Investigations of the Role of the Main Light-harvesting Chlorophyll-Protein Complex in Thylakoid Membranes. Reconstitution of Depleted Membranes from Intermittent-light-grown Plants with the Isolated Complex

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ABSTRACT The functions of the light-harvesting complex of photosystem II (LHC-II) have been studied using thylakoids from intermittent-light-grown (IML) plants, which are deficient in this complex. These chloroplasts have no grana stacks and only limited lamellar appression in situ. In vitro the thylakoids showed limited but significant Mg^{2+}-induced membrane appression and a clear segregation of membrane particles into such regions. This observation, together with the immunological detection of small quantities of LHC-II apoproteins, suggests that the molecular mechanism of appression may be similar to the more extensive thylakoid stacking seen in normal chloroplasts and involve LHC-II polypeptides directly.

To study LHC-II function directly, a sonication-freeze-thaw procedure was developed for controlled insertion of purified LHC-II into IML membranes. Incorporation was demonstrated by density gradient centrifugation, antibody agglutination tests, and freeze-fracture electron microscopy. The reconstituted membranes, unlike the parent IML membranes, exhibited both extensive membrane appression and increased room temperature fluorescence in the presence of cations, and a decreased photosystem I activity at low light intensity. These membranes thus mimic normal chloroplasts in this regard, suggesting that the incorporated LHC-II interacts with photosystem II centers in IML membranes and exerts a direct role in the regulation of excitation energy distribution between the two photosystems.

One of the major developments in contemporary membrane biology is the correlation between ultrastructure and function. The photosynthetic membrane is especially attractive for such studies since much is already known about the light-harvesting and excitation-transfer mechanisms and the nature and sequence of the electron-transport components. The thylakoid membrane in mesophyll cells, though a continuum within a single plastid, is structurally organized into appressed (grana stack) and unappressed regions, and the two photosystems are largely segregated by this organization; photosystem II (PSII[1]) occurs most frequently in appressed, and photosystem I (PSI), in unappressed membranes (1, 2). Ultrastructural studies, based largely on freeze-fracture electron microscopy, have confirmed the differences between these regions (1, 3).

Of the pigment-protein complexes isolated from thylakoids, the light-harvesting chlorophyll *a/b* protein complex (LHC-II) so far has been the focus of most attention. Besides being the antennae array associated with PSII, LHC-II is apparently the key structural element in the cation-induced appression of thylakoids (4, 5–8) and has also been correlated with the cation-induced redistribution of excitation energy towards

[1] *Abbreviations used in this paper:* DCIP, 2,6-dichlorophenolindophenol; IML, intermittent illumination; LHC-II, light-harvesting chlorophyll *a/b* protein complex; PSI (II), photosystem I (II).

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PSII in thylakoids (9–11), although direct evidence for the latter is lacking. This multifunctional nature of LHC-II is nicely exemplified where plants are grown under an intermittent illumination (IML) regime. The chloroplasts here are deficient in LHC-II and, although photosynthetically competent, are low in chlorophyll *b* (12) and exhibit only rudimentary membrane appression (9, 13), a higher than normal light intensity requirement for whole chain electron flow (9), and a lack of the cation-induced changes in chlorophyll fluorescence found in normal thylakoids (9).

A technique of great potential usefulness for studies of structure-function relationships is the in vitro incorporation of purified components into membranes. Fusion of diacyl lipid liposomes into mitochondrial membranes was recently introduced by Hackenbrock and colleagues (14) and was later employed by Siegel et al. (15) to investigate effects of phosphatidyl choline incorporation into thylakoid membranes. The incorporation of mitochondrial cytochrome c oxidase into erythrocyte membranes (16) and reconstituted vesicles (17) has been accomplished by fusion with the cytochrome c oxidase proteoliposomes; however, there are as yet no reports of the incorporation of purified hydrophobic proteins into chloroplast membranes.

Prompted by these developments, we questioned whether a detailed investigation of the in situ function of LHC-II might be feasible, based on the controlled insertion of purified LHC-II into IML thylakoids. Such a study, including new information on the biochemical and ultrastructural properties of IML membranes, is reported here. The almost complete incorporation of LHC-II into IML membranes has been achieved such that the cation-induced changes in the regulation of excitation energy distribution and membrane appression are restored. The direct involvement of LHC-II in these processes is therefore demonstrated.

MATERIALS AND METHODS

Plant Material and Preparation of Thylakoids: Barley seeds (*Hordeum vulgare*, cv. Clipper) were germinated in darkness in vermiculite supplemented with half-strength Hoagland's solutions. After 6 d the seedlings were transferred either to continuous illumination (for control plants) or to an intermittent-light regime of 120-min dark interrupted by 2-min light cycles. Light intensity was ~170 and 70 $\mu E\ m^{-2}\ s^{-1}$ for control and IML plants, respectively, and the temperature was 20–23°C.

Thylakoids were prepared by osmotic lysis of intact plastids. Leaves (10–20 g), cut into 1 cm segments, were blended for 3 s with a Polytron PTA-35 probe (setting 7; [Kinematica Gmb H, Switzerland]) in a medium containing 0.4 M sorbitol, 10 mM sodium ascorbate, 1 mM EDTA, 0.2% BSA, and 50 mM Tricine-NaOH, pH 7.8. The homogenate was filtered through 10 layers of miracloth and centrifuged for 5 min at 1,000 *g*, then the pellet was gently resuspended in grinding medium and the centrifugation repeated (to prevent photo-effects, IML material was handled under dim green light to this point). This procedure yielded largely intact plastids (as judged by comparative phase-contrast and fluorescence microscopy), essentially free of other cell fractions. Chloroplasts were lysed by resuspending in 10 mM NaCl and 2 mM Tricine-NaOH, pH 7.5, and incubating on ice for 15 min; thylakoids were then pelleted by centrifuging at 10,000 *g* for 10 min and finally resuspended in 50 mM sorbitol, 10 mM NaCl, and 2 mM Tricine-NaOH, pH 7.5. Unless used immediately, samples were stored at −196°C.

Isolation of LHC-II and Reconstitution of LHC-II Proteoliposomes: Control plants were used. LHC-II was isolated essentially as described previously (6). LHC-II proteoliposomes were reconstituted by the freeze-thaw-sonication method using the natural mixture of purified chloroplast diacyl lipids (monogalactosyl diglyceride/digalactosyl diglyceride/sulfoquinovosyl diglyceride/phosphatidyl glycerol, 53:26:15:6) at a lipid/protein weight ratio of 5:1 (6).

Incorporation of Purified LHC-II into IML Membranes: IML thylakoids were suspended in 10 mM NaCl and 5 mM Tricine-NaOH, pH 7.5, at 0.1 mg chlorophyll/ml and sonicated for 30 s. LHC-II at 1 mg chlorophyll/ml was similarly sonicated after stirring for 2 h at 4°C with Bio Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) to remove bound Triton X-100 (6). After the solution was mixed at the appropriate chlorophyll ratios and incubated on ice for 15 min, it was frozen-thawed twice by plunging into liquid nitrogen for 1 min and then thawed in room temperature water. The solution was then sonicated for another 30 s.

Fluorescence Spectroscopy: Low-temperature (77°K) fluorescence emission and excitation spectra were recorded with Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with a DCSU-2 corrected spectra unit (Perkin-Elmer, Norwalk, CT). Thylakoids were frozen in a medium containing 50 mM sorbitol, 60% (vol/vol) glycerol, and 5 mM Tricine-NaOH, pH 7.5.

Mg^{2+}-induced changes in room-temperature chlorophyll fluorescence were measured using a locally constructed apparatus. Actinic light from a quartz-iodide lamp was passed through a calflex-C heat filter and a Corning 4-96 blue filter to give excitation between 350 and 580 nm. The beam was focused on one arm of a bifurcated optic fiber bundle (Schott Optical Glass, Inc., Duvyer, PA) which led to the reaction cuvette. The fluorescence emission was received by the photomultiplier through the second arm of the optic fiber bundle after passing through Schott RG620, RG645 (Schott Optical Glass, Inc.), and Balzer K65 red filters (Balzers, Hudson, NH), which allows a spectrum between 620 and 685 nm. The intensity of the actinic light beam at the surface of the vessel was 500 $\mu E\ m^{-2}\ s^{-1}$.

Immunochemistry: Antibodies raised against the purified LHC-II complex from spinach (18), which cross-react with the barley complexes (reference 7, see also Results) were used. For thylakoid agglutination tests, 5 μl of undiluted antiserum and 5 μl of thylakoid suspension (containing 1 mg protein/ml) were mixed at 20°C and photographed under Normarski optics after 5 min. Western blotting immunoelectrophoresis was carried out as described previously (18).

Electron Microscopy: Samples (either chloroplast pellets or small segments from secondary leaves) were fixed and sectioned as described previously (7). For freeze-fracture analysis, thylakoid samples were combined with equal aliquots of 60% (vol/vol) glycerol and pelleted by centrifugation for 90 min at 150,000 *g*. When added, 5 mM $MgCl_2$ was included with the glycerol. Samples at 4°C were frozen in propane cooled to liquid-nitrogen temperature and fractured at −110°C in a Balzers BAF 600 freeze-etch apparatus. The surface was then shadowed with platinum-carbon at an angle of 45°. Replicas were examined in a Hitachi H-500 electron microscope.

Other Methods: Lithium dodecylsulfate (LDS)-polyacrylamide gel electrophoresis was performed essentially as described (7) using an acrylamide gradient of 10–22%. Samples were dissociated by heating at 100°C for 3 min in a medium containing 30% glycerol, 1% dithiothreitol, 30 mM Tris-HCl, pH 6.8, and LDS at a detergent/protein ratio of 6:1. Runs were carried out overnight at 4°C and at a constant current of 8 mA/gel. Staining and destaining were carried out as described previously (7).

PSI electron transport was measured in a medium containing 50 mM sorbitol, 5 mM Tricine-NaOH, pH 7.8, 10 mM NaCl, 0.1 mM 2,6-dichlorophenolindophenol (DCIP), 1 mM sodium ascorbate, 0.1 mM methylviologen, 2.5 mM NH_4Cl, and 15 μM diuron. For whole chain electron transport, DCIP, diuron, and ascorbate were omitted. Oxygen consumption was measured at 25°C with a Rank O_2 electrode (Rank Brothers Cambridge, England)—chlorophyll was at 20 μg/ml and a quantum flux density of 1,000 $\mu E\ m^{-2}\ s^{-1}$. When effects of Mg^{2+} were investigated, limiting red light of varying intensity was provided by passing the incident beam through a 630-nm cut-off filter and a graded series of Balzers neutral density filters.

Protein (19) and chlorophyll *a* and *b* concentrations (20) were estimated as described. For samples with chlorophyll *a*/*b* ratios >6.0, ratios were determined from low-temperature ethanol fluorescence emission spectra (21) after careful removal of residual water in the sample with ethanol (22).

RESULTS

General Properties of IML Plastids

The IML grown barley plants used here had characteristics similar, in general, to those reported for other species (9, 12, 23). Thylakoids from plants exposed to 12 cycles (24 h) of IML had a chlorophyll *a*/*b* ratio of 28, but this decreased to the values found in thylakoids from control plants (*a*/*b* of 3–4) after 72 IML cycles, or upon 3–4 h subsequent exposure to continuous light. The majority of results presented here were obtained with thylakoids from plants exposed to 24

cycles of IML which had chlorophyll *a/b* ratios of 14–18. IML thylakoids possessed substantial photochemical activity; the rates for uncoupled whole chain (H_2O – methylviologen) and PSI (DCIP – methylviologen) electron transport were 368 and 1,283 μmol mg chlorophyll^{-1} h^{-1}, respectively, for IML thylakoids, compared with 61 and 385 μmol mg chlorophyll^{-1} h^{-1} for control thylakoids.

Light-harvesting Complex

As seen by Coomassie Blue staining of gels, barley IML thylakoids were depleted in polypeptides of the chlorophyll *a/b*-containing LHC-II complex (Fig. 1*A*), in line with previous findings (9). The prominent bands in the 23–26-kdalton region cannot be seen in thylakoids from 24 IML cycle plants, but begin to appear after 48 cycles (Fig. 1*A*, lanes *1* and *2*). They are more prominent in IML plants exposed to 4 h of continuous illumination (lane *4*), during which time substantial chlorophyll *b* is synthesized (see above). Electron micrographs of the 24-IML-cycle leaves revealed that the plastids were generally agranal (results not shown); some areas of membrane appression and overlap were apparent but these rarely involved more than two membranes together. The limited appression of IML thylakoids has been noted previously (9, 13) but was assumed to be of a different nature to the more extensive stacking in control chloroplasts because of the apparent absence of LHC-II polypeptides. This question has been reinvestigated here by studying the isolated thylakoids using both electron microscopy and immunological techniques.

During isolation of thylakoids from IML plants, membrane contact is lost and the lamellae form separate vesicles of varying size (Fig. 2*A*). Addition of cations (10mM $MgCl_2$) to these vesicles causes intermembrane contact with limited areas of close membrane appression (Fig. 2*B*), reminiscent of that in vivo. This only involved two to three membranes at a time whereas similar treatment of thylakoid membranes from control plants resulted in extensive membrane stacking (not shown). This limited appression can also be seen by freeze-fracture electron microscopy (Fig. 2, *C–E*). Untreated IML membranes were seen as small distinct vesicles with clearly distinguishable protoplasmic face (PF) and exoplasmic face (EF) fracture faces on which membrane particles appeared to be randomly distributed (see also references 24 and 25). Upon additions of cations, limited membrane contact was clearly evident in some vesicles (arrowed regions, Fig. 2, *C–E*). Where appression occurred, the contact regions usually became more flattened and had a notably higher density of EF particles, sometimes arranged in ordered arrays (see Fig. 2*D* in particular). By contrast, the EF particles in nonappressed regions, and in vesicles with no appression, remained randomly distributed. Similar changes in lateral segregation of EF particles were seen upon addition of cations to unstacked control thylakoids but here, of course, much larger areas of membrane appression occur (3). In normal thylakoids the larger EF particles are thought to be PSII core complexes associated, to varying degrees, with LHC-II units (24); in the presence of cations a marked lateral redistribution of the complexes occurs with PSII concentrating in the stacked regions and PSI being largely confined to unappressed membranes (1, 26). Clearly, the results in Figs. 2, *C* and *D* suggest that a similar reorganization of PSII particles may occur in IML thylakoids during membrane appression, but to a much more limited extent.

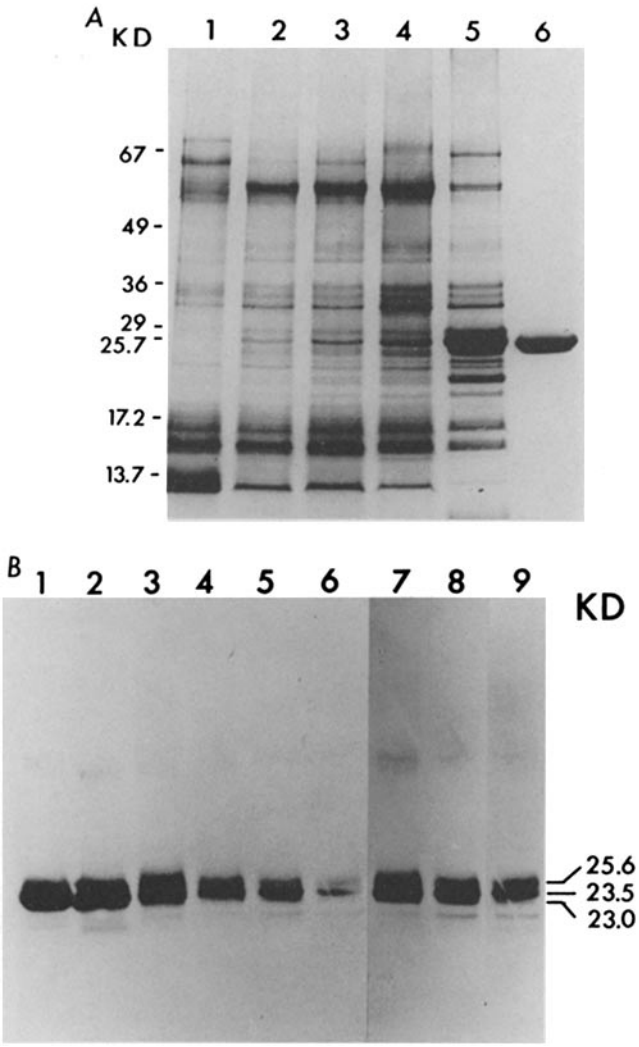
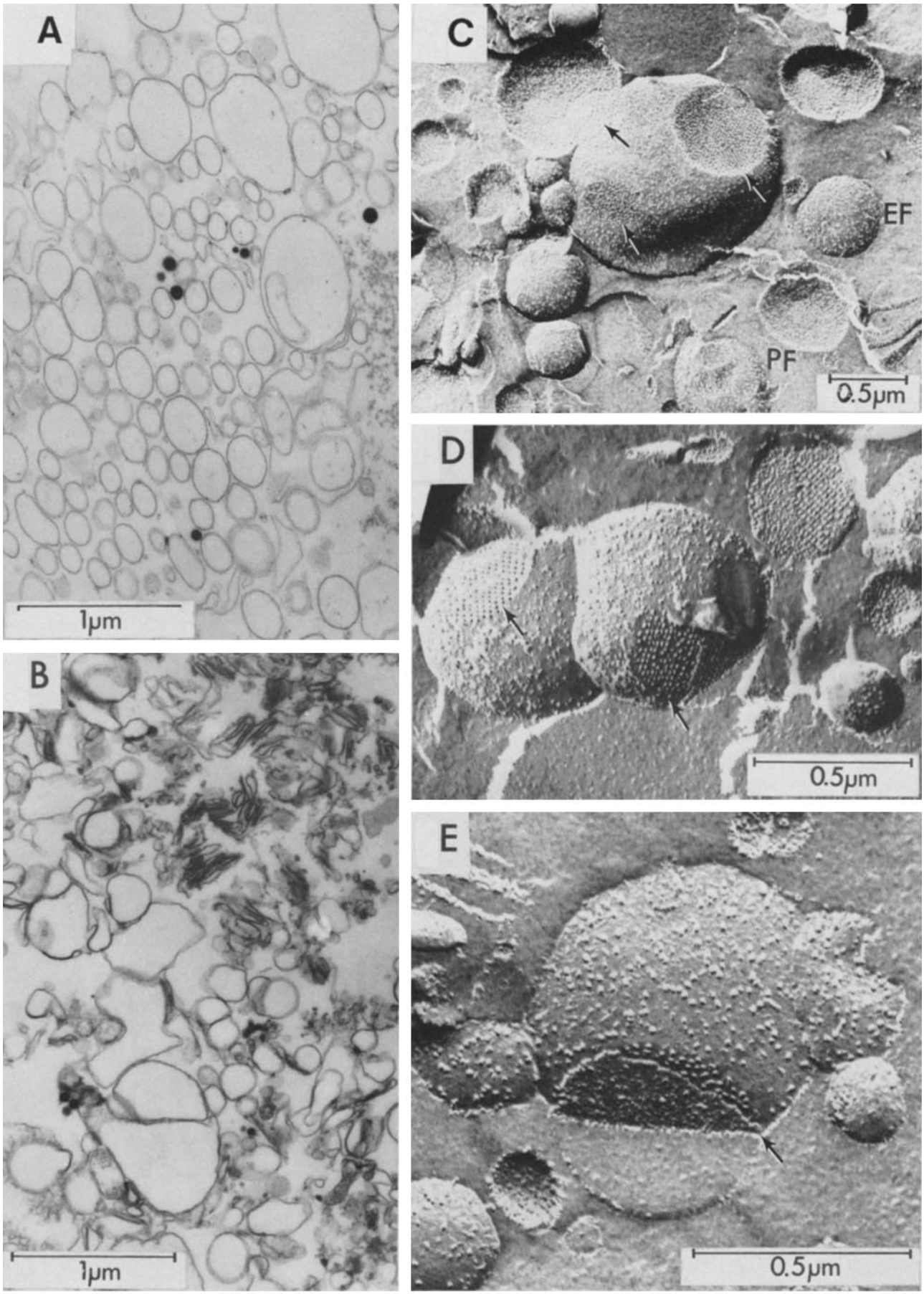


FIGURE 1 (*A*) LDS PAGE of thylakoid polypeptides from IML and continuous-light barley. Lane *1*, 24-cycle-IML thylakoids; lane *2*, 48-cycle-IML thylakoids; lane *3*, 84-cycle-IML thylakoids; lane *4*, thylakoids from 24-cycle-IML plants subsequently exposed to continuous illumination for 4 h; lane *5*, continuous light thylakoids; lane *6*, purified LHC-II. Molecular weight standards used were BSA (67 kdaltons; [*KD*]), fumarase (49 kdaltons), lactic dehydrogenase (36 kdaltons), carbonic anhydrase (29 kdaltons), chymotrypsinogen (25.7 kdaltons), myoglobin (17.2 kdaltons), ribonuclease (13.7 kdaltons). (*B*) Detection of LHC-II polypeptides in IML thylakoids by Western blot immunoelectrophoresis. LDS PAGE and Western blotting were performed as described in the text. Lane *1*, purified LHC-II; lane *2*, thylakoids from continuous light plants; lane *3*, 24-cycle-IML thylakoids subsequently exposed to 4 h continuous light; lane *4*, 48-cycle-IML thylakoids; lane *5*, 24-cycle-IML thylakoids; lane *6*, 12-cycle-IML thylakoids. Lanes *7–9* are identical to lanes *4–6* except that the x-ray film was exposed for 24 rather than 12 h.

Since thylakoid appression is usually linked with the presence of LHC-II we looked more closely at the occurrence of this complex in early-stage IML thylakoids using the sensitive immunoelectrophoretic technique of Western blotting (27, 28). A monospecific antiserum raised against spinach LHC-II, which cross-reacts with the barley complex (7), was used. Although LHC-II poypeptides could not be visualized in stained gels of even 24-cycle IML thylakoids (Fig. 1*A* and reference 9), at least three such polypeptides were detected immunologically, even in 12-cycle IML membranes (Fig. 1*B*,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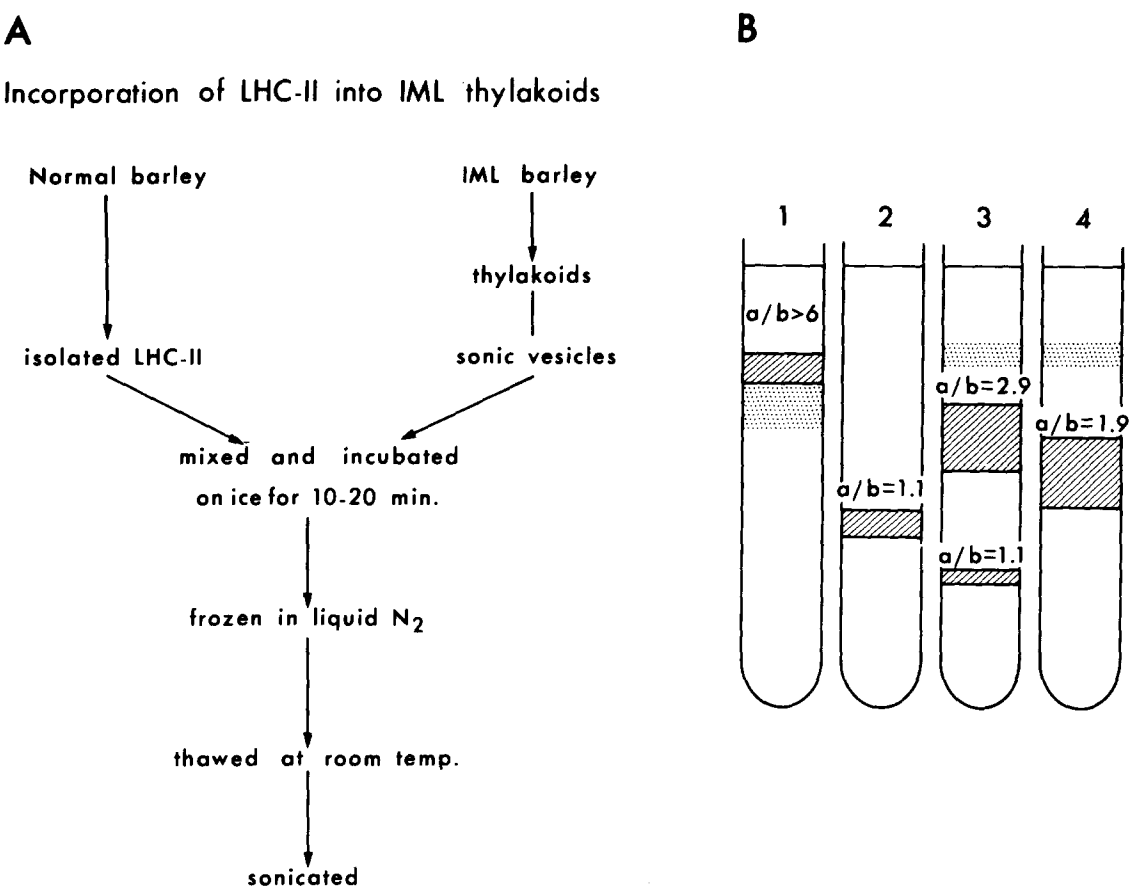

FIGURE 3 Incorporation of isolated LHC-II into IML thylakoid membranes. (*A*) Scheme of reconstitution procedure (see Materials and Methods for details); (*B*) gradient centrifugation of various IML thylakoid and LHC-II fractions. 0.1–2.0 M sucrose gradient was used; centrifugation was for 15 h at 100,000 *g*. Numbers above bands are the chlorophyll *a/b* ratios. Tube *1*, sonicated IML thylakoids; tube *2*, isolated LHC-II; tube *3*, sonicated IML thylakoids incubated with LHC-II for 15 min; tube *4*, sonicated IML thylakoids mixed with LHC-II, frozen in liquid nitrogen, thawed at room temperature, and resonicated. Sonication was carried out for 30 s at 0°C under N_2. For further details, see text.

lanes *6* and *9*). These findings, together with other reports of the presence of LHC-II mRNA and trace amounts of the polypeptides in IML plants (23, 29), show that LHC-II apoproteins are present, but in much smaller amounts than in control membranes (compare lane *1* and *6*, Fig. 1*B*). We therefore suggest that the small degree of membrane appression seen in vivo in IML plastids could be mediated by LHC-II polypeptides as in normal thylakoids and that membrane appression is limited in extent by the very small quantities of LHC-II proteins present.

Incorporation of Isolated LHC-II into IML Thylakoids

IML thylakoids do not display the cation-induced changes in excitation energy distribution found in normal chloroplasts (11, 30). As such, they offer a good opportunity for directly investigating the role of LHC-II in these changes, by artificial insertion of the isolated complex into the depleted membranes.

Incorporation of LHC-II was achieved by the freeze-thaw procedure (6, 31) as outlined in Fig. 3*A* and was monitored by density gradient centrifugation (Fig. 3*B*). Virtually complete incorporation was achieved; only one major green band appeared on gradients (tube *4*), with a density and chlorophyll *a/b* ratio (1.9) intermediate between those of IML thylakoids (tube *1*) and LHC-II (tube *2*). Simply mixing LHC-II and IML membranes yielded partial incorporation and two green bands on gradients (tube *3*); whereas the upper band represented IML membranes containing incorporated LHC-II, the lower band had a chlorophyll *a/b* ratio of only 1.1 and was probably unincorporated LHC-II, the sedimentation of which had been slightly altered presumably by aggregation. In the experiment of Fig. 3*B*, LHC-II and IML thylakoids were mixed in the ratio 4:1 (chlorophyll:chlorophyll); by changing this ratio, the density and chlorophyll *a/b* ratio of the LHC-II/IML band obtained after centrifugation could be altered in a predictable way. An important feature of the experimental protocol is that sonicated IML membranes must be used—why this is essential for LHC-II incorporation is unclear but

FIGURE 2 Thylakoids from IML barley. Thylakoids were isolated in the presence of low salt and then incubated with 5 mM $MgSO_4$ as described in Materials and Methods. (*A* and *B*) Electron micrographs of IML vesicles in absence (*A*) and presence (*B*) of Mg^{2+}; (*C–E*) freeze-fracture micrographs of IML vesicles in the presence of Mg^{2+}. × 32,000 (*A* and *B*); 30,000 (*C*); 62,000 (*D*); 82,000 (*E*).

the reason may be related to increased surface energy due to the high curvature of the small sonicated vesicles.

Low-temperature fluorescence measurements made on the green bands from gradients (Fig. 3*B*) support the conclusion that LHC-II is incorporated into IML membranes. In Fig. 4*A*, the fluorescence spectra of IML thylakoids are compared with those from continuous light (control) plants. The IML thylakoids lack both the 736-nm emission peak of control thylakoids and any appreciable peak in the 475-nm chlorophyll *b* region of the excitation spectrum (E_{436}/E_{475} = 4.1 versus 1.7 in control thylakoids). Fig. 4*B* shows the spectra of the LHC-II/IML band of tube *3* (Fig. 3*B*) and of purified LHC-II. The short wavelength emission peak of the reconstituted system at 683 nm was intermediate between that of LHC-II (681 nm) and that of control thylakoids (684 nm). The excitation spectrum for this emission also begins to resemble that of control thylakoids, with a value of the E_{436}/E_{475} ratio of 1.4.

In chloroplasts, the long wavelength emission at ~735 nm is believed to emanate from the light-harvesting assembly associated with PSI (32, 33). However, in IML thylakoids the polypeptides of this antennae, like those of PSII, are known to be deficient (34), and as a result the 736-nm emission is largely absent in IML membranes of barley (Fig. 4*A*) and wheat (35) or is considerably reduced and shifted to shorter wavelengths in pea (35) and cucumber (34). Incorporation of purified LHC-II, which has poor emission at 736 nm, would not be expected to restore this peak (Fig. 4*B*).

For independent confirmation of LHC-II incorporation into IML membranes, agglutination tests were performed using an antiserum specific for LHC-II (see Materials and Methods). Whereas untreated IML thylakoid vesicles were not significantly agglutinated, in line with the deficiency of LHC-II, the membranes reconstituted with this complex were heavily agglutinated by the antiserum, but not by the preimmune serum (results not shown). Moreover, the higher the LHC-II/IML thylakoid ratio during incorporation the greater the extent of agglutination; for example, using an arbitrary

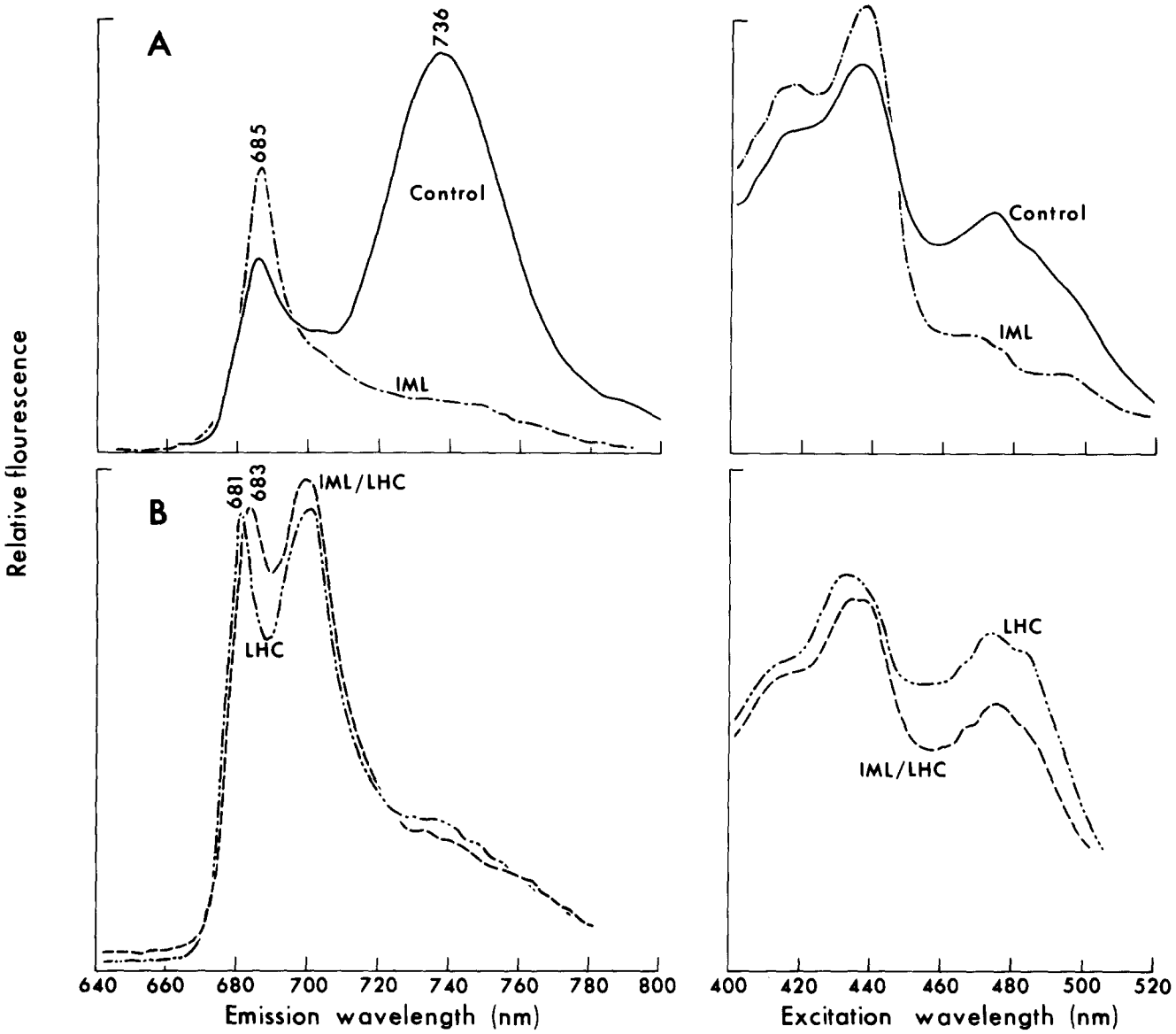

FIGURE 4 Corrected 77°K fluorescence emission and excitation spectra of barley thylakoids and isolated LHC-II. (*A*) –·–, IML thylakoids; ——, continuous light thylakoids. (*B*) – – –, reconstituted LHC-II/IML membranes (from tube *4*, Fig. 6*B*); –·–·–, isolated LHC-II.

rating system of 0 to $^{+}5$ to designate agglutination, a 3:1 chlorophyll ratio of LHC-II/IML thylakoids was rated $^{+}3$ whilst a 5:1 ratio was $^{+}5$. These experiments clearly demonstrate LHC-II incorporation but do not allow conclusions on its orientation; the complex is known to traverse thylakoid membranes and have antigenic sites on both surfaces (18).

The freeze-fracture profile of IML membranes revealed some clear ultrastructural changes after LHC-II incorporation. Unlike IML thylakoids, where the two fracture faces are readily distinguished by the much higher particle density on the PF face (Fig. 5*A* and references 24, 25), the PF and EF faces were difficult to distinguish after LHC-II incorporation and contained a proportion of particles in low relief (Fig. 5*B*, arrowed vesicles) which were of the same size (average 7.2 nm), and sometimes became segregated into the same quasi-crystalline arrays (Fig. 5*B*, *inset*) as seen with purified LHC-II in diacyl lipid membranes (Fig. 5*D*). This suggests that a proportion of the LHC-II remains self-associated after incorporation into IML membranes.

Effect of Mg^{2+} on Energy Distribution to PSI and PSII and on Ultrastructure in Reconstituted IML Membranes

In the presence of diuron, which blocks electron flow on the reducing side of PSII, any change in the room temperature fluorescence of thylakoids is thought to result from an altered input of excitation energy into PSII reaction centers (36). As noted many times by others, addition of Mg^{2+} to normal thylakoids caused a large increase in steady-state fluorescence (Fig. 6, experiment *1*), indicating that cations cause some redistribution of absorbed light energy in favor of PSII. Add-

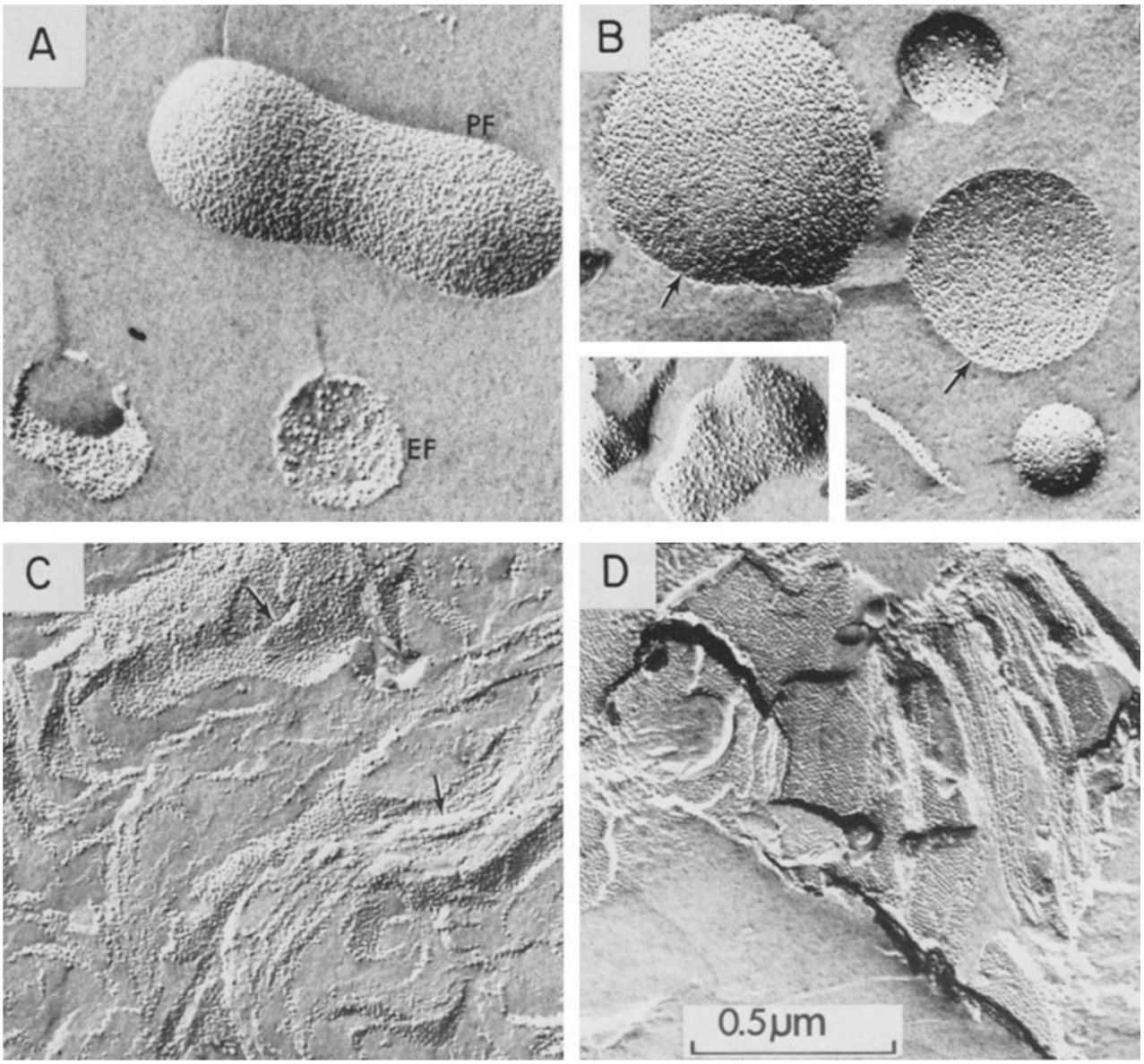

FIGURE 5 Freeze-fracture analysis of LHC-II incorporation into IML thylakoids. (*A*) 24-cycle-IML thylakoids; (*B*) sonicated IML vesicles frozen-thawed in the presence of LHC-II. The freeze-thaw procedure causes some fusion of membranes to yield bigger vesicles. To observe larger membrane surfaces, these larger vesicles were not resonicated. (*C*) LHC-II/IML membrane vesicles were resonicated after freeze-thawing, then isolated in the presence of 40 mM KCl. (*D*) LHC-II incorporated into diacyl lipid liposomes and isolated in the presence of 40 mM KCl. × 62,000.

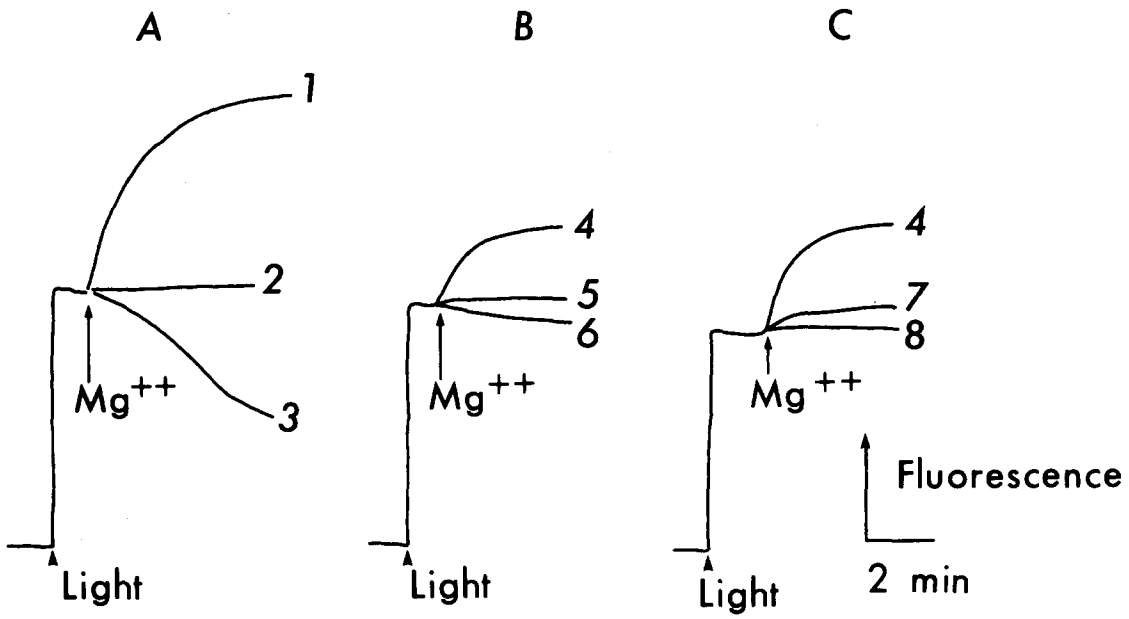

FIGURE 6 Room-temperature chlorophyll fluorescence yield from barley thylakoids. Thylakoids are suspended in a medium containing 50 mM sorbitol, 10 mM NaCl, 2 mM Tricine-NaOH, pH 7.5, and 20 μM diuron. Where indicated, 5 mM Mg^{2+} was added. Experiment *A*: *1*, continuous-light thylakoids; *2*, untreated IML thylakoids; *3*, isolated LHC-II. Experiment *B*: *4*, sonicated IML thylakoids mixed with LHC-II (1:4, chlorophyll/chlorophyll); *5*, untreated IML thylakoids mixed with LHC-II; *6*, LHC-II incorporated into diacyl lipid liposomes. Experiment *C*: *4*, sonicated IML mixed with LHC-II (1:4, chlorophyll/chlorophyll); *7*, sonicated IML thylakoids mixed with trypsin-treated LHC-II; *8*, as for *4*, but treated with tryspin prior to measurements. Trypsin treatment consisted of incubating LHC-II or thylakoids (1 mg protein/ml) at 25°C for 10 min in the presence of 25 μg/ml diphenylcarbamoylchloride-treated trypsin, then isolating the sample by centrifugation. Other details are provided in Materials and Methods.

ing Mg^{2+} to IML thylakoids had no such effect (Fig. 6, experiment *2*; see also reference 37). However, when IML membranes were reconstituted with LHC-II, Mg^{2+} caused the fluorescence yield to rise (Fig. 6, experiment *4*), although not to the extent observed with control thylakoids. Simply mixing LHC-II with unsonicated IML thylakoids (which gives no LHC-II incorporation) did not produce the Mg^{2+} effect (experiment *6*), and adding Mg^{2+} to a suspension of isolated LHC-II alone actually caused a large quenching of fluorescence (experiment *3*), perhaps due to the cation-induced aggregation of the complex (38). When Mg^{2+} was added to LHC-II incorporated into diacyl lipid liposomes (see Materials and Methods), no increase in fluorescence yield was seen (experiment *5*), indicating that mere insertion of LHC-II into the bilayer membrane did not contribute to the rise seen with reconstituted IML thylakoids. Variation of the LHC-II/IML ratio during reconstitution (Fig. 7) revealed that 1–2 LHC-II chlorophylls per IML thylakoid chlorophyll provided the maximal stimulation of the Mg^{2+}-induced fluorescence rise. Although we attach no special importance to this stoichiometry, it nonetheless compares reasonably with the approximate proportion (~50%) of chlorophyll associated with LHC-II in normal thylakoids (39).

Mild trypsin treatment of thylakoids, or isolated LHC-II, is known to remove specifically a 2-kdalton surface fragment from LHC-II; in thylakoids, this results in loss of the cation regulation of energy distribution between the two photosystems (8). When LHC-II was subjected to mild trypsin digestion prior to incorporation into IML membranes, or when the reconstituted membranes themselves were trypsin treated, the Mg^{2+}-induced fluorescence rise was either sharply reduced or abolished completely (Fig. 6, experiment *7* and *8*).

In normal thylakoids, the cation-induced redistribution of

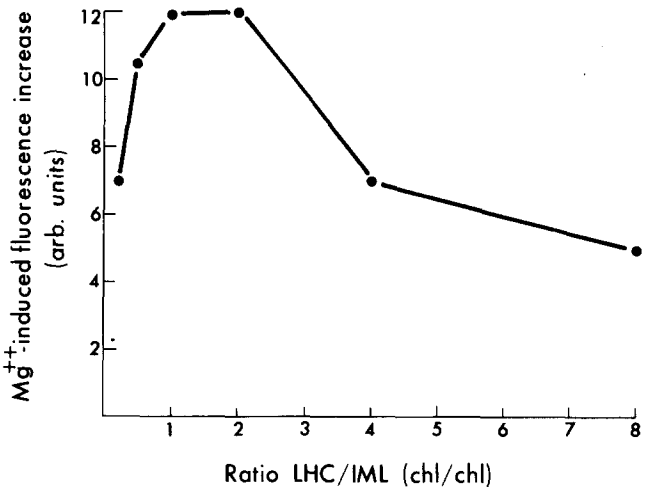

FIGURE 7 Effect of ratio of LHC-II/IML thylakoids on Mg^{2+}-induced rise in room temperature chlorophyll fluorescence. Experimental details are given in Fig. 6 and the text.

energy towards PSII is matched by a corresponding decrease in PSI electron transport at limiting light intensity (10, 40). We also observed this, with thylakoids from control barley plants (data not shown). In IML thylakoids, however, Mg^{2+} had no such effect on PSI activity; in fact, Mg^{2+} consistently stimulated it at all light intensities (Table I). The mechanism underlying this stimulation is unclear but may indicate a direct effect of Mg^{2+} on PSI in the absence of energy redistribution (40). Whatever the reason, the opposite effect was observed where LHC-II was incorporated into the membranes; 5 mM Mg^{2+} now decreased PSI activity but only at a low light intensity. The reconstituted system therefore mimics

TABLE I

Effect of Mg^{2+} on PSI Activity of IML Thylakoids

Light intensity	IML	IML + Mg^{2+}	IML/LHC	IML/LHC + Mg^{2+}	Inhibition of IML/LHC by Mg^{2+}
$\mu E\ m^{-2}\ s^{-1}$		$\mu mol\ O_2 \cdot h^{-1} \cdot mg\ chl^{-1}$ *			%
32	—	—	96 (± 12)	58 (± 10	39
65	142 (± 15)	199 (± 16)	144 (± 15)	101 (± 12)	28
800	544 (± 31)	778 (± 31)	480 (± 12)	507 (± 11)	—

* Rates shown are means ± SD, $n = 4$. When present, $MgSO_4$ was added to 5 mM.

normal thylakoids in this regard and thus provides cogent evidence that LHC-II mediates the cation-regulated distribution of excitation energy between the two photosystems.

Freeze-fracture analysis of the reconstituted LHC-II/IML thylakoids revealed marked changes in membrane interactions in the presence of cations. What were dispersed, unilamellar vesicles at low salt (Fig. 5 *B*) were aggregated and partly fused by 40 mM K^+ (Fig. 5 *C*) or 5 mM Mg^{2+}; appressed membranes were evident (arrowed regions), sometimes in multilamellar stacks, and many particles of 7–8 nm were seen in these regions. No obvious segregation of particles into appressed areas was apparent, however, in contrast to control IML membranes (compare Fig. 5 *B* with Fig. 2, *C–E*). The fusion and multilamellar appression observed here is reminiscent of that seen upon cation addition to reconstituted LHC-II-diacyl lipid proteoliposomes (Fig. 5 *D*) and is further cogent support for an already considerable body of evidence (5–8, 41) suggesting a direct structural role of LHC-II in cation-induced thylakoid appression.

DISCUSSION

We have demonstrated that the purified LHC-II complex can be incorporated into deficient thylakoids from IML plants such that the cation-induced changes in membrane appression and energy distribution seen in normal thylakoids are restored. In similar studies Hunter et al. (42) have demonstrated the reconstitution of photosynthetic electron transport and excitation transfer following cholate-dialysis reconstitution of the antennae and reaction center complexes with membranes from a bacteriochlorophyll-less mutant of *Rhodopseudomonas sphaeroides*. Here, we have developed a sonication-freeze-thaw procedure that avoids the perturbing effects of detergents, and have unambiguously demonstrated LHC-II incorporation by density gradient centrifugation, antibody agglutination tests and freeze-fracture analysis.

One of the significant difficulties in the in vitro incorporation of purified proteins into membranes is the possibility of some orientational "scrambling" of the inserted complex (43). That this may also occur here is suggested by the increasing similarity of the two fracture faces following LHC-II incorporation (Fig. 5 *B*). However, this evidence alone is not definitive since even in natural membranes (where the transmembrane asymmetry of LHC-II is preserved) the presence of LHC-II units that are not associated with the core complex apparently results in their more even partitioning between the two fracture faces, thus making them appear more similar (44). Although some scrambling is probable under our reconstitution conditions, the potent effect of trypsin in inhibiting the Mg^{2+}-induced increase in room temperature fluorescence (Fig. 6) suggests that the proportion of the complex that is functional after incorporation is present with the right-side-out orientation.

The absence of the large (>16 nm) EF particles in appression regions is an important morphological difference between the reconstituted system and normal thylakoids. It should be remembered, however, that these large particles are thought to represent the association of four LHC-II units with a core complex of ~8 nm and that smaller particles corresponding to the attachments of only one or two LHC-II units also occur (25). The fact that the 16-nm particles are not observed in the reconstituted membranes in the presence of Mg^{2+} should not be considered evidence against interactions occurring between the incorporated LHC-II and the core assembly. Rather, such interactions presumably occur but may not be sufficiently stable to maintain a tetrameric association. Indeed, the freeze-fracture profile strongly suggests that, at any instant in time, a proportion of the LHC-II either remains free or is self-associated after incorporation, a circumstance that has also been observed in normal thylakoids where exogenous lipids are incorporated (15). Also, we should recognize that the core PSII complex of IML membranes may be structurally incomplete and may be deficient in the polypeptide(s) required for stable interactions with LHC-II. Although we have no evidence for this at present, our studies of PSI biogenesis in IML thylakoids have clearly shown that whilst PSI is quite active in early stage IML plastids, several of the polypeptide subunits are missing and their subsequent incorporation into the complex is asynchronous (46). Whatever the precise reasons for the absence of the large EF particles in our reconstituted system, that the Mg^{2+}-induced increase in room-temperature fluorescence, and the low light inhibition of PSI activity, are partially restored shows that the incorporated LHC-II is directly involved in the regulation of excitation energy distribution in these membranes.

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REFERENCES

1. Arntzen, C. J. 1978. Dynamic structural features of chloroplast lamellae. *Curr. Top. Bioenerg.* 8:111–160.
2. Anderson, J. M., and B. Andersson. 1982. The architecture of photosynthetic membranes: lateral and transverse organization. *Trends Biochem. Sci.* 7:288–292.
3. Staehelin, L. A., P. A. Armond, and K. R. Miller. 1977. Chloroplast membrane organization at the supramolecular level and its functional implications. *Brookhaven Sym. Biol.* 28:278–315.
4. Miller, K. R., and R. A. Cushman. 1979. A chloroplast membrane lacking photosystem II. Thylakoid stacking in the absence of the photosystem II. *Biochim. Biophys. Acta.* 546:481–497.

5. Mullet, J. E., and C. J. Arntzen. 1980. Simulation of grana stacking in a model membrane system. Mediation by a purified light-harvesting pigment-protein complex from chloroplasts. *Biochim. Biophys. Acta.* 589:100–117.
6. Ryrie, I. J., J. M. Anderson, and D. J. Goodchild. 1980. The role of the light-harvesting chlorophyll a/b-protein complex in chloroplast membrane stacking. *Eur. J. Biochem.* 107:345–354.
7. Ryrie, I. J. 1983. Immunological evidence for apoproteins of the light-harvesting chlorophyll-protein complex in chlorophyll b-less mutant of barley. *Eur. J. Biochem.* 131:149–155.
8. Steinback, K. E., J. J. Burke, and C. J. Arntzen. 1979. Evidence for the role of surface-exposed segments of the light-harvesting complex in cation-mediated control of chloroplast structure and function. *Arch. Biochim. Biophys.* 195:546–557.
9. Armond, P. A., C. J. Arntzen, J. M. Briantais, and C. Vernotte. 1976. Differentiation of chloroplast lamellae. Light harvesting efficiency and grana development. *Arch. Biochem. Biophys.* 175:54–63.
10. Barber, J. 1980. An explanation for the relationship between salt-induced thylakoid stacking and the chlorophyll fluorescence changes associated with changes in spillover of energy from photosystem II to photosystem I. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 118:1–10.
11. Leto, K., and C. J. Arntzen. 1981. Cation-mediated regulation of excitation energy distribution in chloroplasts lacking organized photosystem II complexes. *Biochim. Biophys. Acta.* 647:107–117.
12. Argyroudi-Akoyunoglou, J. H., and G. A. Akoyunoglou. 1970. Photo-induced changes in the chlorophyll a to chlorophyll b ratio in young bean plants. *Plant Physiol.* 46:247–249.
13. Argyroudi-Akoyunoglou, J. H., Z. Feleki, and G. Akoyunoglou. 1971. Formation of two chlorophyll-protein complexes during greening of etiolated bean leaves. *Biochem. Biophys. Res. Commun.* 45:606–614.
14. Hackenbrock, C. R. 1981. Lateral diffusion and electron transfer in the mitochondrial inner membrane. *Trends Biochem. Sci.* 7:151–154.
15. Siegel, C. O., A. E. Jordan, and K. R. Miller. 1981. Addition of lipid to the photosynthetic membrane: effects of membrane structure and energy transfer. *J. Cell Biol.* 91:113–125.
16. Gad, A. E., R. Broza, and G. D. Eytan. 1979. Fusion of cells and proteoliposomes: incorporation of beef heart cytochrome oxidase into rabbit erythrocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 102:230–234.
17. Miller, C., P. Arvan, J. N. Telford, and E. Racker. 1976. Ca^{++}-induced fusion of proteoliposomes: dependence on transmembrane osmotic gradient. *J. Membr. Biol.* 30:271–282.
18. Andersson, B., J. M. Anderson, and I. J. Ryrie. 1982. Transbilayer organization of the chlorophyll-proteins of spinach thylakoids. *Eur. J. Biochem.* 123:465–472.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
20. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiol.* 24:1–15.
21. Boardman, N. K., and S.W. Thorne. 1971. Sensitive fluorescence method for the determination of chlorophyll a/b ratios. *Biochim. Biophys. Acta.* 253:222–231.
22. Fuad, N., D. A. Day, I. J. Ryrie, and S. W. Thorne. 1982. A photosystem II light-harvesting chlorophyll-protein complex with a high fluorescence emission at 736 nm. *Photobiochem. Photobiophys.* 5:255–262.
23. Viro, M., and K. Kloppsteck. 1982. Expression of genes for plastid membrane proteins in barley under intermittent light conditions. *Planta (Berl.).* 154:18–23.
24. Arntzen, C. J., P. A. Armond, J. M. Briantais, J. J. Burke, and W. P. Novitzky. 1977. Dynamic interactions among structural components of the chloroplast membrane. *Brookhaven Symp. Biol.* 28:316–337.
25. Armond, P. A., L. A. Staehelin, and C. J. Arntzen. 1977. Spatial relationships of photosystem I, photosystem II, and the light harvesting complex in chloroplast membranes. *J. Cell. Biol.* 73:400–418.
26. Andersson, B., and J. M. Anderson. 1980. Lateral heterogeneity in the distribution of chlorophyll-protein complexes of the thylakoid membranes of spinach chloroplasts. *Biochim. Biophys. Acta.* 593:427–440.
27. Bittner, M., P. Kupferer, and C. F. Morris. 1980. Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. *Anal. Biochem.* 102:459–471.
28. Towbin H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
29. Cuming, A. C., and J. Bennett. 1981. Biosynthesis of the light harvesting chlorophyll a/b protein. Control of messenger RNA activity by light. *Eur. J. Biochem.* 118:71–80.
30. Davis, D. J., P. A. Armond, E. L. Gross, and C. J. Arntzen. 1976. Differentiation of chloroplast lamellae. Onset of cation regulation of excitation energy distribution. *Arch. Biochem. Biophys.* 175:64–70.
31. Kasahara, M., and P. Hinkle. 1977. Reconstitution and purification of the D-glucose transporter from human erythrocytes. *J. Biol. Chem.* 252:7384–7390.
32. Mullet, J. E., J. J. Burke, and C. J. Arntzen. 1980. Chlorophyll proteins of photosystem I. *Plant Physiol.* 65:814–822.
33. Haworth, P., Watson, J. L., and C. J. Arntzen. 1983. The detection, isolation and characterization of a light-harvesting complex which is specifically associated with photosystem I. *Biochim. Biophys. Acta.* 724:151–158.
34. Mullet, J. E., J. J. Burke, and C. J. Arntzen. 1980. A developmental study of photosystem I peripheral chlorophyll proteins. *Plant Physiol.* 65:823–827.
35. Brown, J. S. 1979. Fluorescence spectroscopy of intermittent light and daylight grown pea and wheat chloroplasts. Carnegie Institute Year Book. 79:176–178.
36. Papageorgiou, G. 1975. Chlorophyll fluorescence: an intrinsic probe of photosynthesis. *In* Bioenergetics of Photosynthesis. Govindjee, editor. Academic Press, Inc., New York. 319–371.
37. Lieberman, J. R., S. Bose, and C. J. Arntzen. 1978. Requirement of the light-harvesting pigment-protein complex for magnesium ion regulation of excitation energy distribution in chloroplasts. *Biochim. Biophys. Acta.* 502:417–429.
38. Burke, J. J., C. J. Ditto, and C. J. Arntzen. 1978. Involvement of the light harvesting complex in cation regulation of excitation energy distribution in chloroplasts. *Arch. Biochem. Biophys.* 187:252–263.
39. Anderson, J. M. 1980. P-700 content and polypeptide profile of chlorophyll-protein complexes of spinach and barley thylakoids. *Biochim. Biophys. Acta.* 591:113–126.
40. Bose, S., J. E. Mullet, G. E. Hoch, and C. J. Arntzen. 1981. Effects of cations on photosystem I electron transport. *Photobiochem. Photobiophys.* 2:45–52.
41. McDonnel, A., and L. A. Staehelin. 1980. Adhesion between liposomes mediated by the chlorophyll a/b light-harvesting complex isolated from chloroplast membranes. *J. Cell Biol.* 84:40–56.
42. Hunter, C. N., R. van Grondelle, N. G. Holmes, and O. T. G. Jones. 1979. The reconstitution of energy transfer in membranes from a bacteriochlorophyll-less mutant of Rhodopseudomonas sphaeroides by addition of light-harvesting and reaction center pigment-protein complexes. *Biochim. Biophys. Acta.* 548:458–470.
43. Eytan, G. D. 1982. Use of liposomes for reconstitution of biological functions. *Biochim. Biophys. Acta.* 694:185–202.
44. Olive, J., F.-A. Wollman, P. Bennoun, and M. Recouvreur. 1981. Ultrastructure of thylakoid membranes in C. reinhardtii: evidence for variations in the partition coefficient of the light-harvesting complex-containing particles upon membrane fracture. *Arch. Biochem. Biophys.* 208:456–467.
45. Ryrie, I. J., and S. Young. 1984. The polypeptides of photosystem 1 in developing thylakoids of intermittent light-grown plants. *Proc. Int. Congr. Photosynth. Res.* In press.