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Analysis of the Light-harvesting Pigment-Protein Complex of Wild Type and a Chlorophyll-*b*-less Mutant of Barley[1]

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ABSTRACT

We have compared chloroplast lamellae isolated from a chlorophyll-*b*-less mutant and wild type barley (*Hordeum vulgare*). The results demonstrate that: (*a*) one of the two major polypeptides comprising the light-harvesting complex (LHC) is present in the chlorophyll-*b*-less mutant; (*b*) higher cation concentrations are required to maintain grana stacks in the mutant; and (*c*) cation effects on excitation energy distribution are present in the chlorophyll-*b*-less mutant but are reduced in amount and are dependent on higher concentrations of cations.

We interpret these data to support the concept that the LHC mediates cation-induced grana stacking and cation regulation of excitation energy distribution between photosystems I and II in chloroplast lamellae. A partial LHC complement in the mutant alters the quantitative cation requirement for both phenomena, but not the over-all qualitative response.

The involvement of cations in the regulation of grana formation and in mediating excitation energy distribution between the two photosystems has been extensively examined in recent years (7, 9, 24). In greening systems, the onset of cation control of energy distribution and the formation of grana stacks appear concomitantly with the synthesis of LHC[3] (4, 6, 15). A correlation between the levels of LHC in chloroplast membranes, cation regulation of grana formation, and cation mediation of excitation energy distribution has been shown for almost all higher plant systems studied (1, 2, 8, 29). This has led to the concept that the light-harvesting complex is the primary membrane constituent which is responsible for cation-mediated changes in these chloroplast membrane processes (1, 7).

The one exception to the above mentioned rule comes from studies of a chlorina mutant of barley which lacks Chl *b*. This mutant has been shown to be missing the pigmented light-harvesting Chl *a*/*b* protein when examined by SDS-polyacrylamide gel electrophoresis (3, 16, 18, 21, 22, 28). When examined by electron microscopy this mutant showed either partial or extensive grana stacking (17, 22). In addition, cations have an effect on the fluorescence yield of isolated chloroplasts from this barley mutant (10), thus indicating cation-controlled energy distribution processes. Since these data pertaining to the barley mutant tend to invalidate the hypothesis that LHC plays a pivotal role in cation-mediated structure-function events, we have reexamined the isolated chloroplasts of the Chl-*b*-less barley mutant. We will demonstrate that the mutant is not totally deficient in the polypeptides of the LHC, and that the partial LHC complement present in the mutant can be correlated to a modified cation concentration requirement for regulation of structural and functional changes in these plastids.

MATERIALS AND METHODS

Chloroplast Lamellae and Submembrane Preparations. Chl-*b*-less and wild type barley (*Hordeum vulgare*) seeds were kindly provided by J. Anderson, CSIRO, Canberra, Australia. The seeds were planted in soil and grown under cool-white fluorescent light (16-hr photoperiod) for 3 to 5 weeks. Washed leaves were homogenized for 15 sec in a Waring Blendor in a grinding medium containing 0.4 M sorbitol, 0.1 M Na-Tricine (pH 7.8), 0.25% (w/v) BSA, and 0.05 M Na-ascorbate. The brei was filtered through four and then 12 layers of cheesecloth and a chloroplast pellet was obtained by centrifugation at 1,000*g* for 10 min. These chloroplasts were resuspended into solutions containing various cation concentrations as indicated below. For isolation of LHC from wild type membranes, the procedure of Burke *et al.* (12) was utilized.

Electron Microscopy. Isolated chloroplasts were resuspended in either: (*a*) 10 mM NaCl, 10 mM Na-Tricine (pH 7.8); (*b*) 10 mM NaCl, 10 mM Na-Tricine (pH 7.8), 3 mM $MgCl_2$; or (*c*) 10 mM NaCl, 10 mM Na-Tricine (pH 7.8), 10 mM $MgCl_2$ and placed on ice for 30 min. Ten per cent glutaraldehyde was then added to a final concentration of 1% (v/v) and membrane fixation was allowed to proceed for 30 min at 4 C. Fixed membranes were pelleted at 10,000*g* for 10 min and resuspended in 1% $KMnO_4$. Following a 2-hr incubation on ice, the membranes were pelleted at 10,000*g* for 10 min, embedded in 2% agar (w/v), dehydrated in graded acetone solution, and embedded in a mixture of low viscosity resins (26). Sections were cut with a diamond knife on an LKB-Huxley Mark 2 ultramicrotome and observed with a Jeol Jcm 100C electron microscope.

SDS-Polyacrylamide Gel Electrophoresis. Analysis of membrane polypeptides using SDS-polyacrylamide gel electrophoresis was carried out using the discontinuous buffer system of Laemmli (20). Electrophoresis was performed in a slab gel apparatus (27) using a 12% (w/v) polyacrylamide separating gel and a 5% (w/v) stacking gel. Membrane proteins were twice extracted with 80% acetone followed by solubilization in a 65 mM Tris-Cl sample buffer (pH 6.8) containing 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, and 2% (w/v) SDS. Samples were boiled for 2 min and applied to the gel sample wells. Electrophoresis was carried out at a constant current of 20 mamp. Gels were stained for protein in a solution containing 0.2% (w/v) Coomassie blue, 50% (v/v) methanol, and 7% (v/v) glacial acetic acid for 30 min, and were destained in 20% methanol, 7% acetic acid.

Polypeptides of the Chl-*b*-less barley mutant co-migrating with the purified LHC polypeptides were further characterized by

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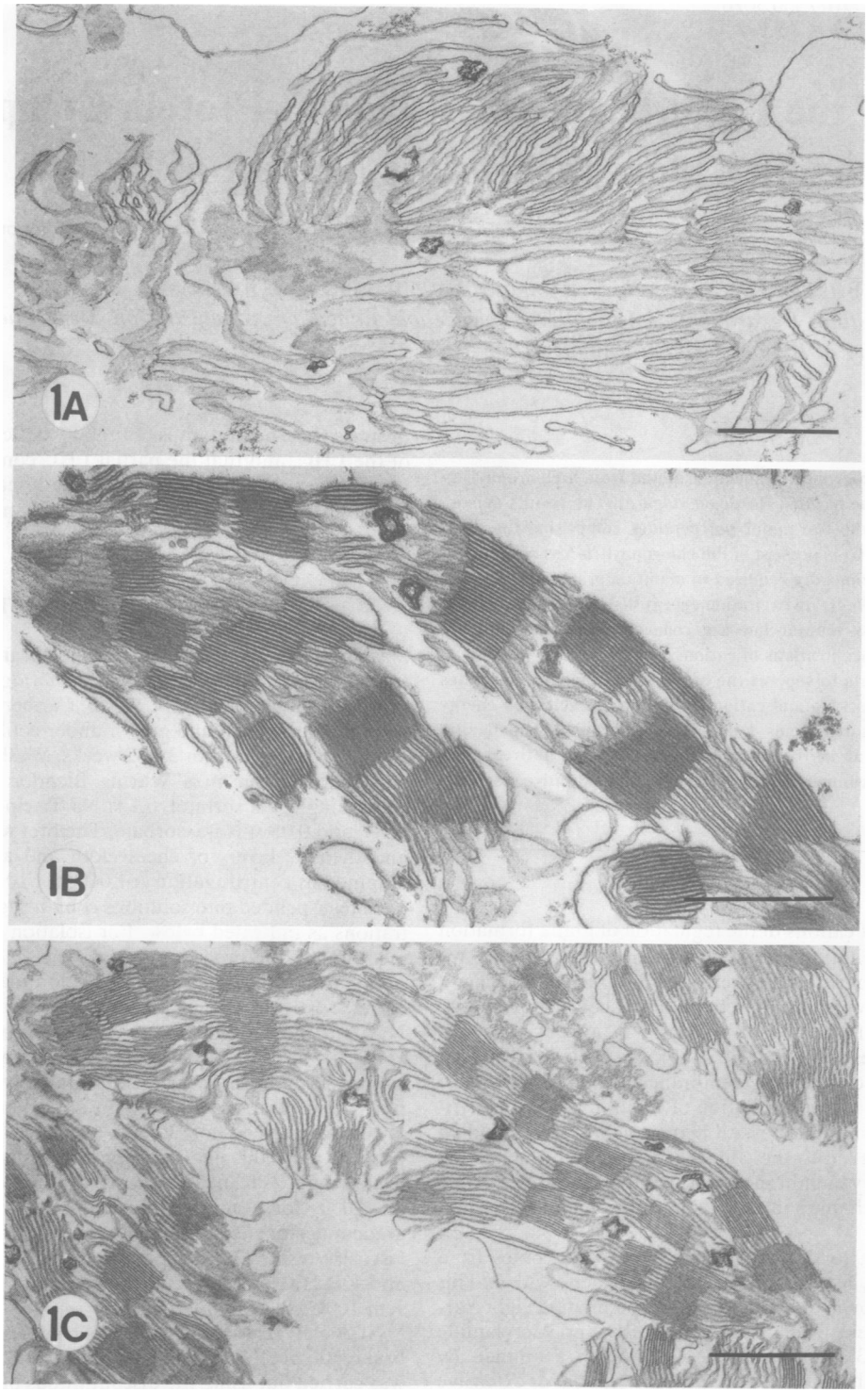
[3] Abbreviation: LCH: light-harvesting complex.



FIGS. 1 and 2. Electron micrographs of wild type and Chl-*b*-less barley chloroplast membranes incubated in varying cation concentrations.

FIG. 1. Thylakoids isolated from wild type barley and resuspended in (A) 10 mM NaCl; (B) 10 mM NaCl, 3 mM $MgCl_2$; and (C) 10 mM NaCl, 10 mM $MgCl_2$. Bars equal 1 μm.

partial proteolysis with Papain (Sigma Chemical Co.[4]) according to the procedure of Cleveland *et al.* (14) for gel slices.

Fluorescence Measurements. For measuring Chl fluorescence, chloroplasts were diluted to 10 μg Chl/ml in 1 mM Na-Tricine (pH 7.8), 10 mM NaCl containing either 0, 3, or 10 mM $MgCl_2$ as indicated in the text. The room temperature fluorescence inductions were obtained as described elsewhere (12). Room temperature and low temperature (77°K) fluorescence emission spectra were measured by the dual channel ratiometric acquisition method using a System 4000 scanning polarization spectrofluorimeter (SLM Instruments, Urbana, Ill.). Samples for low temperature spectra were frozen in 0.5-mm (i.d.) capillary tubes.

[4] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

RESULTS

Cation Concentration Requirements for Grana Stacking. Isolated chloroplasts from the Chl-*b*-less and wild type barley incubated in either 10 mM Na^+, or 10 mM Na^+ plus 3 mM Mg^{2+}, or 10 mM Na^+ plus 10 mM Mg^{2+} were examined with the electron microscope. Figure 1 is a series of micrographs of wild type chloroplast membranes incubated in the various cation concentrations. Wild-type thylakoids suspended in 10 mM Na^+ (Fig. 1A) lost all grana stacking and appeared as long, parallel membrane sheets. Distinct regions of membrane appression (grana) were maintained in the wild type membranes incubated in either 3 or 10 mM Mg^{2+} (Fig. 1, B and C, respectively).

Membranes of the Chl-*b*-less chloroplasts (Fig. 2) appear unstacked and highly vesiculated when incubated in 10 mM Na^+ (Fig. 2A). When incubated in 3 mM Mg^{2+}, the mutant membranes are largely unstacked with only a few regions of membrane appression (Fig. 2B). More distinct regions of membrane appression are evident when the mutant membranes are incubated in 10 mM Mg^{2+} (Fig. 2C). The membranes in these stacked regions

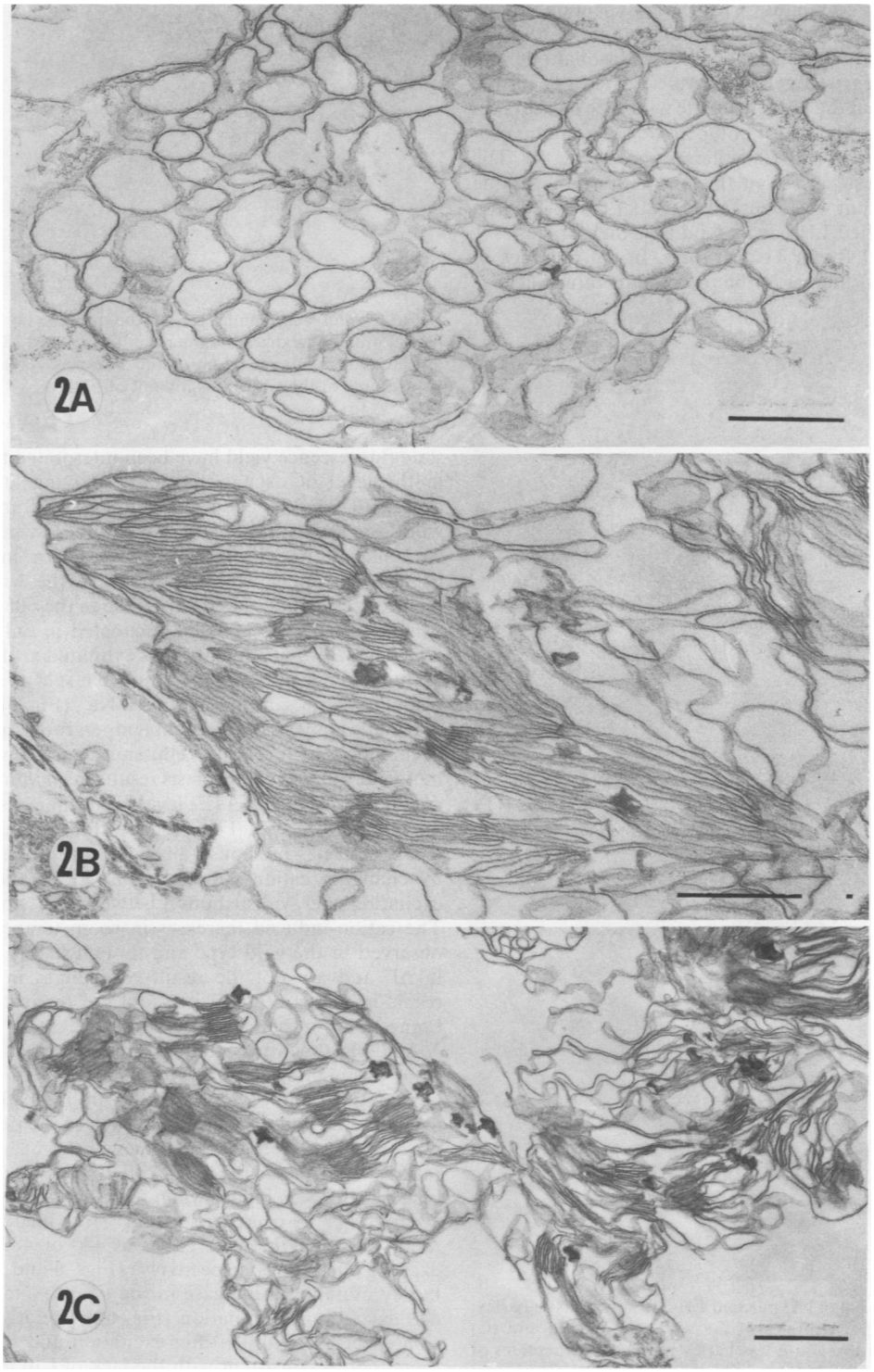


FIG. 2. Thylakoids isolated from the Chl-*b*-less barley mutant and resuspended in (A) 10 mM NaCl; (B) 10 mM NaCl; 3 mM $MgCl_2$, and (C) 10 mM NaCl, 10 mM $MgCl_2$. Bars equal 1 μm.

appear swollen when compared with the control membranes under similar conditions which were shown in Figure 1C.

Polypeptides of Light-harvesting Complex in Mutant and Wild Type Membranes. Polyacrylamide slab gel electrophoretic separation of lipid-extracted wild type chloroplast membranes, Chl-*b*-less barley chloroplast membranes, and the purified light-harvesting complex (LHC) from wild type membranes is shown in Figure 3. In agreement with earlier studies (12) the purified LHC preparation contained three polypeptides; these migrate with apparent mol wt of 23, 25, and 28 kdaltons. Analysis of the Chl-*b*-less membrane sample revealed the presence of two polypeptides (23 and 28 kdaltons) which co-migrate with the polypeptides of the purified LHC (Fig. 3C). To establish the identity of the co-migrating peptide bands, analysis by a one-dimensional peptide-mapping technique of excised gel slices were carried out on the two major polypeptides of the LHC from the wild type membranes (23 and 25 kdaltons) and the major polypeptide (23 kdaltons) co-migrating in this region of the Chl-*b*-less sample (Fig. 4). The polypeptide partial digest pattern of the 23-kdalton polypeptide from the Chl-*b*-less mutant was identical to that of the 23-kdalton polypeptide of the purified LHC from wild type membranes.

Fluorescence Yield at Room Temperature. In the presence of DCMU, cation-induced changes in the room temperature steady-state fluorescence yield have been interpreted to indicate a change in the level of excitation energy arriving at the PSII reaction centers (9, 23, 25). The room temperature fluorescence spectra of wild type and Chl-*b*-less barley chloroplast samples incubated in varying cation concentrations are shown in Figure 5. In the presence of either 3 or 10 mM Mg^{2+}, the wild type chloroplast membranes exhibit an 80% increase in the 685 nm fluorescence as compared with the lamellae incubated in only 10 mM Na^+ (Fig. 5A). The Chl-*b*-less membranes exhibited an 18 and 35% increase in fluorescence intensity for 3 and 10 mM Mg^{2+}, respectively, when compared with samples in 10 mM Na^+ (Fig. 5B).

Kinetic analysis of the room temperature Chl *a* fluorescence has been shown to reveal two emission components; onset of illumination of isolated chloroplasts results in an immediate fluorescence rise to a constant level (F_0 level), and then a slower increase to a maximal (F_M) level. The variable fluorescence ($\Delta F = F_M - F_0$) component has previously been shown to be modified selectively by changing cation concentrations in parallel with changes in excitation energy distribution between the two photosystems (11). The cation-induced increases in room temperature fluorescence observed in the wild type and mutant barley are due to changes in ΔF and are not the result of changes in F_0 (Table I). The resultant increase in ΔF is 2-fold greater in the wild type membranes than in the Chl-*b*-less lamellae.

At 77°K, the ratio of fluorescence emitted at 685 nm (originating from the pigment bed serving PSII) to that emitted at 730 nm (originating from PSI) provides an indication of the relative excitation energy distribution to each of the photosystems; cations were previously shown to influence the 685/730 peak emission ratios (9, 11, 13, 23). The F_{685}/F_{730} emission peak ratio for wild type plastids was higher when the chloroplasts were suspended in 10 mM Mg^{2+} as compared to Mg^{2+}-free medium, and when excitation wavelengths were either at 440 or 480 nm (preferentially exciting Chl *a* or *b*, respectively) (Figs. 6 and 7). In the Chl-*b*-less barley mutant, an increase in the F_{685}/F_{730} ratio was observed in 440 excitation illumination (Fig. 6). A relatively indistinct emission spectrum with 480 nm excitation and lack of cation effects thereupon is consistent with the lack of Chl *b* in the mutant (Fig. 7).

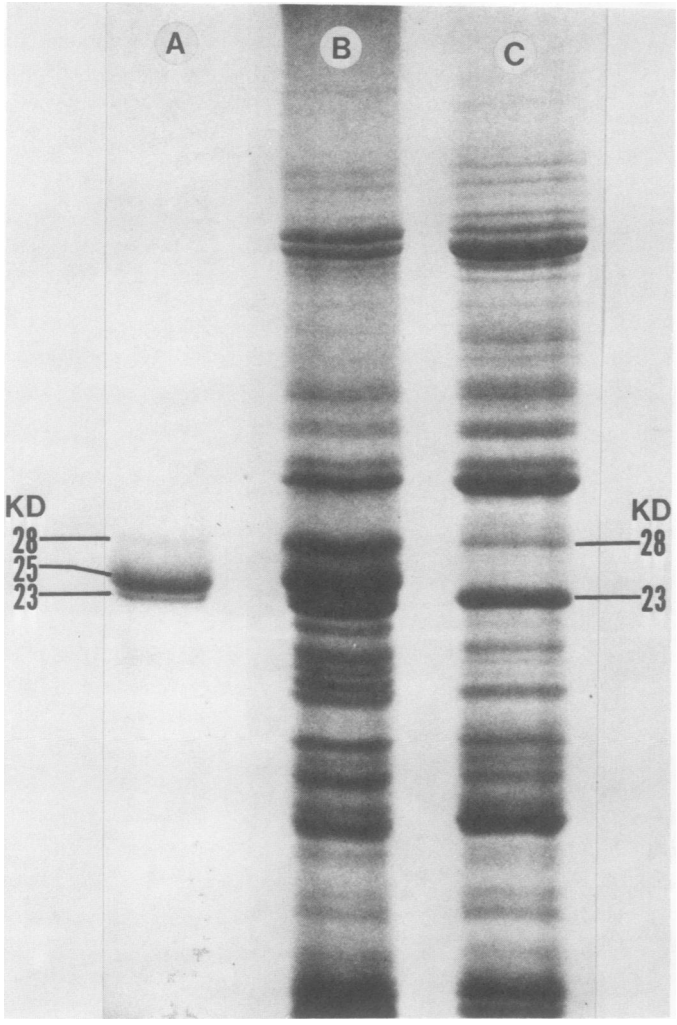


FIG. 3. Polyacrylamide gel of (A) purified LHC from wild type barley; (B) thylakoid membrane polypeptides of the wild type barley; and (C) membrane polypeptides of the Chl-*b*-less barley mutant. Polypeptides of 28, 25, and 23 kdaltons were obtained for the purified LHC. Mutant membranes exhibit two co-migrating polypeptides at 28 and 23 kdaltons.

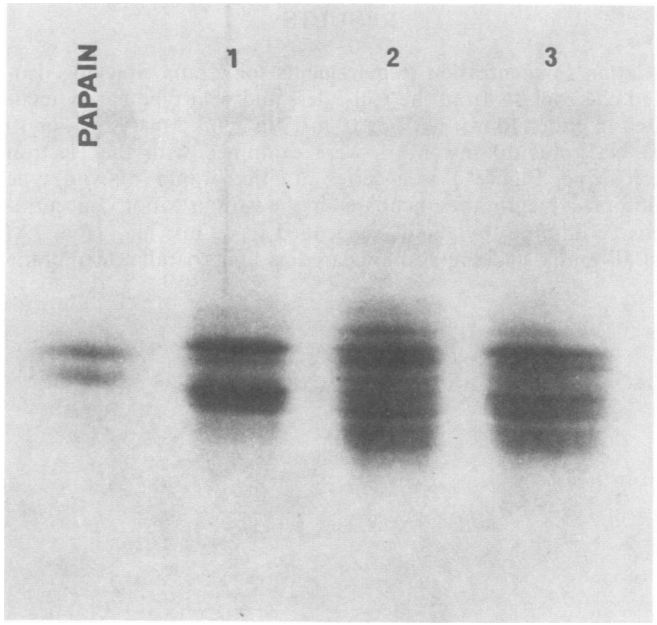


FIG. 4. Polyacrylamide gel of the proteolytic enzyme, papain, the partial proteolytic digest fragments of the 25- and 23-kdalton polypeptides of LHC from wild type barley (1 and 2, respectively) and of the 23-kdalton polypeptide of the Chl-*b*-less mutant of barley.

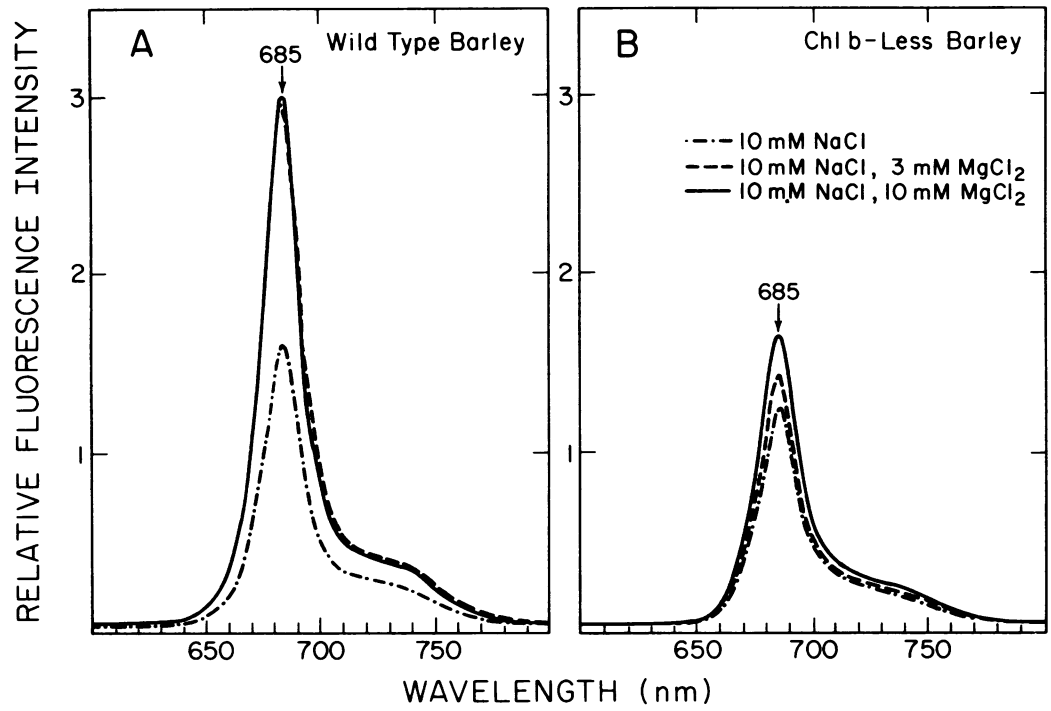


FIG. 5. Room temperature fluorescence spectra of wild type and Chl-*b*-less barley chloroplast samples incubated in varying cation concentrations. Reaction mixtures (2.0 ml) contained 10 mM NaCl, 10 mM Na-Tricine (pH 7.8), 50 mM sorbitol, and 20 μg Chl. Mg^{2+} concentrations in the reaction mixtures are indicated.

Table I. Cation effects on the room temperature chlorophyll a fluorescence of wild type and chlorophyll-b-less barley chloroplasts. Reaction mixtures (2.0 ml) contained 10 mM NaCl, 10 mM Na-Tricine (pH 7.8), 50 mM sorbitol, and 20 μg of Chl. Mg^{2+} concentrations in the reaction mixtures are indicated.

Sample	Concn Mg^{2+}	F_0	F_M	ΔF	$\frac{\Delta F\ (+Mg^{2+})}{\Delta F\ (-Mg^{2+})}$
	(mM)				
Wild type chloroplasts	0	10.0	11.7	1.7	---
	3	9.5	16.9	7.4	4.3
	10	9.3	15.3	6.0	3.5
Chl-b-less chloroplasts	0	10.0	13.1	3.1	---
	3	8.9	14.8	5.9	1.9
	10	9.1	15.9	6.8	2.2

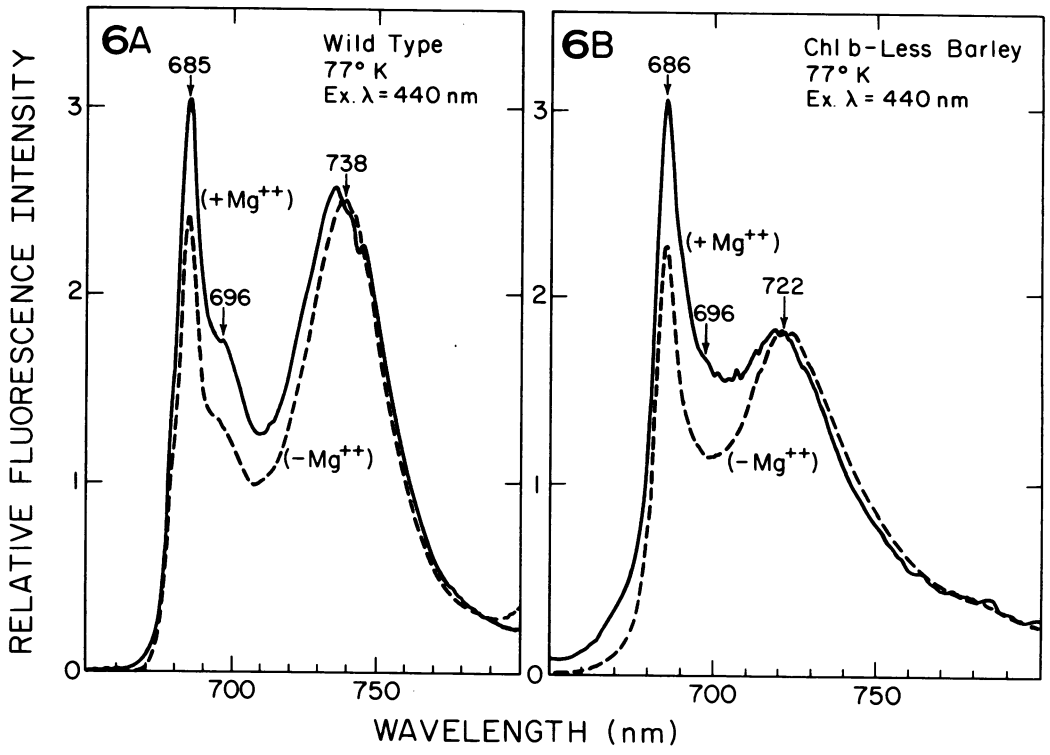
DISCUSSION

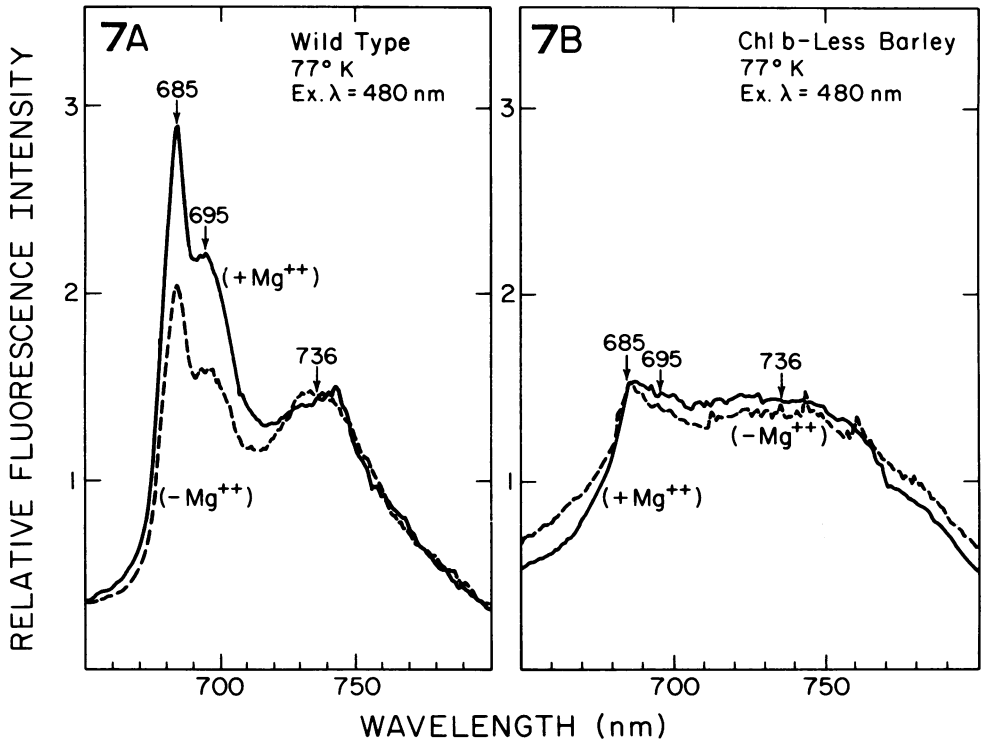
Varying cation concentrations have been shown to alter the macrostructural arrangement of the chloroplast thylakoids (5, 7, 19) and to regulate excitation energy between PSI and II (7, 9, 23). The component of the membrane which mediates these processes has been suggested to be the LHC. This hypothesis is based upon the observations that greening or mutant plastids which lack or are deficient in the LHC are correspondingly deficient in cation effects on grana stacking or upon energy distribution (4, 6, 8, 15, 29). The exception to these studies was the report of a Chl-*b*-less barley mutant which has no pigmented LHC (2, 16, 18, 21, 22, 28) but which contains grana stacks (17, 22) and which demonstrated cation effects on energy distribution (as measured by fluorescence techniques) (10). In order to evaluate this apparent discrepancy further, we have reinvestigated the Chl-*b*-less mutant with regard to cation effects on its structure and function.

As a starting point for these investigations, we have reanalyzed the polypeptide composition of wild type and mutant barley chloroplasts. Previous investigators have stated either that the LHC polypeptides are absent in the mutant (3, 16, 22, 28) or that some LHC polypeptides might be present (18, 21). Uncertainties in interpretation arose from the fact that there could be additional polypeptides in both the mutant and wild type membranes which co-migrate with the LHC polypeptides.

We have isolated the LHC from wild type membranes by techniques previously described (12). Since two of the three polypeptides of the LHC co-migrated with polypeptides of the mutant membranes, we have used partial proteolytic digestion and electrophoretic fingerprinting to characterize the homology of the putative 23-kdalton LHC polypeptide in the mutant with the 23-kdalton component of the isolated complex; the data (Fig. 4) clearly show the identity of the polypeptides. This demonstrates that the Chl-*b*-less mutant contains one of the major LHC polypeptides.

Cation Effects on Function and Structure. Several lines of evidence have indicated that the LHC is required for Mg^{2+}-induced regulation of energy distribution between PSI and II. These have included studies which showed parallel appearance of the LHC and cation-mediated effects in greening chloroplasts and in partial loss of the LHC and cation effects in mutant systems. In addition, evidence for this observation was provided by a study on the effect of antibodies prepared against the purified LHC





FIGS. 6 and 7. Cation effects on 77°K fluorescence of wild type and Chl-*b*-less barley chloroplasts. Reaction mixtures were as in Figure 5. Where indicated, Mg^{2+} concentration was 10 mM (as $MgCl_2$).

FIG. 6. Excitation wavelength was 440 nm (preferentially exciting Chl *a*).

FIG. 7. Excitation wavelength was 480 nm (preferentially exciting Chl *b*).

upon Mg^{2+}-induced changes of photosynthetic electron transport partial reactions, variable fluorescence of Chl *a*, and 77°K emission spectra of control chloroplasts prepared under "low salt" conditions (12).

An earlier study by Boardman and Thorne (10) indicated that a small cation effect on energy distribution could be detected in the barley mutant chloroplasts. Our study demonstrates a substantial cation effect in the *b*-less chloroplasts and suggests that earlier reports of little or no cation effects resulted from low salt pretreatment of the membranes prior to analysis. We have shown that the

effect is on the variable component of room temperature fluorescence and is accompanied by alterations in the 77°K fluorescence emission spectrum. These data indicate that cation regulation of excitation energy distribution is present in the barley mutant chloroplasts, although to a lesser extent than in the wild type membranes. We found that the cation concentration dependence on this regulatory process was markedly different in the mutant system; greater than 3-fold higher concentrations of cations were required to elicit the fluorescence changes in comparing the mutant to wild type membranes.

The involvement of grana stacking has frequently been related to cation regulation of membrane function (5, 7). In agreement with this, we have found that the barley mutant chloroplasts require higher concentrations of divalent cations to maintain grana stacks in isolated chloroplasts than do the wild type membranes, and that the stacks which are maintained seem less distinct than those of the wild type. These data show that the Chl-*b*-less mutant contains one of the major polypeptides which comprises part of the LHC structural subunit of wild type membranes. We conclude that the partial complement of the LHC in the mutant membranes results in a modified cation requirement for both functional and structural parameters. The data are consistent with our earlier suggestions (7, 8) that the LHC mediates cation-induced grana stacking and excitation energy distribution between PSI and II.

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