Biochimica et Biophysica Acta, 637 (1981) 107–117
Elsevier/North-Holland Biomedical Press

BBA 48096

CATION-MEDIATED REGULATION OF EXCITATION ENERGY DISTRIBUTION IN CHLOROPLASTS LACKING ORGANIZED PHOTOSYSTEM II COMPLEXES

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(Received March 24th, 1981)

Key words: Chlorophyll-protein complex; Photosystem II mutant; Excitation energy distribution; Cation effect; (Chloroplast)

Despite the total loss of Photosystem II activity, thylakoids isolated from the green nuclear maize mutant *hcf-3 contain normal amounts of the light-harvesting chlorophyll *a/b* pigment-protein complex (LHC). We interpret the spectroscopic and ultrastructural characteristics of these thylakoids to indicate that the LHC present in these membranes is not associated with Photosystem II reaction centers and thus exists in a 'free' state within the thylakoid membrane. In contrast, the LHC found in wild-type maize thylakoids shows the usual functional association with Photosystem II reaction centers. Several lines of evidence suggest that the free LHC found in thylakoids isolated from *hcf**-3 is able to mediate cation-dependent changes in both thylakoid appression and energy distribution between the photosystems: (1) Thylakoids isolated from *hcf**-3 and wild-type seedlings exhibit a similar Mg^{2+}-dependent increase in the short/long wavelength fluorescence emission peak ratio at 77 K. This Mg^{2+} effect is lost following incubation of thylakoids isolated from either source with low concentrations of trypsin. Such treatment results in the partial proteolysis of the LHC in both membrane types. (2) Thylakoids isolated from both *hcf**-3 and wild-type seedlings show a similar Mg^{2+} dependence for the enhancement of the maximal yield of room temperature fluorescence and light scattering; both Mg^{2+} effects are abolished by brief incubation of the thylakoids with low concentrations of trypsin (3) Mg^{2+} acts to reduce the relative quantum efficiency of Photosystem I-dependent electron transport at limiting 650 nm light in thylakoids isolated from *hcf**-3. (4) The pattern of digitonin fractionation of thylakoid membranes, which is dependent upon structural membrane interactions and upon LHC in the thylakoids, is similar in thylakoids isolated from both *hcf**-3 and wild-type seedlings. We conclude that the surface-exposed segment of the LHC, but not the LHC-Photosystem II core association, is necessary for the cation-dependent changes in both thylakoid appression and energy distribution between the two photosystems, and that the LHC itself is able to transfer excitation energy directly to Photosystem I in a Mg^{2+}-dependent fashion in the absence of Photosystem II reaction centers. The latter phenomenon is equivalent to a cation-induced change in the absorptive cross-section of Photosystem I.**

Introduction

Maximal quantum efficiency of photosynthetic electron transport requires controlled distribution of excitation energy between the two photosystems. The mechanism regulating the distribution of excitation energy in vivo [1–3] can be closely mimicked in broked chloroplasts by altering the cation content of the suspending medium [3–7]. Typically, addition of 2–5 mM divalent cations or 100–150 mM monovalent cations to a suspension of cation-depleted thylakoids results in an increase in the quantum efficiency of Photosystem II [5,8–10], increased amplitude of variable fluorescence [5,11,12], increased energy transfer among Photosystem II units [4] and

Abbreviations: SDS, sodium dodecyl sulfate; Tricine, *N*-tris-(hydroxymethyl)methylglycine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; Chl, chlorophyll.

an increase in the 685/735 nm fluorescence emission peak ratio at 77 K [5,8,10], while concomitant decreases in quantum yield are seen for Photosystem I photoreactions at limiting light [5,8–10]. The sum total of these changes is taken as evidence for the regulation of excitation energy distribution via a 'spillover' mechanism (for reviews see refs. 13 and 14), where cations regulate the transfer of excitation energy from Photosystem II to Photosystem I.

Several lines of evidence suggest that the light-harvesting pigment-protein complex (LHC) which is structurally and functionally associated with Photosystem II is the primary membrane component responsible for mediating cation-dependent changes in both grana stacking and energy distribution. Studies with mutants lacking LHC [6,15–19] and with greening pea leaves containing varying amounts of the LHC [20–23] show a strong correlation between the amount of LHC integrated into the thylakoid, the development of grana stacks [19,23–25] and, where examined, the extent of cation-mediated changes in energy distribution [6,15,17,20,22]. Purified LHC retains the ability to aggregate in the presence of cations [8,26,27] and can mediate cation-dependent membrane appression when inserted into phospholipid vesicles [26–28]. The ability of cations to modulate grana stacking and energy distribution in thylakoid membranes is lost following treatment of the membranes with low concentrations of trypsin [10,26,29]; such treatment cleaves a surface-exposed segment of the LHC [10,30,31], indicating that this exposed segment is necessary for cation-mediated structural and functional changes in isolated chloroplasts. Similarly, trypsin or pronase treatment of the isolated LHC prevents cation-mediated aggregation of the isolated complex [26,28], while trypsin treatment of LHC inserted into phospholipid vesicles blocks cation-induced membrane appression in these artificial membranes [27]. Finally, antibodies raised against purified LHC block cation-mediated changes in energy distribution in thylaoid membranes [8].

Although the correlation between the presence of LHC in the membrane and the ability of cations to induce membrane appression seems clear, there are still questions concerning the mechanism by which LHC is able to mediate cation-dependent changes in excitation energy distribution. At the present time at least two hypotheses can be put forward. (1) Cations alter the physical interaction between LHC subunits and the Photosystem II reaction center [32], resulting in an increased probability of energy transfer between Photosystem II units and the LHC; this energy cycling would compete with energy transfer from Chl a_{II} to Chl a_I, resulting in decreased spillover [33,34]. According to this view, direct energy transfer from LHC to Chl a_I plays a minor role in energy distribution [34,35]. (2) Alternatively, the LHC itself may play a significant role in transferring energy directly to Photosystem I, with cations affecting the properties of chromophores within the LHC in such a way that the probability of energy transfer directly from LHC to Photosystem I is decreased upon the addition of cations.

In order to differentiate between these two possibilities, we have selected as a test system a maize mutant in which interactions between Photosystem II (Chl a_{II}) and the LHC are interrupted due to the inability to assemble Photosystem II core complexes [36]. This mutant (*hcf**-3), which was previously shown to be totally deficient in Photosystem II activity, nevertheless contains wild-type amounts of the LHC and a highly active Photosystem I [37,38]. The effect of cations upon the control of energy distribution in these membranes is presented in this manuscript.

Materials and Methods

Plant material. The maize (*Zea mays* L.) stock segregating the single gene nuclear mutation *hcf**-3 used in this study was derived by crossing F_3 plants propagated from the original accession material (Neuffer E-846) onto suitable stock followed by selfing of the F_1 to yield an improved seed source. The new genetic background introduced by crossing did not alter the characteristics of the mutant as reported in Ref. 37. In each experiment genetic control was provided by isolating chloroplasts from high-fluorescent and normal seedlings obtained from the same ear. For all experiments kernels were planted in vermiculite moistened with half-strength Hoagland's solution and seedlings were grown to the three-leaf stage in a controlled-environment chamber under cool-white fluorescent illumination (200 $\mu E/m^2$ per s, 16 h photoperiod).

Chloroplast isolation. Chloroplasts (primarily

class II) were isolated from seedlings by grinding 10 g of washed leaf material in a Waring blender at low speed with 100 ml of grinding medium containing 0.1 M Na^+-Tricine (pH 7.8), 0.4 M sorbitol, 10 mM NaCl and 5 mM $MgCl_2$. Approx. 100 mg of solid polyvinylpolypyrrolidone were added to the medium just prior to grinding. The brei was filtered through four layers of Miracloth and a pellet collected by centrifugation at $1500 \times g$ for 10 min. This initial pellet was washed in several ways as described below. Prior to assay, the chlorophyll concentration of the final stock suspension was determined by the method of Mackinney [39].

Trypsin incubation. In all experiments involving trypsin digestion, chloroplasts were osmotically shocked and unstacked by washing the initial pellet three times in washing buffer containing 10 mM Na^+-Tricine, pH 7.8, and 10 mM NaCl. Following resuspension in the washing buffer, aliquots of the final suspension were diluted with washing buffer to a chlorophyll concentration of 100 μg/ml. Trypsin (TPCK trypsin, 232 units/mg, Millipore Corp.) was added as a concentrated stock solution to give a final trypsin concentration of 0.2–0.6 μg/ml and the incubation was allowed to proceed in the dark for 10 min. Digestion was terminated by addition of a 20–50-fold excess of trypsin inhibitor (type I-S, from soybean, Sigma Chemical Co.). The membranes were either diluted further with washing buffer for fluorescence assay or rapidly pelleted for electrophoretic analysis of lamellar polypeptides as described below.

Gel electrophoresis. SDS-polyacrylamide gradient gel electrophoresis was performed as previously described [37]. Osmotically shocked thylakoids were prepared and subjected to trypsin digestion (0.6 μg trypsin/ml) as described above. Following 10 min dark incubation with trypsin and the subsequent addition of trypsin inhibitor, thylakoids were collected from 1 ml aliquots by rapid centrifugation in a cold microcentrifuge and lamellar polypeptides solubilized immediately in heated SDS-containing sample buffer to inactivate residual trypsin. Samples were quickly heated to 100°C for 2 min to further dissociate chlorophyll-protein complexes. The gels were calibrated with respect to molecular weight using phosphorylase A (92 000 daltons), bovine serum albumin (67 000 daltons), ovalbumin (45 000 daltons), carbonic anhydrase (29 000 daltons), chymotrypsinogen (25 000 daltons) and cytochrome *c* (12 400 daltons) as molecular weight standards.

77 K fluorescence emission spectra. Low-temperature fluorescence emission spectra were recorded using a System 4000 scanning polarization spectrofluorimeter (SLM Instruments, Urbana, IL) operating in a ratiometric acquisition mode. Exciting light was provided at 440 nm with a half bandwidth of 4 nm and the fluorescence emission scanned in 0.5 nm increments from 650 to 800 nm with a half bandwidth of 1 nm. Acquisition, storage and mathematical manipulation of uncorrected spectra were performed by an on-line Hewlett-Packard 9825 desk-top computer linked to a Hewlett-Packard 7225A plotter. Stroma-free thylakoids were prepared and incubated with trypsin at a final concentration of 0.6 μg/ml as described above. Control chloroplasts were incubated under similar conditions in the absence of trypsin. After the addition of trypsin inhibitor, aliquots were diluted to 10 μg Chl/ml with 10 mM Na^+-Tricine (pH 7.8), 100 mM Sorbitol, 10 mM NaCl and 50% (v/v) glycerol either with or without 5 mM $MgCl_2$ and incubated in the dark for at least 15 min at room temperature prior to freezing in 0.5 mm inner diameter capillary tubes.

Room temperature fluorescence and light scattering. Mg^{2+}-induced changes in room temperature chlorophyll fluuorescence and light scattering were monitored simultaneously in a locally constructed apparatus incorporating fiber optic bundles to separate and detect the two signals. Fluorescence was detected using a system similar to that previous described [8], with the principal modification being the use of a bifurcated light pipe for both the excitation (broad-band blue light; Corning 4-96 filter) and detection of the fluorescence signal. The exciting light was of sufficient intensity to close all reaction centers as determined by the inability of DCMU to cause a further increase in the fluorescence yield. Light scattering at 180° was measured through a 560 nm interference filter (Pomfret Research Optical, Inc.) with an Aminco J10-280 photomultiplier microphotometer equipped with an S-20 response phototube. Both the fluorescence and the light-scattering signals were displayed on an Exployer III digital oscilloscope (Nicolet Instrument Corp.) and the signals stored for further analysis using a Hewlett-Packard 9825 desk-top computer.

Trypsin-treated thylakoids were prepared as described above using 0.2 μg trypsin/ml; after addition of trypsin inhibitor an aliquot was diluted to 15 μg Chl/ml for assay.

Photosystem I photoreactions. Photosystem I-mediated electron transport from $DCIPH_2$ to methyl viologen was monitored using a Clark-type electrode connected to a locally constructed solid-state amplifier. Chloroplasts were isolated as described above and the initial pellet was washed twice in 10 mM Na^+-Tricine (pH 7.8), 50 mM sorbitol and 10 mM NaCl with or without 5 mM $MgCl_2$. At the time of measurement, aliquots from the stock suspensions were diluted to 10 μg Chl/ml in 1 ml of reaction mixture containing 10 mM Na^+-Tricine (pH 7.8), 50 mM sorbitol, 10 mM NaCl, 0.33 mM DCIP, 0.5 mM methyl viologen, 0.5 mM NaN_3, $5 \cdot 10^{-6}$ M DCMU, $1 \cdot 10^{-7}$ M gramicidin D and 1 mM sodium ascorbate, pH 7.8, in the presence or absence of 5 mM $MgCl_2$. The reaction solution was magnetically stirred and temperature regulated at 20°C. Limiting light of varying intensity was provided by passing light from a Vickers intense lamp through a 650 ± 10 nm interference filter (Pomfret Research Optics, Inc.) and a graded series of Balzers neutral density filters.

Chloroplast fractionation. For detergent fractionation experiments, chloroplasts were isolated as described above except that the grinding medium contained 10 mM $MgCl_2$. The initial pellet was washed once in a buffer containing 10 mM Na^+-Tricine (pH 7.8), 0.1 M sorbitol, 10 mM NaCl and 5 mM $MgCl_2$. Following centrifugation, the pellet was resuspended in a sufficient volume of wash buffer to give a final chlorophyll concentration of 0.3 mg Chl/ml, solid recrystallized digitonin was added to a final concentration of 10 mg/ml, and the suspension was stirred at 0°C in the dark for 10 min. Following incubation with digitonin the suspension was centrifuged at 1000 × *g* for 10 min to remove unfractionated lamellae, 10000 × *g* for 30 min to collect a 'heavy' (D-10) pelleted fraction, and 144000 × *g* for 1 h to recover a 'light' (D-144) pelleted fraction.

Results

Gel electrophoresis

Thylakoids isolated from the high-fluorescent maize mutant *hcf**-3 exhibit the loss of a major lamellar polypeptide with an apparent molecular weight of 32000 (Fig. 1). The loss of this polypeptide was not accompanied by the loss of polypeptides in the 40000–50000 dalton region of the gel which have been previously identified as components of the Photosystem II reaction center complex [40,41]. In a previous study it was found that *hcf**-3 thylakoids are nearly devoid of the large exoplasmic face freeze-fracture particles. These membrane subunits are thought to represent the Photosystem II reaction center-core complex in association with peripherally arranged subunits of the LHC [14]. We have postulated that the 32000 dalton polypeptide is

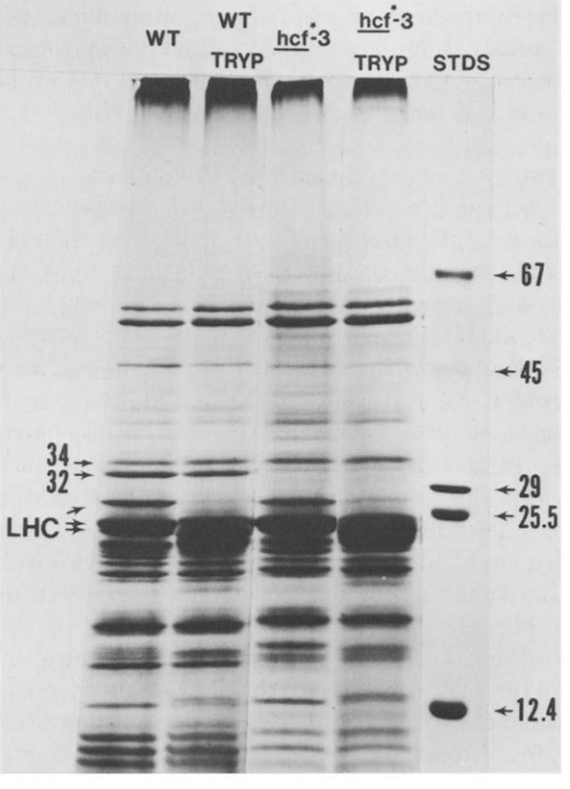

Fig. 1. SDS-polyacrylamide gradient gel electrophoresis of lamellar polypeptides isolated from *hcf**-3 and wild-type (WT) maize seedlings. Trypsin (TRYP) treatment (0.6 μg trypsin/100 μg Chl per ml) of divalent cation-depleted thylakoids was carried out. Control samples were similarly incubated at room temperature in the absence of trypsin. Lanes were loaded on an equal chlorophyll basis. Molecular masses are given on either side of gel scan in kdaltons.

essential for the assembly of functionally active Photosystem II reaction centers [36,37]. However, despite the loss of Photosystem II activity in *hcf**-3, the polypeptide profiles presented in Fig. 1 indicate that these membranes contain a normal complement of the major lamellar polypeptides identified as components of the LHC [8]. Incubation of unstacked thylakoids obtained from both wild-type and *hcf**-3 seedlings with low concentrations of trypsin resulted in the complete loss of the upper and the partial proteolysis of the lower two LHC polypeptides, as has been reported earlier with similarly treated pea chloroplasts [10]. Accompanying this proteolysis was the appearance of several closely spaced polypeptides with mobilities just slightly greater than those exhibited by the lower LHC polypeptides prior to trypsin treatment; in Fig. 1 this results in the appearance of a single broad band with an apparent average molecular weight approx. 2000 less than that of the lower LHC polypeptides prior to proteolysis.

Low-temperature fluorescence emission spectra

Stroma-free wild-type thylakoids resuspended in divalent cation-free media exhibited a typical fluorescence emission spectrum [44], with emission maxima at 685 and 734 nm and a prominent shoulder at 696 nm (Fig. 2). Incubation of these membranes with 5 mM Mg^{2+} resulted in an increase in the fluorescence yield at 685 nm relative to that at 734 nm. It should be noted that the spectra presented in Fig. 2 were normalized via a computer routine to give equal emission yields at 734 nm. As observed previously [26], dark incubation of the thylakoids with low concentrations of trypsin prior to fluorescence assay largely eliminated the cation-induced changes in fluorescence yield.

The 77 K fluorescence emission spectrum obtained with divalent cation-depeleted thylakoids isolated from *hcf**-3 exhibited two features which differed from the control; the short-wavelength emission maximum was shifted from 685 to 680 nm and the shoulder at 696 nm was more prominent. In spite of these alterations, addition of Mg^{2+} to the low-salt membranes caused an enhancement of the short-wavelength emission yield when compared to the far-red emission maximum at 733 nm. The extent of this enhancement was equal in magnitude to that observed upon adding Mg^{2+} to divalent cation-depleted

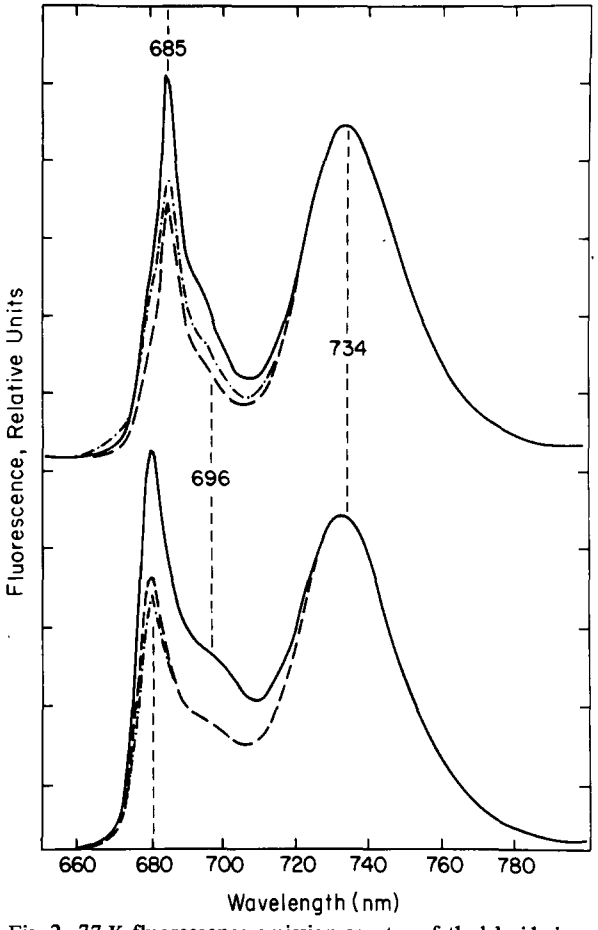

Fig. 2. 77 K fluorescence emission spectra of thylakoids isolated from *hcf**-3 (bottom) and wild-type (top) seedlings. Divalent-cation depleted thylakoids were assayed directly or subsequent to incubation with Mg^{2+} for 15 min in the dark. When performed, incubation of divalent-cation depleted thylakoids with trypsin (dark, 10 min) preceded the addition of Mg^{2+}. Emission peaks were normalized to 734 nm via computer routine. (———) 5 mM Mg^{2+}; (– – –) no Mg^{2+}; (· · · · ·) 10 min incubation with trypsin, followed by the addition of 5 mM Mg^{2+}.

wild-type membranes. As in the wild-type control, this cation-induced change was nearly eliminated following incubation of the membranes with low concentrations of trypsin prior to fluorescence assay.

Effect of cations upon room temperature fluorescence and light scattering

The simultaneous measurements of cation-induced light scattering and chlorophyll fluorescence emission at 20°C in wild-type thylakoids is shown in Fig. 3a.

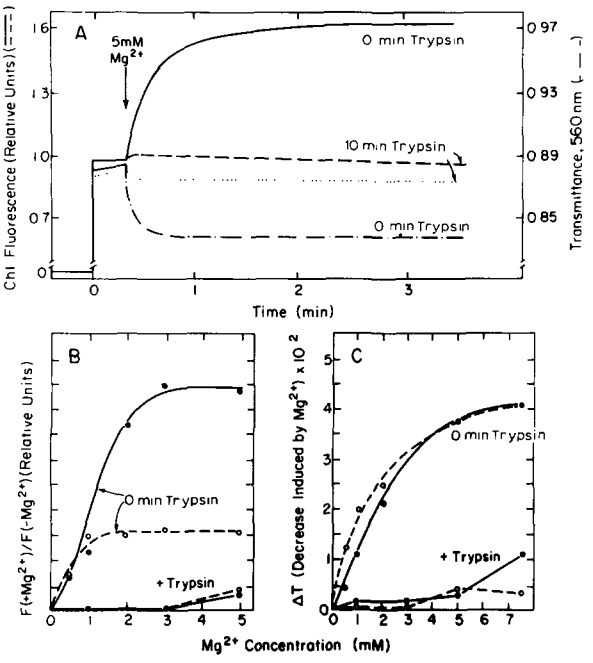

Fig. 3. Changes in light scattering (ΔT, 560 nm) and the maximal level of room temperature fluorescence following addition of Mg^{2+} to divalent-cation depleted thylakoids obtained from *hcf**-3 and wild-type seedlings. Where indicated, thylakoids were incubated with trypsin and, subsequently, trypsin inhibitor, prior to assay. (A) Simultaneous measurement of the maximal fluorescence yield and transmittance following addition of 5 mM Mg^{2+} to divalent-cation depleted wild-type thylakoids. Chl, 15 μg/ml. (B) Mg^{2+} concentration dependence for the cation-stimulated increase in maximal fluorescence yield seen in divalent cation-depleted thylakoids obtained from *hcf**-3 (– – –) and wild-type (———) seedlings. Chl, 15 μg/ml. (C) Mg^{2+} concentration dependence for the cation-dependent decrease in transmittance (ΔT, 560 nm) observed in samples similar to those in (B). (– – –) *hcf**-3, (———) wild-type. Chl, 3.4 μg Chl *b*/ml.

Support for the hypothesis that cation-induced changes in light scattering reflect alterations in the pattern of grana stacking comes from the work of Gross and Prasher [42] who demonstrated that changes in turbidity (i.e., 180° light scattering) in response to the addition of cations reflect changes in the extent of grana stacking as judged by electron microscopy. Wollman and Diner [43] have recently reported a similar correlation between the extent of 90° light scattering and thylakoid appression in *Chlamydomonas;* furthermore, these authors state that light-scattering changes are not seen following the addition of 10 mM Mg^{2+} to divalent cation-depleted thylakoids prepared from ac-5, a *Chlamydomonas* mutant, which does not undergo membrane stacking upon addition of Mg^{2+} [24]. Cation-induced changes in the fluorescence yield have been interpreted to be indicative of changes in energy distribution between the photosystems [13,14]. As shown in Fig. 3a the addition of 5 mM Mg^{2+} to thylakoids obtained from control seedlings resulted in an increase in the maximal level of fluorescence (indicating preferential energy distribution to Photosystem II) and a concomitant decrease in the 180° light-scattering signal (indicative of increased lamellar appression). As noted by others [42,43], we observed both a fast and a slow phase in the scattering signal, while the fluorescence yield increase exhibited only a slow phase. A similar response to the addition of Mg^{2+} was seen with thylakoids isolated from *hcf**-3 (kinetic data not shown). The magnitude of the Mg^{2+}-induced fluorescence and scattering changes was greatly reduced in both wild-type and *hcf**-3 thylakoids following brief dark incubation of the chloroplasts suspension with trypsin prior to assay.

The cation requirement for Mg^{2+}-induced fluorescence increase in chloroplast isolated from *hcf**-3 and control seedlings is shown in Fig. 3b. Chloroplasts isolated from both sources exhibited a cation-dependent increase in fluorescence, although the amplitude of the fluorescence increase, and the concentration of Mg^{2+} required for half-maximal effects, were smaller in the mutant. The fluorescence increase induced by Mg^{2+} was eliminated in both wild-type and mutant chloroplasts by treatment of the membranes with trypsin prior to assay.

As shown in Fig. 3c, chloroplasts isolated from *hcf**-3 and wild-type seedlings exhibited a nearly identical Mg^{2+} requirement for changes in 180° light scattering (measured as a decrease in transmission). In both cases, the cation-inducible scattering signal was largely eliminated following incubation of unstacked thylakoids with low concentrations of trypsin. In these experiments samples were utilized which contained equal amounts of Chl *b*; when normalized to total chlorophyll, the Mg^{2+}-inducible scattering signal observed with mutant thylakoids was larger than that seen with control plastids, although the cation concentration requirement to elicit the change remained the same. It is uncertain whether the difference in

the amplitude of the scattering change reflects an alteration in the chlorophyll/protein ratio in mutant plastids or whether it is a consequence of the altered pattern of stacking seen in these plastids [36,38].

Photosystem I photochemistry

Despite the total loss of Photosystem II activity, thylakoids isolated from *hcf**-3 exhibit high rates of Photosystem I-dependent electron flow [37,38]. This has enabled us to measure the effect of Mg^{2+} on the elative quantum efficiency of Photosystem I-dependent photochemistry. Chloroplasts isolated from *hcf**-3 were osmotically shocked in either the presence or absence of 5 mM Mg^{2+} and the rate of Photosystem I-dependent electron transport from $DCIPH_2$ to methyl viologen was measured in the rupturing medium at various subsaturating quantum flux densities of actinic 650 nm light. These data, when plotted in double-reciprocal form (Fig. 4), indicated that the relative quantum efficiency of Photosystem I at limiting light was lower in the presence of Mg^{2+} than in its absence (abscissa intercept), although the extrapolated maximal rates of electron transport were the same in both cases (ordinate intercept). Similar data (not shown) were obtained with thylakoids prepared in identical fashion from wild-type control seedlings.

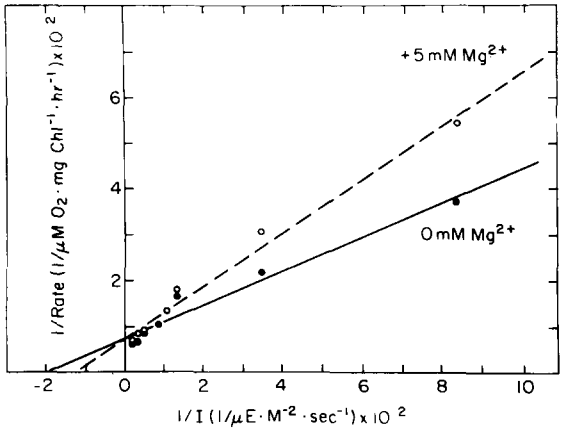

Fig. 4. Double-reciprocal plot of the rate of Photosystem I-dependent electron transport ($DCIPH_2$ → methyl viologen) as a function of incident quantum flux density (650 nm light) measured in thylakoids prepared from *hcf**-3. Chloroplasts were isolated in the presence of 5 mM Mg^{2+} and subsequently both washed and assayed in the presence of 5 mM Mg^{2+}. Rates, regression lines and correlation coefficients (r) were calculated and plotted by computer routine. (———) $r = 0.98$, (– – –) $r = 0.99$.

Digitonin fractionation

Digitonin has been utilized to disrupt wild-type chloroplast lamellae into membrane subfractions: a heavy, Photosystem II-enriched preparation (D-10) with a low Chl *a*/*b* ratio, and a light, Photosystem I-enriched fraction with a high Chl *a*/*b* ratio. The pattern of disruption is known to depend upon both the cation concentration of the medium and the LHC content of the membranes prior to fractionation, implying that a critical concentration of the LHC is required for successful fractionation with this detergent [32]. Since thylakoids isolated from *hcf**-3 contain wild-type amounts of the LHC as judged by gel electrophoresis (Fig. 1), we have compared the ability of digitonin to fractionate chloroplasts derived from both *hcf**-3 and wild-type control seedlings. In spite of the low Chl *a*/*b* ratio seen in chloroplasts obtained from *hcf**-3, these chloroplasts, when isolated and incubated in cation-containing media, were readily fractionated into light and heavy subfractions following incubation with digitonin (Table I). The extent of Chl *b* enrichment in the D-10 fraction and Chl *a* enrichment in the D-144 fraction was comparable in both plastid types. We note that the Chl *b* distribution into the D-144 fraction is higher for these maize chloroplast samples than is usually observed for spinach or pea chloroplasts. This is a general feature of these C-4 type chloroplasts; this pattern does not detract from the fact that the *hcf**-3 chloroplasts do undergo normal type detergent fractionation. Gel electrophoresis of the samples indicated that the D-10 fractions of both *hcf**-3 and wild-type membranes were enriched in the LHC and 40 000–50 000 dalton 'Photosystem II' polypeptides.

Discussion

Organization of LHC subunits in hcf-3*

In the present study, we have examined the ability of the LHC to mediate cation-dependent changes in both membrane appression (stacking) and excitation energy distribution under two conditions, one in which the LHC exhibits a functional and structural association with Photosystem II reaction center-core

TABLE I

DIGITONIN FRACTIONATION OF THYLAKOIDS OBTAINED FROM *hcf**-3 AND WILD-TYPE SEEDLINGS

Thylakoids were prepared and fractionated as described in Materials and Methods. Following incubation with digitonin, heavy (10 000 × *g*) and light (144 000 × *g*) pelletable fractions were collected by centrifugation. Enrichment, calculated from the measured Chl *a*/*b* ratios, is given as the percent increase in the Chl *b* (D-10) or Chl *a* (D-144) content of the respective fractions as compared to unfractionated thylakoids.

Chloroplast source	Chl *a*/*b* ratio			Enrichment	
	Chloroplasts	D-10	D-144	Chloroplasts/ D-10	Chloroplasts/ D-144
Wild-type	3.5	2.8	4.1	125	117
*hcf**-3	2.5	1.95	3.3	128	132

complexes (wild-type thylakoids) and a second one in which the LHC is present in the absence of active Photosystem II reaction centers (*hcf**-3 thylakoids). It is important to elucidate the structural organization of the LHC subunits in *hcf**-3 thylakoids, for at least two arrangements of the subunits can be envisioned in these membranes: (1) since *hcf**-3 thylakoids apparently contain polypeptides of the same size class as the reaction center of Photosystem II [37], the LHC subunits could be physically associated with an inactive Photosystem II core complex, or, (2) the loss of Photosystem II activity in these thylakoids could be accompanied by the physical loss of organized Photosystem II reaction centers, with the LHC existing as free subunits within the membrane. The following observations favor the latter hypothesis: (a) Thylakoids isolated from *hcf**-3 exhibit the selective loss of the large particles commonly seen on exoplasmic freeze-fracture faces in regions of membrane appression [36]. These particles are believed to be pigment-protein complexes consisting of a central Photosystem II reaction center-core complex in association with peripherally arranged subunits of the LHC [14]. This identification has recently been strengthened by the observation that thylakoids from Photosystem II-deficient mutants of tobacco [45], barley [46] and a *Chalmydomonas* mutant (F34) lacking polypeptides postulated to be an integral part of the Photosystem II reaction center complex [47,48] all exhibit the selective loss of the exospasmic face particles. The loss of these particles in the *Chlamydomonas* mutant F34 was accompanied by an increase in particle density on the complementary protoplasmic face fracture face, which Wollman et al. [48] suggest is due to the appearance of LHC subunits on both fracture faces when the association between the LHC and Photosystem II reaction centers is lost. Our examination of *hcf**-3 thylakoids revealed a similar increase in the protoplasmic face particle density. (b) The shift of the short-wavelength 77 K fluorescence emission maximum from 685 to 680 nm in thylakoids isolated from *hcf**-3 (Fig. 2) also suggests that the LHC subunits present in these membranes are altered, presumably due to weak or absent energetic coupling with Photosystem II complexes. Several authors have demonstrated that detergent-derived LHC preparations devoid of Photosystem II reaction center polypeptides exhibit low-temperature fluorescence emission maxima near 680 nm [27,49,51,52]. This spectral shift is also seen in intact thylakoids which contain LHC but lack Photosystem II reaction centers; both the y-1 mutant of *Chlamydomonas* greened in the presence of chloramphenicol (which permits the de novo synthesis of the LHC while preventing the synthesis of Photosystem II reaction centers [31,50]) and the F34 *Chlamydomonas* mutant described above exhibit a 5 nm blue shift of the short-wavelength low-temperature fluorescence emission maximum [43, 49]. In contrast to these gross disruptions, subtle changes in the physical coupling between the LHC and Photosystem II reaction centers, such as those caused by addition of Mg^{2+} to divalent cation-depleted thylakoids [32], do not affect the position of the short-wavelength 77 K fluorescence emission maximum. We conclude that the 5 nm blue shift of

the short-wavelength fluorescence emission maximum at 77 K is seen when the physical and functional association between Photosystem II reaction centers and the LHC subunits is grossly disrupted. We interpret the structural, electrophoretic and spectral data to suggest that the Photosystem II reaction center fails to organize in *hcf**-3 thylakoids due to the loss of the 32000 dalton lamellar polypeptide, and that under these conditions the LHC exists as 'free' subunits within the thylakoid membrane. At least a portion of each subunit remains exposed at the membrane surface, as judged by the susceptibility of these subunits to partial proteolytic digestion when *hcf**-3 thylakoids are subjected to mild trypsin digestion (Fig. 1).

Energy transfer and membrane appression

The coexistence of 'free', oriented (with respect to the membrane surface) LHC subunits and an active Photosystem I in *hcf**-3 thylakoids makes these membranes a useful model system for testing the ability of free LHC subunits to mediate cation-dependent changes in both lamellar appression and excitation energy distribution. We have examined Mg^{2+}-induced changes in low-temperature fluorescence emission spectra (where the short-wavelength emission maximum originates primarily from pigments associated with Photosystem II [34]) and room temperature fluorescence yield (where the bulk of the emission again arises predominently from the LHC [34,51,52]) as measures of cation-dependent changes in excitation energy distribution. Data presented in Figs. 2 and 3 show that the addition of Mg^{2+} to divalent cation-depeleted thylakoids isolated from either *hcf**-3 or control seedlings caused an increase in the fluorescence yield from the LHC, suggesting that Mg^{2+} acts to decrease energy transfer from the LHC to Photosystem I in both cases. These cation effects were largely abolished in both wild-type and *hcf**-3 thylakoids following incubation of the membranes with low concentrations of trypsin, indicating that the LHC is the component mediating these changes. It should be noted that the extent of the Mg^{2+}-induced room temperature fluorescence increase was smaller in *hcf**-3 thylakoids than that observed with wild-type thylakoids. Studies with the isolated LHC demonstrate that the high fluorescence yield seen with the solvated complex is rapidly quenched upon addition of Mg^{2+} [8]; perhaps a similar quenching process is competing with the fluorescence increase due to energy redistribution in *hcf**-3 thylakoids.

As discussed by Williams [13], the Mg^{2+}-induced increase in Photosystem II fluorescence yield could be due to factors other than a redistribution of excitation energy from the LHC to Photosystem I. However, as shown in Fig. 4, Mg^{2+} acts to decrease the quantum efficiency of Photosystem I-dependent photochemistry in *hcf**-3 thylakoids at limiting 650 nm light, providing strong evidence that cations do, in fact, act to control the distribution of excitation energy in these membranes.

As shown in Fig. 3c, thylakoids obtained from *hcf**-3 and wild-type seedlings exhibited a nearly identical trypsin-sensitive cation dependence for the onset of thylakoid appression. Furthermore, in the presence of Mg^{2+}, digitonin fractionation of both membrane types resulted in the recovery of both heavy and light subchloroplast particles (Table I), which would not be expected if the LHC content, or the ability of the LHC to maintain thylakoid appression, was not similar in both plastid types. Coupled with the observation that thylakoids from *hcf**-3 are extensively stacked [36,38], these data indicate that the free LHC subunits present in *hcf**-3 are capable of mediating thylakoid appression.

The ability of free LHC to mediate energy transfer to Photosystem I is in apparent conflict with the assumption of Butler [34] that the rate of direct energy transfer from the LHC to Photosystem I is small. According to this view, the major effect of Mg^{2+} is to increase the rate of energy coupling between LHC and Chl a_{II} while decreasing the rate of energy transfer from Chl a_{II} to Chl a_I, the net effect being an increase in the yield of variable fluorescence and a decrease in spillover from Photosystem II to Photosystem I. While we cannot dispute the occurrence of energy transfer from Chl a_{II} to Chl a_I in the absence of the LHC [53], this energy transfer has not been shown to be cation dependent. Our data are consistent with the concept that cations act to increase the relative number of incoming quanta remaining in the LHC pigment bed, and that cation-dependent energy transfer from the LHC to Photosystem I can occur in the absence of a functional Photosystem II reaction center-core complex. The qualitative and quantitative similarity of the cation

effects in both wild-type and *hcf**-3 thylakoids suggests that significant energy transfer from the LHC to Photosystem I can also occur in normal membranes, leading to in vivo control of excitation energy distribution through changes in the effective optical cross-section of each photosystem, as suggested by Bonaventura and Meyers [2].

Acknowledgements

This research was initiated in the USDA-SEA photosynthesis laboratory at Urbana, IL, and supported, in part, by DOE Contract No. EE-77-5-02-4475-A00.

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