast membranes and for reconstituting chlorophyll-protein complexes isolated from SDS polyacrylamide gels. Photosynth. Res. In press.

Our work suggests that different complexes of integral proteins in chloroplast membranes have different lipid requirements for optimal activity, or perhaps for partial regulation of endogenous activity.

Our results suggest that more attention should be paid to the lipid component not only in reconstitution experiments but also in lipid-dilution experiments as pioneered by Schneider et al. (35, 36) with mitochondrial membranes and adapted by Siegel et al. (37) and Millner et al. (38) for thylakoids. Siegel et al. found that addition of either PC or phosphatidyl glycerol to thylakoids caused severe fragmentation of the membranes and extensive aggregation of the chl a/b LHC into semicrystalline arrays. Our work suggests that at least the chl a/b LHC aggregation could be eliminated by use of the uncharged DG, provided the concentration of Mg ++ is kept low. Lipid-dilution experiments are underway in our laboratory to determine if in vivo thylakoid structure and function can in fact be maintained after addition of DG.

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